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Thèse N° :

Contribution à l'étude des cellules Natural Killer dans le contexte de la double-greffe de sang de cordon

JURY

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Liste des abréviations

ABM : Agence de la BioMédecine

ADAM : A Disintegrin And Metalloprotease

ADCC : Antibody Dependant Cellular Cytotoxicity

ADN : Acide DesoxyriboNucléique

ATG : Anti-Thymocyte Globulin

β2m : Beta-2 microglobuline

BAT-3 : HLA-B Associated Transcript 3

BCR : B cell Receptor

BiKEs : Bi-specific Killer-cell Engagers

CAR : chimeric antigen receptor

CD : Cluster de Différenciation

CMH : Complexe Majeur d'Histocompatibilité

CHILP : Common Helper-like ILCs Lymphoid Progenitor

CLP : Common lymphoid progenitor

CMV : Cytomegalovirus

CPA : Cellule Présentatrice d'Antigène

CSH : Cellules Souches Hématopoïétiques

CSM : Cellules Souches Mésenchymateuses

DC : Dendritic Cell

DLI : Donor Lymphocyte Infusion

DNAM-1 : DNAX Accessory Molecule 1

dUCBT : double Umbilical Cord Blood Transplantation

EBV : Epstein Barr Virus

GCV : Ganciclovir

G-CSF : Granulocyte Colony Stimulating Factor

GM-CSF : Granulocyte/Macrophage Colony Stimulating Factor

GvH : Graft-versus-Host

GvHD : Graft-versus-Host-Disease

GvL : Graft-versus-Leukemia

HIV : Human Immunodeficiency Virus

HLA : Human Leukocyte Antigen

HSPC : Protéoglycanes héparane sulfate

HSV-TK : Herpes Simplex Virus Thymidine Kinase

HR : High Resolution

HSCT : Hematopoietic Stem Cell Transplantation

HSV : Herpes Simplex Virus

ICAM : InterCellular Adhesion Molecule

IFN : Interferon

Ig : Immunoglobuline

IL : Interleukine

ILC : Innate Lymphoid Cell

ILT : Immunoglobulin-Like Transcript

iNK : Immature Natural Killer cell

IPD : Immuno Polymorphism Database

ITAM : Immunoreceptor Tyrosine-based Activation Motif

ITIM : Immunoreceptor Tyrosine-based Inhibition Motifs

ITSM : Immunoreceptor Tyrosine-based Switch Motif

KACL : Keratinocyte-Associated C-type Lectin

KIR : Killer Cell Immunoglobulin-like Receptor

LR : Low Resolution

LTi : Lymphoïd tissue inductor

LAL : Leucémie Aigüe Lymphoïde

LAM : Leucémie Aigüe Myéloïde

LAMP : Lysosomal-associated membrane protein

LFA-1 : Leukocyte Function-Associated Antigen 1

LLT : Lectin-Like Transcript

LMC : Leucémie Myéloïde Chronique

LRC : Leukocyte Receptor Complexe

MIC-A, -B : MHC class I related protein A, B

MLL5 : Mixed Lineage Leukemia-5

MMP : Matrix Metalloproteinase

MPR : Mannose-6-Phosphate Receptor

NCR : Natural Cytotoxicity Receptor

NK : Natural Killer

NKC : Natural Killer cell Complex

NKG2 : Natural Killer Group 2

NKP : Natural Killer Progenitor

NOD : None Obese Diabetic

NRM : Non Relapse Mortality

OS : Overall Survival

PAMP : Pathogen-Associated Molecular Pattern

PBMC : Peripheral Blood Mononuclear Cell

PCR : Polymerase Chain Reaction

pDC : Plasmacytoïdes Dendritic Cell

PFS : Progression Free survival

PLZF : Promyelocytic Leukaemia Zinc-Finger

PRR : Pattern Recognition Receptor

RAE : Retinoic Acid Early inducible

RIC : Reduced Intensity Conditionning

SAP : SLAM Associated Protein

SC : Sang de Cordon

SCID : Severe Combined Immuno-Deficient

SIDA : Syndrome de l'ImmunoDéficience Acquise

SLAM : Signaling Lymphocyte Activation Molecule

SNP : Single Nucleotid Polymorphism

STR : Short Tandem Repeat

sUCBT : single Umbilical Cord Blood Transplantation

TAP : Transporter Associated with antigen Processing

TCR : T cell Receptor

TGFβ : Transforming Growth Factor Beta

TLR : Toll Like Receptor

TNF : Tumor Necrosis Factor

TRAIL : TNF Related Apoptosis Inducing Ligand

TriKEs : Tri-Specific Killer-cell Engagers

TRM : Transplant Related Mortality

UCB : Umbilical Cord Blood

UCBT : Umbilical Cord Blood Transplantation

ULBP : UL16 Binding Protein

VHC : Virus de l'Hépatite C

VIH : Virus de l'Immunodéficience Humaine

VNTR : Variable Number Tandem Repeats

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INTRODUCTION GENERALE

I. Immunité innée et adaptative

1. Les acteurs de l'immunité innée et adaptative

L'immunité est l'ensemble des mécanismes de défense d'un organisme contre les éléments étrangers, en particulier les agents infectieux (virus, bactéries ou parasites). Elle est assurée par deux systèmes qui collaborent pour protéger l'organisme contre les pathogènes : l'immunité innée et l'immunité adaptative, que nous allons brièvement présenter dans ce paragraphe.

L'immunité innée, dite naturelle, constitue la première ligne de défense de l'organisme. Mise en place très rapidement, elle comprend des barrières anatomiques comme les couches épithéliales de la peau et les muqueuses. Certaines protéines du système du complément peuvent rendre les bactéries sensibles à la phagocytose par les macrophages, induire des réponses inflammatoires ou encore s'assembler pour tuer directement certains pathogènes en créant des pores à travers leur membrane. Certaines cellules de l'immunité innée expriment naturellement un ensemble de récepteurs membranaires, les PRR (Pattern Recognition Receptors), qui vont reconnaître des molécules caractérisant des groupes entiers de pathogènes : les PAMP (Pathogen-Associated Molecular Pattern). La détection de micro-organismes étrangers peut induire une réponse inflammatoire impliquant des cytokines inflammatoires (IL-1, IL-6, TNFα) et différentes chimiokines permettant de recruter les acteurs de l'immunité sur les sites d'inflammation. Enfin, des acteurs cellulaires comme les polynucléaires, les macrophages, les cellules dendritiques, les mastocytes et les cellules Natural Killer (NK) assurent une reconnaissance rapide des pathogènes, bien que non spécifique. Les cellules NK seront abordées plus en détails ultérieurement.

En parallèle, les pathogènes ayant traversé les barrières de l'immunité innée peuvent être reconnus par les cellules de l'immunité adaptative. Les acteurs principaux de l'immunité adaptative sont les lymphocytes T et B, exprimant un très large répertoire de récepteurs

d'antigènes. Les récepteurs des lymphocytes B (ou BCR) peuvent reconnaître un antigène isolé, induisant leur activation et leur différenciation en cellules effectrices, les plasmocytes. Ils sont spécialisés dans la sécrétion d'immunoglobulines solubles, les anticorps spécifiques des antigènes rencontrés, constituant les molécules effectrices de la réponse dite humorale. Au contraire, les récepteurs des lymphocytes T (ou TCR) reconnaissent seulement des peptides antigéniques présentés par des cellules présentatrices de l'antigène (CPA) via des protéines de surface : les molécules du Complexe Majeur d'Histocompatibilité que nous allons décrire dans le paragraphe suivant.

2. Le Complexe Majeur d'Histocompatibilité au centre de la réponse immunitaire

Le Complexe Majeur d'Histocompatibilité (MCH) a été découvert pour son rôle déterminant dans l'acceptation ou le rejet de la greffe, un rejet étant inévitable entre deux individus non apparentés. La première molécule d'histocompatibilité identifiée chez l'homme avait été repérée comme un antigène leucocytaire en 1958 par Jean Dausset, récompensé pour ses travaux par le prix Nobel de physiologie et médecine en 1980. Le CMH humain fut ainsi nommé HLA (Human Leukocyte Antigen). Notons également que le CMH murin est nommé H-2.

Chez l'homme, le complexe génique des molécules HLA est situé sur le bras court du chromosome 6. Les gènes HLA sont organisés en régions codant trois classes de molécules : les molécules HLA de classe I, de classe II et de classe III, illustrées dans la Figure 1. Les gènes HLA sont transmis en haplotype, ce qui correspond à l'ensemble des gènes HLA présents sur un même chromosome. Chaque individu hérite ainsi d'un haplotype HLA du père et d'un haplotype HLA de la mère. De plus, les gènes HLA sont dits co-dominants, les molécules codées par chaque haplotype étant co-exprimées à la surface cellulaire. Le système HLA est le complexe génique le plus polymorphe du génome humain.

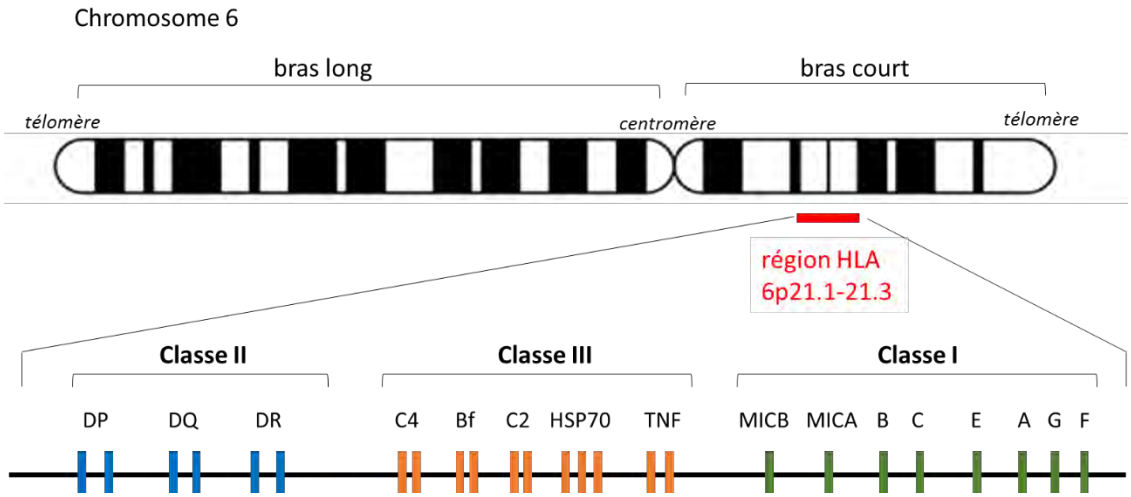

Figure 1 : Complexe génique des molécules HLA
Situé sur le bras court du chromosome 6, le complexe HLA est constitué de gènes HLA de classe I, de classe II et de classe III. La région HLA de classe I comprend les gènes HLA classiques (HLA-A, -B, -C) et non classiques (HLA-E, -F, -G) ainsi que les gènes MIC-A et MIC-B. La région HLA de classe II comprend les gènes HLA classiques (HLA-DP, DQ, DR) et non classiques, non représentés sur cette illustration. La région HLA de classe III comprend les gènes codant des protéines du complément (C2, C4), les protéines de choc thermiques (HSP70) et les TNF. Inspiré de Mehra and Kaur 2003

Les gènes HLA de classe I codent des molécules présentes sur toutes les cellules nucléées et spécialisées dans la présentation d'antigène endogènes aux lymphocytes T $CD8^+$. Parmi les molécules HLA de classe I classiques, on compte les molécules HLA-A, HLA-B et HLA-Cw. Il existe d'autres molécules HLA de classe I dites non classiques (HLA-E, -F, -G) qui présentent une structure proche mais sont peu polymorphes, et enfin des molécules apparentées aux molécules HLA de classe I comme MIC-A et MIC–B. Les gènes HLA de classe II codent des molécules exclusivement exprimées sur les CPA professionnelles et présentent des antigènes exogènes aux lymphocytes T $CD4^+$ qui vont activer et stimuler la réponse immunitaire. Les molécules HLA de classe II comprennent les molécules classiques HLA-DR, HLA-DP et HLA-DQ. Enfin, des gènes HLA de classe III codent des molécules qui n'interviennent pas dans la présentation de l'antigène mais codent différents composants du système du complément et des molécules impliquées dans l'inflammation comme les TNF.

Les molécules HLA de classe I et de classe II diffèrent dans leur fonction mais aussi dans leur structure primaire comme illustré dans la figure 2.

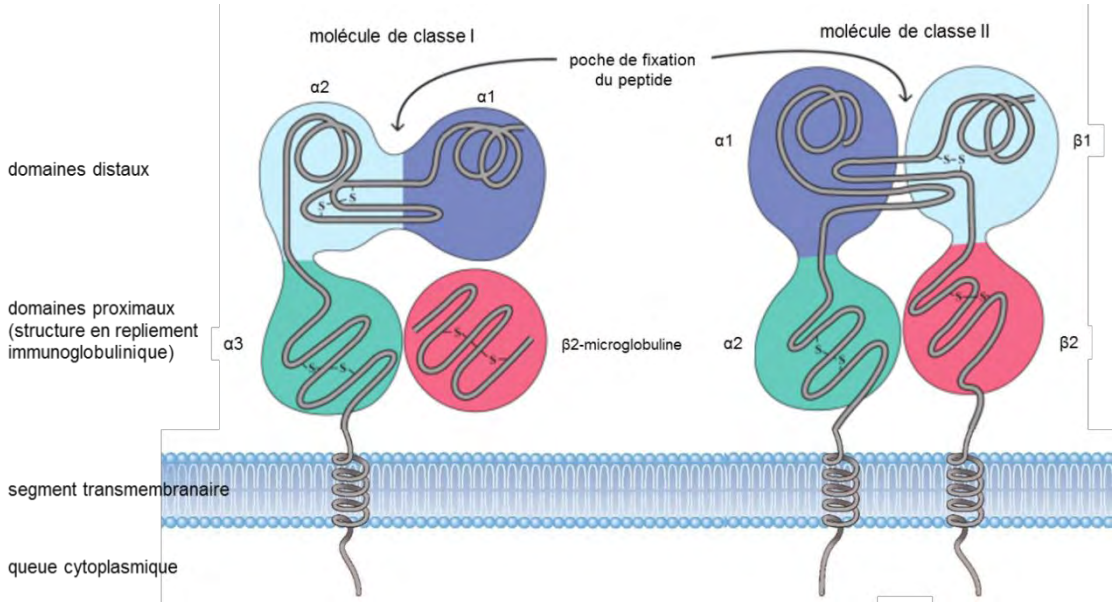

Figure 2 : Structure des molécules HLA de classe I et de classe II
Les molécules HLA sont des glycoprotéines présentant des domaines proximaux ayant une structure en domaine d'immunoglobulines. Les molécules HLA de classe I sont constituées d'une chaîne α à trois domaines extracellulaires. Les domaines α1 et α2 forment la poche de fixation au peptide et le domaine α3 est associé à la protéine β2-microglobuline. Les molécules HLA de classe II sont constituées de deux chaines α et β comportant deux domaines extracellulaires chacune. Les domaines distaux α1 et β1 délimitent la poche de fixation du peptide (Immunologie, Owen, Punt et Stranfort, 2013)

Les molécules HLA de classe I contiennent une chaine lourde α, associée de façon non-covalente à une chaîne légère, la β2-microglobuline. La chaine α est organisée en 3 domaines externes (α1, α2, α3) et un domaine transmembranaire. Les domaines α1 et α2 interagissent étroitement constituant la poche de liaison du peptide permettant la fixation d'un peptide antigénique de 8 à 10 acides aminés.

Les molécules HLA de classe II sont quant à elles constituées de deux chaînes différentes α et β présentant chacune deux domaines extracellulaires (α1/α2, β1/β2) et un domaine transmembranaire. La poche peptidique formée par les domaines α1 et β1 peut se lier à de plus grands peptides (13-18 acides aminés). L'association avec le peptide stabilise la molécule HLA, permettant ainsi le transport de ce complexe vers la membrane plasmique.

L'interaction entre le peptide et la molécule HLA est peu spécifique, permettant aux molécules HLA de présenter des milliers de peptides différents. Il faut noter que le polymorphisme génétique HLA est principalement situé au niveau des domaines α1, α2, (exons 2 et 3) des gènes HLA de classe I et de la chaîne β1 (exon 2) des gènes HLA de classe II.

Le rôle de présentation de peptides par les molécules HLA est primordial pour la sélection des lymphocytes T au niveau du thymus, dite sélection thymique. Les lymphocytes T ayant une affinité intermédiaire pour les molécules HLA du soi sont sélectionnés (sélection positive). Les cellules ayant une faible affinité pour les molécules HLA du soi ne reçoivent pas de signal positif nécessaire à leur survie et restent quiescentes. Les lymphocytes T présentant une trop forte affinité pour les molécules HLA du soi sont potentiellement autoréactifs et sont éliminés par apoptose (sélection négative). Ce processus assure ainsi la tolérance au soi des lymphocytes T circulants.

Les molécules HLA assurent également l'activation des cellules T en périphérie. Les lymphocytes T sont dits naïfs jusqu'à leur interaction avec un antigène présenté par une molécule HLA, devenant par la suite des cellules effectrices capables d'éliminer les cellules du non soi, infectées par un pathogène ou les cellules tumorales. On distingue plusieurs types de lymphocytes T (LT). Les LT-αβ $CD8^+$, également appelés lymphocytes T cytotoxiques, se caractérisent par l'expression du cluster de différentiation CD8 et du TCR-2, et ont des fonctions cytolytiques. Les LT-αβ $CD4^+$, qui donneront des LT « helper » (ou auxiliaires), régulent la réponse immunitaire adaptative par l'activation d'autres cellules immunitaires. La différenciation des lymphocytes T $CD4^+$ naïfs peut se produire en deux directions : TH1 ou TH2 en fonction des cytokines et des signaux de co-stimulation. La production de cellules TH1 mène à une immunité cellulaire, tandis que la production de cellules TH2 mène à une immunité humorale (Janeway et al. 2001). Enfin, les LT-γδ sont des lymphocytes T

particuliers caractérisés par l'expression d'un TCR-1 associé à un CD3 mais n'exprimant ni CD4, ni CD8. Ils sont beaucoup plus rares que les LT dits conventionnels (1-10% des lymphocytes T circulants) et ne sont pas restreints aux molécules HLA. Les LT γδ sont également producteurs de cytokines et cytotoxiques et ils peuvent présenter des peptides antigéniques aux LT αβ $CD4^+$ et $CD8^+$ (Brandes Science 2005). Ils expriment d'autres récepteurs et assurent en particulier des fonctions immunorégulatrices (Kalyan Cell Mol Immunol 2013) interagissant avec d'autres cellules de l'immunité comme les cellules dendritiques (Devilder JI 2006). Après rencontre avec le peptide antigénique, les LT conventionnels peuvent se différencier en cellules dites « mémoires » capables d'assurer une réponse immune à long terme.

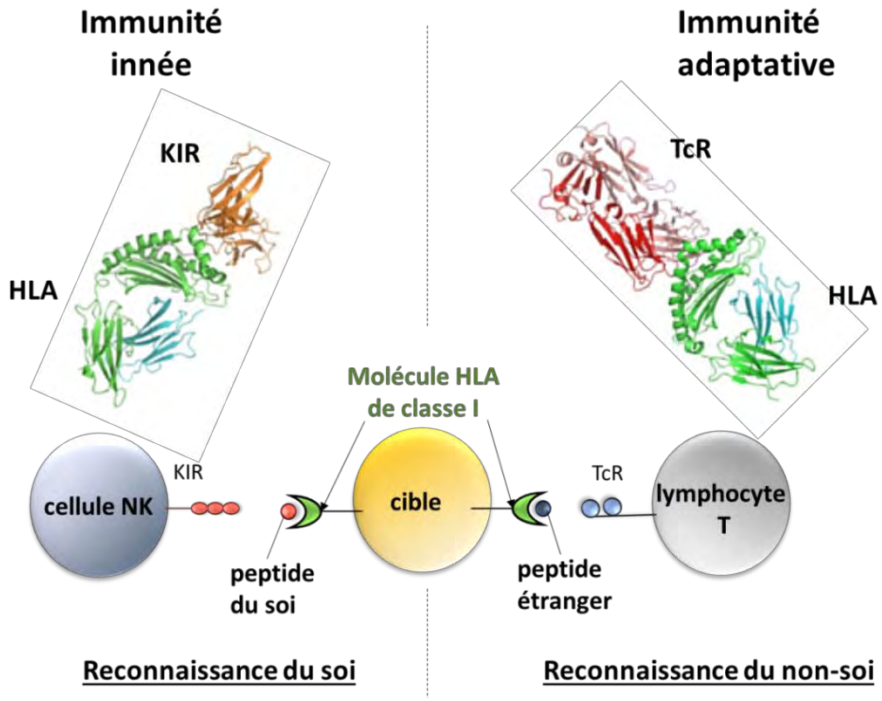

Figure 3 : Molécules HLA de classe I au centre de l'immunité innée et adaptative
Les molécules HLA de classe I sont spécifiquement reconnues par les récepteurs KIR et les récepteurs des lymphocytes T (TCR). Elles peuvent présenter des peptides du soi aux cellules NK (reconnaissance du soi) et des peptides étrangers aux lymphocytes T (reconnaissance du non-soi) induisant l'activation des réponses immunitaires pour protéger l'organisme des pathogènes.

Dans l'ensemble, les molécules HLA de classe I ont un rôle central dans la réponse immunitaire (figure 3), assurant d'une part l'activation les lymphocytes T et la reconnaissance du non-soi par la présentation d'un peptide étranger, et d'autre part la reconnaissance du soi via la présentation de peptides du soi aux cellules Natural Killer, un aspect qui sera abordé en détails dans la partie « II. Cellules NK ».

3. Les cellules lymphoïdes innées (ILC)

Les cellules Natural Killer ont longtemps été considérées comme la seule population lymphoïde de l'immunité innée capable de répondre à l'invasion de pathogènes. Ces dernières années, de nombreuses études ont prouvé l'existence de différentes populations de Cellules Lymphoïdes Innées (ILC) au niveau des muqueuses, qui réagissent rapidement suite à des signaux de stress provenant de tissus infectés, à des composés microbiens et des cytokines du tissu environnant et produisent une grande variété de cytokines régulant le développement de la réponse immune. Elles se distinguent des lymphocytes T et B n'exprimant pas de récepteurs spécifiques de l'antigène, n'expriment pas de marqueur de lignée (LIN^-) mais beaucoup de cytokines associées aux lymphocytes T helper.

Les ILCs sont catégorisées en 3 groupes majeurs selon leur dépendance à des facteurs de transcriptions et leur similarité fonctionnelle avec les lymphocytes T helper. Les ILC du groupe 1 (ILC1) ont été les premières ILCs identifiées, comprenant les cellules productrices de cytokines de type Th1 comme l'IFNγ, incluent les cellules Natural Killer et sont dépendantes des facteurs de transcription T-bet et/ou Eomes (Spits et al. 2013). Elles peuvent stimuler les phagocytes, assurant ainsi une activité antimicrobienne et contrôlant efficacement les infections intracellulaires. Les ILC2 produisent de grandes quantités de cytokines de type Th2, IL-5 et IL-13 (Moro et al. 2010; Neill et al. 2010) et leur différenciation dépend du facteur de transcription GATA-3 (Hoyler et al. 2012; Yagi et al. 2014). Ces cellules induisent

l'activation et la migration des cellules dendritiques, et ainsi la différenciation des cellules T Th2 (Halim et al. 2014). Elles jouent également un rôle important dans l'immunité de la pathogénèse de l'asthme et des allergies (Nussbaum et al. 2013). Les ILC3, importantes sources de cytokines de type Th17 comme l'IL-22 et l'IL-17A, dépendent du facteur de transcription RORγt (Serafini et al. 2014) et comprennent différentes sous-populations dont les cellules inductrices de tissu lymphoïdes (LTi). Elles sont impliquées dans la défense contre les infections bactériennes intestinales (Diefenbach 2013) et expriment les transcrits des molécules HLA de classe II, présentant les peptides exogènes aux LT $CD4^+$ (Hepworth et al. 2013).

Des avancées conséquentes dans l'identification des facteurs de transcription requis pour leur développement ont montré que, comme les cellules B et T, les ILCs se développent à partir d'un progéniteur commun lymphoïde (CLP) et des facteurs de transcription spécifiques dirigent la génération des différents sous-types d'ILCs. Contrairement aux cellules NK conventionnelles, les ILC1, ILC2 et ILC3 expriment l'IL-7Rα et dépendent de GATA-3 (Yagi et al. 2014). Les cellules NK conventionnelles qui nous intéressent tout particulièrement dans nos axes de recherche, sont indépendantes des facteurs T-bet et GATA-3, mais dépendantes du facteur Eomes, et ne semblent remplir donc que partiellement les critères du groupe ILC1 dont l'appartenance reste aujourd'hui débattue.

Un progéniteur commun aux ILC dites Helper-Like ou CHILP a récemment été identifié, exprimant le régulateur transcriptionnel inhibiteur de liaison à l'ADN Id2 (Hoyler et al. 2012; Diefenbach et al. 2014). La différenciation de CHILP induit chaque membre des trois groupes d'ILCs, à l'exception des cellules NK ce qui suggère leur différenciation à partir d'un progéniteur distinct dit NKP (Klose et al. 2014). Bien que toutes les ILCs semblent provenir d'une population exprimant le facteur de transcription *promyelocytic leukaemia zinc-finger* ($PLZF^+$), les cellules LTi dérivent d'un précurseur indépendant $PLZF^-$ (Constantinides et al.

2014; Constantinides et al. 2015). Enfin, le facteur NFIL3 a été identifié comme régulateur critique du progéniteur CHILP, requis pour la différenciation des ILCs et des cellules NK (Seillet et al. 2014) et exerce sa fonction avant l'engagement des sous populations ILC (Xu et al. 2015). Dans l'ensemble, ces données corroborent l'existence d'un progéniteur commun aux cellules NK et au progéniteur des ILCs, Id2^{-} NFIL3^{+}, nommé CILP. La figure 4 propose une représentation non exhaustive des stades de développement des ILCs.

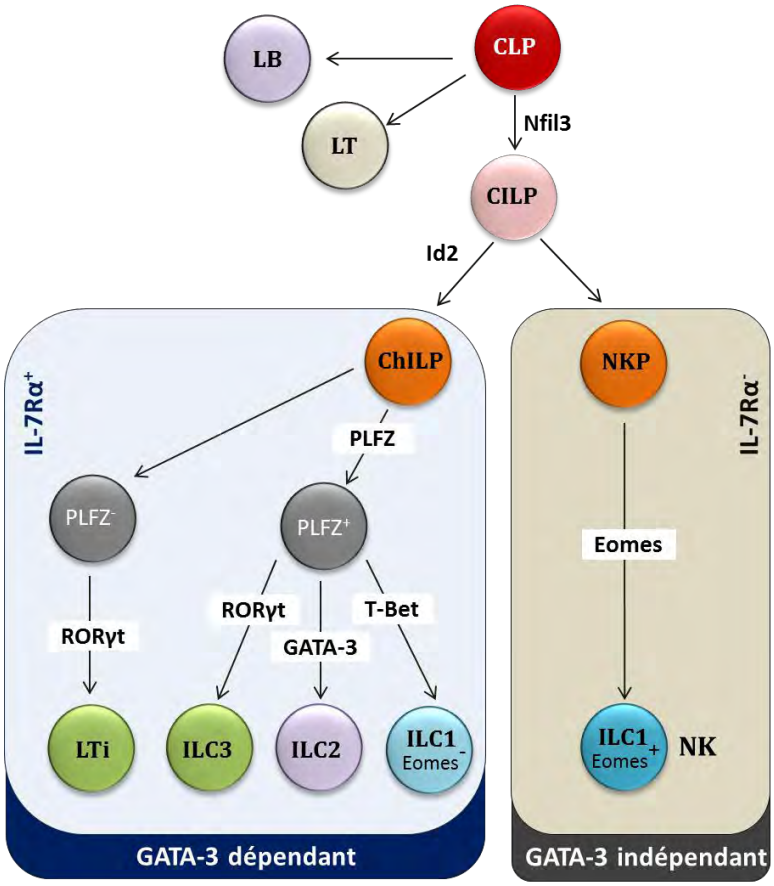

Figure 4 : Cellules Lymphoïdes Innées (ILC)
Le développement des ILCs à partir d'un progéniteur lymphoïde commun (CLP) requiert une suppression du signal de devenir des cellules lymphoïdes alternatives, médié par le facteur Id2. Des précurseurs distincts engendrent les ILCs GATA-3-dépendantes, exprimant l'IL-7Rα, et les ILCs GATA-3-indépendantes. Le Progéniteur Commun aux ILC dites Helper-Like (CHILP) induit la différenciation de chaque membre des groupes d'ILCs, à l'exception des cellules NK. CHILP est régulé par un précurseur dépendant du Nfil3. Le facteur de transcription PLZF divise ensuite la progéniture de CHILP en ILC1, ILC2 et ILC3 PLZF-dépendantes, et en cellules inductrices de tissu lymphoïdes (LTi) PLZF-indépendantes. Les ILC1, ILC2 et ILC3 dépendent respectivement des facteurs de transcription T-bet, GATA-3 et RORγt. Les cellules NK partagent des caractéristiques des ILC1 mais sont GATA-3-indépendantes et dépendent du facteur de transcription Eomes.

Il demeure une réelle complexité des sous populations d'ILC et une meilleure compréhension de leurs fonctions et de leurs modes d'action est essentielle pour identifier leur implication dans les immunités innée et adaptative et leur rôle dans les pathologies. Les ILCs pourraient ainsi être ciblées pour activer ou bloquer les réponses immunitaires précocement dans le cadre de la vaccination, l'immunothérapie et les maladies inflammatoires.

II. Les Cellules Natural Killer

En 1975, Rolf Kiessling et ses collaborateurs ont réalisé des expériences chez des souris BALB/c immunodéficientes et pouvant facilement développer des tumeurs, afin d'évaluer les capacités des lymphocytes T à tuer des cellules leucémiques Moloney contre lesquelles les souris avaient été préalablement immunisées. Ces expériences mirent en évidence une nouvelle population de cellules, présentes chez les souris saines, et capables de lyser ces cellules tumorales sans immunisation préalable, contrairement aux lymphocytes T. C'est ainsi que furent découvertes les cellules dites naturellement tueuses, ainsi nommées Natural Killer (NK) (Kiessling et al. 1975). Plus tard, Joe Phillips et Lewis Lanier ont caractérisé cette population lymphocytaire qui ne présente pas de réarrangement génétique de ses récepteurs de surface et ne reconnait pas les cibles via un récepteur CD3. Ces cellules, reconnues comme une population distincte des lymphocytes T, sont capables de tuer des cellules tumorales sans restriction par les molécules CMH et sans immunisation préalable (Phillips and Lanier 1986).

Dans cette partie, nous allons présenter les caractéristiques principales des cellules NK, les récepteurs qui modulent leur activation, les étapes de leur développement, les modèles d'éducation, présenter leurs fonctionnalités et leur implication dans les infections virales, les maladies auto-immunes et les cancers.

1. Caractéristiques générales

Constituant 5 à 20% des lymphocytes du sang périphérique, les cellules Natural Killer sont de larges lymphocytes granuleux phénotypiquement définies par l'expression du marqueur CD56 et l'absence d'expression du marqueur CD3 (Robertson and Ritz 1990). Le récepteur NKp46 a été identifié plus tard à la surface de cellules NK au repos ou activées, et impliqué dans l'activation de la lyse des cellules cibles (Sivori et al. 1997; Pessino et al. 1998). Les cellules NK sont des composants de l'immunité innée ayant un rôle majeur dans la défense de l'hôte contre les pathogènes, dans l'immunité anti-virale et anti-tumorale comme nous le décrirons dans les prochains paragraphes (Vivier et al. 2011). Elles expriment un grand nombre de récepteurs activateurs et inhibiteurs et la balance des signaux transmis par ces récepteurs régulent leur activation.

1.1. Deux sous-populations NK : $CD56^{bright}$ et $CD56^{dim}$

Deux populations distinctes basées sur la densité de l'expression du récepteur CD56 ont été identifiées. Approximativement 90% des cellules NK humaines expriment faiblement le récepteur CD56 ainsi que le récepteur CD16, dites $CD56^{dim}CD16^{+}$, tandis qu'une minorité de cellules NK (10%) expriment fortement le récepteur CD56, et peu ou pas de récepteur CD16, dites $CD56^{bright}$, $CD16^{low/neg}$. Ces populations présentent d'importantes différences de potentiel cytotoxique et de capacité de production de cytokines. Elles constituent des stades différents de développement comme nous le décrirons ultérieurement. Les cellules NK $CD56^{bright}$ sont plus abondantes dans les tissus lymphoïdes secondaires comme les nœuds lymphoïdes et les amygdales. Elles présentent de fortes capacités de prolifération, de production de cytokines (TNFβ, IFNγ, IL-10) mais une faible cytotoxicité naturelle exprimant peu ou pas de FcγRIII. Elles expriment des récepteurs aux chimiokines CCR7, CCR5 qui

permet leur recrutement au niveau des tissus inflammés et des sites tumoraux (Stojanovic and Cerwenka 2011). Elles sécrètent également les facteurs de croissance GM-CSF et G-SCF (Smyth et al. 2002) assurant le « homing » ou retour vers les organes lymphoïdes secondaires. Elles s'accumulent très tôt dans le sang après greffes de cellules souches de moelle osseuse ou de sang périphérique (Dulphy et al. 2008). Des cellules NK $CD56^{bright}$ purifiées peuvent se développer en cellules NK $CD56^{dim}$ *in vitro* et dans des systèmes *in vivo* indiquant qu'elles constituent les précurseurs directs des sous-populations $CD56^{dim}$ (Chan et al. 2007). Au contraire, les cellules NK $CD56^{dim}$ constituent la population majoritaire dans le sang. Elles présentent une forte cytotoxicité naturelle mais ne produisent que faiblement des cytokines (Cooper et al. 2001). Elles sont caractérisées par l'expression du récepteur CD16 et des récepteurs KIR qui seront décrits dans la partie dédiée «2. Les récepteurs des cellules NK». Plus récemment, l'étude de la densité d'expression du récepteur CD94 a permis d'identifier un intermédiaire entre les sous-populations NK $CD56^{bright}$ et $CD56^{dim}$. Cette étude décrit d'une part les sous-populations $CD94^{high}/CD56^{bright}$ présentant la plus faible expression de récepteurs CD16 et de KIR, et d'autre part les sous-populations $CD94^{high}/CD56^{dim}$, plus cytotoxiques que les sous-populations $CD94^{high}/CD56^{bright}$ mais exprimant faiblement les granzymes. Enfin, les sous-populations $CD94^{low}/CD56^{dim}$ présentent une forte cytotoxicité naturelle et une plus forte expression de perforine et de granzymes (Yu et al. 2010).

Les caractéristiques des deux sous-populations NK $CD56^{bright}$ et $CD56^{dim}$ sont résumées dans la Figure 5.

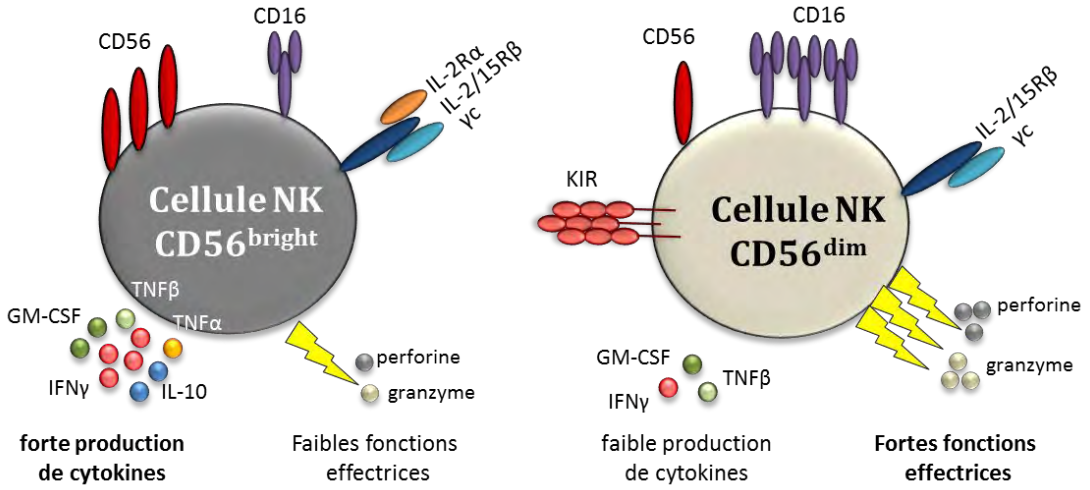

Figure 5 : Les sous-populations NK CD56^{bright} et CD56^{dim}
Deux populations NK se distinguent par la densité d'expression du marqueur CD56. Les NK CD56^{bright} produisent beaucoup de cytokines (TNFα/β, IFNγ, IL-10) mais sont peu cytotoxiques. Les CD56^{dim} expriment fortement le récepteur CD16 et les récepteurs KIR, sont très cytotoxiques mais produisent peu de cytokines. (Inspiré de Cooper Blood 2001)

Les cellules NK peuvent ainsi s'activer et induire la lyse de cellules cibles dans différents contextes que nous allons décrire dans les paragraphes suivants comme illustré par la figure 6.

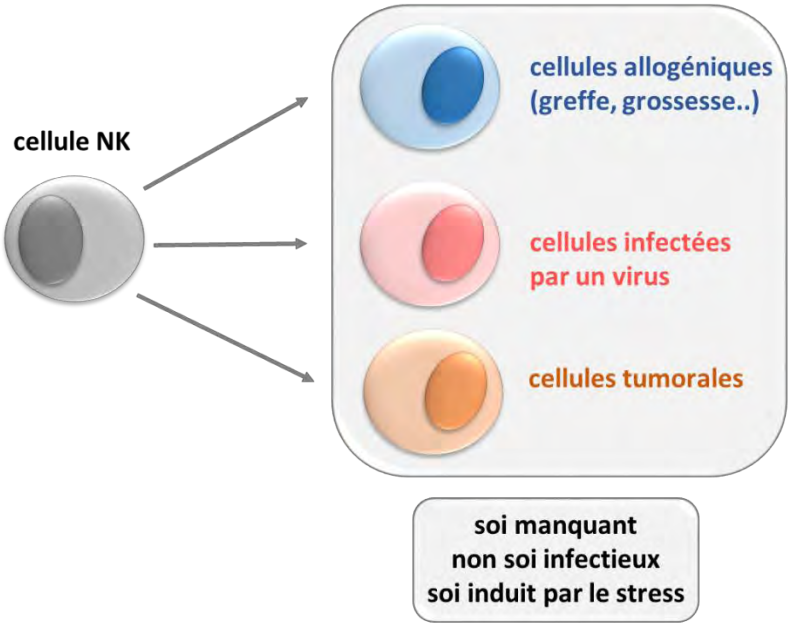

Figure 6 : Rôle des cellules Natural Killer
Les cellules Natural Killer sont capables de reconnaître le soi-manquant, le non-soi infectieux et le soi induit par le stress comme dans un contexte allogénique (greffe, grossesse), tumoral ou infectieux (inspiré de Vivier Immunol Letters 2006)

1.2. Lyse spontanée

a) Reconnaissance du soi manquant

A la fin des années 1980, Klas Kärre a observé que les cellules NK tuaient préférentiellement des cellules tumorales présentant un défaut d'expression en molécules CMH de classe I. Des expériences *in vivo* de transfection chez des souris C57BL ont en effet montré que seules les souris inoculées avec des tumeurs (RMA) exprimant des molécules CMH de classe I développent des tumeurs, contrairement aux souris inoculées avec des lignées CMH de classe I déficientes (RMA-S) (Kärre et al. 1986). Après déplétion des cellules NK, cette différence est abolie, les deux groupes de souris développant une tumeur. Les auteurs ont également observé que les cellules exprimant les molécules CMH de classe I étaient résistantes à la lyse par les cellules NK. Le postulat émis par Klaas Kärre en 1986 était que les cellules NK sont capables de reconnaître l'absence du soi au niveau des cellules présentant un défaut d'expression des molécules CMH de classe I comme les cellules tumorales. C'est la naissance de la théorie du « missing self » ou reconnaissance du soi manquant. A l'époque, cette théorie très controversée allait à l'encontre du dogme considérant que les cellules NK fonctionnaient comme les lymphocytes T, reconnaissant la présence d'antigènes étrangers. On notera que les auteurs ont également mis en évidence que le rejet du soi manquant se produit dans tous les organes testés exceptés dans le cerveau, un organe où l'expression des molécules CMH de classe I est maintenue à un niveau très bas (Ljunggren and Kärre 1985). Les cellules NK sont ainsi responsables de la détection du soi manquant du à une diminution de l'expression des molécules HLA de classe I qui peut être observée dans le contexte des infections virales, le contexte tumoral et le contexte allogénique comme la greffe de cellules souches ou la grossesse. La détection du soi manquant induit l'activation des cellules NK, dites alloréactives, médiée par des récepteurs spécifiques qui seront décrits ultérieurement.

Par la suite, la découverte des récepteurs spécifiques des molécules CMH de classe I (les récepteurs KIR et LY49 chez l'homme et la souris respectivement) a corroboré cette hypothèse (Long et al. 2001). Différents récepteurs sont impliqués dans la reconnaissance des molécules HLA de classe I du soi. Les récepteurs KIR reconnaissent en particulier les molécules HLA-B et HLA-C, le récepteur ILT2 reconnait l'ensemble des molécules HLA de classe I et les hétérodimères CD94/ NKG2A et CD94/NKG2C reconnaissent la molécule HLA-E qui seront présentés dans le paragraphe 2.

Cependant, la détection de l'absence des molécules HLA du soi n'est pas suffisante pour induire la lyse par les cellules NK. Un signal positif est également nécessaire, via l'engagement de récepteurs activateurs NK par des ligands exprimés à la surface de la cellule cible. Ainsi, l'activation de la cytotoxicité naturelle des cellules NK est gouvernée par l'intégration des signaux activateurs et inhibiteurs médiés par une grande variété de récepteurs de surface.

b) Reconnaissance du non-soi infectieux

Dans le contexte des infections virales, certains ligands infectieux peuvent mimer les molécules HLA de classe I et être reconnus par les cellules NK. La première démonstration de la reconnaissance d'une molécule virale par un récepteur NK activateur a été faite d'une part dans le cadre de l'infection à cytomégalovirus (CMV), plus particulièrement avec l'identification de la molécule virale m157 comme ligand du récepteur Ly49H chez la souris (Arase et al. 2002). D'autre part, le peptide de la molécule UL40 du CMV peut se lier à la molécule HLA-E assurant son expression à la surface cellulaire et son interaction avec le récepteur NKG2A (Tomasec et al. 2000). Enfin, la protéine UL18 du CMV, homologue des molécules HLA de classe I, fixe la β2microglobuline et interagit avec le récepteur ILT2 (Chapman et al. 1999).

c) Reconnaissance du soi induit par le stress

En plus de leurs capacités à reconnaître l'absence du soi, les cellules NK expriment des récepteurs capables de reconnaître des molécules ayant été induites suite à un stress comme une infection ou une agression du tissu. Les récepteurs des cellules NK peuvent détecter ces modifications ce qui amène le concept de reconnaissance du soi induit par le stress (Bauer et al. 1999). Ce mode de détection est basé sur la reconnaissance de molécules dont l'expression est à peine détectable dans des conditions physiologiques mais qui sont induites après une infection virale, infection bactérienne ou dommage tissulaire. L'expression de molécules comme MIC-A et MIC-B ou les ULBP peut ainsi être induite dans ces conditions (Gasser et al. 2005; Raulet and Guerra 2009), et sont reconnues par le récepteur activateur NKG2D induisant l'activation des cellules NK, ce qui sera décrit dans la partie dédiée aux différents récepteurs NK.

1.3. Cytotoxicité Cellulaire Dépendante de l'Anticorps

Les cellules NK sont capables de reconnaître et tuer les cellules cibles recouvertes d'anticorps (dites opsonisées) par cytotoxicité dépendante de l'anticorps ou ADCC (Antibody Dependent Cellular Cytotoxicity). Les cellules NK expriment le récepteur CD16 (ou FcRIIIA) qui est un récepteur de faible affinité pour le fragment Fc de certaines immunoglobulines G (IgG). Son association avec les protéines adaptatrices CD3ζ ou FcεRIγ assure la transduction du signal (Lanier et al. 1989; Lanier et al. 1991). L'ADCC est régulée par les récepteurs inhibiteurs Fc exprimés par les cellules présentatrices d'antigène, l'absence de ces récepteurs inhibiteurs améliorant l'efficacité de l'ADCC *in vivo* (Clynes et al. 2000). Des études ont mis en évidence le rôle protecteur de l'ADCC dans le contrôle de l'infection du Virus de l'Immunodéficience Humaine (VIH) de type 1. Il a été reporté que l'ADCC médiée par les cellules NK était sévèrement compromis dans les infections chroniques liées au VIH comparé

à des sujets sains (Liu et al. 2009b). Des anticorps monoclonaux dirigés contre le récepteur CD16 peuvent inhiber ou induire des réponses cellulaires et font l'objet de nombreuses immunothérapies que nous présenterons dans la partie « 7.2. Les immunothérapies NK ». Un lien entre la lyse spontanée et l'ADCC a été suggéré par une étude de patients atteints de neuroblastome après immunothérapies induisant l'ADCC. La reconnaissance des molécules HLA de classe I par les récepteurs inhibiteurs induit l'inhibition des cellules NK dites éduquées, permettant aux cellules NK dites non éduquées d'assurer l'ADCC (Tarek et al. 2012). La notion d'éducation sera développée ultérieurement, dans le paragraphe dédié « 5. Education ». La figure 7 illustre la modulation de l'activation des cellules NK par les récepteurs activateurs et inhibiteurs dans les différents contextes présentés.

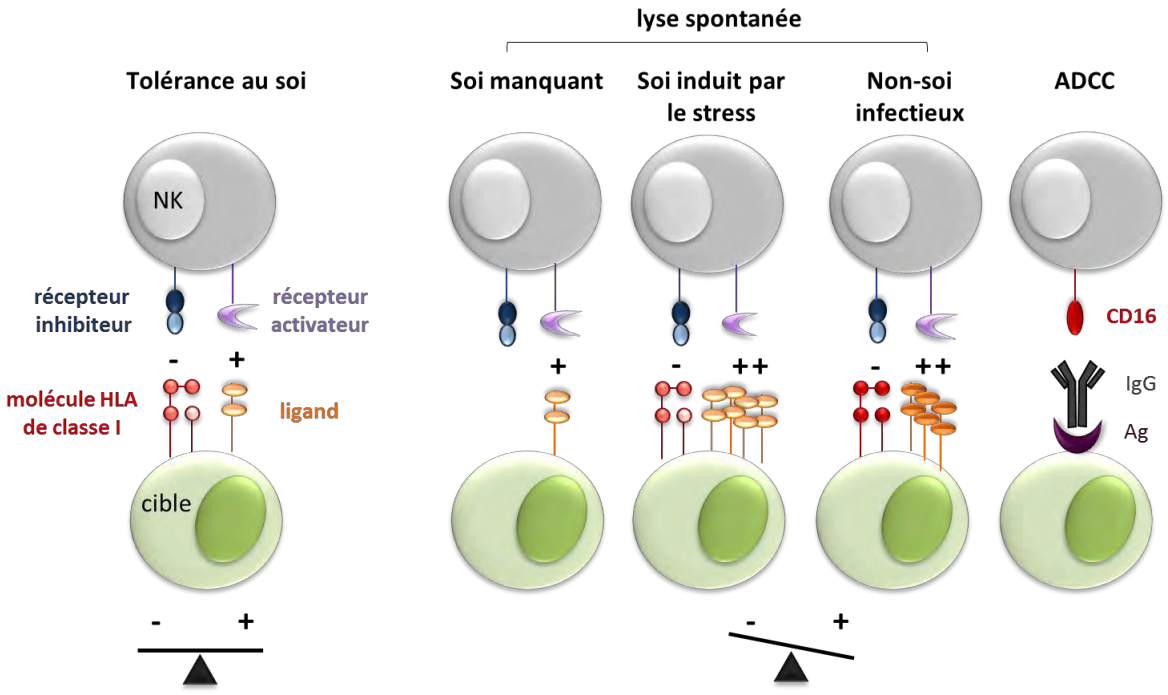

Figure 7 : Activation des cellules NK régulée par des signaux inhibiteurs et activateurs
La tolérance au soi est assurée par un équilibre entre les signaux inhibiteurs et activateurs. En absence d'expression de molécules HLA de classe I du soi, ou « soi manquant » le non-engagement des récepteurs inhibiteurs induit l'activation de la cellule NK et la lyse spontanée de la cellule cible. Suite à un stress cellulaire, certains ligands des récepteurs activateurs sont induits conduisant à la lyse spontanée. Dans le cas d'une infection virale, l'expression de protéines virales reconnues par les récepteurs activateurs peut être induite ce qui conduit à la lyse spontanée. L'ADCC est observée suite à l'engagement du récepteur CD16 avec le fragment Fc d'une IgG qui induit l'activation de la cellule NK et la lyse cellulaire.

2. Les récepteurs des cellules NK

Les fonctions effectrices des cellules NK sont régulées par un ensemble de récepteurs activateurs et inhibiteurs parmi lesquels les récepteurs de type Immunoglobuline (KIR, ILT/LIR), les récepteurs hétérodimères de type lectine CD94/NKG2 (A, -B, -C et E), l'homodimère NKG2D, les récepteurs de cytotoxicité naturelle (NKp46, NKp44, NKp30), ainsi que les récepteurs DNAM-1, 2B4 et CD161. Les cellules NK expriment différentes combinaison de récepteurs ce qui induit une grande variété de spécificités. Dans ce paragraphe, nous allons décrire de façon non-exhaustive les propriétés des principaux récepteurs comme illustré dans la figure 8.

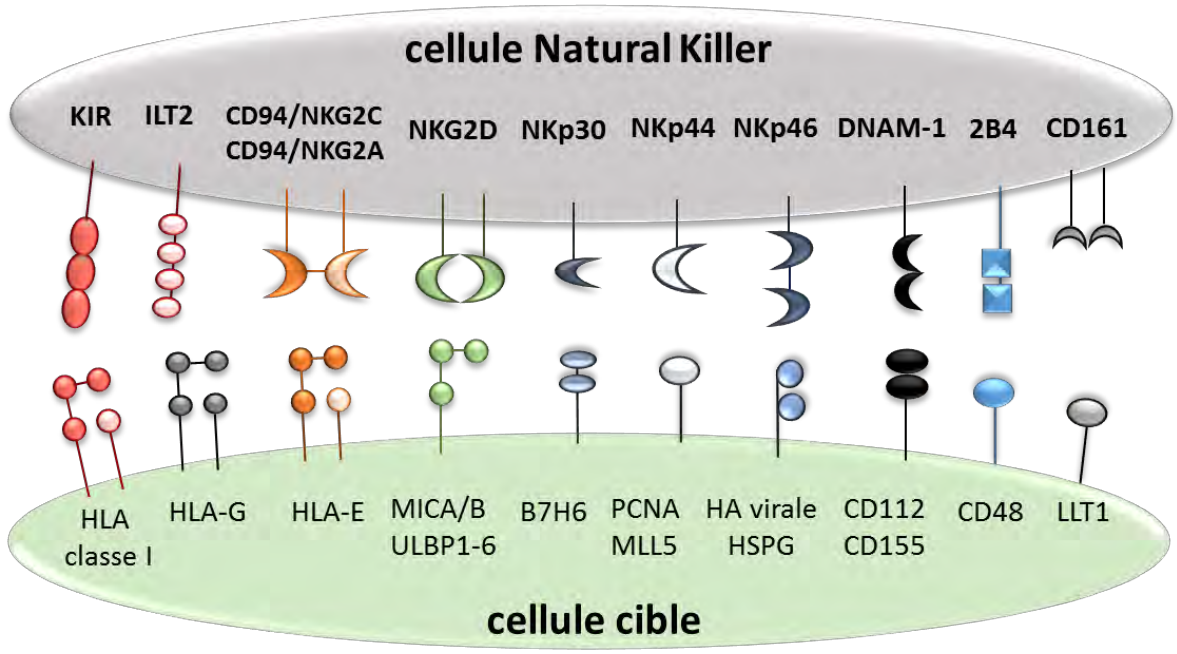

Figure 8 : Récepteurs exprimés à la surface des cellules Natural Killer
L'activation des cellules NK est régulée par un ensemble de récepteurs activateurs et inhibiteurs présentant des spécificités définies. Les récepteurs KIR et ILT2 reconnaissent les molécules HLA de classe I, les hétérodimères CD4/NKG2A et CD94/NKG2C se lient à la molécule HLA-E. Les récepteurs NKG2D, NB4 et DNAM-1 reconnaissent respectivement les molécules MIC/ULBP, CD48 et CD112/CD155. Les récepteurs de cytotoxicité naturelle NKp30, NKp44 et NKp46 se lient respectivement aux molécules B7H6, PCNA/MLL5 et HSPG. Enfin, le récepteur CD161 reconnait la molécule LLT1. (Inspiré de Chan Cell Death and Differenciation 2014).

2.1. Les récepteurs Killer Cell Immunoglobulin-like Receptors (KIRs)

Les récepteurs KIR sont principalement exprimés à la surface des cellules NK (Moretta et al. 1990) et de certaines populations de lymphocytes T (Phillips et al. 1995) chez l'homme, mais également chez les primates (Sambrook et al. 2005). Chez la souris, les homologues des récepteurs KIR sont les récepteurs Ly49 comprenant des récepteurs activateurs (Ly49D et H) (Mason et al. 1997) et inhibiteurs (Ly49A, C, E, G et I) (Mason et al. 1996; Nakamura et al. 1999). Nous allons dans cette partie décrire les caractéristiques de ces récepteurs au niveau protéique et génétique.

a) Récepteurs KIR – structure protéique et nomenclature

Les KIR sont des protéines de la superfamille des Immunoglobulines comme les récepteurs des cellules T (TCR), les molécules HLA, les molécules CD4, CD3 et CD8. Les récepteurs KIR sont constitués de 306 à 406 acides aminés et nommés en fonction de leur structure (Marsh et al. 2003). Les récepteurs KIR peuvent présenter 2 ou 3 domaines extra-cytoplasmiques Immunoglobulin-like (Ig) (KIR2D et KIR3D). Les KIR2D sont composés des domaines extracellulaires D1 et D2 alors que les KIR3D sont composés des domaines D0, D1 et D2. Les récepteurs KIR sont constitués d'un domaine intra-cytoplasmique qui peut être long pour les KIR inhibiteurs (KIR2DL/3DL, L pour Long), ou court pour les KIR activateurs (KIR2DS/3DS, S pour short). Les récepteurs activateurs présentent des résidus chargés dans leur domaine transmembranaire permettant l'association à la protéine adaptatrice DAP12 qui possède des motifs ITAM (Immunoreceptor Tyrosine-Based Activation Motif), entraînant la phosphorylation de tyrosines, et ainsi le recrutement des tyrosines kinases Syk et ZAP-70 (Lanier et al. 1998a; McVicar et al. 1998). Au contraire, les récepteurs inhibiteurs présentent un ou deux motifs ITIM (Immunoreceptor Tyrosine-Based Inhibition Motifs). Ces motifs contiennent des tyrosines qui seront phosphorylées après engagement avec le récepteur,

conduisant au recrutement des phosphatases SHP-1 et SHP-2 et à l'inhibition des signaux transmis par les récepteurs activateurs (Plas et al. 1996; Binstadt et al. 1996). Notons toutefois que le récepteur KIR2DL4 se distingue des autres KIR car il ne contient pas les domaines D1 et D2 mais un arrangement D0-D2. De plus, il est constitué d'un long domaine intra-cytoplasmique présentant un motif ITIM mais peut également s'associer à l'adaptateur FcεRIγ possédant lui un motif ITAM. Ceci confère au KIR2DL4 la possibilité de transduire un signal inhibiteur ou activateur. (Kikuchi-Maki et al. 2005). La structure des récepteurs KIR est résumée dans la Figure 9.

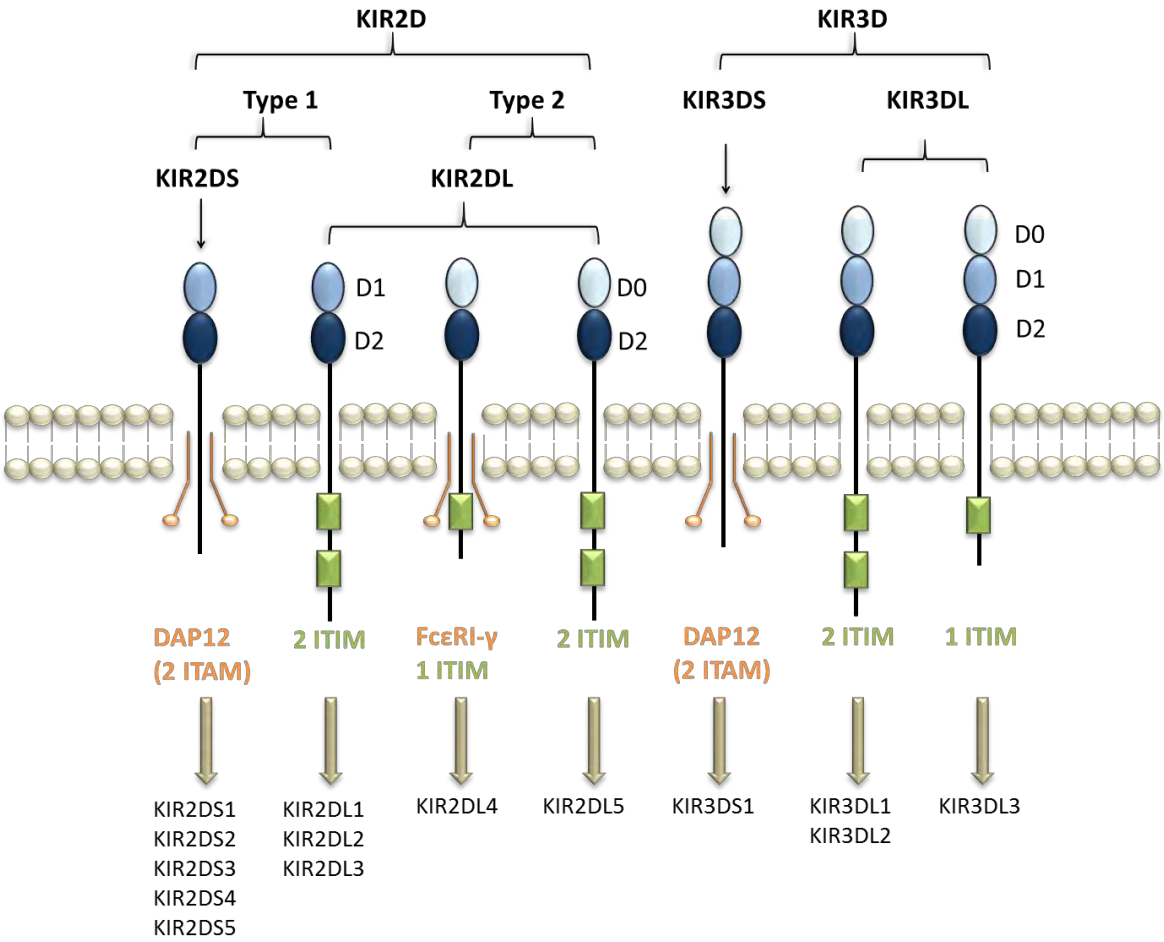

Figure 9 : Structure des récepteurs KIR
Les récepteurs KIR sont des protéines constituées de deux ou trois domaines extracellulaires Ig-like (KIR2D ou KIR3D), pouvant présenter une partie intracytoplasmique courte (KIR2DS, KIR3DS) ou longue (KIR2DL, KIR3DL). Les récepteurs de type 1 possèdent les domaines D1 et D2 tandis que les récepteurs de type 2 possèdent les domaines D0 et D2. Les récepteurs KIR3D possèdent 3 domaines D0, D1 et D2. Les récepteurs KIR2DS, KIR3DS et KIR2DL4 s'associent à des protéines adaptatrices qui assurent un signal activateur. Les KIR2DL et 3DL présentent un ou deux motifs ITIM dans leur partie intracytoplasmique induisant l'inhibition des signaux activateurs (inspiré de la base de données IPD-KIR)

b) Ligands des récepteurs KIR

Les molécules HLA de classe I sont les ligands des récepteurs KIR (Wagtmann et al. 1995). En particulier, les molécules HLA-Cw sont divisées en deux groupes, C1 et C2, selon la nature de l'acide aminé présent en position 80 (Mandelboim et al. 1996). Les récepteurs KIR2DL2/KIR2DL3 reconnaissent les molécules HLA-Cw du groupe C1 présentant une asparagine en position 80 (C1 : codés par les allèles HLA-C*01, C*03, -C*07, -C*08, -C*12, -C*13, -C*14 et –C*16). De plus, le récepteur KIR2DL1 est spécifique des molécules du HLA-Cw groupe C2, présentant une lysine en position 80 (C2 : codés par les allèles HLA-C*02, -C*04, -C*05, -C*06, -C*15, -C*16 :02, -C*17, et -C*18) (Colonna et al. 1993; Wagtmann et al. 1995). L'interaction du récepteur KIR2DL1 avec les molécules HLA-C2 d'une part et du récepteur KIR2DL2 avec les molécules HLA-C1 d'autre part semblent être plus fortes que l'interaction du récepteur KIR2DL3 avec les molécules HLA-C1 (Winter et al. 1998). L'affinité de ces récepteurs KIR2D peut varier d'un individu à l'autre et s'observe à deux niveaux. D'une part, l'affinité d'un récepteur pour le ligand peut varier selon la nature de l'allèle. Par exemple, le récepteur codé par l'allèle KIR2DL1*010 a une plus forte capacité d'inhibition d'une cible exprimant le ligand C2 comparé au récepteur codé par l'allèle KIR2DL1*004 (Bari et al. 2009). D'autre part, un même récepteur KIR2D peut avoir des affinités variables selon différentes molécules d'un même ligand. Les récepteurs KIR2DL2 et KIR2DL3 peuvent également reconnaître certaines molécules du groupe C2 comme les molécules codées par les allèles HLA-C*05:01 et HLA-C02:02 (Moesta et al. 2008). Ainsi, notre équipe a montré que les molécules codées par les allèles HLA-C*04 (groupe C2) sont mieux reconnues par les récepteurs KIR2DL2/KIR2DL3 que certains ligands du groupe C1 (David et al. 2013). L'observation de la diminution de la fréquence des cellules NK KIR2DL1^{+} chez les individus KIR2DL2^{+} est notamment dû au fait que ces deux récepteurs partagent des spécificités (Schönberg et al. 2011b).

Le récepteur KIR3DL1 reconnaît l'épitope HLA-Bw4 (acides aminés 80-83) (Cella et al. 1994) porté par certaines molécules HLA-A (A23, A24, A32) (Thananchai et al. 2007; Stern et al. 2008) et de nombreuses molécules HLA-B (B27, B37, B38, B44, B47, B49, B51, B52, B53, B57, B58, B59, B63, B77) avec une affinité variable selon la nature du ligand (Gumperz et al. 1995; Pende et al. 1996; Foley et al. 2008). Enfin, le récepteur KIR3DL2 reconnaît les molécules HLA-A3 et -A11 (Döhring et al. 1996).

Les anticorps spécifiques des récepteurs KIR reconnaissent aussi bien les formes inhibitrices que les formes activatrices en raison d'une homologie de séquence pour les parties extracellulaires. Cet aspect explique que la spécificité des récepteurs KIR activateurs est moins bien connue que celle des récepteurs KIR inhibiteurs. Néanmoins, l'obtention de certains anticorps permettant de différencier les deux formes et la possibilité de trier des cellules NK KIR^+ au niveau clonal a permis de mieux les caractériser. Le récepteur KIR2DS1 reconnaît notamment les molécules HLA-Cw du groupe C2 comme son homologue inhibiteur KIR2DL1 (Stewart et al. 2005; Chewning et al. 2007), et notre équipe a également confirmé ces résultats (Morvan et al. 2008). Bien que la spécificité du récepteur KIR2DS2 ne soit pas bien connue, notre équipe a mis en évidence d'une part que les clones NK $KIR2DS2^+$ sont réactifs vis-à-vis de lignées 221 exprimant les molécules HLA du groupe C1 et d'autre part le rôle synergique des récepteurs KIR2DS2 et KIR2DL1 vis-à-vis de cibles C1C1 (David et al. 2013). Le récepteur KIR2DS4 reconnaît certaines molécules HLA-C comme HLA-Cw4 avec une faible affinité (Katz et al. 2001) et la molécule HLA-A11 (Graef et al. 2009). En effet, ce récepteur serait le produit d'une conversion génique du récepteur KIR3DL2 (Graef et al. 2009). Il a été récemment montré que l'interaction du KIR2DS4 avec le ligand HLA-C*04 induit l'expression du récepteur CCR7, impliqué dans la migration des cellules NK vers les nœuds lymphoïdes (Pesce et al. 2015). Le récepteur KIR2DS3 n'est quant à lui pas exprimé à la surface des cellules NK et son rôle fonctionnel reste à ce jour

indéterminé (VandenBussche et al. 2009). Bien que l'interaction entre le récepteur KIR3DS1 et les molécules HLA-Bw4 ne puisse pas directement être mesurée dans des conditions physiologiques, notre équipe a mis en évidence une plus forte fréquence des cellules NK KIR3DS1^{+} chez les individus Bw4^{+} comparé aux individus Bw4 sans toutefois avoir démontré une interaction fonctionnelle du KIR3DS1 avec les ligands Bw4 (Morvan et al. 2009). Différentes études génétiques ont mis en évidence une corrélation entre la présence du gène KIR3DS1 et le motif Bw4 I80 dans le contrôle de certaines infections virales (Martin et al. 2002a; Boulet et al. 2008; Guerini et al. 2011). Une étude récente a montré que le KIR3DS1 peut interagir avec certaines molécules HLA-Bw4 dans le contexte de l'infection au virus de l'immunodéficience humaine (VIH) (O'Connor et al. 2015). Ces résultats suggèrent une activation peptide-spécifique du KIR3DS1 pouvant se produire lorsque les épitopes présentés par la cible sont altérés comme dans des contextes d'infection, de stress ou de transformation. Bien que ces données suggèrent une spécificité du KIR3DS1 pour les molécules Bw4 dans un contexte particulier, cette interaction n'a toutefois pas été clairement démontrée dans des conditions physiologiques. Les spécificités des récepteurs KIR inhibiteurs et activateurs sont illustrées dans la figure 10.

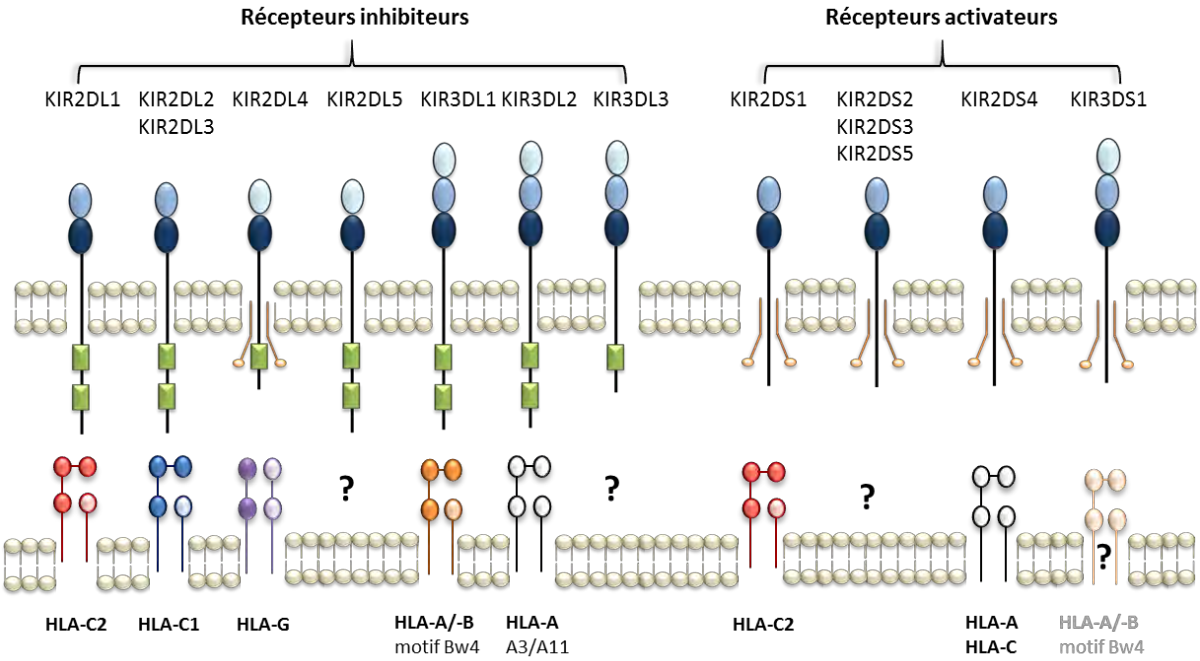

Figure 10 : Les récepteurs KIR et leurs ligands
Les récepteurs KIR2DL1 et KIR2DS1 reconnaissent les molécules HLA-C du groupe C2. Les récepteurs KIR2DL2 et KIR2DL3 reconnaissent les molécules HLA-C du groupe C1. Le récepteur KIR2DL4 reconnait la molécule HLA-G. Le récepteur KIR3DL1 reconnait le motif Bw4 présent chez certaines molécules HLA-A et HLA-B. Les récepteurs KIR3DL2 et KIR2DS4 reconnaissent les molécules HLA-A11. Le KIR3DL2 reconnait également la molécule HLA-A3. Les spécificités des récepteurs KIR2DL5, KIR3DL3, KIR2DS2, KIR2DS3, KIR2DS5 et KIR3DS1 restent à déterminer.

Il a été mis en évidence que la nature du peptide présenté par les molécules HLA de classe I impacte l'affinité de l'interaction KIR/HLA dans des conditions physiologiques (Cassidy et al. 2014) ou suite à une infection virale (Colantonio et al. 2011; Fadda et al. 2012; van Teijlingen et al. 2014). En particulier, les capacités de liaison des récepteurs KIR2DL2 et KIR2DL3 aux molécules HLA-Cw dépendent de la nature du peptide présenté. Trois classes de peptides ont été identifiées selon la force de l'interaction du complexe HLA-peptide avec le récepteur KIR (forte, faible ou nulle) et qui est également corrélée avec leur potentiel d'inhibition. Un peptide dit antagoniste présenté par la molécule HLA de classe I peut recruter un KIR inhibiteur au niveau de la zone de contact avec la cible mais n'induit pas de signal inhibiteur, régulant ainsi les signaux inhibiteurs (Fadda et al. 2010; Borhis et al. 2013). Récemment, Salim Khakoo et ses collaborateurs ont également montré que les

cellules NK KIR2DL3^{+} sont plus sensibles au changement du contenu en peptide que les cellules NK KIR2DL2^{+} (Cassidy et al. 2015).

c) Les gènes KIR

Les gènes KIR sont localisés au niveau du chromosome 19q13.4 humain (Trowsdale 2001). A ce jour, 16 gènes KIR ont été identifiés (Middleton and Gonzelez 2010). Dans cette région nommée LRC (Leucocyte Receptor Complex), on retrouve 8 gènes codant les récepteurs KIR inhibiteurs (2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 3DL1, 3DL2, 3DL3), 6 gènes codant les récepteurs KIR activateurs (2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DS1) et 2 pseudogènes (2DP1, 3DP1) (Uhrberg et al. 1997a). Les gènes KIR ont une taille variable et peuvent présenter jusqu'à 9 exons comme le récepteur KIR3DL1 illustré dans la figure 11.

Au sein d'une population, la diversité génotypique des gènes KIR s'effectue à différents niveaux. D'une part, le nombre et la nature des gènes KIR varient entre individus à l'exception de 3 gènes KIR qui sont présents chez tous les individus (KIR2DL4, KIR3DL2 et KIR3DL3) (Single et al. 2008). Selon la nature des gènes KIR présents, les individus peuvent être définis en différents haplotypes KIR, les haplotypes A et B (Uhrberg et al. 1997). Les gènes de structure dit « framework » KIR3DL3, KIR3DL2 et KIR2DL4 sont présents dans chaque haplotype. Les individus d'haplotype KIR A ne possèdent qu'un seul gène KIR activateur (KIR2DS4) et 4 gènes KIR inhibiteurs (KIR2DL1, KIR2DL3, KIR3DL1, KIR3DL2). Les individus d'haplotype KIR B possèdent un nombre variable de gènes KIR (de 7 à 14) avec un ou plusieurs gènes KIR activateurs (KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5, KIR3DS1) et des gènes KIR inhibiteurs spécifiques (KIR2DL2, KIR2DL5) qui sont propres à cet haplotype, comme illustré dans la Figure 11.

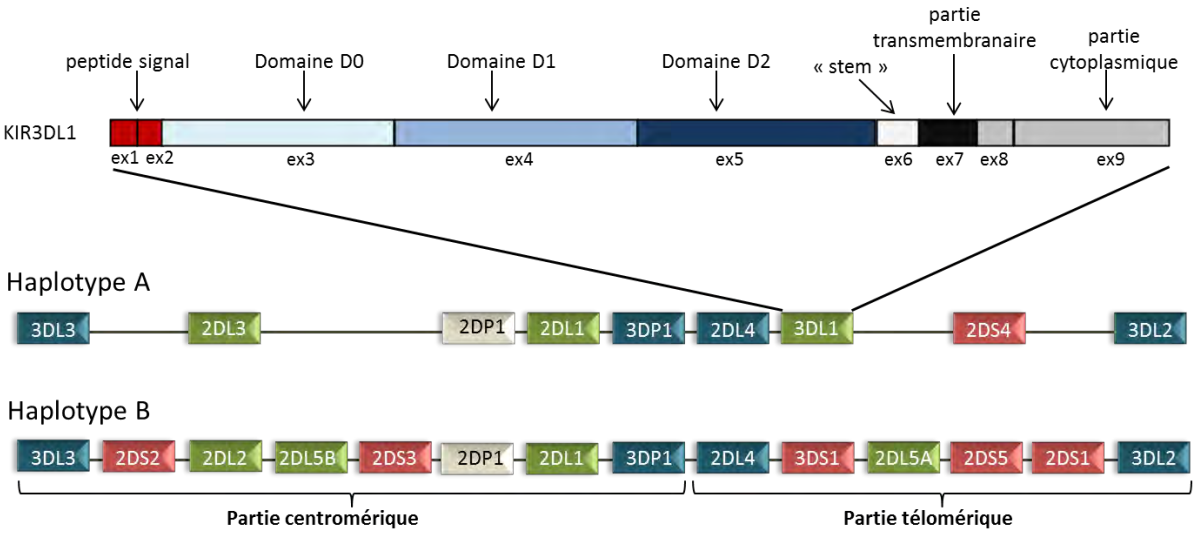

Figure 11 : Organisation des gènes KIR et haplotypes KIR
Le gène KIR3DL1 illustré ici comprend 9 exons. Les deux premiers codent le peptide signal, les exons 3,4 et 5 codent respectivement les domaines Ig extracellulaires D0, D1 et D2. L'exon 6 code la partie « stem » qui lie les domaines extracellulaires et transmembranaires. L'exon 7 code la partie transmembranaire et les exons 8 et 9 codent la partie intracytoplasmique. Selon le contenu en gènes KIR, deux sortes d'haplotype KIR ont été décrits, dont deux exemples représentatifs sont illustrés ici. L'haplotype du groupe A possède un seul KIR activateur, le KIR2DS4, représenté en rouge et des KIR inhibiteurs, représentés en vert. Les haplotypes du groupe B peuvent posséder plusieurs gènes KIR activateurs et des KIR inhibiteurs. Les gènes conservés et les pseudogènes sont représentés en bleu et gris respectivement.

Les individus d'haplotype KIR B possèdent ainsi un plus grand nombre de combinaisons de gènes KIR différents par rapport aux individus d'haplotype A, assurant une plus grande variabilité (Martin et al. 2004). Les génotypes KIR peuvent être sous-divisés en génotypes AA, AB ou BB (Hsu et al. 2002). Différentes études ont montré que les fréquences des haplotypes et génotypes KIR varient selon les ethnies, mettant en évidence que les gènes KIR peuvent constituer des outils génétiques d'étude des populations (Denis et al. 2005; Frassati et al. 2006). L'haplotype A est notamment le plus commun dans les populations Japonaises où 56% des individus portent le génotype AA (Yawata et al. 2002b). Pour les populations Caucasiennes, la répartition des haplotypes A et B est équitable (Yawata et al. 2002a).

De plus, la variation du nombre de copies (CNV) de gènes KIR peut être source de diversité. Les individus peuvent porter plus de deux copies d'un gène KIR comme il a été montré pour le KIR3DL1 et le KIR3DS1 (Martin et al. 2003). Le locus KIR présente en effet

un très grand nombre de copies de gènes (Vendelbosch et al. 2013a). Pelak et ses collègues ont montré l'implication immunologique des CNV en mettant en évidence qu'un individu ayant un plus grand nombre de gènes KIR3DS1 présente une meilleure résistance au VIH (Pelak et al. 2011). D'autre part, la présence de plusieurs copies du gène KIR2DL3 a également été corrélée à une élimination plus efficace du virus de l'hépatite C (VHC) (Khakoo et al. 2004). Plus récemment, une étude a mis en évidence que le potentiel cytotoxique des cellules NK KIR^+ est d'autant plus important que le nombre de copies de gènes KIR est grand (Béziat et al. 2013b).

Le polymorphisme des gènes KIR est donc dû à la diversité du contenu en gènes mais également au polymorphisme allélique. H. Shilling a montré que l'étendue du polymorphisme allélique différencie les génotypes KIR présentant le même contenu en gènes KIR, cet effet étant plus marqué pour les génotypes A (Shilling et al. 2002). En effet, un large polymorphisme allélique est observé pour certains gènes KIR, en particulier pour les gènes KIR inhibiteurs. En 2015, 753 allèles KIR étaient répertoriés dans la base de données IPD/KIR (The Immuno Polymorphism Database http://www.ebi.ac.uk/ipd) (Robinson et al. 2010). Une nomenclature basée sur la nomenclature HLA a été mise en place pour différencier les différents allèles d'un même gène KIR. Le nom du gène est suivi d'un astérix et de la désignation numérique de l'allèle (ex KIR2DL1*). Les trois premiers digits permettent de décrire les allèles qui diffèrent dans les séquences protéiques (ex KIR2DL1*002). Les $4^{ème}$ et $5^{ème}$ digits d'une part et les $6^{ème}$ et $7^{ème}$ digits d'autre part différencient les allèles qui diffèrent par une ou plusieurs substitutions dans les régions codantes (ex KIR2DL1*00201) et dans les régions non codantes (ex KIR2DL1*0020102) respectivement.

Notons que KIR3DS1/KIR3DL1, KIR2DL2/KIR2DL3 et KIR2DS4/1D sont considérés comme allèles du même gène. Le gène KIR3DL1 présente en particulier un important

polymorphisme allélique (110 allèles décrits) réparti sur les 9 exons, contrairement aux gènes HLA de classe I pour lesquels le polymorphisme allélique est localisé majoritairement au niveau des exons 2 et 3. Le large polymorphisme allélique du récepteur KIR3DL1 a également un impact phénotypique et fonctionnel (Yawata et al. 2006; O'Connor et al. 2007). En effet, selon l'allèle, le niveau d'expression du récepteur KIR3DL1 identifié à l'aide des anticorps commercialisés DX9 et Z27 à la surface des cellules NK varie (Gardiner et al. 2001). Il peut être fortement exprimé, dit « high » pour les récepteurs codés par les allèles KIR3DL1*001, KIR3DL1*002, KIR3DL1*008 et KIR3DL1*015, faiblement exprimé dit « low » pour les récepteurs codés par les allèles KIR3DL1*005 et KIR3DL1*007, et non exprimé à la surface des cellules NK, dit « null » pour le récepteur codé par l'allèle KIR3DL1*004 qui a notamment été très bien décrit (Pando et al. 2003; Thomas et al. 2008; Taner et al. 2011). Le polymorphisme du KIR3DL1 est illustré dans la figure 12.

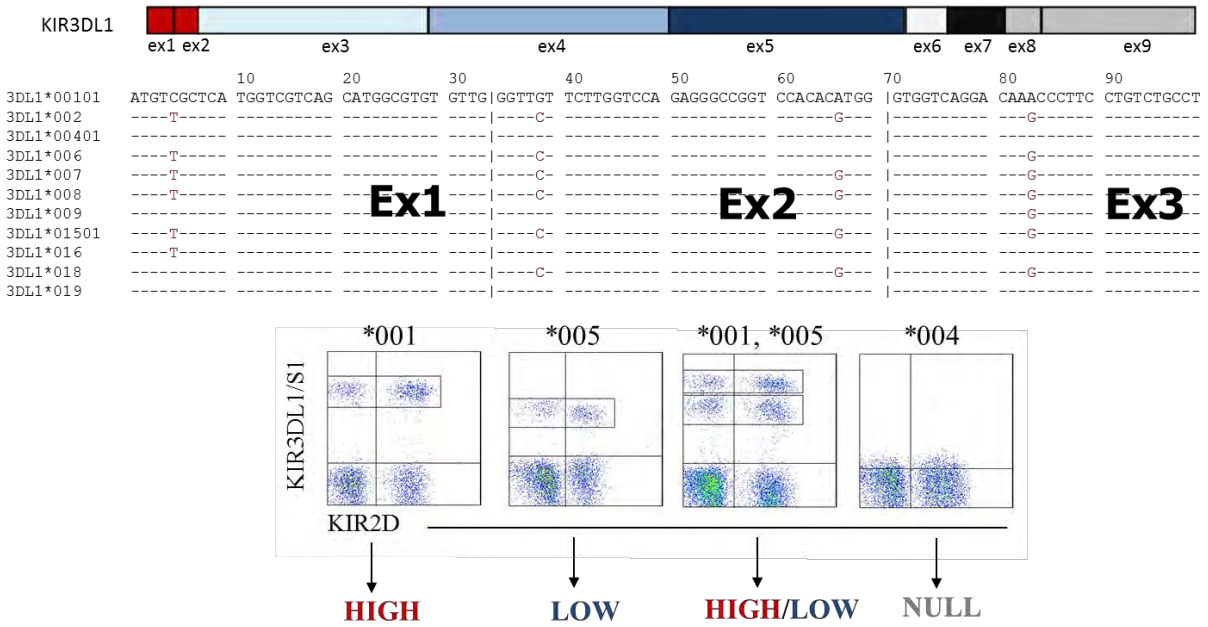

Figure 12 : Polymorphisme allélique : exemple du KIR3DL1
Le gène KIR3DL1 présente un important polymorphisme allélique réparti sur les 9 exons. 110 allèles ont été décrits. Les séquences nucléotidiques de certains allèles KIR au niveau des 3 premiers exons sont illustrées ici. Le polymorphisme allélique du KIR3DL1 a un impact phénotypique et fonctionnel. Selon l'allèle KIR3DL1 présent, le récepteur peut être fortement ($3DL1^{high}$), faiblement ($3DL1^{low}$) ou non exprimé ($3DL1^{null}$) à la surface des cellules NK. Les density plots représentent les populations NK KIR3DL1 ciblées par la combinaison d'anticorps anti-KIR2D/anti-KIR3DL1/S1 d'individus exprimant les allèles KIR3DL1*001 (high) et/ou KIR3DL1*005 (low) et KIR3DL1*004 (null)

Récemment, l'équipe de K. Hsu a développé une technique de typage par PCR multiplex afin de détecter la présence des différents allèles KIR3DL1 (Boudreau et al. 2014) L'intérêt porté au phénotype et à la fonctionnalité des cellules NK KIR^{+} doit donc également prendre en compte la nature des allèles KIR présents et leur potentiel impact sur l'alloréactivité NK.

Différentes techniques de génotypages KIR ont été développées, basées sur l'amplification de l'ARN ou l'ADN d'un individu par PCR. Le choix de la méthode de typage va dépendre du matériel disponible en termes de qualité et de quantité, de la résolution nécessaire (générique ou allélique) ou encore du nombre de génotypages à réaliser. La première technique développée est la PCR-SSP (Sequence Specific Primer) (Uhrberg et al. 1997b) et reste la plus utilisée à ce jour (Norman et al. 2002). Cette méthode est facile, rapide et différents kits sont disponibles mais ne permettent toutefois qu'une détection limitée des allèles. Les PCR-SSP duplex (Ashouri et al. 2009) ou multiplex (Sun et al. 2004) ont été développées par la suite pour réduire la quantité d'ADN utilisée et augmenter le nombre de typages. La PCR-SSP n'est toutefois pas la technique la plus adaptée pour le typage de multiples échantillons. La PCR-SSO (Sequence Specific Oligoprobe) (Crum et al. 2000; Cook et al. 2003) ou la "reverse" PCR-SSO (Nong et al. 2007) utilisant une plateforme Luminex peuvent être utilisées pour le génotypage KIR d'un grand nombre d'échantillons simultanés. Les méthodes de PCR-SBT (Sequence Based Typing) permettent le typage allélique des gènes KIR mais sont généralement spécifiques d'un seul locus (Zhu et al. 2006; Schellekens et al. 2008; Hou et al. 2012) incluant notamment les méthodes de séquençage Sanger.

d) Le Répertoire KIR

L'étude du répertoire NK KIR et donc des récepteurs KIR doit d'une part prendre en compte le contenu en gènes KIR, la nature des allèles KIR et les mécanismes de régulation épigénétiques. L'analyse du répertoire KIR peut en effet passer par l'analyse du nombre de

copie de gènes KIR (CNV) précédemment citée (Vendelbosch et al. 2013b). Les transcrits ARN des gènes KIR peuvent également être évalués par RT-PCR quantitative (McErlean et al. 2010). Des mécanismes génétiques transcriptionnels et post-transcriptionnels gouvernent l'expression des gènes KIR, mais ne sont pas tous bien connus. L'expression des KIR est régulée par des mécanismes épigénétiques, notamment la méthylation de l'ADN de la région promotrice des gènes KIR (Chan et al. 2003). Ainsi, les gènes KIR sont actifs si les régions CpG situées au niveau des promoteurs sont non-méthylées, et silencieux lorsqu'elles sont méthylées. D'autre part, l'étude phénotypique du répertoire NK KIR est réalisée par cytométrie de flux. Différents anticorps commerciaux permettent d'étudier les sous-populations NK KIR. Notre équipe a également développé des anticorps permettant de discriminer les KIR activateurs des KIR inhibiteurs (David et al. 2009)

Les récepteurs KIR sont exprimés de façon stochastique, aléatoire, à la surface des cellules NK. De plus, les cellules NK présentent une expression clonale des récepteurs KIR, ceci impliquant que tous les KIR présents dans le génome d'un individu ne seront pas tous exprimés à la surface de chaque cellule NK (Valiante et al. 1997) comme illustré par la figure 13.

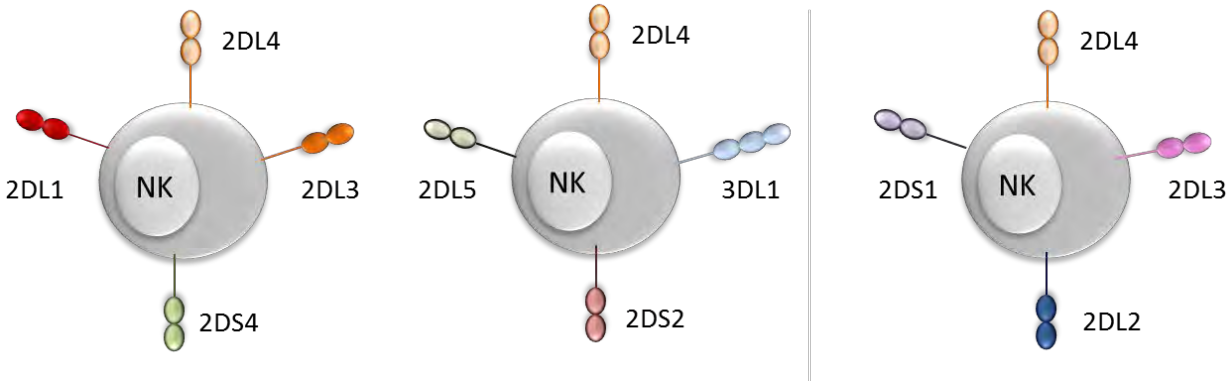

Figure 13 : Expression clonale des récepteurs KIR sur les cellules NK
Les cellules NK présentent une expression clonale des récepteurs KIR engendrant une grande diversité de spécificités. Bien que certains récepteurs comme le récepteur KIR2DL4 soient exprimés sur chaque cellule NK, différents clones NK peuvent être présents chez un individu. Ici, 3 clones représentatifs sont illustrés exprimant différentes combinaisons de récepteurs KIR activateurs et inhibiteurs exprimés (Inspiré de Parham 2005)

A l'exception des récepteurs KIR2DL4 et KIR3DL3, tous les récepteurs KIR présentent une expression clonale (Markus Uhrberg 2005). Ce profil implique l'existence d'une grande diversité de clones NK ayant leurs propres spécificités et fonctionnalités.

La façon dont les cellules NK acquièrent ce répertoire KIR distribué de façon clonale reste débattue. Différentes théories ont été proposées pour expliquer la formation du répertoire des cellules NK KIR. Certains groupes proposent un modèle basé sur une acquisition dite séquentielle des KIR, l'un après l'autre, et fortement impacté par l'expression des molécules HLA de classe I autologues (Schönberg et al. 2011b; Sleiman et al. 2014). Une étude Japonaise réalisée chez des individus d'haplotype A a rapporté une forte influence des molécules HLA-Cw sur la distribution des KIR inhibiteurs (Yawata et al. 2008a). En parallèle, d'autres groupes ont au contraire proposé que la diversité du répertoire NK KIR est générée par une acquisition aléatoire des récepteurs KIR indépendamment de la présence ou l'absence des molécules HLA de classe I (Andersson et al. 2009a; Sternberg-Simon et al. 2013). Les travaux de notre équipe ont suggéré une expression consécutive des récepteurs KIR2D sur les cellules NK KIR3DL1^{+} (Gagne et al. 2013). Nous avons également montré que l'expression des récepteurs KIR2DL2, KIR2DS1 et KIR2DS2 limite l'acquisition du récepteur KIR2DL1 (David et al. 2013) suggérant que le contenu en gènes KIR impacte plus la formation du répertoire NK que l'environnement HLA de classe I autologue en termes de ligands KIR. L'impact des molécules HLA de classe I du soi sur les fréquences des populations KIR reste donc débattu.

Différentes études suggèrent également que l'histoire immunologique des individus, et en particulier les infections virales vont structurer le répertoire NK de façon HLA-dépendante. Les infections virales impactent différemment le répertoire NK : des études ont rapporté l'expansion d'une population NK NKG2C^{+} après infection à Cytomégalovirus (Béziat et al. 2013a; Schlums et al. 2015) et d'une population CD56^{dim} NKG2A^{+} KIR^{-} NK après infection à

B-EBV . D'autres facteurs comme l'âge (Lutz et al. 2005; Le Garff-Tavernier et al. 2010) et les facteurs environnementaux (Lu et al. 2007; Mian et al. 2008a) semblent également impacter le répertoire NK. Dans ce contexte, j'ai revisité la formation du répertoire NK en étudiant le répertoire de cellules NK de sang de cordon ombilical. L'ensemble de ces données seront présentées ultérieurement dans la partie résultats.

2.2. Immunoglobulin-like transcripts (ILT)

D'autres récepteurs de la superfamille des Immunoglobulines sont exprimés à la surface des cellules NK. Contrairement aux KIR qui sont restreints aux cellules NK et T, les transcrits Ig-like (ILT) sont exprimés à la surface de nombreuses cellules d'origine myéloïde et lymphoïdes dont les cellules NK, les lymphocytes T, les lymphocytes B, les monocytes, les macrophages et les cellules dendritiques (Samaridis and Colonna 1997). Ils sont également appelés LIR pour « Leukocyte Ig-like receptors » ou MIR pour « monocyte-macrophage inhibitory receptor ». L'acronyme LILR est désormais utilisé pour standardiser la nomenclature des récepteurs de cette famille. Ces récepteurs partagent des homologies avec les récepteurs KIR et leurs gènes codants sont localisés au niveau du complexe LRC sur le chromosome 19q13.4, proche des gènes KIR. Contrairement aux KIR, les gènes codants les récepteurs ILT présentent un polymorphisme beaucoup plus limité. A ce jour, 13 récepteurs ILT ont été identifiés. La plupart des molécules ILT sont des protéines transmembranaires présentant 2 ou 4 domaines Ig extracellulaires. Toutefois, seuls les récepteurs ILT2 et ILT4 reconnaissent les molécules HLA de classe I. Le récepteur ILT2 (CD85 ou LIR1) est le seul exprimé sur les cellules NK (Colonna et al. 1997). Il possède 4 domaines Ig extracellulaires et une portion cytoplasmique contenant 4 motifs ITIM (Immunoreceptor Tyrosine-Based Inhibition Motifs) assurant la transduction d'un signal inhibiteur. Il peut se lier à la plupart des molécules HLA de classe I (HLA-C*0702, -C*0602, -C*0301 et HLA-B*2702), étant

spécifique du domaine α3 de la chaîne lourde, ainsi qu'aux molécules HLA non classiques HLA-G et HLA-E (Navarro et al. 1999). ILT2 se lie à la molécule HLA-G avec une affinité 3 à 4 fois plus forte qu'aux molécules HLA de classe I classiques ce qui suggère un rôle dominant de l'interaction ILT2/HLA-G dans la régulation des fonctions des cellules NK (Shiroishi et al. 2003). La molécule HLA-G étant exprimée à la surface de différentes cellules tumorales, l'interaction ILT2/HLA-G confère aux cellules tumorales une protection à la lyse médiée par les cellules NK (Adrián Cabestré et al. 1999).

2.3. Les hétérodimères CD94/NKG2

Les récepteurs NKG2 sont des glycoprotéines de membrane de type II, dit « lectine-like ». Chez l'homme, on compte 5 récepteurs NKG2 : NKG2A/B, -C, -E/H et -F, dont les gènes codants sont situés sur le chromosome 12 (12p12.3-p13.2) au niveau du complexe génique NK (NKC). Ces récepteurs sont exprimés à la surface cellulaire sous forme d'hétérodimères avec le récepteur CD94, reliés par un pont disulfide (Lazetic et al. 1996). Les récepteurs NKG2 se lient à la molécule HLA de classe I non classique HLA-E (Braud et al. 1998; Borrego et al. 1998). Toutefois, le complexe NKG2A/CD94 a une plus grande affinité pour le HLA-E que le complexe CD94/NKG2C (Valés-Gómez et al. 1999) Ce ligand est exprimé par les cellules du trophoblaste, les cellules endothéliales vasculaires et les cellules dendritiques. La molécule HLA-E présente des peptides dérivés de la séquence signale d'autres molécules HLA de classe I (Lee et al. 1998) et son expression est augmentée par certaines cytokines (IFNγ, TNFα) (Coupel et al. 2007).

Le polymorphisme des gènes NKG2 est bien plus limité que celui observé pour les gènes KIR. L'analyse du polymorphisme a montré que l'ensemble de ces gènes est relativement conservé. En effet, aucun polymorphisme n'est observé pour le NKG2A et les allotypes

différent par pas plus de 2 substitutions en acides aminés, pour le NKG2C notamment (Shum et al. 2002).

Parmi ces récepteurs, le NKG2A porte 2 motifs ITIM assurant une fonction inhibitrice au complexe NKG2A/CD94. Au contraire, le complexe NKG2C/CD94 s'associe à la protéine adaptatrice DAP12 transmettant un signal activateur (Carretero et al. 1997; Lanier et al. 1998b). Le récepteur NKG2F s'associe également à DAP12 mais pas avec la molécule CD94 et n'est pas exprimé à la surface cellulaire. DAP12 s'associe aux récepteurs NKG2E et NKG2C avec la même efficacité (Call et al. 2010) et les deux récepteurs présentent des séquences transmembranaires identiques. En conséquence, NKG2E est souvent cité conjointement avec le NKG2C. Récemment, une étude a montré la rétention intracellulaire du complexe NKG2E/CD94/DAP12 (Orbelyan et al. 2014).

2.4. Récepteur NKG2D

Certains récepteurs sont impliqués par la reconnaissance du soi induit par le stress cellulaire par les cellules NK. Parmi ces récepteurs, le NKG2D est un récepteur transmembranaire lectine-like de type 2, membre de la famille NKG2 codé par le complexe génique NK sur le chromosome 2 humain. Non couplé au CD94, il est exprimé sous forme d'homodimère chez la souris et chez l'homme sur toutes les cellules NK (Raulet 2003) mais également sur les cellules T (Bauer et al. 1999). Il s'associe avec la protéine DAP10 (Wu et al. 1999) qui possède des motifs ITAM assurant la transduction du signal activateur. Son interaction avec ses ligands active la cytotoxicité des cellules NK contre leurs cibles (Jamieson et al. 2002). Les ligands du NKG2D sont des protéines du soi, homologues des molécules HLA de classe I. Chez l'homme, ils incluent notamment MIC-A, MIC-B et jusqu'à 6 protéines liant l'UL16 différentes ou ULBP. Chez la souris, les ligands incluent les protéines RAE (retinoic acid early inducible), H60 et MULT-1 (ULBP-like transcript-1)

(Diefenbach et al. 2001). Les ligands du NKG2D sont peu ou pas exprimés à la surface des cellules saines mais fréquemment surexprimés suite à un stress cellulaire associé avec une infection virale ou une transformation maligne comme nous l'avons abordé précédemment. La lyse des cellules leucémiques médiée par les cellules NK NKG2D^{+} a en effet été rapportée (Salih et al. 2003). Le polymorphisme du gène NKG2D est limité. Le gène NKG2D présente un polymorphisme au niveau d'un simple nucléotide (SNP) dans la région transmembranaire, une substitution d'une guanine en adénine, situé proche du site de liaison de la protéine adaptatrice DAP10 (Shum et al. 2002). D'autre part, Hayashi et ses collaborateurs ont identifié plusieurs haplotypes d'allèles au niveau du NCR, formés à partir de 3 ou 5 SNP principalement localisés dans la région du gène NKG2D. Chaque bloc d'haplotype contient deux SNP majeurs qui ont été reliés à une cytotoxicité naturelle faible (LNK1 et LNK2) ou forte (HNK1 et HNK2). L'haplotype HNK1 étant associé à une diminution du risque de cancer (Hayashi et al. 2006) et à une meilleure survie globale et mortalité liée à la greffe chez des patients japonais après greffe de moelle osseuse (Espinoza et al. 2009). Les haplotypes sont associés avec différents niveaux d'expression du NKG2D à la surface des cellules NK et T induisant une variabilité interindividuelle de la réponse cytotoxique (Imai et al. 2012).

2.5. Récepteur CD161

Le récepteur CD161 ou NKR-P1A appartient également à la superfamille des lectines de type C. Contrairement à ce qui est observé chez la souris, un seul gène est connu chez l'homme et situé sur le chromosome 12. Il peut se comporter comme un récepteur activateur sur les lymphocytes T et comme un récepteur inhibiteur sur les cellules NK (Lanier et al. 1994). Il est exprimé à la surface des cellules NK immatures avant l'acquisition du marqueur CD16 et du marqueur CD56 (Bennett J Exp Med1996) comme nous le présenterons dans la description des étapes de développement des cellules NK. Il est également surexprimé sur les

cellules NK matures suite à l'action de l'IL-12 (Azzoni et al. 1998). Le LLT1 (lectin-like transcript-1) a été identifié comme ligand physiologique du CD161 chez l'homme, dont l'interaction peut inhiber la cytotoxicité et la production de cytokines des cellules NK (Aldemir et al. 2005). Au contraire, l'engagement du LLT1 avec le CD161 sur les lymphocytes T $CD8^+$ inhibe la production de TNFα (Germain et al. 2011). Le développement d'un anticorps anti-LLT1 a permis de montrer l'expression de LLT1 sur les cellules dendritiques activées et les cellules B suggérant le rôle du CD161 dans le cross-talk entre les cellules NK et les cellules présentatrices de l'antigène (Rosen et al. 2008).

Parmi le panel de récepteurs activateurs inhibiteurs exprimés à la surface des cellules NK, certains sont plus particulièrement impliqués dans la défense antivirale et anti-tumorale comme les récepteurs de cytotoxicité naturelle, DNAM-1 et le 2B4 ou régulent leur activation comme les TLR qui vont être brièvement présentés dans les prochains paragraphes.

2.6. Les récepteurs de cytotoxicité naturelle (NCR)

Les Récepteurs de Cytotoxicité Naturelle (ou NCR) jouent un rôle clé dans la reconnaissance des cellules infectées par un virus et des cellules tumorales. Les récepteurs NKp46, NKp44, NKp30 sont les principaux NCRs qui ont été découverts à la fin des années 1990. Ces récepteurs ont initialement été regroupés pour leur capacité de lyse des cellules tumorales. Cependant, malgré une fonctionnalité similaire, ils ne partagent que peu de similarités du point de vue de leur séquence en acides aminés ou de leur structure. Notons que la reconnaissance et l'élimination optimale de cellules cibles est assurée lorsque plusieurs NCR agissent simultanément (Augugliaro et al. 2003). Ce sont des protéines transmembranaires de type I appartenant à la superfamille des Immunoglobulines composées de 1 ou 2 domaines extracellulaires Ig-like responsables de la liaison au ligand. Ils présentent

également un domaine transmembranaire avec un acide aminé chargé positivement qui interagit avec une protéine adaptatrice de signalisation contenant un motif ITAM. Nous décrirons ici les principaux NCR activant les fonctions anti-tumorales des cellules NK, grâce à la reconnaissance de ligands exprimés par les cellules tumorales.

a) Récepteur NKp46

Le récepteur NKp46 est le premier NCR qui fut identifié chez l'homme, et est également nommé NCR1 (Sivori et al. 1997; Pessino et al. 1998). Les homologues du NKp46 ont été identifiés chez le rat (Falco et al. 1999), la souris (Biassoni et al. 1999) et le singe (De Maria et al. 2001). Le gène codant pour le récepteur NKp46 est localisé sur le chromosome 19 (19q13.42) au sein du LRC. Ce récepteur est présent sur les cellules NK mais également sur certaines populations ILCs, sur les lymphocytes Tαβ intestinaux et les lymphocytes Tγδ après activation par l'IL-15 (Meresse et al. 2006; Tang et al. 2008). Chez l'homme, le récepteur NKp46 est exprimé sur les cellules NK quel que soit leur état d'activation. Cette protéine de 46kDa est caractérisée par 2 domaines extracellulaires C2 de type Ig-like et peut s'associer aux protéines adaptatrices CD3ζ et FcRγ qui possèdent respectivement 2 et 3 motifs ITAM, assurant la transduction du signal activateur. Les ligands du NKp46 ne sont pas totalement définis mais semblent être très variés. Différentes études ont mis en évidence que le récepteur NKp46 reconnait certaines hémagglutinines virales (Mandelboim et al. 2001) et les protéoglycanes à héparane sulfate associés à la membrane (HSPG), pouvant être surexprimés par les cellules tumorales (Arnon et al. 2004; Bloushtain et al. 2004; Hecht et al. 2009). Les cellules NK $NKp46^{+}$ sont également impliquées dans le développement de diabète de type I, les cellules β pancréatiques ayant été identifiées comme ligands du NKp46 chez l'homme et la souris (Gur et al. 2011; Gur et al. 2013). Une étude récente a montré qu'un traitement anticorps murin NCR1.15 anti-NKp46 suffisait à retarder significativement le développement

précoce de diabète chez la souris, suggérant ainsi de nouvelles stratégies thérapeutique (Yossef et al. 2015).

b) Récepteur NKp44

Le récepteur NKp44 est un récepteur aux propriétés uniques dont l'expression est restreinte aux cellules NK activées capables de médier une réponse cytotoxique (Vitale et al. 1998). Il peut également être exprimé sur des cellules NK déciduales (Vacca et al. 2008) et des sous-populations de cellules dendritiques plasmacytoïdes dans l'amygdale (Fuchs et al. 2005). Cette glycoprotéine de 44 kDa présente un seul domaine extracellulaire et une lysine dans la région transmembranaire assurant l'interaction avec la protéine adaptatrice DAP12, portant un motif ITAM transducteur du signal d'activation. Le gène NKp44 est situé dans la région HLA de classe III sur le chromosome 6 (6p21.1).

Bien qu'il fût découvert à la fin des années 1990, ses ligands ont été extrêmement difficiles à identifier. La première molécule identifiée comme le ligand du NKp44 est le PCNA (Proliferating Cell Nuclear Antigen) (Rosental et al. 2011) qui est surexprimé à la surface de cellules tumorales ainsi que sur certaines cellules saines comme les trophoblastes déciduaux (Korgun et al. 2006). L'interaction du NKp44 avec le PCNA pourrait contribuer à la tolérance du fœtus dans le premier trimestre de la grossesse.

Comme les autres NCR, le NKp44 constitue un médiateur de la cytotoxicité NK et contribue à l'immunosurveillance tumorale. Son expression est responsable d'une forte augmentation de la lyse de nombreux types de de cellules cancéreuses (neuroblastome, lymphome de burkitt, cancer du côlon, de la prostate ou des poumons). Récemment, une isoforme atypique de la protéine MLL5 (mixed lineage leukemia-5) aussi connue comme la lysine méthyltransférase 2^{E}, a été identifiée comme ligand du NKp44 (Baychelier et al. 2013). Elle représente une forme tronquée de MLL5. Contrairement à MLL5 présent dans le noyau et le cytosol, ce

ligand est détecté à la surface cellulaire mais il reste à déterminer comment il est exposé à la surface des cellules tumorales bien que son expression à la surface des T $CD4^+$ soit induite par un peptide du VIH dérivé de gp41 (Vieillard et al. 2005). Enfin, le NKp44 est impliqué dans l'immunité antivirale et reconnait notamment les hémagglutinines du virus influenza (Arnon et al. 2001).

c) Récepteur NKp30

Comme le récepteur NKp44, le gène codant le récepteur NKp30 est situé dans la région HLA de classe III sur le chromosome 6 (6p21.1). Il code pour une glycoprotéine de 30 kDa exprimée sur toutes les cellules NK matures (Pende et al. 1999). Il peut également être exprimé sur les cellules T de sang de cordon après exposition à l'IL-15 (Li et al. 2011), sur les T $V\delta1^+$ en présence d'IL-12 et IL-15 après engagement du TCR (Correia et al. 2011). Le récepteur NKp30 présente un seul domaine extracellulaire et une arginine chargée dans la région transmembranaire qui peut interagir avec les protéines adaptatrices CD3ζ et FcRγ, portant des motifs ITAM transducteurs du signal d'activation. Le seul ligand correspondant à une protéine de surface transmembranaire est B7-H6 de la famille B7 (Brandt et al. 2009) possédant 2 domaines Ig extracellulaires et présent à la surface de cellules tumorales, de monocytes proinflammatoires $CD16^+CD14^+$ et des neutrophiles après stimulation des « Toll-Like Receptor » (TLR). La protéine de tégument du CMV humain pp65 a été identifiée comme ligand dérivé du virus pour le récepteur NKp30. En effet, la protéine de fusion NKp30-Fc peut se lier au pp65 de cellules infectées et l'interaction NKp30-pp65 induit l'inhibition de la cellule NK (Arnon et al. 2005). Enfin, BAT3 (HLA-B associated transcript3) aussi connu sous le nom de BCL2-associated athanogene, peut se fixer au NKp30, et est présent sur les cellules tumorales, les cellules dites « stressées » et les cellules dendritiques (Pogge von Strandmann et al. 2007). Comme les autres NCR, le récepteur NKp30 peut

également reconnaître certaines hémagglutinines virales des poxirus comme les virus de la vaccine et de l'ectromélie (Jarahian et al. 2011).

2.7. DNAM-1 (DNAX accessory molecule-1)

La cytotoxicité des cellules NK vis-à-vis des cellules tumorales ou infectées par un virus impliqué également la molécule d'adhésion transmembranaire DNAM-1 (DNAX accessory molecule-1) qui est caractérisée par deux domaines Ig-like extracellulaires et une portion intracytoplasmique contenant des résidus tyrosine impliqués dans la signalisation (Shibuya et al. 1996). Cette molécule est constitutivement exprimée à la surface des cellules T, NK, des macrophages et de certaines sous populations de lymphocytes B chez la souris et chez l'homme. Son engagement induit la cytotoxicité et la production de cytokines par les cellules T et NK.

Ses ligands identifiés sont les nectines et les membres de la famille nectine-like CD155 (PVR, necl-5) et CD112 (PVRL2, nectin-2 (Bottino et al. 2003; Pende et al. 2005a). Ils sont fréquemment exprimés à la surface de cellules infectées par un virus ou de cellules transformées comme les cellules tumorales. Les cellules cibles surexprimant ces ligands présentent une expression altérée des molécules HLA de classe I. Ainsi, en absence d'interactions efficaces médiées par des récepteurs inhibiteurs, l'engagement de DNAM-1 résulte en l'induction de la cytotoxicité des cellules NK. Toutefois, PVR et Nectin-2 sont également exprimés sur certaines cellules saines comme les cellules épithéliales et endothéliales qui sont protégées de la lyse médiée par les cellules NK grâce à l'expression des molécules HLA de classe I. L'implication du récepteur DNAM-1 dans la surveillance tumorale fut mise en évidence chez des souris DNAM-1-déficientes (Iguchi-Manaka et al. 2008) et sera décrit ultérieurement dans le paragraphe « 9. Cellules NK et cancer ».

2.8. Le récepteur 2B4

Le récepteur 2B4 (ou CD244) est un membre de la famille des récepteur SLAM (Signaling Lymphocyte Activation Molecule). Contrairement aux autres membres de la famille SLAM qui sont exploités par les virus, 2B4 contribue à l'immunité anti-virale. Il est exprimé par toutes les cellules NK, les cellules T γδ, les basophiles, les monocytes et une sous-population de cellules T $CD8^+$ αβ (Garni-Wagner et al. 1993; Valiante and Trinchieri 1993). Il interagit avec son ligand, la molécule Ig-like CD48 (Brown et al. 1998) qui est exprimée sur les cellules lymphoïdes et myéloïdes, et dont l'expression est augmentée en réponse à l'IFNγ et l'IFNα/β (Tissot et al. 1997). La molécule CD8 présente des motifs basés sur une tyrosine similaire aux ITIM appelés ITSM pour « Immunoreceptor Tyrosine-based Switch Motif». Les signaux d'activation sont transmis via la protéine SAP (SLAM-Associated Protein) qui recrute la tyrosine kinase Fyn (Latour et al. 2003). Les cellules NK expriment d'autres molécules adaptatrices liées à SAP dont EAT-2 qui ne se fixe pas à la kinase Fyn (Veillette et al. 2009). SAP et EAT-2 ont une action combinée induisant l'activation des cellules NK comme le montre l'inhibition plus marquée de 2B4 dans le cas d'une double déficience SAP/EAT-2 chez la souris (Dong et al. 2012). Il est maintenant bien connu que l'engagement du 2B4 avec son ligand peut induire un signal inhibiteur aussi bien qu'un signal activateur. En effet, des mutations de SAP altèrent les fonctions antivirales des lymphocytes T et des cellules NK chez des patients atteints de syndrome lymphoprolifératif (Nakajima et al. 2000).

Les cellules NK expriment donc un ensemble de récepteurs activateurs et inhibiteurs régulant leur activation et assurant la reconnaissance du soi manquant, du non-soi infectieux et du soi induit par le stress. D'autres molécules présentes à leur surface cellulaire modulent leur activation et peuvent être impliquées dans leur interaction avec d'autres cellules du

système immunitaire comme les TLR ou les molécules de co-stimulation, ou encore des molécules qui assurent leur migration aux niveaux des sites tumoraux et des sites d'inflammation comme les récepteurs aux chimiokines qui vont être brièvement décrits dans les paragraphes suivants.

2.9. Les récepteurs TLR (Toll Like Receptor)

Les récepteurs TLR (Toll like Receptors) sont essentiels dans la reconnaissance des pathogènes et stimulent également les capacités cytotoxiques des cellules NK. Exprimés par différentes cellules de l'immunité innée dont les cellules NK, les macrophages et les cellules dendritiques, les TRL recrutent les lymphocytes circulants au lieu de l'infection pour limiter la prolifération des pathogènes (Kawai and Akira 2010). Les cellules NK expriment différents TLR selon leur état d'activation (Chalifour et al. 2004) dont le TLR2 se lie à des produits d'origine bactérienne (Esin et al. 2013). Ces récepteurs sont également impliqués dans le « cross-talk » entre les cellules NK et les cellules dendritiques. En effet, suite à l'engagement avec le TLR3 sur les cellules dendritiques, l'IL-2 relarguée augmente la cytotoxicité des cellules NK qui acquièrent la capacité de tuer les cellules dendritiques immatures assurant la survie sélective des DC matures. La stimulation des TLR augmente également leur capacité de production de TNFα et IFNγ qui stimule encore plus la maturation des cellules NK et ainsi l'induction de réponses de type Th1 (Agaugué et al. 2008). Via l'activation des TLR, les cellules NK peuvent ainsi exercer un contrôle régulateur dans les premières étapes des réponses de l'immunité innée contre des agents pathogènes (Sivori et al. 2004).

2.10. Les molécules et récepteurs de co-stimulation

Les cellules NK au repos ou activées peuvent exprimer différentes molécules de co-stimulation à leur surface comme le CD2, le LFA-1, le CD69 etle VLA-4 impliquées dans

leur activation (Robertson et al. 1990; Barber et al. 2004). Suite à une inflammation, les cytokines relarguées par les neutrophiles ou les macrophages peuvent induire l'expression ou la surexpression des ligands de différentes molécules de co-stimulation comme le CD58 (ligand du CD2), le VCAM-1 (ligand du VLA-4), le CD54 (ligand du LFA-1) et les CD80 et CD86 (ligands des CD28 et CTLA4). L'expression du CD28 à la surface des cellules NK a également été démontrée par plusieurs études chez la souris (Nandi et al. 1994) et chez l'homme (Roger et al. 1996), la reconnaissance du CD80 induisant l'activation de la cellule NK. Le changement de conformation de la molécule LFA-1 dans une configuration ouverte, active est impliqué dans la formation de la synapse immune entre cellules effectrice et cible (Liu et al. 2009a). La liaison du LFA-1 avec ICAM-1 est notamment essentiel pour la polarisation des granules cytotoxiques assurant la lyse efficace des cellules cibles (Bryceson et al. 2005). LFA-1 peut former une paire avec le récepteur DNAM-1 et la stimulation de DNAM-1 sur les cellules NK induit le changement de conformation du LFA-1 ce qui stimule la formation de conjugués (Bryceson et al. 2009). La lyse des cellules peut également être activée par le CD40, se liant au CD40L qu'elles expriment (Carbone et al. 1997). De plus, la molécule OX40L peut être induite à la surface des cellules NK activées par l'IL-2, IL-12 ou IL-15 qui interagissent ainsi avec les lymphocytes T $CD4^+$ exprimant la molécule OX40, contribuant à leur prolifération et la production d'IFNγ (Kashii et al. 1999).

2.11. Les récepteurs de cytokines et de chimiokines

Les cellules NK expriment constitutivement différents récepteurs aux cytokines impliquées dans leur développement et la régulation de leur fonctionnalité. Parmi ces récepteurs, on trouve les récepteurs à l'IFNα/β, l'IL-2, l'IL-10, l'IL-12, l'IL-15 et l'IL-18 (Carson et al. 1995; Carson et al. 1997; Hyodo et al. 1999; Kawamura et al. 2003). Les cellules NK expriment également des récepteurs aux chimiokines, molécules qui régulent leur

migration et assurent leur recrutement sur les sites d'inflammation et les sites tumoraux (Robertson et al. 2000). Le CCR1 et CCR5 assurent la migration des cellules NK aux sites d'inflammation et d'infections virales (Allavena et al. 1994). Le CCR7 et la L-sélectine (CD62L) sont impliqués dans l'entrée des cellules dans les organes lymphoïdes secondaires. Ils sont fortement exprimés à la surface des cellules NK $CD56^{bright}$ mais non exprimés à la surface des NK $CD56^{dim}$ (Frey et al. 1998; Berahovich et al. 2006). Le CXCL12 assure la rétention cellulaire des cellules souches hématopoïétiques dans la moelle osseuse. L'expression de son récepteur, le CXCR4, diminue au cours de la maturation des cellules NK assurant leur migration à la périphérie (Mayol et al. 2011). Le récepteur CX3CR1 est également exprimé par les cellules NK. Son expression est régulée négativement et positivement par l'IL-15 et l'IL-2 respectivement (Barlic et al. 2003). Le ligand du CX3CR1 est la molécule CX3CL1 également connue sous le nom de fractalkine, qui recrute les cellules NK activée sur les sites tumoraux (Lavergne et al. 2003). Enfin, les chimiokines CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CXCL10, et CX3CL1 semblent promouvoir le relargage de granules cytotoxiques des cellules NK au repos augmentant ainsi la cytotoxicité des cellules NK vis-à-vis de lignées K562 dépourvues de molécules HLA de classe I (Robertson 2002).

Le compartiment humain de cellules NK est très hétérogène et comprend des cellules se trouvant à différents stades de développement et de maturation que nous allons brièvement présenter dans le paragraphe suivant. Nous développerons ensuite le processus d'éducation de ces cellules assurant la reconnaissance du soi manquant et du non soi tout en maintenant la tolérance au soi.

3. Développement des cellules NK

Les cellules NK se développent dans la moelle osseuse à partir d'un progéniteur commun aux cellules lymphoïdes (CLP) comme cité précédemment. Des intermédiaires de

développement NK $CD34^-$ ont récemment été mis en évidence dans la moelle osseuse et également dans le foie (Kim et al. 2002), le thymus (Di Santo and Vosshenrich 2006) et dans les ganglions lymphatiques (Freud et al. 2005), suggérant que la maturation des cellules NK peut se poursuivre dans les tissus extramédullaires. Bien que de nombreux facteurs de croissance soient nécessaires à leur développement, l'interleukine 15 (IL-15) est considérée comme la cytokine la plus importante pour l'homéostasie des cellules NK. En effet, elle induit la différenciation et la maturation de cellules souches $CD34^+$ en $CD56^{bright}$ (Carson et al. 1994; Mrózek et al. 1996). Pour pouvoir répondre à l'IL-2 et à l'IL-15, les cellules NK doivent exprimer la chaîne commune α (ou CD132) et l'IL-2Rβ (ou CD122).

Différents stades de développement ont ainsi été identifiés notamment par Freud et Caligiuri (Freud and Caligiuri 2006) et sont illustrés dans la figure 14 :

- les progéniteurs des cellules NK ou « pro-NK » (stade 1), sont phénotypiquement caractérisés $CD34^+$ $CD45RA^+$ $CD94^-$ $CD117^-$. Ces cellules n'expriment pas de CD122 et ne répondent donc pas à l'IL-2 et l'IL-15.

- les précurseurs NK ou « pré-NK » (stade 2) sont $CD34^+$ $CD45RA^+$ $CD94^-$ $CD117^+$. Ces cellules ont acquis le marqueur CD122 et sont donc capables de répondre à l'IL-2 et l'IL-15. Le CD161 est le seul récepteur NK exprimé à ce stade.

- les cellules NK immatures ou « iNK » (stade 3) sont $CD34^-$ $CD94^-$ $CD117^+$. Leur phénotype est marqué par la perte des récepteurs CD34 et CD45RA. Ces cellules ont acquis le récepteur NKp44. A ce stade, les cellules ne présentent aucun récepteur inhibiteur CD94/NKG2A ou récepteurs KIR. Elles ne sont pas capables de cytotoxicité naturelle à ce stade.

- les cellules $CD56^{bright}$ (stade 4) sont $CD34^-$ $CD94^+$ $CD117^+$. Ce stade est marqué par l'acquisition du CD94. Les cellules ont également acquis l'expression du NKp46, NKG2A, NKG2D et présente une faible expression en CD117 et CD127. Elles produisent de l'IFNγ et

sont capables de cytotoxicité naturelle via l'expression de perforine et de Fas-ligand (Fas-L) (Freud J Exp med 2006).

- Les cellules CD56^{dim} (stade 5) CD94^{+} CD16^{+} KIR^{+} semblent constituer la population cellulaire la plus mature, marquée par l'acquisition finale du CD16 et des récepteurs KIRs. Elles sont plus cytotoxiques contre les cellules cibles que les CD56^{bright} et prolifèrent en réponse à une stimulation par l'IL-2 et l'IL-15 grâce à la signalisation de l'hétérodimère IL-2Rβγ/IL-15Rα. Les CD56^{dim} sécrètent peu d'IFNγ, et ce même après activation par les cytokines.

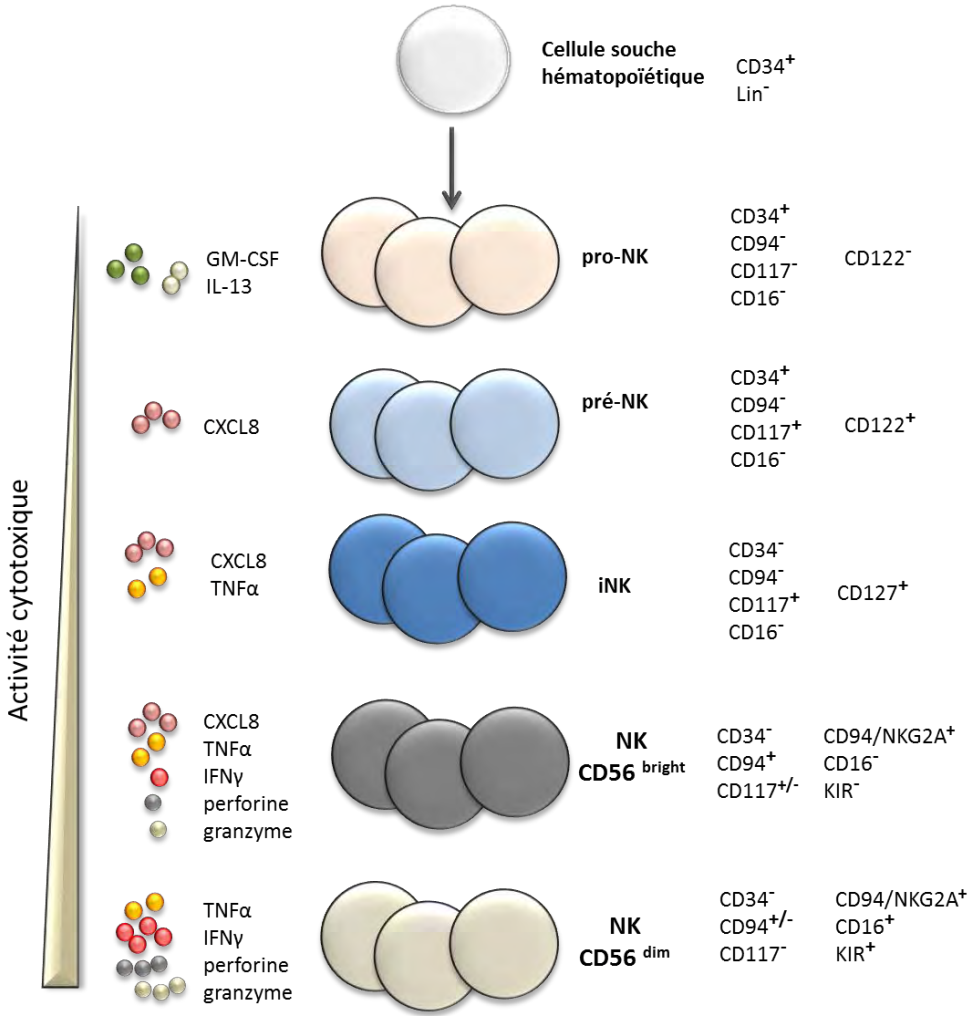

Figure 14 : Etapes de développement des cellules NK humaines
Issues des cellules souches hématopoïétiques, les cellules NK passent par différents stades de développement. Après des stades précoces de pro-NK et pré-NK, les cellules NK acquièrent leur fonctionnalité et leur capacité de production de cytokines. L'acquisition des récepteurs KIR et du CD16 marquent le dernier stade de maturation, conférant aux cellules NK un potentiel cytotoxique (inspiré de Montaldo et al. 2013)

4. Education des cellules NK

Dans les années 1960, le phénomène de « résistance hybride » a été identifié en greffe de moelle osseuse chez la souris, observant un rejet des greffons de cellules souches parentaux homozygotes par des souris hybrides (Cudkowicz and Stimpfling 1964). Les règles de transplantation de l'époque, selon lesquelles le rejet du greffon requiert la présence de molécules de CMH étrangères sur le greffon comparées à celles présentes sur l'hôte, n'ont pas permis de prédire ces résultats. L'implication des cellules NK dans la résistance hybride a par la suite été montrée dans les années 1970 (Kiessling et al. 1977). L'introduction d'un transgène de souche H-2D^{d} dans une souris de souche H-2^{b} induit une résistance au rejet du greffon médiée par les cellules NK d'un donneur de fond génétique différent, montrant que les molécules du CMH affectent le rejet du greffon (Ohlén et al. 1989). Par la suite, la découverte des récepteurs inhibiteurs Ly49 chez la souris, spécifiques des molécules du CMH de classe I a permis d'identifier la nature de l'interaction des cellules NK avec les molécules du CMH (Karlhofer et al. 1992).

Les cellules saines expriment différentes combinaisons et différents niveaux d'expression de molécules CMH de classe I qui peuvent s'engager avec les récepteurs inhibiteurs et activateurs des cellules NK. Plusieurs modèles ont été proposés pour expliquer le développement fonctionnel et la tolérance au soi des cellules NK. Le terme d'éducation des cellules NK fait référence à un processus différent de celui de la maturation lymphocytaire T ou B (Starr et al. 2003).

Il a initialement été reporté que toutes les cellules NK matures expriment au moins un récepteur spécifique pour une molécule de CMH du soi (Valiante et al. 1997). Cette notion dite du « au moins un » est basée sur l'idée que si l'engagement de ce récepteur est suffisant pour outrepasser la stimulation que les cellules NK reçoivent quand elles rencontrent un ligand activateur sur les cellules saines, la tolérance au soi est alors établie (Raulet et al.

1997). Cette hypothèse a été corroborée par une étude réalisée chez la souris suggérant que les cellules NK accumulent des récepteurs Ly49 inhibiteurs additionnels jusqu'à ce qu'elles soient capables d'engager une molécule du CMH du soi (Dorfman and Raulet 1998). La figure 15 résume les différents modèles d'éducation des cellules NK qui vont être présentés dans ce paragraphe.

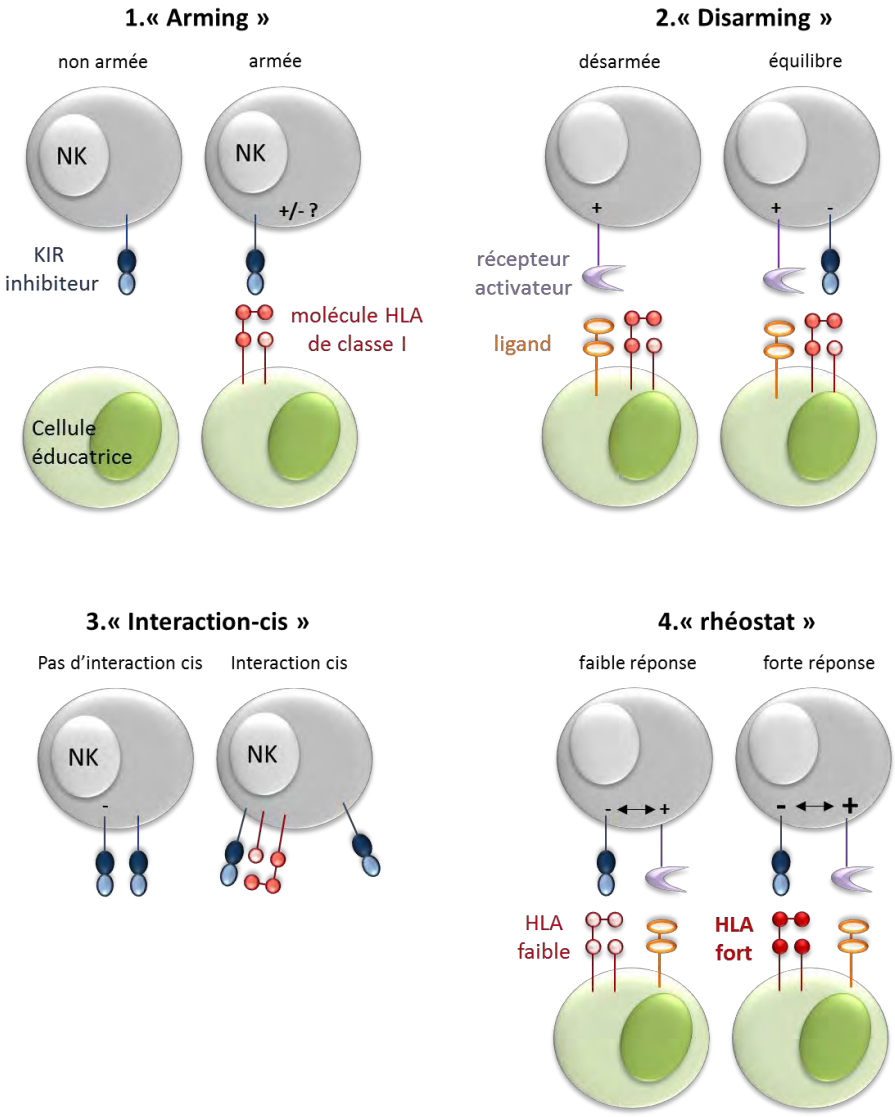

Figure 15 : Modèles d'éducation des cellules NK
1. « arming » : l'engagement des récepteurs inhibiteur avec la molécule HLA de classe I induit l'acquisition des capacités fonctionnelles des cellules NK 2. « disarming » : en l'absence de l'engagement des récepteurs inhibiteurs, les cellules NK deviennent anergiques, désarmées. Les cellules redeviennent réactives suite à l'engagement avec les molécules HLA de classe I. 3. « interaction-cis » : les récepteurs inhibiteurs peuvent se lier aux molécules HLA de classe I sur la même membrane cellulaire et activer les cellules NK. 4. « rhéostat » : les capacités des réponses des cellules NK dépendent de la force du signal inhibiteur (inspiré de Höglund and Brodin 2010).

L'engagement d'un récepteur inhibiteur avec une molécule CMH du soi peut éduquer les cellules NK qui deviennent fonctionnellement compétentes. C'est le modèle du « licensing » ou « arming » (Kim et al. 2005; Yokoyama and Kim 2006). Ce modèle implique qu'une forte capacité de réponse des cellules NK est induite par l'engagement des récepteurs inhibiteurs avec les molécules CMH de classe I du soi au cours de leur maturation. Le non-engagement de récepteurs inhibiteurs au cours du développement induirait ainsi la génération d'une population dit anergique, de faible capacité de réponse : on parle de cellules NK non éduquées (« unlicensed »). Suite à la rencontre avec des cellules présentant un défaut d'expression des molécules CMH de classe I, les cellules NK éduquées ne sont plus sensibles aux signaux inhibiteurs, induisant la cytotoxicité NK et la production de cytokines. Différentes expériences réalisées chez la souris et chez l'homme ont établi le rôle des molécules CMH de classe I rendant les cellules NK fonctionnelles (Liao et al. 1991; Höglund et al. 1991). Les travaux d'Anfossi et ses collaborateurs sont en accord avec ce modèle, montrant que les cellules NK humaines doivent rencontrer les molécules HLA de classe I du soi pour acquérir leurs capacités fonctionnelles et devenir réactives (Anfossi et al. 2006). Ils proposent que l'éducation des cellules NK KIR^+ leur permet sélectivement de discriminer les cellules saines présentant des niveaux d'expression normaux de molécules HLA de classe I de celles présentant une diminution du niveau d'expression des molécules HLA de classe I, faisant ainsi référence à la théorie du « missing self ».

Par la suite, des travaux ont montré l'existence de cellules NK chez la souris et chez l'homme dépourvues de récepteurs inhibiteurs spécifiques des molécules CMH du soi (Fernandez et al. 2005). Ces cellules sont incapables de tuer des cibles CMH de classe I-déficientes comparées aux cellules NK exprimant des récepteurs spécifiques du CMH de classe I du soi. Elles sont qualifiées de désarmées ou anergiques. Kim et ses collaborateurs ont rapporté que les cellules NK de souris déficientes en Ly49C, récepteur inhibiteur spécifique

du CMH du soi de souris B6 de CMH de classe I H2b, sont fonctionnellement altérées tandis que les cellules NK Ly49C$^+$ peuvent répondre à une stimulation par un anticorps anti-NK1.1, dont l'effet est comparable à une stimulation par une cellule tumorale (Kim et al. 2005). Il a ainsi été proposé qu'une stimulation *in vivo* persistante par des signaux activateurs induise l'anergie des cellules NK présentant de très faibles capacités des réponses. C'est le modèle de « disarming » (Fernandez et al. 2005; Raulet and Vance 2006). Ceci suggère que ces cellules NK présentent une forme de tolérance au soi. Ainsi, les NK de souris déficientes en CMH de classe I ne reçoivent pas de signaux inhibiteurs, contrecarrant les signaux activateurs, et sont rendues anergiques. Chez les souris « saines », les cellules NK sont équipées de récepteurs inhibiteurs spécifiques du soi, ce qui assure la tolérance au soi. Le processus de « disarming » implique que l'hyporéactivité est activement induite contrairement au modèle de « licensing » pour lequel la forte capacité de réponse des cellules NK est induite par des interactions avec les molécules CMH de classe I.

Un troisième modèle est basé sur la capacité des récepteurs Ly49 à se lier au CMH de classe I en « cis », c'est-à-dire sur la membrane cellulaire elle-même (Chalifour et al. 2009). Dans ce cas, les interactions entre ligands et récepteurs séquestrent les récepteurs empêchant leur relocalisation au niveau de la synapse immunologique. Ainsi, l'influence des récepteurs inhibiteurs est diminuée et les cellules NK ont de meilleures capacités de réponse. De plus amples études sont nécessaires afin de déterminer si tous les récepteurs NK sont capables de s'engager avec les molécules du CMH de classe I en cis.

La capacité des cellules NK à avoir des capacités des réponses de lyse spontanée diminuées ou augmentées selon la force du signal inhibiteur suggère que le phénomène d'éducation est quantitatif et a donné lieu à un quatrième modèle (Brodin and Höglund 2008; Joncker et al. 2009). Le modèle du rhéostat affirme que les capacités de réponses des cellules NK peuvent être activées (modèle « arming ») ou désactivées (modèle « disarming ») mais de

façon quantitative et non qualitative et binaire selon le signal inhibiteur reçu (Brodin et al. 2009). Ce modèle est cohérent avec les modèles « arming » et « disarming » et pourrait également être compatible d'un point de vue mécanistique avec le modèle d'interaction-cis.

L'importance des récepteurs KIR inhibiteurs dans l'éducation des cellules NK a été bien établie chez l'homme. Notamment, les ligands des récepteurs KIR2DL1/2 (Anfossi et al. 2006), KIR3DL1 (Kim et al. 2008; Yawata et al. 2008a) et NKG2A (Fauriat et al. 2008; Andersson et al. 2009b) doivent être exprimés sur les cellules de l'hôte afin que les cellules NK acquièrent leur fonctionnalité et répondent à la stimulation par la cellule cible. L'influence des KIR activateurs doit également être étudiée pour évaluer leur implication dans la tolérance des cellules NK.

Notre équipe a précédemment mis en évidence que les cellules NK KIR2DS1^{+} sont fonctionnelles en terme de dégranulation et de production d'IFNγ uniquement chez des individus C2^{-}). Le récepteur KIR2DS1 diminue en effet les capacités de réponses des cellules NK envers des cellules cibles chez des donneurs homozygotes pour son ligand HLA-C du groupe C2. Ceci est observé aussi bien chez les cellules NK exprimant exclusivement le KIR2DS1 que celles co-exprimant des récepteurs inhibiteurs KIR2DL3 et NKG2A (Fauriat et al. 2010). Ces résultats suggèrent une modulation de l'éducation des cellules NK via les KIR activateurs qui pourrait être un mécanisme contribuant à la tolérance au soi.

De façon intéressante, le traitement aux cytokines peut partiellement surpasser le statut hyporéactif des cellules NK non éduquées (Fernandez et al. 2005). En effet l'addition d'IL-2 peut notamment rendre les cellules NK non éduquées fonctionnelles (Kim et al. 2005). D'autre part différentes études suggèrent que les cellules NK ont besoin d'un « priming » afin d'optimiser leurs fonctions effectrices. En effet, dans le cadre d'infections virales, différentes cytokines comme les interférons de type I et l'IL-15 sont souvent produites et peuvent stimuler les cellules NK (Biron et al. 1999). De plus, dans certaines infections bactériennes,

l'IL-15 peut être trans-présentée par les cellules dendritiques et peut activer les cellules NK et potentialiser leurs fonctions effectrices (Lucas et al. 2007). De la même façon, il a été montré que les cellules NK éduquées et non éduquées produisent de l'IFNγ dans les mêmes proportions en réponse à l'infection *in vivo* de Listeria monocytogenes (Fernandez et al. 2005). Tarek *et al* ont montré que l'inhibition médiée par les récepteurs KIR inhibiteurs suite à l'interaction avec les molécules HLA de classe I constitue un signal assez fort pour surpasser les signaux délivrés dans le processus de l'éducation (Tarek et al. 2012). Ces observations sont concordantes avec une précédente étude chez la souris montrant que les cellules NK non éduquées sont critiques pour la réponse contre le CMV murin (Orr et al. 2010). Enfin, des cellules NK KIR non éduquées dans leur environnement autologue semblent également alloréactives dans le contexte des greffes de cellules souches hématopoïétiques (Haas et al. 2011). L'ensemble de ces résultats suggèrent que les cellules NK non éduquées pourraient être stimulées dans des conditions de stress comme les infections virales, bactériennes ou certains cancers, et devenir fonctionnellement compétentes indépendamment des molécules CMH de classe I du soi.

5. Cellules NK « mémoires »

L'une des caractéristiques de l'immunité adaptative est la capacité de mémoire immunologique des lymphocytes T et B spécifiques de l'antigène. Cette mémoire est présente d'une part de façon passive par la recirculation d'anticorps et d'autre part, par une immunité active à long terme via l'expansion et la réactivation et lymphocytes T mémoires spécifiques suite à une seconde exposition à l'antigène (Crotty and Ahmed 2004). Plusieurs études ont toutefois montré que cette capacité de mémoire immunologique n'est pas restreinte aux cellules de l'immunité adaptative et que les cellules NK peuvent changer de comportement suite à une activation préalable.

Dans un modèle de souris déficiente en lymphocytes T et B (SCID Rag2$^{-/-}$), O'Leary et ses collaborateurs ont démontré la capacité des cellules NK à médier une réponse d'hypersensibilité au contact des haptènes. D'autre part, un transfert adoptif de cellules NK ayant été en contact avec les haptènes dans des souris naïves induite une réaction d'hypersensibilité suite à l'activation par ces haptènes. Cette réponse était particulièrement observée lors du transfert de cellules NK du foie exprimant les récepteurs Ly49 (O'Leary et al. 2006). Lanier et ses collaborateurs ont également montré l'existence d'une population NK mémoire dans l'infection à CMV chez des souris de souche C57BL/6. Les cellules NK exprimant le récepteur Ly49H, spécifique de la protéine virale m157, prolifèrent jusqu'à 100x dans la rate et 1000x dans le foie après infection à CMV, et persistent plusieurs mois dans l'organisme. Le transfert adoptif de ces cellules NK Ly49H^{+} à une souris naïve, suivi d'une infection virale, induit une forte expansion cellulaire et une protection immunitaire (Sun et al. 2009). Une autre étude a montré que des cellules NK pré-activées in vitro par des cytokines transférées dans des souris naïves sont capables de migrer dans différents organes et prolifèrent dans le foie, la rate et les organes lymphoïdes. Ces cellules présentent des capacités de réponse plus forte après réactivation par ces cytokines et persistent pendant plus d'un mois dans l'organisme (Cooper et al. 2009).

Ces observations ont été confirmées chez l'homme. Une étude a mis en évidence que les cellules NK pré-activées avec des cytokines, suivie par une période de repos, présentent une fonctionnalité augmentée suite à une re-stimulation avec ces mêmes cytokines. Ce phénotype est préservé après division cellulaire, confirmant que cette capacité mémoire est transmise aux cellules progénitrices. Le phénotype mémoire a été corrélé à l'expression de CD94, NKG2A, NKG2C et CD69 et une absence d'expression du CD57 et des KIR chez les CD56dim (Romee et al. 2012). L'ensemble de ces données suggèrent que les cellules NK dites « memory-like » peuvent moduler leur capacité de réponse suite à une activation

préalable et présentent des capacités de mémoire immunologique. D'autres études sont nécessaires afin d'évaluer les propriétés fonctionnelles et d'identifier les caractéristiques phénotypiques de ces cellules afin d'envisager leur utilisation thérapeutique.

6. Fonctionnalité des cellules NK

Les cellules Natural Killer ont initialement été définies par leur capacité à tuer une cellule cible sans nécessité d'immunisation préalable (Kiessling et al. 1975; Herberman et al. 1975), comme nous l'avons décrit dans le paragraphe **2.1 Lyse spontanée**. Contrairement aux lymphocytes T cytotoxiques, les cellules NK n'ont pas besoin de recombinaison somatique de leurs récepteurs pour « détecter » une cellule cible. La fonctionnalité d'une cellule NK est régulée par une balance de signaux positifs et négatifs transmis par de nombreux récepteurs de surface qui seront décrits par la suite.

Les cellules Natural Killer permettent une élimination des cellules infectées par des virus, des cellules tumorales et des cellules allogéniques par cytotoxicité à médiation cellulaire dépendante des anticorps (**paragraphe 2.2 ADCC**), cytotoxicité naturelle (**paragraphe 2.1 Lyse spontanée**), production de cytokines ou encore par interactions cellulaires notamment avec les cellules dendritiques que nous allons décrire dans les paragraphes suivants.

6.1. Cytotoxicité

Les cellules NK stockent des protéines cytotoxiques dans des lysosomes sécrétoires, des organelles exocytiques spécialisées, aussi connues sous le nom de granules lytiques. La reconnaissance d'une cible aberrante va polariser l'exocytose des lysosomes sécrétoires qui relarguent les molécules cytotoxiques au niveau de la synapse immunologique. Les protéines cytotoxiques majeures sont les perforines, les granzymes A et B, et les granulysines. Des

études de knock-out de la perforine chez la souris ont montré que la perte de la perforine supprime la mort cellulaire dépendante des granules (Voskoboinik et al. 2006). Les perforines facilitent l'entrée des granzymes dans le cytoplasme de la cellule en formant des pores dans la membrane plasmique de façon calcium-dépendante (Voskoboinik et al. 2015). En absence de perforine, le granzyme B peut toutefois être introduit dans la cellule en formant un complexe avec le récepteur au mannose-6-phosphate (MPR) (Motyka et al. 2000). Les granzymes induisent la mort de la cellule cible par apoptose principalement par la voie des caspases (Shi et al. 1992; Metkar et al. 2003). Ce processus d'exocytose de granules lytiques est aussi appelé dégranulation. Enfin, les granulysines sont des protéines cytolytiques présentes dans les granules qui agissent contre les tumeurs et les microbes (Stenger et al. 1998).

Les protéines de la famille des LAMP (Lysosomal-Associated Membrane Protein) sont des protéines hautement glycosylées représentant 50% des protéines qui tapissent la membrane des granules cytolytiques, et la protègent de l'action des enzymes lytiques. Les molécules LAMP- 1, ou CD107a, sont exprimées à la surface des cellules NK stimulées avec des cellules dépourvues de molécule HLA classe I, en corrélation avec la sécrétion de cytokines et la lyse de cellules cibles (Alter et al. 2004). Le marqueur CD107a constitue ainsi un marqueur très sensible de dégranulation des cellules NK et sa présence est souvent évaluée lors de tests fonctionnels de l'activité des cellules NK par cytométrie de flux.

L'apoptose médiée par les perforines/granzymes s'avère être est une voie importante utilisée par les cellules NK pour éliminer des cellules infectées par un virus et des cellules tumorales (van den Broek et al. 1995; Trapani and Smyth 2002).

Les cellules NK peuvent également tuer les cellules cibles via l'engagement de ligands des récepteurs de mort appartenant à la famille des TNF (Tumor Necrosis Factor), FASL, TNFα et TRAIL avec leurs récepteurs respectifs (FAS, TNF-R et TRAIL-R) présents sur les cellules cibles induisant la mort par apoptose de ces cellules cibles (Liu et al. 1995). L'étude

de ligands Fas chimériques par J-L Taupin et ses collaborateurs a mis en évidence que le signal apoptotique médié par le ligand Fas dépend également de l'adaptation conformationnelle du récepteur à son ligand. Cette étude a montré le potentiel anti-leucémique de ces ligands Fas chimériques dans un modèle de tumeur (Daburon et al. 2013). Plusieurs études ont mis en évidence le rôle de l'apoptose médiée par TRAIL dans différents systèmes tumoraux (Takeda et al. 2001; Cretney et al. 2002). Notons que l'expression des ligands des récepteurs de mort peut être induite par des cytokines comme l'IL-2, l'IL-15 ou l'IFN de type I (Screpanti et al. 2005). Les mécanismes de cytotoxicité des cellules NK sont illustrés dans la Figure 16.

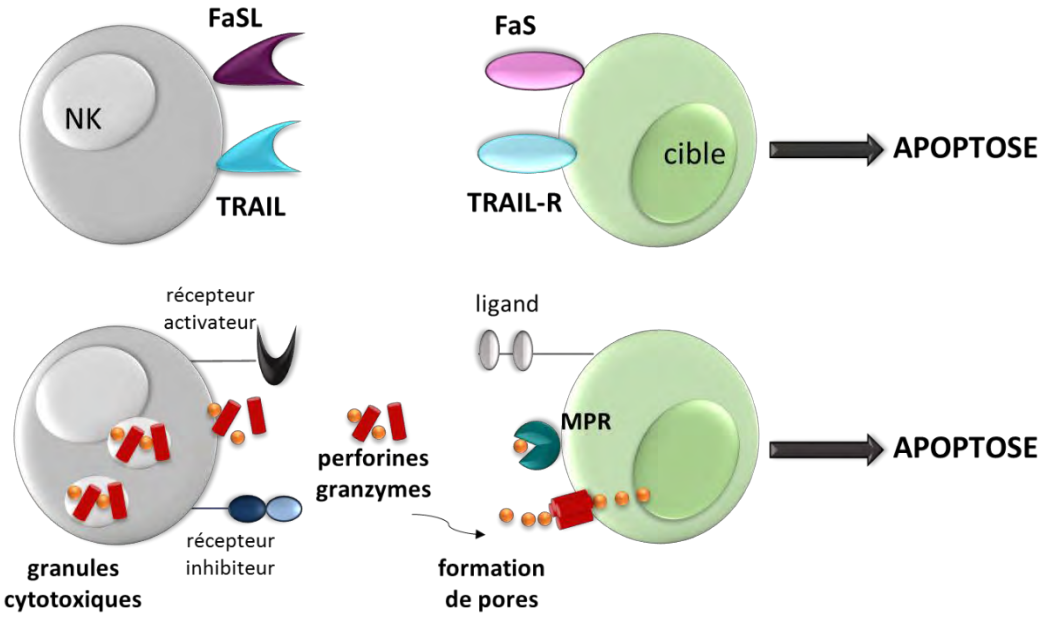

Figure 16 : Cytotoxicité des cellules NK
Les cellules NK peuvent induire la lyse de cellules cibles par plusieurs mécanismes. Elles expriment des molécules comme FaSL et TRAIL, ligands reconnus par leurs récepteurs respectifs FaS et TRAIL-R, des récepteurs de mort exprimés par les cellules cibles qui induit l'apoptose de ces cellules. Les cellules NK peuvent également sécréter des granules cytotoxiques contenant les perforines et granzymes. Après libération dans l'espace intracellulaire, les perforines s'associent pour former des pores permettant le passage des granzymes. Les granzymes peuvent également se lier au récepteur MPR après endocytose. L'entrée des granzymes induit une cascade d'évènements déclenchant l'apoptose des cellules cibles par la voie des caspases.

6.2. Production de cytokines et de chimiokines

En plus de leur capacité majeure de cytotoxicité, les cellules NK peuvent produire des cytokines suite à leur activation. La cytokine majeure produite par les cellules NK est l'IFNγ qui participe à la régulation de la réponse adaptative. En effet, cette cytokine induit la maturation des cellules dendritiques et initient les cellules T adaptives helper de type 1 (Th1), stimulant ainsi les fonctions effectrices des lymphocytes T cytotoxiques (Martín-Fontecha et al. 2004). Les cellules NK peuvent également sécréter différents facteurs proinflammatoires comme le TNFα (tumor necrosis factor), des cytokines immunorégulatrices comme l'IL-5, IL-8, IL-10, IL-13 et des facteurs de croissance comme le GM-CSF (Granulocyte/Macrophage Colony Stimulating Factor), le G-CSF (Granulocyte Colony Stimulating Factor) et l'IL-3 (Cooper et al. 2001).

Suite à leur activation, les cellules NK peuvent également produire des chimiokines comme le CCL3/MIP-1α, le CCL4/MIP-1β, et le CCL5/RANTES. Les chimiokines sont des molécules impliquées dans le recrutement des différents acteurs de l'immunité sur les sites tumoraux et inflammatoires.

6.3. Prolifération

Plusieurs cytokines peuvent stimuler la prolifération des cellules NK : l'IL-15, l'IL-2 et l'IL-12. L'IL-15 est considérée comme la cytokine la plus importante pour l'homéostasie des cellules NK. En effet, elle peut induire la prolifération et la survie des cellules NK matures (Lodolce et al. 1998). Les cellules dendritiques constituent la source majeure d'IL-15 et sont ainsi particulièrement impliquées dans la prolifération des cellules NK (Lucas et al. 2007). L'IL-21 améliore également la prolifération des cellules NK cultivées en présence d'IL-2 et IL-15 (Lim et al. 2014). Toutefois, en l'absence d'IL-15, l'IL-12 peut stimuler la prolifération des cellules NK (Sun et al. 2009). L'IL-2 est majoritairement utilisée i*n vitro*

afin de cultiver les cellules NK et assurer leur expansion. Il est également possible de réaliser la co-culture de cellules NK avec des lignées lymphocytaires immortalisées comme les lignées B-EBV et également des lignées tumorales (Luhm et al. 2002; Clémenceau et al. 2006). L'IL-21 est également impliquée dans l'expansion des cellules NK (Parrish-Novak et al. 2000). Récemment, l'IL-21 a été utilisée avec le 4-1BBL pour amplifier les cellules NK à partir de cellule mononucléées du sang périphérique (PBMC) de donneurs sains, proposant une stratégie de prolifération ne nécessitant par l'introduction de cellules stimulatrices ou la préalable purification des cellules NK (Li et al. 2015).

7. Cellules NK et infections virales

Les cellules NK jouent également un rôle important dans la défense de l'organisme contre les infections virales. Ce rôle a initialement été mis en évidence chez l'homme par l'étude d'un patient déficient en cellules NK et présentant une infection à herpès virus malgré la présence de lymphocytes T et B (Biron et al. 1989). Les cellules NK expriment différents récepteurs qui vont être impliqués dans la reconnaissance des virus. Dans les paragraphes suivants, nous allons présenter certaines infections virales dans lesquelles les cellules NK sont impliquées comme le virus de l'hépatite C (VHC), le virus de l'immunodéficience humaine (VIH) et le cytomégalovirus (CMV).

7.1. Cellules NK et Virus de l'Hépatite C (VHC)

Les cellules NK défendent l'organisme contre les infections virales telles que l'hépatite C. Le virus de l'hépatite C (VHC) est une infection mondiale commune, pouvant entraîner à la fois une infection aiguë et une infection chronique dont la gravité est variable. L'infection chronique peut évoluer vers la cirrhose ou le carcinome hépatocellulaire. Il n'existe pas de vaccin contre l'hépatite C à ce jour. La fréquence des cellules NK est plus

élevée dans le foie par rapport au sang périphérique dans le cadre des infections chroniques du virus de l'hépatite C et de l'hépatite B (Bonorino et al. 2009). L'étude menée par S.Khakoo a montré une association entre l'homozygotie HLA-C2 et la persistance virale. De plus, le gène KIR3DS1 a un faible effet protecteur en combinaison avec certaines molécules Bw4. Au contraire, la présence du gène KIR2DL3 et des molécules HLA-C1 influence de façon positive la résolution de l'infection à VHC (Khakoo et al. 2004). Khakoo et ses collaborateurs ont en effet montré que la présence du gène KIR2DL3 et de son ligand HLA-C1 est impliquée dans l'élimination du virus. D'autre part, l'interaction KIR2DL3/HLA-C1 serait plus protectrice que l'interaction KIR2DL2/HLA-C1 dans l'infection à VHC. Ces données suggèrent que certaines interactions inhibitrices KIR-HLA sont importantes pour le contrôle de l'immunité antivirale et que des réponses inhibitrices confèrent une protection contre le VHC. Le rôle possible des cellules NK dans l'immunobiologie du VHC est supporté par des études plus récentes mettant en évidence l'activation des cellules NK chez les sujets atteints d'infection VHC aigue associée à l'augmentation de l'expression du récepteur NKG2D et de la production d'IFNγ (Amadei et al. 2010), ainsi que la dégranulation et l'expression de TRAIL augmentée chez les patients atteints de VHC chronique (Oliviero et al. 2009).

7.2. Cellules NK et Virus de l'Immunodéficience Humaine (VIH)

Plusieurs études génétiques et fonctionnelles ont associé certains allotypes HLA et KIR avec une diminution de la progression du Virus de l'Immunodéficience Humaine (VIH). Les individus homozygotes pour le motif Bw4 sont plus résistants face au développement du syndrome d'immunodéficience acquise (SIDA) (Flores-Villanueva et al. 2001). Martin et ses collaborateurs ont conduit une étude sur plus de 1000 patients nord-américains infectés par le VIH mettant en évidence que la présence simultanée du gène KIR3DS1 et des allèles HLA Bw480I est associé à une progression retardée du SIDA (Martin et al. 2002a). En l'absence

des allèles HLA Bw4-80Ile, la seule présence du gène KIR3DS1 est plutôt associée à une aggravation de la pathologie, ce qui suggère une interaction épistatique entre les deux. Le mécanisme sous-jacent pourrait correspondre à la reconnaissance directe par le récepteur activateur KIR3DS1 de molécules HLA-Bw4 présentant des peptides viraux dérivés du VIH. L'étude des groupes d'allèles KIR3DL1 a montré un effet protecteur de certains allèles sur la progression de la maladie et la charge virale, en particulier le KIR3DL1*004. Ce résultat est d'autant plus étonnant que cet allèle code pour une protéine présentant une rétention intracellulaire, suggérant toutefois un réel rôle fonctionnel de ce récepteur. Cette étude suggère également que la présence du gène inhibiteur KIR3DL1 avec le ligand Bw4 associé chez les individus infectés par le VIH assure une protection contre la progression du SIDA, notamment les allèles KIR3DL1 fortement exprimés (high) avec les molécules HLA-Bw4I80. Cependant, des données ont montré que les cellules NK KIR induisent une pression immunologique sur le VIH et que le virus peut échapper à cette pression immune en sélectionnant des polymorphismes de séquence. On observe ainsi un polymorphisme des acides aminés associés aux KIR dans la séquence du VIH-1 d'individus atteints d'infection chronique, réduisant ainsi l'activité antivirale des cellules NK KIR^+ (Alter et al. 2011).

7.3. Cellules NK et co-infection VIH/VHC

Il est intéressant de noter qu'approximativement 15 à 30% des patients atteints de VIH présentent une co-infection VHC. La co-infection VIH/VHC induit une progression plus rapide de la fibrose du foie comparé à la simple infection VHC (Mohsen et al. 2003; Monforte et al. 2008). En effet, les pathologies du foie liées à l'infection VHC sont devenues la cause majeure de mortalité chez les individus infectés par le VIH. Un intérêt grandissant est porté aux cellules NK qui présentent une activité anti-fibrotique par l'induction de l'apoptose des cellules hépatiques activées HSC (hepatic stellate cells) (Radaeva et al. 2006). Cette fonction

anti-fibrotique est médiée par le récepteur activateur NKG2D et les ligands de mort TRAIL et FAS-L (Glässner et al. 2012). Les cellules NK d'individus co-infectés VIH /VHC peuvent toutefois présenter une absence d'expression du récepteur CD56 et des fonctions altérées (Gonzalez et al. 2008). Plusieurs études ont mis en évidence que les lymphocytes T $CD4^+$ stimulent l'activité anti-fibrotique des cellules NK (Fehniger et al. 2003). Une dérégulation des fonctions des cellules T $CD4^+$ a été observée chez des patients co-infectés VIH/VHC, pouvant résulter en l'accélération de la progression de la fibrose (Glässner et al. 2013).

7.4. Cellules NK et Cytomégalovirus (CMV)

Le cytomégalovirus est un virus de la famille des herpès virus qui n'a généralement pas d'implication clinique majeure chez les individus sains immunocompétents. Toutefois, il est responsable d'une forte morbidité et mortalité chez les individus dits immunodéficients ou immunodéprimés, en particulier après greffes de cellules souches. La réplication du CMV est contrôlée par les cellules NK et les cellules T. Le CMV présente cependant de nombreux mécanismes d'échappement aux cellules T, impliquant différentes protéines virales permettant d'inhiber l'expression des molécules HLA de classe I à la surface des cellules infectées par le virus. Ces cellules sont ainsi rendues plus susceptibles à la lyse NK. Toutefois, différents mécanismes d'échappement du CMV à la lyse des cellules NK ont également été identifiés. Le virus peut d'une part inhiber l'expression des ligands pour certains récepteurs activateurs NK comme MIC-A (ligand du NKG2D) via la protéine gpUL142 (Chalupny et al. 2006) ou les CD112 (nectin-2) et CD155 (PVR), (ligands de DNAM-1 et Tactile) via la protéine gpUL141 (Tomasec et al. 2005; Prod'homme et al. 2010; Magri et al. 2011). La protéine gpUL16 peut également reconnaitre les ligands MIC-B, ULBP1, ULBP2, et ULBP5 afin de bloquer leur interaction avec le récepteur NKG2D (Rölle et al. 2003). Le virus induit l'expression de la protéine gpUL18, homologue des molécules

HLA de classe I, qui se lie au récepteur inhibiteur ILT2 assurant l'inhibition de la lyse NK (Prod'homme et al. 2007). D'autre part, le CMV code une autre glycoprotéine gpUL40 dont le peptide signal se lie à la molécule HLA-E permettant de maintenir son expression à la surface de la cellule. La molécule HLA-E est le ligand du complexe CD94/NKG2A, ainsi les cellules NK NKG2A$^+$ sont inhibées par la liaison avec le HLA-E protégeant ainsi les fibroblastes infectés de la lyse NK (Tomasec et al. 2000; Ulbrecht et al. 2000; Wang et al. 2002). Enfin, la protéine pp65 du tégument du CMV peut se fixer au NKp30 et inhiber la transduction du signal activateur (Arnon et al. 2005).

Différents travaux ont décrit l'expansion de cellules NK NKG2C$^+$ chez des individus CMV séropositifs (Béziat et al. 2012; Foley et al. 2012; Béziat et al. 2013a). L'équipe de Lopez Botèt a mis en évidence une amplification de la population NK NKG2C$^+$ KIR$^+$ chez des individus CMV$^+$, indépendamment du génotype KIR ou de la sérologie positive pour d'autres herpès virus (EBV, HSV-1). Au contraire, chez les individus CMV séronégatifs, la fréquence de cellules NK NKG2C$^+$ est faible (Gumá et al. 2004). Récemment, notre équipe a montré que l'expression des récepteurs KIR2D sur les cellules NK est indispensable pour une meilleure dégranulation et production d'IFNγ au contact de cellules dendritiques infectées par le CMV (Djaoud et al. 2013).

8. Cellules NK et maladies auto-immunes

Dans le cadre de la réponse immune innée, différents médiateurs inflammatoires sont sécrétés dont l'IL-1, IL-6, IL-12, Il-18 et le TNF. Ces molécules peuvent induire le développement d'une auto-immunité destructrice due à une activation inappropriée de cellules immunitaires. Les maladies auto-immunes comme la sclérose multiple, l'arthrite rhumatoïde et le diabète de type 1 sont caractérisées par une inflammation chronique, la destruction d'un tissu et le dysfonctionnement des organes correspondants. Elles sont généralement causées

par une activation inappropriée de cellules T ou B autoréactives résultant en l'altération d'un tissu, d'un organe ou d'un type cellulaire. Les maladies auto-immunes sont initiées par étapes, incluant le relargage d'antigènes du soi dans l'organe cible, l'activation dans les organes secondaires et le retour dans l'organe cible avec la destruction du tissu. Outre leur potentiel anti-viral et anti-leucémique, les cellules NK sont fréquemment présentes durant la progression de maladies autoimmunes. Les cellules NK peuvent ainsi agir à différents niveaux. Différentes études ont mis en évidence le rôle protecteur ou promoteur des cellules NK dans l'auto-immunité selon le modèle étudié. Takahashi et ses collaborateurs ont suggéré un rôle protecteur des cellules NK chez les patients atteints de sclérose multiple, présentant une forte expression de Fas et semblent réguler l'expression d'IFNγ des lymphocytes T (Takahashi et al. 2004). L'équipe de Zhang a également montré le rôle protecteur des NK dans l'encéphalomyélite autoimmune, leur déplétion aggravant la pathologie (Zhang et al. 1997). Au vue de leurs capacités de production d'IFNγ, un effet promoteur des cellules NK sur l'autoimmunité semble également probable. Nous avons précédemment cité le rôle des cellules NK et plus précisément du récepteur NKp46 dans le développement du diabète de type 1 (Gur et al. 2010). Notons également que l'augmentation des combinaisons KIR2DS2/ligand combinée à une diminution des combinaisons KIR inhibiteurs/ligand est associée à un risque additionnel de développer la maladie (van der Slik et al. 2003). Différentes associations ont en effet été identifiées entre le risque de développer une maladie autoimmune et les génotypes KIR/HLA. La sclérodermie a été associée avec la présence du gène KIR2DS2 et à l'absence de son homologue inhibiteur KIR2DL2 (Momot et al. 2004). Martin et ses collaborateurs ont corrélé la présence des gènes activateurs KIR2DS1 et KIR2DS2 et la susceptibilité à l'arthrite psoriasique (PsA) (Martin et al. 2002b). Les patients ayant une déficience pour la protéine TAP (transporter-associated antigen processing), impliquée dans l'assemblage des molécules HLA, présentent une forte diminution de

l'expression des molécules HLA de classe I, les rendant susceptibles à la lyse par les cellules NK (de la Salle et al. 1994; de la Salle et al. 1999). Toutefois, aucun phénomène d'auto-immunité médiée par les cellules NK n'est observé chez ces patients. En effet, les cellules TAP-déficientes sont tuées par des cellules NK TAP^+ mais pas par des cellules NK autologues (Vitale et al. 2002). Les cellules des individus TAP déficients ont développé des mécanismes pour compenser le manque d'inhibition médiée par les molécules HLA de classe I et prévenir la lyse des cellules autologues. Néanmoins, les cellules NK d'individus TAP déficients sont capables d'ADCC et sont cytotoxiques après stimulation par de l'IL-2 (Zimmer et al. 1998).

9. Cellules NK et cancer

L'activité anti-tumorale des cellules NK a été mise en évidence au cours d'expériences ayant pour objectif d'évaluer les capacités de lymphocytes T à tuer des cibles tumorales contre lesquelles les souris avaient été préalablement immunisées (Kiessling et al. 1975). En effet, les cellules NK déterminent le rejet d'une grande variété de tumeurs dans des modèles murins et participent à la surveillance tumorale. La diminution de l'expression des molécules HLA de classe I par les cellules tumorales implique également une réponse anti-tumorale des cellules NK (Verheyden et al. 2009). La fonction d'ADCC des cellules NK est très utilisée en immunothérapie pour traiter différents types de cancers, seule ou associée à d'autres approches basées sur l'utilisation d'anticorps spécifiques d'une tumeur.

Différentes études ont décrit des génotypes KIR et HLA associés à une susceptibilité à certains cancers comme le mélanome (Naumova et al. 2005), les leucémies (Verheyden et al. 2004) ou le lymphome d'Hodgkin (Besson et al. 2007). Une large étude génétique des KIR et des ligands HLA chez des enfants atteints de leucémie aigüe lymphoïde (LAL) a par exemple montré l'association de l'homozygotie HLA-Bw4 avec l'augmentation du risque de la

maladie et a suggéré une variation selon les groupes ethniques. La fréquence des génotypes KIR AA est également augmentée chez ces patients (de Smith et al. 2014). Un effet protecteur du gène KIR3DS1 associé aux molécules $Bw4^{80Ile}$ contre le développement du carcinome hépatocellulaire a également été identifié (López-Vázquez et al. 2005). L'étude des génotypes KIR et HLA peut être utilisée pour identifier la susceptibilité des patients à la récurrence de cette maladie (Tanimine et al. 2014). Le rôle potentiel des cellules NK dans la surveillance du carcinome hépatocellulaire a également été suggéré, et l'utilisation de leurs capacités cytotoxiques pourrait améliorer le pronostic de ces patients (Tanimine and Ohdan 2015).

A travers la description du panel de récepteurs exprimés par les cellules NK, nous avons précédemment cité l'importance des récepteurs activateurs NKG2D, DNAM-1 et des NCR dans l'effet anti-leucémique médié par les cellules NK (Pende et al. 2001; Pende et al. 2005a). Ces récepteurs reconnaissent des ligands peu exprimés par les cellules saines mais surexprimés par les cellules tumorales. L'élimination des cellules tumorales exprimant les ligands du NKG2D de façon NKG2D-dépendante a été documentée *in vitro* et dans des expériences de transplantation de cellules tumorales (Pende et al. 2001). Un mécanisme d'échappement des cellules tumorales à la lyse NK $NKG2D^{+}$ a été identifié. Les cellules tumorales produisent une molécule MIC-A soluble qui induit la sous-expression du récepteur NKG2D des cellules T circulantes infiltrant les tumeurs, impactant la réponse T anti-tumorale (Groh et al. 2002). Plusieurs groupes ont confirmé que le récepteur NKp46 est impliqué dans la reconnaissance et la lyse de cellules tumorales par les cellules NK comme les cellules de mélanome (Lakshmikanth et al. 2009), de carcinome (Glasner et al. 2012), de neuroblastome (Sivori et al. 2000) et les cellules leucémiques (Nowbakht et al. 2005). Les capacités anti-tumorales du NKp46 ont été utilisées pour développer un récepteur chimérique basé sur le NKp46, rétroviralement exprimé par des lymphocytes T, assurant la lyse de diverses cibles tumorales (Tal et al. 2014). Le rôle du récepteur NKp44 dans la lyse de cellules tumorales a

été démontré par des études de blocage à l'aide d'anticorps spécifiques (Moretta et al. 2001; Koch et al. 2013). La reconnaissance des cellules tumorales est partiellement médiée par la liaison aux protéoglycanes héparane sulfate (HSPC) (Hershkovitz et al. 2007; Hecht et al. 2009). La molécule CD15, ligand du récepteur DNAM-1, est particulièrement surexprimée dans le cas de pathologies malignes. Le blocage des interactions de DNAM-1 avec ses ligands réduit les capacités des cellules NK à tuer les cellules tumorales *in vitro* (Pende et al. 2005a). L'implication de DNAM-1 dans la surveillance tumorale a été mise en évidence chez des souris déficientes en DNAM-1 (Iguchi-Manaka et al. 2008) ainsi que son rôle dans le contrôle des métastases tumorales (Chan et al. 2010). Des études à partir de modèles de cancer *in vivo* ont mis en évidence l'expansion de tumeurs en absence de DNAM-1 (Lakshmikanth et al. 2009). Enfin, l'importance de DNAM-1 dans l'immunosurveillance du myélome multiple a également été démontrée grâce au développement d'un modèle murin de myélome multiple (Guillerey et al. 2015), pouvant être la base de nouvelles stratégies thérapeutiques.

III. Les greffes de cellules souches hématopoïétiques

Les greffes de cellules souches hématopoïétiques (CSH) constituent l'une des immunothérapies les plus utilisées dans le monde. Elles visent principalement à rétablir la fonction hématopoïétique chez des patients présentant une lésion ou un dysfonctionnement du système hématopoïétique.

Dans cette partie, nous allons présenter les greffes de CSH, expliciter le rôle des molécules HLA dans la sélection des donneurs et les conditionnements utilisés. Nous présenterons ensuite les réactions allogéniques entre hôte, greffon et cellules leucémiques, introduirons les complications pouvant être rencontrées après greffes et détaillerons le rôle des cellules NK en greffes de CSH. Nous accorderons une attention particulière à un « type »

de greffe, la greffe de sang de cordon, qui a fait l'objet de mes travaux de thèse et présenterons enfin les immunothérapies cellulaires T et NK utilisées en greffes de CSH.

Les greffes de CSH sont en particulier utilisées pour remplacer le système immunitaire de patients atteints de maladies hématologiques malignes comme les leucémies, les lymphomes et les myélomes. La première greffe de CSH a été rapportée par Donnall Thomas en 1957 (Thomas et al. 1957) qui fut récompensé pour ses travaux par le prix Nobel de physiologie et médecine en 1990. Les traitements de chimiothérapie et radiothérapie utilisés pour éliminer les cellules cancéreuses sont très agressifs et détruisent également les cellules immunitaires et hématopoïétiques. Ainsi, l'objectif des greffes de CSH est de permettre l'administration de doses élevées de chimiothérapie potentiellement curatives tout en assurant l'hématopoïèse du patient. Chez les patients atteints d'une maladie non maligne comme l'anémie aplasique sévère, l'anémie de Fanconi, la thalassémie ou la drépanocytose, le but est de remplacer une moelle osseuse défectueuse ou défaillante.

Les greffes de CSH peuvent également être utilisées pour induire la tolérance en greffes de tissu ou d'organes (Billingham et al. 1953; Ildstad and Sachs 1984; Sachs et al. 2014). En effet, l'injection de cellules souches est utilisée pour induire un état de tolérance et éviter le rejet de l'organe transplanté comme le rein (Kawai et al. 2008; Scandling et al. 2008).

1. Compatibilité HLA et sélection d'un donneur de CSH

Selon la source de CSH, les greffes peuvent être catégorisées en greffe autologue, si le donneur est le patient lui-même, ou allogénique, si le donneur est un autre individu de fond génétique HLA différent, apparenté ou non apparenté. Il y a 3 sources majeures de CSH : la moelle osseuse, le sang périphérique et le sang du cordon ombilical (Figure 17).

Les cellules souches médullaires proviennent de la moelle osseuse contenue dans les os du sternum, du crâne, des hanches, des côtes et de la colonne vertébrale. A l'état physiologique, la concentration sanguine en cellules souches est très faible. Toutefois, l'utilisation de facteurs de croissance comme le G-CSF permet la mobilisation des cellules souches médullaires, c'est-à-dire leur passage de la moelle osseuse au sang périphérique. Les cellules sont ensuite prélevées par aphérèse et peuvent être utilisées dans le cadre d'une greffe de cellules souches périphériques (CSP). Enfin, le sang de cordon contient également des CSH, en plus faible quantité, et peut être prélevé à la naissance pour une future greffe de CSH dite greffe de sang de cordon (UCBT) (Figure 17).

La rareté des CSH peut être une limitation en greffes de CSH, en particulier en greffes de sang de cordon (Ballen et al. 2013). La meilleure connaissance de la biologie des CSH devrait permettre d'améliorer l'efficacité des greffes en augmentant le nombre de CSH et leur fonction. En particulier, différentes études ont montré que les CSH et les progéniteurs hématopoïétiques dans la moelle osseuse et le sang de cordon prolifèrent dans des conditions hypoxiques avec seulement 3% de dioxygène (Bradley et al. 1978; Danet et al. 2003) alors que les CSH sont collectées à l'air ambiant soit environ 20% de dioxygène. La conservation des CSH dans des conditions physiologiques hypoxiques semble maintenir quiescence des CSH et favoriser leur prolifération (Ivanovic 2009; Vlaski et al. 2014; Mantel et al. 2015). Ces conditions devraient être considérées pouvant être bénéfique au devenir des greffes de CSH.

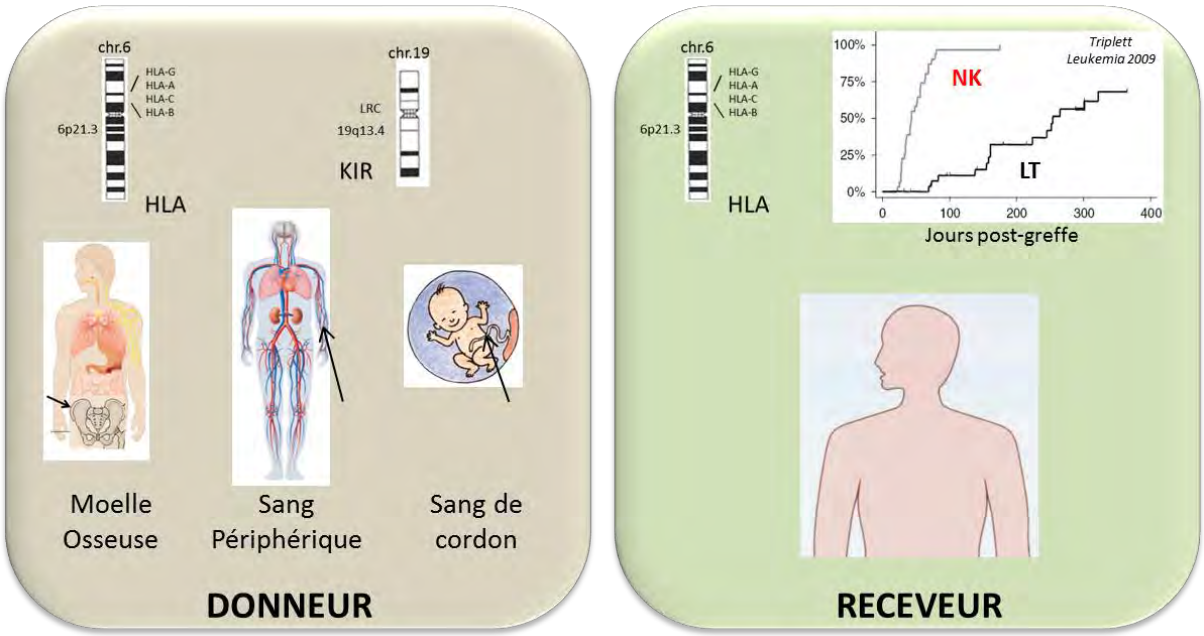

Figure 17 : Greffes de cellules souches hématopoïétiques
Il existe 3 sources majeures de CSH : la moelle osseuse, le sang périphérique et le sang de cordon. Le choix du donneur est basé sur la compatibilité HLA, le meilleur donneur étant un donneur compatible HLA 10/10 pour les loci HLA-A, -B, -C, -DRB1 et -DQB1 avec le receveur. Les cellules NK sont les premières populations à reconstituer l'hématopoïèse après greffes de CSH

Les molécules HLA ont un rôle déterminant dans le devenir des greffes de CSH. Dans le cas d'une greffe allogénique, il est nécessaire de trouver un donneur dit compatible pour les gènes HLA afin de limiter l'alloréactivité des cellules T (Figure 17). En effet, il a été mis en évidence que certaines incompatibilités HLA entre donneur et receveur augmentent les risques d'échec de la greffe De plus, le risque de complications après greffes de CSH augmente avec le nombre d'incompatibilités HLA (Petersdorf et al. 2004; Lee et al. 2007; Petersdorf 2015).

Le système HLA est le complexe génique le plus polymorphe du génome humain. A ce jour, plus de 13000 variants alléliques différents ont été identifiés (source base de données IPD HLA 2015), ce qui traduit la grande diversité du système HLA. Les premières techniques de typage des molécules HLA exprimées en surface (sérologiques) sont vite devenues limitées pour couvrir cette diversité. Les techniques de typages de biologie moléculaire basées sur

l'amplification de séquences polymorphiques d'ADN par PCR se sont ensuite développées et ont permis de palier à ces limites techniques. Le typage des gènes HLA dit « high resolution » discrimine les allèles dans chaque sérotype (exemple HLA-A*02 :01) permettant ainsi de lever les ambiguïtés. L'amélioration des techniques de typages a ainsi permis d'évaluer l'impact des incompatibilités HLA alléliques sur le devenir de la greffe (Petersdorf et al. 2001).

Une étude Japonaise réalisée sur une large cohorte de 1298 greffes a notamment montré que la présence d'une seule incompatibilité allélique HLA-A, -B, -C, -DRB1 ou -DQB1 constitue un facteur de risque de GvHD. De plus, la présence de plusieurs incompatibilités alléliques (HLA-A, -B et/ou -C) est corrélée à une diminution de la prise de greffe et les incompatibilités HLA-A et HLA-B induisent une diminution de la survie globale (Morishima et al. 2002).

L'impact des incompatibilités HLA-A, -B, -C, DRB et DQB1 alléliques sur la survie, la GvHD aigüe et la rechute a également été évalué sur une cohorte française de 334 patients après greffe de moelle osseuse non T déplétée de donneur non apparenté montrant que la présence de plusieurs incompatibilités impacte négativement la survie et la GvHD aigüe sévère (Loiseau et al. 2007).

Une incompatibilité HLA-C entre patient et donneur semble particulièrement corrélée à une forte incidence de la GvHD aïgue et à une augmentation de la mortalité après HSCT (Woolfrey et al. 2011). Plus récemment, l'étude de l'intensité moyenne de fluorescence des allotypes HLA-C chez des patients présentant une incompatibilité HLA-C avec le donneur après HSCT non apparentée a été corrélée au devenir de la greffe. Une plus forte expression HLA-C est associée à une augmentation de l'incidence de la GvHD aigue et de la mortalité, confirmant que certaines incompatibilités HLA-C doivent être évitées lors de la sélection du donneur (Petersdorf et al. 2014).

En greffe de moelle osseuse ou de CSP non apparentées, un donneur présentant une compatibilité HLA 10/10 au niveau allélique avec le receveur pour les loci HLA-A, -B, -C, -DRB1 et -DQB1 sera préférentiellement choisi. En absence d'un donneur compatible HLA 10/10, un donneur compatible HLA 9/10 peut être sélectionné, les incompatibilités HLA-DQB1 n'ayant pas d'impact sur le devenir de la greffe non apparentée (Flomenberg et al. 2004; Lee et al. 2007).

Seulement 30% des patients en attente de greffe de CSH trouvent un donneur familial génotypiquement HLA identique (Ballen et al. 2008). Pour les 70% de patients restants, un donneur non apparenté va être recherché dans les registres nationaux et internationaux de donneurs de cellules souches hématopoïétiques. Les greffes de CSH constituent un traitement pour différentes pathologies malignes dont 36.2% de leucémies aigües myéloïdes (LAM), 12.8% de leucémies aigües lymphoïdes (LAL), 9.8% de myélodysplasies et 1.8% de leucémies myéloïdes chroniques (LMC) selon le dernier rapport de l'agence de biomédecine de 2013. En France, 1872 greffes allogéniques ont été réalisées en 2013 avec 1090 greffes d'origine non apparentées, dont 25.2% de greffes de MO, 64.6% de greffes de CSP et 10.2% de greffes de sang de cordon. A Nantes, 35 greffes apparentées et 44 greffes non apparentées ont été réalisées dont 7 greffes de MO, 26 greffes de CSP et 11 greffes de sang de cordon. Les 3 sources de cellules souches hématopoïétiques restent complémentaires et aucune source n'est abandonnée à l'heure actuelle bien que l'utilisation des greffes de sang de cordon soit en légère diminution ces dernières années.

Dans le cas des greffes HLA haploidentiques, le donneur est un membre de la famille partageant un haplotype HLA avec le receveur, ainsi partiellement compatible. L'utilisation d'un donneur haploidentique présente plusieurs avantages comme la possibilité de sélectionner le meilleur donneur sur la base de l'âge, du sexe et du statut infectieux, ainsi que l'accès au donneur en cas d'échec de la greffe ou pour mettre en place une thérapie cellulaire

après greffe. Cette stratégie de greffe est longtemps restée associée à une forte mortalité non liée à la rechute et maladie du greffon contre l'hôte, malgré les améliorations apportées par la déplétion en cellules T et l'infusion de très fortes de doses de CSH $CD34^+$ (Aversa et al. 2012; Velardi 2013). A l'heure actuelle, les cliniciens reconsidèrent les greffes haploidentiques grâce à la mise en place de nouvelles stratégies comme la non-déplétion des lymphocytes T associée à de fortes doses de cyclophosphamide après greffe. Ceci permet de préserver les potentielles cellules effectrices contenues dans le greffon tout en réduisant considérablement l'incidence et la sévérité des réactions du greffon contre l'hôte (Bolaños-Meade et al. 2012; Luznik et al. 2012).

Dans le cadre de ma thèse, je me suis plus particulièrement intéressée aux greffes de sang de cordon, qui seront présentées dans le paragraphe « 5.Les greffes de sang de cordon ».

2. Conditionnements

Dans les jours qui précèdent une greffe de CSH allogénique, le patient reçoit un traitement immunosuppresseur pour empêcher le rejet de greffe, éliminer les cellules malignes et créer une niche de CSH dans la moelle osseuse permettant la prise de la greffe. Afin d'éliminer les cellules cancéreuses, les patients peuvent recevoir de larges doses de chimiothérapie (cyclophosphamide, Endoxan^{TM}, sérum anti-lymphocytaire (ATG), busulfan) et/ou une irradiation corporelle totale à haute dose (8-10 Gray) (TBI). Cependant, ces traitements sont très agressifs et les cellules saines de la moelle osseuse du patient sont également détruites. Dans ce cas, le conditionnement a un effet dit myéloablatif, d'où son appellation « conditionnement myéloablatif ».

Afin de diminuer la toxicité liée à ce conditionnement, les greffes avec un conditionnement dit réduit ou atténué, sans myéloablation, ont été mises en place et constituent à ce jour plus de la moitié des greffes allogéniques. En effet, 59.1% des allogreffes de CSH sont réalisées

après un conditionnement d'intensité réduite en 2013, selon le rapport l'ABM. Dans le cas de conditionnement réduit, sont administrés essentiellement des agents chimiothérapeutiques comme la fludarabine, pouvant être associée à une autre molécule (busulfan, aracytine) ou à une irradiation corporelle à faible dose (2-3 gray). L'objectif est d'une part la création d'une chimère hématopoïétique, un état de tolérance où coexistent les cellules du donneur et du receveur, assurant l'effet immunologique du greffon contre les cellules tumorales du patient et d'autre part d'induire un état d'immunosuppression qui permet au patient de supporter la greffe en minimisant le risque de rejet.

Le devenir des greffes de CSH est évalué en prenant en compte différents facteurs : la survie globale (OS), la rechute, la mortalité liée à la greffe (TRM), la mortalité non liée à la rechute (NRM), la maladie du greffon contre l'hôte (GvHD), qui sera décrite ultérieurement, la survenue d'infections virales et la prise de greffe .

3. Détermination de la prise de greffe

La prise de greffe est évaluée par la reconstitution des neutrophiles et des plaquettes associée à l'analyse du chimérisme donneur/receveur. La prise totale de greffe est considérée si le patient est sorti de neutropénie et présente plus de 90% des cellules du donneur. Un chimérisme mixte ou partiel est mis en évidence par la présence de cellules du patient et du donneur. Enfin, la reconstitution des cellules du patient en absence des cellules du donneur met en évidence la non-prise de la greffe.

La détermination du statut du chimérisme après greffes de CSH est réalisée par l'évaluation de la présence de marqueurs polymorphes. L'amplification des marqueurs VNTR (Variable number tandem repeats) ou STR (short tandem repeats) qui sont des régions hautement polymorphiques a longtemps été considéréaprèse comme la méthode standard d'analyse du chimérisme (Lion et al. 2001; Chalandon et al. 2003). D'autres méthodes ont été développées

basées sur l'amplification de SNP (single nucleotid polymorphism) par PCR quantitative en temps réel et ont amélioré la sensibilité de ces techniques (Alizadeh et al. 2002; Maas et al. 2003). Ces variants bi-alléliques ne diffèrent que par un simple nucléotide. L'utilisation d'une méthode précise est de grande importance pour détecter l'échec de la prise de la greffe ou un chimérisme mixte, qui est notamment associé à une augmentation de l'incidence de la rechute (Barrios et al. 2003; Lamba et al. 2004). Dans le cadre de mon projet, j'ai évalué l'utilisation des gènes KIR comme marqueurs du chimérisme hématopoïétique après greffes de sang de cordon et présenterai ces données ultérieurement dans la partie **Résultats.**

4. Les réactions allogéniques

Les greffes de CSH impliquent le transfert des cellules du donneur à un receveur et engendrent naturellement des réactions allogéniques des cellules du greffon dirigées contre les cellules de l'hôte d'une part et contre les cellules leucémiques d'autre part. Nous allons présenter ces réactions et leurs acteurs et présenter brièvement les complications post greffes dans les paragraphes suivants comme illustré dans la figure 18.

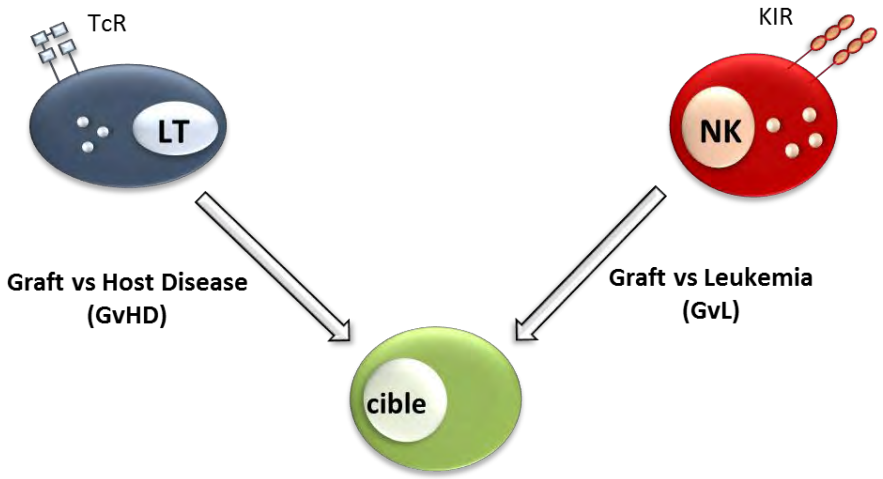

Figure 18 : Réactions allogéniques T et NK
Après greffes de CSH, les cellules T sont responsables de la réaction du greffon contre l'hôte (GvHD) et les cellules NK peuvent médier l'effet anti-leucémique (réaction du greffon contre la leucémie, GvL)

4.1. Réaction du greffon contre l'hôte (GvHD)

Le greffon de CSH est riche en cellules allogéniques T immunocompétentes qui peuvent identifier les cellules et tissus du receveur comme étrangers : on parle de la maladie du greffon contre l'hôte ou GvHD (Graft-versus-Host Disease) (Krenger et al. 1997). En effet, les cellules T reconnaissent les antigènes du receveur comme étranger ce qui induit leur activation et la destruction tissulaire par des cytokines inflammatoires (TNF, IL-1), les perforines/granzymes ou encore la voie des Fas/FasL. Ainsi, les disparités des gènes HLA entre le donneur et le receveur sont un important facteur de risque. Les organes principalement ciblés sont la peau, le foie et le tube digestif. Les manifestations et la sévérité de la GvHD sont très variables et sont influencées par les proportions de cellules régulatrices T dont la maturation dépend des cytokines de l'environnement (Henden and Hill 2015). Les patients ayant reçu une greffe de CSH peuvent développer une GvHD aigue ou une GvHD chronique. La GvHD aigue survient dans les 3 premiers mois post-greffe et présente des manifestations cutanées, hépatiques et digestives. Selon la sévérité, la GvHD aigüe peut être classée en 4 grades (I à IV). La GvHD chronique survient au-delà de 100 jours après greffe. Les receveurs présentant une GvHD aiguë ont d'avantage de risque de développer une GvHD chronique. Elle se présente sous forme limitée ou extensive. Elle peut affecter presque n'importe quelle partie du corps et peut causer des dommages permanents à un organe. Elle entraîne la persistance d'un déficit immunitaire responsable d'infections graves parfois tardives. La physiopathologie de la GvHD chronique est moins bien connue que la GvHD aigüe qui implique les lymphocytes T du donneur, résultant en une activité cytolytique médiée par les perforines, granzymes, FaS ligand et diverses cytokines inflammatoires. La GvHD chronique est un syndrome semblable aux maladies auto-immunes, se développant graduellement et impliquant également les lymphocytes T du donneur activés par les cellules présentatrices de l'antigène.

Dans ces circonstances, différents traitements ont été étudiés notamment la déplétion du greffon en cellules T diminuant l'incidence de la GvHD aigüe mais augmentant l'incidence de rejet et le risque de rechute post-greffe. Après greffe de CSH, un traitement immunosuppresseur est donné au patient afin de prévenir le rejet de greffe et prévenir ou traiter la GvHD. Différentes molécules peuvent être utilisées, seules ou associées, comme des inhibiteurs de calcineurine, la cyclosporine A et le tacrolimus, le méthotrexate et le mycophénolate mophétil (Ho and Cutler 2008). Les corticostéroïdes sont généralement utilisés en traitement d'attaque de la GvHD mais sont toutefois corrélés à de plus fort risques d'infections tardives. Les patients atteints de GvHD aigue réfractaires aux stéroïdes sont traités par différents agents biologiques dirigés contre les lymphocytes T. Il existe différents anticorps dirigés contre les récepteurs CD3 (OKT3) (Knop et al. 2005), CD25 (Daclizumab) Przepiorka et al. 2000) ou l'ATG (MacMillan et al. 2002 ; Atta et al. 2014). L'ATG est utilisé à des doses très variables selon les centres (Hsu et al. 2001) et ses effets sont controversés notamment en greffes de sang de cordon. Une étude réalisée chez 207 enfants atteints de pathologies hématologiques malignes recevant une greffe de sang de cordon a montré que les patients non traités à l'ATG présentaient une reconstitution des plaquettes plus rapide, un plus faible risque de rechute et une meilleure survie comparée à ceux ayant reçu de l'ATG (Zheng et al. 2015). Récemment, l'utilisation de l'ATG après sUCBT ou dUCBT (n=226 et n=435 respectivement) chez des patients adultes après conditionnement réduit a été corrélée à une augmentation de l'incidence de la NRM et une diminution de l'incidence de la survie globale (Pascal et al. 2015).

L'injection de lymphocytes T régulateurs de sang de cordon après expansion *ex vivo* a également permis de diminuer l'incidence de la GvHD aiguë (Brunstein et al. 2011). Bien que l'implication des lymphocytes T dans la physiologie de la GvHD soit bien connue, de récentes

études supportent le rôle des cellules B dans le développement de la maladie (Zhang et al. 2006). En effet, les lymphocytes B sont d'excellentes cellules présentatrices de l'antigène et expriment des molécules de co-stimulation nécessaire à l'activation des lymphocytes T. Ainsi, la déplétion en cellules B médiée par un anticorps anti-CD20 (rituximab) est utilisée dans le traitement de la GvHD chronique, et plus particulièrement dans le cas de corticorésistance (Ratanatharathorn et al. 2000; Kharfan-Dabaja and Cutler 2011).

L'implication des cellules dendritiques dans la physiopathologie de la GvHD est basée sur des modèles murins dans lesquels le manque d'activité des DC de l'hôte empêche le développement de GvHD (Shlomchik et al. 1999). Toutefois, l'implication des DC dans la GvHD reste très controversée. Des études ont reporté qu'une reconstitution altérée des DC augmente l'incidence de la GvHD aigüe (Mohty et al. 2005). Fagnoni et ses collaborateurs ont mis en évidence qu'un grand nombre de DC plasmacytoïdes (pDC) est retrouvé chez des patients atteints de G*v*HD aiguë (Fagnoni et al. 2004). Cependant, une autre étude a montré qu'un faible nombre de pDC au moment de la greffe favorise la G*v*HD aiguë (Reddy et al. 2004). Plus récemment, Chen et ses collaborateurs ont montré que la déficience des DC en micro ARN 155 induit un GvHD moins sévère après greffes de CSH (Chen et al. 2015). L'inhibition des miARN-155 pro-inflammatoires pourrait être considérée comme une potentielle cible thérapeutique.

Le traitement de la GvHD aigüe est poursuivi pendant au moins 6 mois puis, en l'absence de GVH, diminué très progressivement jusqu'à l'arrêt total.

1.1. Réaction du greffon contre la leucémie (GvL)

Le terme de réaction du greffon contre la leucémie (Graft-versus-Leukemia, GvL) décrit la réponse immunitaire de cellules dérivées du donneur dans le greffon attaquant les cellules tumorales. Cette réaction permet de maintenir le patient en état de rémission des

pathologies hématologiques après greffe de CSH. L'effet GvL a été initialement suggéré par Barnes et Loutit en 1956 d'après des observations dans un modèle murin (Barnes et al. 1956). L'effet bénéfique de la GvHD sur la rechute leucémique après greffe de CSH a été identifié très tôt dans des modèles murins (Barnes et al. 1956) et rapidement appliqué chez l'homme (Mathé et al. 1965, Weiden et al. 1979). Chez des patients souffrant de GvHD chronique, cette rechute réduite était également associée à une meilleure survie (Weiden et al. 1981). Le rôle des cellules T est devenu évident après l'introduction de la déplétion des cellules T du greffon en prévention de la GvHD. En effet, malgré un effet bénéfique sur la GvHD, on observe une augmentation de la rechute leucémique. L'implication des cellules T $CD4^+$ et $CD8^+$ dans la réaction de GvL a été largement étudiée dans des modèles animaux et chez l'homme (Korngold and Sprent 1987; Truitt and Atasoylu 1991).

Toutefois, les cellules NK sont les premières cellules lymphoïdes à reconstituer l'hématopoïèse du patient après greffe de CSH, avant les lymphocytes T. Elles ont été identifiées pour leur capacité de lyse des cellules tumorales sans immunisation préalable et ont une forte activité anti-leucémique, sans induction d'effet GvHD. En effet, le défaut d'expression de molécules HLA de classe I ou l'expression de ligands pour les NCR rend les cellules leucémiques vulnérables à la lyse NK. Les cellules NK constituent ainsi les seules cellules responsables de l'effet GvL dans les premiers mois après greffes de CSH.

L'effet GvL médié par les cellules NK a initialement été mis en évidence dans des modèles murins de leucémie (Uharek et al. 1993, Glass et al. 1996, Zeis et al. 1997). Ruggeri et ses collaborateurs furent les premiers à évaluer le potentiel anti-leucémique des cellules NK du donneur contre les cellules leucémiques issues de patients atteints de LAM ou de LMC *in vitro*, après greffe de CSH HLA incompatible de donneur apparenté. Ils ont mis en évidence que la totalité des leucémies myéloïdes, mais seulement quelques LAL, furent tuées par les clones NK alloréactifs, selon les règles de reconnaissance des molécules HLA classe I par les

récepteurs KIR inhibiteurs (Ruggeri et al. 1999). Par la suite, l'équipe a montré dans des modèles murins NOD /SCID que les cellules NK peuvent éliminer les cellules leucémiques de LAM humaines in vivo (Ruggeri et al. 2002). D'autre part, l'étude rétrospective de 57 patients atteints de LAM et 35 patients atteints de LAL après greffes HLA haploidentiques fortement T déplétées a mis en évidence l'alloréactivité des cellules NK dans le sens graft-versus-host (GvH) en présence d'incompatibilités KIR-HLA (Ruggeri et al. 2002). Les blastes de LAL restent résistants à la lyse NK. Ce potentiel anti-leucémique a d'autre part un effet bénéfique sur l'incidence de la GvHD aigüe puisque les cellules NK ciblent également les cellules présentatrices de l'antigène du receveur, diminuant ainsi la GvHD médiée par les lymphocytes T (Shlomchik et al. 1999). Ainsi, l'alloréactivité NK diminue l'incidence de la rechute de la LAM et protège également contre la GvHD (Ruggeri et al. 2002 ; Farag et al. 2002).

Pende et ses collaborateurs ont également montré que les cellules NK lysent différents types de cellules tumorales via l'engagement des récepteurs de cytotoxicité naturelle (NCR) et du NKG2D (Pende et al. 2001). Il a été mis en évidence que le blocage des récepteurs inhibiteurs augmente l'activité anti-leucémique des cellules NK dans un modèle de leucémie aigüe murine (Koh et al. 2001)

L'objectif thérapeutique est de mettre en place des protocoles permettant de prévenir l'effet néfaste de la GvHD tout en préservant l'effet anti-leucémique GvL. Des exemples de procédés de modification des cellules T et NK seront décrits dans le paragraphe « Immunothérapies en greffe de CSH ».

1.2. Les complications post greffe

L'activation des cellules du receveur envers le greffon peut induire un rejet de greffe. Toutefois, le risque de rejet reste faible grâce à l'important traitement immunosuppresseur reçu par le patient lors du conditionnement et après la greffe.

D'autre part, différentes infections peuvent être causées à cause de la sévère neutropénie et de l'immunodéficience parfois prolongée du patient post greffe de CSH (Staphylococcus, Herpes Virus, CMV). Le plus important risque d'infection lié aux greffes de CSH allogéniques provient d'infections antérieures contractées par le patient. Ces infections refont surface après greffes allogéniques à cause des médicaments immunosuppresseurs donnés au patient. Un risque plus faible d'infection provient également du donneur, qui est testé avant de faire le don de CSH. De plus, la présence d'une GvHD chronique favorise la survenue d'infections.

L'infection à CMV constitue une complication majeure après greffes de CSH. L'effet de l'infection à CMV sur la rechute a initialement été découvert par Lönnqvist et ses collaborateurs en 1986. Cette étude réalisée sur une cohorte de 72 patients montre que les patients présentant une infection à CMV après greffe de moelle osseuse ont une plus faible incidence de rechute (Lönnqvist et al. 1986). Plusieurs groupes ont confirmé la corrélation entre l'infection à CMV et la rechute après greffe de cellules souches hématopoïétiques HLA-identiques chez des patients présentant une LAM (Elmaagacli et al. 2011; Manjappa et al. 2014), une LMC (Ito et al. 2013) ou chez des patients présentant des pathologies multiples (Green et al. 2013). Toutefois, l'effet de la réactivation du CMV sur la rechute est plus important dans le cas de LAM et est influencé par le conditionnement utilisé lors des greffes de CSH (Manjappa et al. 2014). La réactivation du CMV après greffes de CSH induit également l'amplification de cellules NK $NKG2C^{+}$ éduquées, capables de contrôler l'infection (Foley et al. 2012). Il a récemment été mis en évidence que la réactivation du CMV influence la reconstitution des cellules NK après greffe de sang de cordon en accélérant

la différenciation de cellules NK matures $NKG2C^+$ $NKG2A^-$ KIR^+. L'infection à CMV induit en effet la maturation rapide des cellules NK après greffe de CSH (Chiesa et al. 2013).

Découvert en 1986, l'herpès Virus Humain 6 (HHV6) appartient à la même sous famille que le CMV. La potentielle pathogénicité du HHV-6 a été explorée ces dernières années chez les patients après greffes de CSH (Singh and Carrigan 1996). Une étude chez 92 receveurs de greffe de MO ou de CSP a montré une incidence de 42.5% d'infection HHV-6 active (Imbert et al. 2000). Les patients infectés présentent une augmentation de la sévérité de la GvHD. Toutefois la contribution active de l'infection HHV-6 à la GvHD reste controversée (Kadakia et al. 1996; Wang et al. 1996). Deux sous types du virus peuvent être trouvés : HHV6-A et HHV6-B. L'HHV6-B est principalement réactivé en greffe de CSH (Frenkel et al. 1994). La greffe de sang de cordon présente une plus forte incidence de l'infection HHV-6B et une plus forte charge virale comparé aux greffes de CSH HLA compatibles non apparentées et pourrait être corrélé au délai de reconstitution des neutrophiles (Chevallier et al. 2010). Ce virus est réactivé dans 60 à 90% des greffes de sang de cordon et est associé à une forte morbidité (Hill et al. 2012). Toutefois, de plus larges études sont nécessaires afin de définir le rôle du virus dans la reconstitution immune.

L'évolution des techniques a permis de générer des lymphocytes T naïfs spécifiques de plusieurs virus notamment l'EBV, le CMV et l'adénovirus afin de lutter contre les infections virales post greffe. Des études précliniques suggèrent la faisabilité de ces approches (Hanley et al. 2009; Hanley et al. 2012).

2. Rôle des cellules NK KIR^+ en greffe de CSH

Après greffes allogéniques de CSH, les cellules NK constituent la première population à reconstituer l'hématopoïèse du patient, avant la réapparition les lymphocytes T (Triplett et al. 2009). L'équipe de Ruggeri a initialement mis en évidence la capacité des cellules NK

alloréactives à tuer les cellules leucémiques de patients atteints de LAM ou LMC *in vitro* (Ruggeri et al. 1999). L'implication des cellules NK dans le devenir des greffes de CSH a fait l'objet de nombreuses études qui présentent toutefois une grande hétérogénéité, le rôle des cellules NK étant évalué en prenant en compte soit les gènes KIR, soit les KIR ligands ou enfin les combinaisons KIR/ligands.

L'absence d'engagement d'un KIR inhibiteur avec son ligand peut entraîner une activation et donc une alloréactivité des cellules NK KIR^+ éduquées au cours de leur développement comme nous l'avons précédemment décrit. Une alloréactivité médiée par les KIR activateurs du donneur peut également être attendue en présence du ligand correspondant chez le patient. Les mécanismes de l'alloréactivité des cellules NK sont illustrés dans la figure 19.

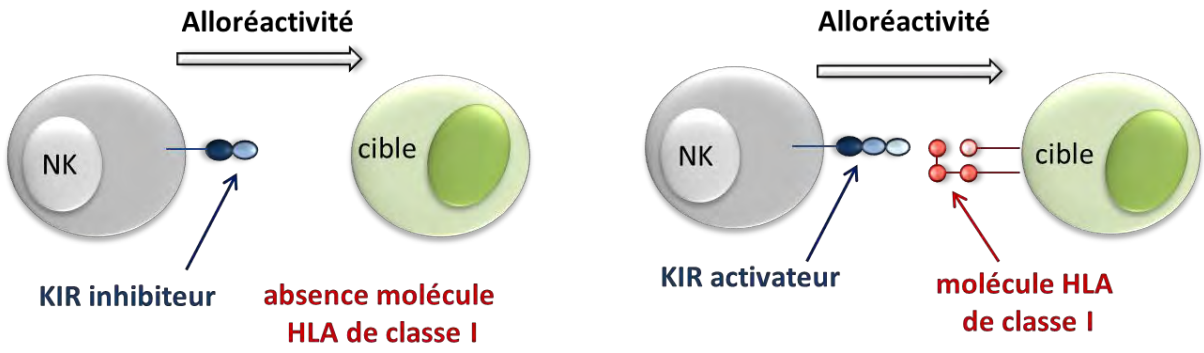

Figure 19 : Mécanisme de l'alloréactivité des cellules NK
L'alloréactivité des cellules NK peut être médiée par un récepteur KIR inhibiteur en absence de la molécule HLA de classe I ligand correspondante ou par un KIR activateur en présence du ligand correspondant.

Des cellules NK KIR non éduquées dans leur environnement autologue semblent néanmoins alloréactives dans le contexte des greffes de CSH, favorable à leur activation (Haas et al. 2011). Cette étude réalisée après greffes de CSH non apparentées met en évidence que les capacités de réponses des cellules NK du receveur post greffe dépendent des ligands du donneur, les cellules NK exprimant un récepteur KIR spécifique d'un ligand HLA du

donneur ayant de plus forte capacités de réponses que les cellules NK exprimant un récepteur spécifique d'un ligand HLA présent uniquement chez le patient.

L'alloréactivité des cellules NK peut être étudiée d'une part d'un point de vue génétique par l'évaluation de la présence des gènes KIR individuellement comparé aux gènes HLA de classe I ou par l'étude des haplotypes KIR. D'autre part, l'alloréactivité des cellules NK KIR d'un individu peut être évaluée par des tests fonctionnels mesurant le potentiel de dégranulation (CD107a), de sécrétion d'IFNγ, d'expression de granzymes ou perforine vis-à-vis d'une lignée cible exprimant ou non le ligand HLA de classe I correspondant.

Différentes études ont été réalisées pour évaluer le rôle des incompatibilités 1) des ligands KIR, 2) des gènes KIR ou 3) des combinaisons KIR/ ligands, que nous allons présenter dans les paragraphes suivant.

Une étude novatrice a montré que l'alloréactivité des cellules NK du donneur dirigée contre le receveur pouvait éliminer l'incidence de la rechute sans augmenter la GvHD (Ruggeri et al. 2002). Dans cette étude, le rôle de l'alloréactivité des cellules NK a été évalué sur une cohorte de patients atteints de LAM (n=57) et LAL (n=35) après greffes de CSH haploidentiques T déplétées. L'analyse de l'impact des incompatibilités HLA de classe I (modèle ligand-ligand) a été réalisée en comparant deux groupes, avec et sans incompatibilités HLA-C, ligands des KIR2DL1 et KIR2DL3. L'équipe a montré l'effet bénéfique de la présence d'incompatibilités KIR ligand dans le sens GvH sur l'incidence de la rechute (0% versus 75% en absence d'incompatibilités), sur l'incidence de la GvHD aigüe (0% versus 13,7% en absence d'incompatibilités) et sur le rejet de greffe (0% versus 15,5% en absence d'incompatibilités) chez les patients atteints de LAM. Ces effets n'ont pas été observés chez les patients atteints de LAL. Les analyses multivariées ont également montré que les incompatibilités KIR ligands constituent un facteur prédicteur de la survie chez les patients atteints de LAM. Leurs travaux *in vivo* ont mis en évidence la capacité des cellules

NK alloréactives à éliminer les cellules leucémiques de LAM humaines induite chez des souris NOD/SCID. Leurs données cliniques suggèrent que l'alloréactivité NK n'induit pas d'augmentation de l'incidence de la GvHD aigüe et assure au contraire une protection contre la GvHD aigüe en éliminant les cellules présentatrices de l'antigène. Chez la souris comme chez l'homme, Ruggeri et ses collaborateurs ont donc mis en évidence l'implication directe de l'alloréactivité NK dans l'éradication des cellules leucémiques assurant une meilleure survie et une protection contre la GvHD aigüe (Ruggeri et al. 2002).

Bien que certaines études n'aient pas réussi à mettre en évidence l'impact significatif des incompatibilités KIR ligands sur la rechute, la mortalité ou la survie (Davies et al. 2002; Farag et al. 2006), d'autres études ont montré que l'effet GvL médié par des incompatibilités KIR ligands est associé à une meilleure survie (Giebel et al. 2003; Kroger et al. 2005; Ruggeri et al. 2007).

De plus, sur une cohorte de 1770 patients ayant reçu une greffe de CSH HLA compatible ou incompatible de donneur non apparenté, K.Hsu *et al* ont montré que l'homozygotie HLA-C ou HLA-B, et donc l'absence de certains KIR ligands, est associée à une diminution de la rechute. L'absence de HLA-Cw2 ou HLA-Bw4 est associée à une diminution de l'incidence de la rechute, et ce quelle que soit la nature de la leucémie (LAM, LAL, LMC) (Hsu et al. 2006). Ces études ont mené certains cliniciens à considérer la sélection des donneurs de CSH sur la base de leurs incompatibilités HLA de classe I avec le receveur en termes de ligand KIR, afin de favoriser les réactions médiées par les cellules NK.

Il faut noter que ces données conflictuelles sont certainement dues à des différences dans les cohortes de patients impliqués, les protocoles de conditionnements, les pathologies et les régimes immunosuppresseurs post greffe.

Différentes études ont évalué l'impact des gènes KIR (modèle KIR-KIR) et notamment des KIR activateurs sur le devenir des greffes de CSH. Notre équipe a réalisé une étude collaborative des disparités des gènes KIR sur le devenir des greffes de CSH chez 264 individus après greffes de CSH non apparentées HLA compatibles (n=164) ou HLA incompatibles (n=100). Les analyses univariées et multivariées montrent une diminution de la survie chez les couples présentant des incompatibilités des gènes KIR3DL1 ou KIR3DS1, avec un effet plus marqué chez les couples HLA compatibles (Gagne et al. 2007; Gagne et al. 2009). Les incompatibilités KIR2DS1 sont associées à une augmentation de l'incidence de la GvHD aigüe pour les couples donneurs-receveurs HLA incompatibles. Au contraire, les incompatibilités KIR2DS3 sont corrélées à une diminution de l'incidence de la GvHD aigüe chez les couples HLA identiques (Gagne et al. 2009). Il est toutefois difficile de spéculer sur une potentielle alloréactivité NK, les ligands pour ces récepteurs n'étant pas connu ou leur expression à la surface des cellules NK n'étant pas déterminée.

Le rôle des gènes KIR a également été évalué sur une large cohorte de 1409 patients atteints de LAM après greffe de CSH. L'équipe a mis en évidence qu'une greffe réalisée à partir d'un donneur d'haplotype KIR B, présentant donc beaucoup de KIR activateurs, est corrélée à une incidence de rechute diminuée et une meilleure survie (Cooley et al. 2009). Pour évaluer quel segment de l'haplotype est impliqué dans ces effets protecteurs, les gènes KIR de l'haplotype B ont été divisés en régions centromériques et télomériques. Les donneurs homozygotes pour les gènes KIR de la région centromérique présentent la plus faible incidence de rechute et la meilleure survie globale. Une étude a également évalué si les génotypes KIR du donneur affectent l'incidence de la rechute après greffes de CSH HLA identique apparentées, et corrélé la présence de certains gènes KIR activateurs (KIR2DS1 et KIR2DS2) chez le donneur à une forte diminution de l'incidence de la rechute (Verheyden et al. 2005).

Par la suite, Stringaris et ses collaborateurs ont révélé que 3 KIR gènes de l'haplotype B du donneur, les KIR2DL5A, KIR2DS1 et KIR3DS1, sont associés à une plus faible incidence de la rechute des patients atteints de LAM après greffes de CSH familiales HLA identiques (Stringaris et al. 2010). D'autre part, la présence du gène KIR2DS1 au niveau du greffon a été associée à un risque de rechute réduit pour les patients LAM après greffes de CSH non apparentée (Venstrom et al. 2012) ou apparentée HLA-compatible (Stringaris et al. 2010). Une étude a également montré que la présence du gène KIR3DS1 est corrélée à une augmentation de l'incidence de la rechute après autogreffe de CSH chez des patients atteints de myélome, tandis que les patients dépourvus du gène KIR3DS1 ont un meilleur pronostic (Gabriel et al. 2010). Il est toutefois difficile de conclure sur l'impact des gènes KIR sur le devenir des greffes de CSH, les différentes études citées ayant été réalisées à partir de cohortes de patients ayant reçu différents types de conditionnements et étant atteints de plusieurs types de pathologies (LAM, LAL, LMC, myélome..).

Par la suite, des études ont montré l'importance de prendre en compte à la fois la présence des gènes KIR chez le donneur et l'absence ou la présence du ou des ligand(s) correspondant(s) chez le receveur (modèle récepteur-ligand) afin d'améliorer la prédiction de la rechute (Leung et al. 2004). Cette étude montre l'augmentation de l'effet anti-leucémique associé avec le nombre d'incompatibilités récepteur-ligand. Les investigations ont majoritairement démontré le potentiel effet anti-leucémique des cellules NK contre les LAM mais pas contre les LAL. L'équipe de Pende a mis en évidence la lyse NK médiée par le récepteur KIR2DS1 de blastes leucémiques exprimant le ligand C2 (Pende et al. 2009). Ce modèle KIR-ligand a été étudié en greffes de CSH apparentées HLA-identique, déplétée en cellules T, pour des patients atteints de LAM. Les résultats montrent un effet bénéfique des combinaisons KIR/KIR ligand sur l'incidence de la rechute leucémique et le devenir des

greffes de CSH (Hsu et al. 2005). Notre équipe a également mis en évidence l'impact des combinaisons KIR/KIR ligands sur le devenir des greffes de CSH non apparentées HLA identiques et HLA non identiques (Gagne et al. 2009). Nous avons en particulier montré que la présence du gène KIR3DL1/KIR3DS1 chez le donneur associée à la présence du ligand Bw4 chez le receveur est corrélée à une plus forte incidence de l'incidence de la rechute chez les couples HLA compatibles et HLA incompatibles.

5. Les greffes de sang de cordon

Les greffes de sang de cordon (UCBT), initialement développées chez l'enfant (Gluckman and Rocha 2008), constituent une alternative aux greffes de CSH de moelle osseuse ou de sang périphérique en l'absence d'un donneur HLA 10/10 identique (Tse et al. 2008a). L'immaturité des cellules immunitaires du sang de cordon permet en effet d'accepter certaines incompatibilités HLA de classe I entre le greffon et le patient. Les greffes de sang de cordon sont associées à une diminution de l'incidence de la GvHD aigüe mais également à une incidence plus faible de prise de greffe ou une prise de greffe retardée, par rapport aux greffes de moelle osseuse et de cellules souches périphériques (Grewal et al. 2003). Différents facteurs peuvent expliquer la plus faible incidence de la GvHD aigüe comme la plus faible dose de cellules T contenues dans une unité de sang de cordon (Haspel and Miller 2008) ainsi que le phénotype naïf et la plus faible fonctionnalité de ces cellules T (D'Arena et al. 1998; Merindol et al. 2010). Chez l'adulte, les greffes sont réalisées à partir de deux unités de sang de cordon co-infusées (dites double-greffes de sang de cordon ou dUCBT) afin d'obtenir un nombre suffisant de cellules nucléées totales et de cellules $CD34^+$ et d'assurer une meilleure prise de greffe (Moscardó et al. 2004; Barker et al. 2005; Scaradavou et al. 2013). Les résultats des dUCBT sont très encourageants, avec une diminution de la mortalité précoce liée à la greffe, une diminution de l'incidence de la rechute pour les patients

présentant des pathologies malignes comparé aux autres sources de CSH (Brunstein et al. 2007; Verneris et al. 2009; Rocha et al. 2010). Cette thérapie a permis une augmentation de l'utilisation de CSH de sang de cordon pour les patients en absence de donneur HLA 10/10 compatible, comme les patients avec des haplotypes HLA rares.

Après dUCBT, seul l'un des 2 cordons contribue à l'hématopoïèse du patient dans 95% des cas (Majhail et al. 2006; Somers et al. 2014). Les explications cliniques et/ou immunologiques de la prise d'un des deux greffons restent cependant énigmatiques ce qui a fait l'objet d'une grande partie de mon travail de thèse, qui sera présenté dans la partie « Résultats ».

5.1. Facteurs de sélection d'une unité de sang de cordon

Le choix d'une unité de sang de cordon repose jusqu'à présent sur plusieurs facteurs principaux : le nombre de cellules nucléées totales et de cellules $CD34^+$ injectées au patient et le niveau de compatibilité des gènes HLA-A, -B (au niveau antigénique) et -DRB1 (au niveau allélique) entre le patient et le cordon. Il est maintenant bien établi que les patients et les unités de sang de cordon doivent être HLA 4 à $6/6^{ème}$ compatible (HLA-A, HLA-B, HLA-DRB1), que le nombre de cellules nucléées totales et de cellules $CD34^+$ injectées doivent être supérieurs à $3x10^7$/kg et $1,5x10^5$ cellules/kg respectivement (Gluckman et al. 1997; Wagner et al. 2002). Ces facteurs sont étroitement associés à la prise du greffon et à la survie du patient (Rodrigues et al. 2009; Barker et al. 2010; Avery et al. 2011; Eapen et al. 2011).

Plusieurs études ont permis d'identifier les facteurs influençant la reconstitution des neutrophiles et des plaquettes après UCBT. Gluckman et ses collaborateurs ont mis en évidence que les patients ayant reçu une unité de sang de cordon contenant moins de 37 millions de cellules nucléées par kg mettent 34 jours à sortir de neutropénie contre 25 jours pour des patients ayant reçu une unité de sang de cordon contenant plus de 37 millions de

cellules nucléées par kg (Gluckman et al. 1997). D'autre part, Rubinstein et ses collaborateurs ont montré que la prise de la greffe est corrélée au nombre total de cellules nucléées par unité de sang de cordon et également au degré d'incompatibilité HLA (Rubinstein et al. 1998) ce qui a par la suite été corroboré par d'autres études (Laughlin 2001; Eapen et al. 2007). L'étude réalisée par Barker et ses collaborateurs a mis en évidence l'effet combiné de la dose de cellules nucléées totales et du niveau de compatibilité HLA. Chez 1061 receveurs de greffe de sang de cordon, l'augmentation de la dose de cellules nucléées totale peut compenser le nombre d'incompatibilités HLA. En effet, aucune différence sur la survie n'est observée chez les patients présentant une incompatibilité HLA associée à une dose de $2{,}5.10^7$/kg ou 2 incompatibilités HLA associées à une dose de $5{,}0.10^7$/kg (Barker et al. 2010). Cette étude a confirmé que l'augmentation de la dose de cellules nucléées totales est corrélée à une prise de greffe plus rapide.

Habituellement, seuls les typages HLA-A, -B (générique) et -DRB1 (allélique) sont pris en compte dans le choix d'une unité de sang de cordon. Cependant des incompatibilités alléliques (HLA-A, -B) et/ou génériques (HLA-C) entre patient/cordon (GvH ou HvG) ou entre cordons (GvG) peuvent être observées, pouvant générer des incompatibilités KIR ligand et une potentielle alloréactivité des cellules NK KIR^+ (Eapen et al. 2011). Une étude comparative a mis en évidence que le taux de survie après greffes de sang de cordon HLA compatible 4 à 6/6 (HLA-A, -B générique et -DRB1 allélique) est comparable à celui obtenu après greffes de moelle osseuse HLA 10/10 identique (HLA-A, -B, -C, DRB1 allélique) chez des enfants (Eapen et al. 2007) et des patients adultes (Eapen et al. 2010) atteints de leucémies aigües.

Certaines incompatibilités HLA-C peuvent constituer un facteur de risque après UCBT. En effet, la présence d'incompatibilités HLA-C génériques combinées ou non avec d'autres incompatibilités HLA-A, -B génériques ou –DRB1 allélique est corrélée à une forte

mortalité liée à la greffe (Eapen et al. 2011). De façon intéressante, Stevens et ses collaborateurs ont étudié l'impact de la direction des incompatibilités HLA-A, -B génériques et HLA-DRB1 allélique sur le devenir des greffes de sang de cordon sur une cohorte de 1202 sUCBT (Stevens et al. 2011). Ils ont mis en évidence que les incompatibilités HLA HLA-A, -B génériques et HLA-DRB1 allélique dans le sens Host-versus-Graft (HvG) dit sens « rejet », entrainent une diminution de la prise de greffe, une augmentation de la rechute et une augmentation de l'échec du traitement, contrairement aux greffes présentant des incompatibilités HLA dans le sens Graft-versus-Host (GvG). Ces résultats suggèrent d'identifier le sens des incompatibilités HLA et de sélectionner de façon prioritaire les unités de sang de cordon présentant des incompatibilités HLA dans le sens Graft-versus-Host (GvH).

D'autres marqueurs génétiques peuvent également être pris en compte dans la sélection d'une unité de sang de cordon, notamment les NIMA (Noninheritied maternal antigens). Ce sont des alloantigènes maternels dont le fœtus n'a pas hérité. Les greffes de sang de cordon dites NIMA compatibles sont associées à une meilleure survie globale (Rocha et al. 2012). D'autre part, Van Rood et ses collaborateurs ont étudié l'impact de l'exposition aux antigènes NIMA sur le devenir des greffes de sang de cordon chez 1121 patients. Cette étude a corrélé la compatibilité NIMA avec une plus faible mortalité globale et mortalité liée à la greffe, ainsi qu'une diminution de l'incidence de la rechute (van Rood et al. 2009). Ces données suggèrent que sélectionner un donneur NIMA compatible augmenterait considérablement le devenir des greffes de sang de cordon.

D'autres facteurs dits « non HLA » sont également pris en compte dans la sélection d'une unité de sang de cordon comme le sexe, la compatibilité ABO et l'origine des banques d'unités de sang de cordon (Ramirez et al. 2012; Romee et al. 2013b).

Etant donné les incompatibilités HLA de classe I fréquentes en UCBT, la présence d'anticorps anti-HLA dirigés contre le greffon peut impacter le devenir des greffes (Takanashi

et al. 2010; Cutler et al. 2011; Dahi et al. 2014). L'analyse de la présence d'anticorps anti-HLA réalisée sur une cohorte de 294 UCBT a corrélé la présence d'anticorps anti-HLA avec une diminution significative de la survie et de la mortalité 1 an après greffes (Ruggeri et al. 2013). Les résultats de cette étude suggèrent donc l'importance de la recherche d'anticorps anti-HLA et d'éviter de sélectionner des unités de sang de cordon contre lesquelles le patient présente des anticorps anti-HLA spécifiques.

De la même façon, en transplantation d'organe, la présence d'anticorps anti-HLA spécifiques du donneur est associée à une augmentation du rejet de greffe (Fidler et al. 2013; Malheiro et al. 2015). En greffe de rein, une étude récente a confirmé l'effet délétère d'anticorps spécifiques du donneurs anti-HLA-C et anti-HLA-DP autant que les anti-HLA-A, -B, -DR et –DQ, justifiant leur inclusion dans les critères de sélection du greffon (Bachelet et al. 2015).

Différents facteurs semblent donc impacter le devenir des greffes de sang de cordon en particulier des facteurs intrinsèques aux unités de sang de cordon comme la dose de cellules et des facteurs pouvant induire des interactions immunologiques comme la compatibilité HLA. Dans le cadre de ma thèse, je me suis intéressée à l'impact des incompatibilités KIR et HLA sur la dominance d'un cordon en dUCBT afin d'envisager la sélection des unités de sang de cordon sur la base de génotype KIR et HLA.

5.2. Réactions cellulaires T et NK et dUCBT

Les dUCBT constituent un contexte particulier dans lequel 3 systèmes immunitaires sont mis en relation. Dans ce contexte de « ménage à 3 » des réactions cellulaires peuvent être observées entre le patient et les unités de sang de cordon comme illustré dans la figure 20.

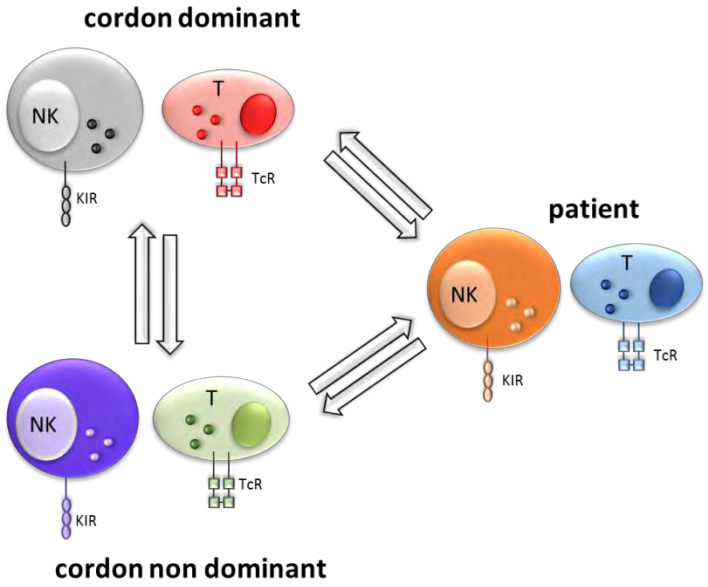

Figure 20 : Les double-greffes de sang de cordon : alloréactivités T et NK
Dans ce ménage à 3 impliquant deux unités de sang de cordon et un patient adulte, des réactions cellulaires médiées par les cellules T et cellules NK sont attendues entre les deux sangs de cordon, dans le sens Graft-versus-Graft et entre chaque cordon et le patient, dans le sens Graft-versus-Host.

Il a été récemment démontré que la prédominance d'un cordon après dUCBT était corrélée à une réponse fonctionnelle des cellules T $CD8^+$ du cordon « gagnant » contre le cordon « perdant » (Gutman et al. 2010). Au contraire des lymphocytes T, la reconstitution des cellules NK est rapide chez les patients pédiatriques et adultes après UCBT. Il a été montré que les incompatibilités KIR ligand dans le sens GvH en UCBT, initialement décrites par l'équipe de Ruggeri, ont soit un effet bénéfique sur la survie et la rechute par l'augmentation de l'effet anti-leucémique GvL (Willemze et al. 2009), soit un effet délétère sur la prise de greffe et la survie par l'augmentation de la GvH aiguë et une plus forte mortalité liée au traitement (Brunstein et al. 2009). En effet, Willemze et ses collaborateurs ont évalué l'impact des incompatibilités KIR ligand chez 218 patients après sUCBT présentant des LAM (n=94) et des LAL (n=124). Ils ont mis en évidence la corrélation des incompatibilités KIR ligand dans le sens GvH avec une diminution de l'incidence de la rechute et une augmentation de la survie globale, avec un suivi médian de 34 mois (Willemze et al. 2009). Au contraire, Brunstein et ses collaborateurs ont réalisé une étude chez 257

receveurs sUCBT et dUCBT après conditionnement d'intensité réduite (RIC) et ont corrélé la présence d'incompatibilités KIR ligand dans le sens GvH avec une augmentation de l'incidence de la GvHD aigüe et une diminution de la survie globale (Brunstein et al. 2009). D'autres études des incompatibilités des ligands KIR présentent également des résultats contradictoires (Tanaka et al. 2013; Garfall et al. 2013). Récemment, une étude réalisée sur une cohorte plus limitée de 83 dUCBT n'a toutefois pas mis en évidence d'association significative entre les combinaisons génétiques KIR et HLA dans les sens GvH et GvG et la dominance d'une unité de sang de cordon (Tarek et al. 2014).

Dans l'ensemble, il semble recommandé de prendre en compte à la fois la présence des gènes KIR et la présence ou l'absence des ligands KIR respectifs dans les sens GvG et GvH afin d'évaluer l'impact d'une potentielle alloréactivité des cellules NK sur le devenir des dUCBT. Les cellules NK pourraient donc jouer un rôle sur le devenir des dUCBT en éliminant les cellules leucémiques mais aussi contribuer à la prise de greffe et à la dominance d'un seul cordon.

L'impact des combinaisons génétiques KIR et HLA sur le devenir des greffes de sang de cordon, et plus particulièrement sur la dominance d'un seul cordon et sur l'incidence de la rechute après dUCBT, fait l'objet d'une étude réalisée au niveau local d'une part et sur une plus large cohorte nationale d'autre part, que je détaillerai dans la partie « Résultats ».

5.3. Greffes de sang de cordon : nouvelles stratégies

Ces dernières années, les greffes de sang de cordon sont devenues une source reconnue de CSH, permettant aux patients sans donneur de moelle ou de sang périphérique HLA identique disponible d'accéder à une greffe de CSH. Toutefois l'utilisation de sang de cordon reste limitée par le faible nombre de cellules progénitrices et cellules $CD34^+$ contenues dans une unité de sang de cordon, induisant un délai de reconstitution. La reconstitution

retardée des neutrophiles prédispose le patient à des infections et la reconstitution retardée des plaquettes prédit une mortalité précoce (Majhail et al. 2009; Ramírez et al. 2011). Dans ce contexte, la mise en place des double-greffes de sang de cordon chez l'adulte a permis d'augmenter le nombre de cellules injectées au patient, la quantité de cellules totales et cellules $CD34^+$ contenue dans une unité étant souvent limitée (Majhail et al. 2006). L'amélioration de la prise de greffe fait l'objet de nombreuses études et différentes stratégies ont été mises en place.

a) Injection intra-médullaire

La prise de greffe retardée observée après UCBT suggère un défaut de « homing » ou retour à la moelle osseuse. En effet, une proportion non négligeable de CSH n'atteint pas la niche dans la moelle osseuse après infusion intraveineuse (van Hennik et al. 1999). Ainsi l'injection directe des CSH directement dans la moelle osseuse (« intrabone marrow injection ») assurerait une meilleure prise de greffe. Une étude réalisée sur 32 patients a permis de suivre l'ensemencement et la répartition des CSH après injection. Dans chaque cas, l'injection induit la reconstitution des CSH ainsi que leur recirculation précoce et un ensemencement homogène dans la moelle osseuse (Frassoni et al. 2008). Les résultats préliminaires ont montré une reconstitution plus rapide des neutrophiles après chimérisme total des cellules du donneur. Une autre étude réalisée 2 ans plus tard par la même équipe sur 75 patients révèle que cette technique est associée à une meilleure prise de greffe, une reconstitution précoce et robuste des plaquettes et une plus faible incidence de GvHD aigue (Frassoni et al. 2010). Notons qu'une diminution de l'incidence de GvHD avait également été reportée dans les premières études comparatives d'injection intraveineuse et intra-osseuse dans des modèles animaux (Fukui et al. 2007).

b) Expansion ex vivo des CSH de sang de cordon

Les cellules $CD34^+$ de sang de cordon ont de grandes capacités de prolifération (Kim et al. 1999). Différentes stratégies de manipulation *ex vivo* des UCB ont émergé au cours des années afin de palier à la faible quantité de cellules progénitrices présentes dans une unité de sang de cordon. Notons qu'un second sang de cordon non manipulé sera toujours co-infusé au patient afin d'assurer la reconstitution hématopoïétique à long terme. Différents protocoles d'expansion *ex vivo* de cellules de sang de cordon ont été mis au point et testés dans le cadre d'essais cliniques comme l'expansion cellulaire médiée par les ligands Notch (Delaney et al. 2010), par des dérivés de la purine (Stem Regenin, SR1) (Boitano et al. 2010) ou des fragments du complément C3 (Ratajczak et al. 2004). Les cellules souches mésenchymateuses (CSM) sécrètent des facteurs solubles proches du microenvironnement de la moelle osseuse. Des cellules de sang de cordon sont cultivées avec des CSM ce qui augmente l'expansion des cellules $CD34^+$ *in vitro* (Zhang et al. 2006). La co-injection d'une unité de sang de cordon non manipulée avec une unité infusée avec des CSM améliore significativement la prise de greffe (de Lima et al. 2012). Des études du chimérisme montre que l'unité amplifiée avec les CSM permet la prise de greffe à court terme tandis que l'unité non manipulée est responsable de la prise à long terme.

c) Amélioration du homing

D'autres techniques furent développées pour spécifiquement améliorer le retour des CSH à la moelle osseuse (« homing »). La culture de cellules de sang de cordon en présence de PGE2 (prostaglandine e2) connue pour réguler l'hématopoïèse (Cutler et al. 2013) ou la fucosylation des ligands de la sélectine pour augmenter l'adhésion des P- et E-sélectine (Xia et al. 2004; Robinson et al. 2012) ont montré des résultats préliminaires très encourageants et font l'objet d'essais cliniques.

d) Co-transplantation : haploidentique-UCB

Une autre approche pour augmenter la dose de CSH est de supplémenter l'unité de sang de cordon avec des CSH provenant d'un donneur adulte familial haploidentique. Ceci assure une reconstitution précoce à partir des cellules du donneur haploidentique jusqu'à la prise des CSH du sang de cordon à long terme, raccourcissant ainsi la période de neutropénie. Cette approche a été développée aussi bien après conditionnement myéloablatif (Fernández et al. 2003; Kwon et al. 2014) que réduit (Liu et al. 2011; Jain et al. 2013) et assure une prise de greffe rapide, une faible incidence de GvHD aigüe et une rémission durable.

L'ensemble de ces stratégies, seules ou combinées, devrait permettre une reconstitution immune plus rapide après UCBT. Toutefois, les mécanismes impliqués dans la dominance d'une seule unité de sang de cordon en dUCBT doivent être identifiés pour mieux comprendre les obstacles, mieux sélectionner les unités placentaires et améliorer le devenir des greffes.

Afin d'améliorer le devenir des greffes de CSH, en particulier augmenter la prise de greffe et limiter les réactions allogéniques néfastes, différentes immunothérapies ont été développées en utilisant les capacités cytotoxiques des cellules T et des cellules NK.

6. Immunothérapies en greffe de CSH

6.1. Les immunothérapies T

Ces dernières années, différentes stratégies pour accélérer la reconstitution immune après greffes de CSH, sans déclencher de GvHD aigüe, ont été développées. L'immunothérapie adoptive principale par les cellules T consiste en l'infusion de lymphocytes du donneur (DLI) après transplantation afin d'assurer une activité anti-leucémique directe pour traiter la rechute chez des patients atteints d'hématologies malignes.

Les cellules T du donneur sont isolées *in vitro*, puis stimulées par des cellules présentatrices de l'antigène. Les lymphocytes T alloréactifs sont sélectionnés sur la base de leur profil cytokinique pour être ensuite amplifiés (expansion clonale). Ces cellules sont finalement transférées au receveur (Bleakley and Riddell 2004). Le conditionnement myéloablatif et l'immunosuppression n'assurent toutefois pas la prise de la greffe. La déplétion en cellules T du greffon améliore la prise de greffe mais une immunosuppression post greffe est nécessaire pour gérer la GvHD aigüe.

Dans ce contexte, la manipulation génétique des lymphocytes T du greffon a été étudiée afin d'éliminer sélectivement les cellules cibles en cas de GvHD aigüe sévère. Un gène suicide peut être introduit, codant pour une protéine capable de convertir une drogue non toxique en produit toxique. De tous les gènes étudiés, le gène suicide « herpes simplex virus thymidine kinase » (HSV-TK) est le plus efficace et a été le premier utilisé dans des essais cliniques (Bonini et al. 1997). Suite à ce transfert de gène, les lymphocytes T deviennent sensibles à une drogue anti-herpès virus : le ganciclovir (GCV). Ainsi, si une GvHD aigüe se déclenche, il est possible de réaliser une déplétion sélective *in vivo* des lymphocytes T par traitement au ganciclovir, qui est converti en sa forme toxique (GCV-3P) induisant l'inhibition de l'élongation de l'ADN de la cellule ciblée une fois incorporé. La faisabilité et l'innocuité de ce traitement ont été confirmées en greffes de moelle osseuse HLA identiques T déplétées, induisant la rapide diminution des cellules modifiées dans la circulation avec une GvHD sensible au ganciclovir (Tiberghien et al. 2001). Récemment, l'efficacité de cette technologie a été montrée après greffes de CSH HLA haploidentiques (Ciceri et al. 2009).

Les lymphocytes T peuvent également être génétiquement modifiés pour rediriger leur spécificité. Les récepteurs chimériques de l'antigène (CAR) ont été initialement créés par l'équipe d'Eshhar pour évaluer si le complexe anticorps pouvait donner une nouvelle spécificité aux cellules T (Eshhar et al. 1993). Ces récepteurs chimériques sont composés d'un

domaine extracellulaire (généralement dérivé des régions variables d'un anticorps) couplé à des domaines de signalisation intracellulaires. Initialement, les CAR présentaient uniquement la chaine CD3ζ du TCR comme domaine de signalisation. Ces récepteurs de première génération n'ont pas donné de résultats concluants à cause d'un manque de persistance dans l'organisme (Jensen et al. 2010). Une seconde génération de CAR a été générée combinant le signal de la chaine CD3ζ du TCR avec le signal d'une molécule co-stimulatrice comme CD137 (41BB). En greffes de CSH, un récepteur a été créé pour reconnaître le récepteur CD19 présent sur les pathologies malignes à cellules B (Ramos et al. 2014). La persistance des CAR a été améliorée en utilisant un régime de lympho-déplétion pour éliminer les cellules endogènes, induisant la rémission de patients atteints de pathologie agressive B (Kochenderfer et al. 2012). L'utilisation d'un vecteur lentiviral pour transférer le gène exprimant le ligand du CD137 (41BBL) après chimiothérapie a également permis d'améliorer cette technique et d'induire la rémission complète pour des patients atteints de leucémie lymphoïde chronique (LLC) (Kalos et al. 2011; Porter et al. 2011). Des résultats encore plus encourageants ont été mis en évidence chez des patients souffrant de LAL (Grupp et al. 2013). Des effets indésirables ont toutefois été rapportés comme des « tempêtes cytokiniques » accompagnant l'utilisation des CAR (Kochenderfer et al. 2012). Des efforts sont donc encore nécessaires afin d'améliorer l'utilisation des CAR en thérapie anti-tumorale et assurer la sécurité de leur utilisation et leur persistance à long terme.

6.2. Les immunothérapies NK

De nombreuses stratégies ont été évaluées afin de stimuler l'activité des cellules NK dans le contexte des greffes de CSH et certaines thérapies cellulaires vont être présentées dans ce paragraphe. Une des techniques mises en place vise à augmenter la sensibilité à l'ADCC médiée par le récepteur CD16. L'activation des cellules NK est associée au clivage

du récepteur CD16 de la membrane cellulaire, processus médié par les métalloprotéinases (MMP) comme ADAM-17 (Romee et al. 2013a). Ainsi, l'utilisation d'inhibiteurs des MMP permet le maintien de l'expression du récepteur CD16 stimulant ainsi la production de cytokines et la lyse par les cellules NK (Zhou et al. 2013).

D'autres méthodes impliquant l'utilisation d'anticorps monoclonaux dirigés contre des antigènes tumoraux ont été développées afin d'induire l'ADCC des cellules NK. Un anticorps anti-CD20, le rituximab, est notamment utilisé dans le traitement des lymphomes B (Kohrt et al. 2012). Une autre technique repose sur les immunoglobulines et consiste à cibler plus de réponses immunitaires en créant des anticorps bi- ou tri-spécifiques. Pour la plupart des anticorps dits bi-spécifiques, le domaine Fv du récepteur CD16 de la cellule NK est fusionné au domaine Fv de marqueurs tumoraux comme le récepteur CD19 (Kipriyanov et al. 2002) ErbB2 (Lu et al. 2008), ou le CD30 (Reusch et al. 2014). Des approches recombinantes modernes fusionnent les domaines Fv créant des constructions nommées BiKEs ou TriKEs (bi or tri-specific killer cell engagers) entièrement dépourvues du domaine Fc. Dans ces conditions, les cellules NK sont directement activées via le récepteur CD16 induisant leur cytotoxicité et la production de cytokines (Gleason et al. 2012). Ainsi, ces anticorps offrent une approche ciblée pour une thérapie anti-tumorale impliquant directement les fonctions effectrices NK.

Une stratégie intéressante pour stimuler les fonctions effectrices des cellules NK est l'activation des cellules NK par les tumeurs. Via l'interaction du récepteur CD2 présent sur les cellules NK avec un ligand tumoral, les cellules NK sont plus cytotoxiques, permettant la lyse des cellules tumorales. Les cellules NK au repos ont besoin d'au moins deux signaux activateurs afin de pouvoir sécréter des cytokines et lyser une cellule cible. Le premier signal d'amorçage dit « priming » peut être apporté par l'IL-2 ou la liaison au récepteur CD2 (Bryceson et al. 2006) et également par un contact avec une cellule tumorale (North et al.

2007). En effet, les signaux médiés par les KIR inhibiteurs peuvent être surmontés par les interactions avec les cellules tumorales. Contrairement à la nécessité d'une exposition continue aux cytokines, les cellules NK sont maintenues dans leur état activé après séparation avec les cellules tumorales. De plus, ces cellules conservent leur capacité à tuer des cellules tumorales NK-résistantes après cryopréservation et décongélation, ce qui favorise leur utilisation dans des protocoles cliniques.

Des études cliniques ont mis en évidence le rôle thérapeutique des cellules NK contre les cellules LAM, en dehors du contexte des greffes de CSH. Le transfert adoptif de cellules NK d'individus HLA haploidentiques préalablement activées par l'IL-2 à des patients atteints de différents cancers ont montré des résultats préliminaires très encourageants (Miller et al. 2005). Dernièrement, de nouvelles approches de déplétion des lymphocytes T régulateurs ont permis d'augmenter l'activité des cellules NK envers les LAM après greffes de CSH HLA haploidentiques (Gasteiger et al. 2013; Bachanova et al. 2014).

OBJECTIFS DES TRAVAUX DE THESE

Les greffes de cellules souches hématopoïétiques (CSH) constituent une thérapie de choix pour de nombreuses hémopathies malignes. Ces dernières années le recours au sang de cordon comme source de CSH a permis de répondre à l'augmentation croissante de patients en attente de greffon en l'absence d'un donneur HLA 10/10 compatible (Ballen et al. 2013). Les double-greffes de sang de cordon (dUCBT) sont réalisées chez l'adulte afin d'obtenir un nombre suffisant de cellules nucléées totales et de cellules $CD34^+$ et seul l'un des deux cordons contribue à reconstituer l'hématopoïèse du patient dans 95% des cas. Cependant, les mécanismes cliniques et/ou immunologiques impliqués dans la dominance d'un seul cordon n'ont pas été clairement identifiés à ce jour. Une meilleure compréhension des mécanismes immunologiques impliqués dans ce « ménage à 3 » permettrait aux cliniciens de mieux sélectionner les deux unités de sang de cordon afin de favoriser la prise de greffe/dominance d'un seul cordon et optimiser l'effet bénéfique anti-leucémique (GvL).

Les cellules NK constituent la première population à reconstituer l'hématopoïèse du patient, réapparaissant dans les premiers mois après greffe avant les lymphocytes T, et sont ainsi les seules cellules responsables de l'effet GvL. Dans le contexte des dUCBT, de potentielles alloréactivités des cellules T et NK sont favorisées puisque les cordons et le patient peuvent présenter des incompatibilités HLA-A , -B, (-C) ou -DRB1. L'immaturité des cellules de sang de cordon permet en effet de tolérer certaines incompatibilités HLA de classe I, pouvant représenter des incompatibilités en termes de ligands KIR. Ainsi, dans ce « ménage à 3 » impliquant plusieurs systèmes immunitaires, notre hypothèse de travail repose sur le postulat que des incompatibilités KIR et/ou HLA de classe I pourraient générer une potentielle alloréactivité des cellules NK et/ou des cellules T pouvant directement impacter le devenir des dUCBT.

En collaboration avec les cliniciens du laboratoire d'hématologie du CHU de Nantes (Dr P. Chevallier, Pr P. Moreau, Dr L. Lodé), nous avons réalisé une étude rétrospective de l'impact des gènes KIR, des ligands KIR et des combinaisons KIR/KIR ligand sur la

dominance d'un seul cordon après dUCBT à partir d'une cohorte locale de dUCBT (**Article n°1**). Cette étude génétique KIR/HLA a été confortée par une analyse statistique rigoureuse (Pr V. Sébille, Dr C. Volteau, Université de Nantes). Afin de confirmer les résultats obtenus, nous avons élargi cette étude au niveau national et réalisé une étude rétrospective multicentrique des combinaisons génétiques KIR/HLA à partir d'un large effectif de dUCBT impliquant 21 laboratoires HLA et les principaux centres de greffes de CSH français, sous l'égide de la SFHI (Pr J.F. Eliaou), la SFGM-TC (Pr I. Yakoub-Agha) et la RFGM (Dr E. Marry). Les données génotypiques, biologiques et cliniques obtenues ont été confrontées dans des analyses statistiques multivariées (Dr C. Le Gall, Methodomics) afin d'évaluer l'impact des combinaisons génétiques KIR/HLA sur le devenir des dUCBT (**Article n°2**).

Pour évaluer la relevance des résultats génotypiques obtenus sur un plan mécanistique, nous avons étudié la biologie et le répertoire NK des cellules de sang de cordon en collaboration avec la maternité du CHU de Nantes (Dr J. Esbelin). Ces cellules présentent un statut immature mais semblent néanmoins acquérir très rapidement des fonctionnalités impactant le devenir des greffes de CSH. Le phénotype des cellules NK de sang de cordon a été étudié par peu de groupes (Le Garff-Tavernier et al. 2010; Schönberg et al. 2011a), et la discrimination entre les récepteurs KIR inhibiteurs et KIR activateurs n'a notamment pas été effectuée jusqu'à présent, en absence d'anticorps spécifiques disponibles, dont nous disposons au laboratoire (David et al. 2009). Pour approfondir leur caractérisation, nous avons réalisé une étude phénotypique et fonctionnelle des cellules NK issues de sangs de cordon (**Article n°3**). Ces données ont été mises en lien avec l'étude du répertoire NK réalisée au laboratoire sur une cohorte d'individus adultes sains (David et al. 2013). Cette étude cellulaire a permis de mieux appréhender l'alloréactivité des cellules NK KIR sous un angle mécanistique.

Ces diverses études nous ont amenés à considérer les gènes KIR comme éventuels marqueurs du chimérisme hématopoïétique après dUCBT. En effet, les techniques

habituellement utilisées pour établir le statut du chimérisme après greffes de CSH peuvent présenter quelques limites notamment pour la détermination précoce du chimérisme. En collaboration avec le laboratoire d'hématologie du CHU de Nantes (Dr P. Chevallier, Dr L. Lodé), la comparaison des profils génétiques KIR des deux unités de sang de cordon et du patient pré et post-greffe sur une cohorte locale de dUCBT, confortée par des données phénotypiques des cellules NK KIR, a mis en évidence l'utilité des gènes KIR comme marqueurs additionnels précoces du chimérisme hématopoïétique après dUCBT (**Article 4**).

L'ensemble de ces travaux sera présenté dans la partie « Résultats » puis discuté dans la partie « Discussion-Perspectives ».

RESULTATS

RESULTATS

ARTICLE N°1 : Etude de l'impact de l'alloréactivité des cellules NK sur la dominance d'un seul cordon en double-greffe de sang de cordon

Impact of Graft-versus-Graft NK cell alloreactivity on single unit dominance after double umbilical cord blood transplantation

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(soumis)

Les double-greffes de sang de cordon sont généralement réalisées en l'absence d'un donneur de moelle osseuse ou de sang périphérique HLA 10/10 compatible (Tse et al. 2008b; Ballen et al. 2013). En effet, l'immaturité des cellules de sang de cordon permet d'accepter certaines incompatibilités HLA de classe I entre le patients et les unités de sang de cordon injectées. Ce protocole de greffe est associée à une plus faible incidence de la maladie du greffon contre l'hôte mais une prise de greffe retardée (Grewal et al. 2003). Dans la majorité des cas, le chimérisme est dérivé d'un seul des 2 unités de sang de cordon (UCB). Toutefois à ce jour, ni la dose de cellules nucléées totales ou de $CD34^+$ injectées, ni la compatibilité des gènes HLA-A, -B, -DRB1 entre patients et cordons ne permettent de prédire quelle unité de sang de cordon sera dominante (Barker et al. 2005). Les cellules NK constituent la première population lymphocytaire à reconstituer l'hématopoïèse du patient dans les premier mois après dUCBT, bien avant la reconstitution des lymphocytes T. De ce fait, ces cellules NK sont les seules responsables de l'effet anti-leucémique Graft-versus-Leukemia comme cela a été initialement reporté après greffes HLA haploidentique T-déplétées (Ruggeri et al. 2002). En dUCBT, le patient et les 2 UCB injectées peuvent présenter des incompatibilités HLA de classe I, en particulier les incompatibilités HLA-C, qui représentent potentiellement des incompatibilités en termes de KIR ligands. Ces incompatibilités KIR/KIR ligands entre le patient et les 2 cordons dans le sens Graft-versus-Host (GvH) et/ou entre les 2 cordons dans le sens Graft-versus-Graft (GvG) peuvent générer une alloréactivité des cellules NK KIR^+ qui pourrait jouer un rôle sur la dominance d'un seul cordon et/ou sur l'incidence de la rechute après dUCBT. Deux études contradictoires ont reporté un effet bénéfique (Willemze et al. 2009) ou délétère (Brunstein et al. 2009) des disparités KIR ligands sur le devenir des dUCBT. Cependant, ces études n'ont pas pris en compte ni les gènes KIR ni les combinaisons KIR/ligands dans les sens Graft-versus-Host (GvH) et Graft-versus-Graft (GvG) pour évaluer l'effet d'une potentielle alloréactivité de cellules NK sur la prise et la rechute. Ainsi, nous

avons étudié la contribution des combinaisons KIR/KIR ligands sur la dominance d'un seul cordon par dUCBT sur un effectif local de 50 dUCBT avec prise de greffe. Nous avons réalisé d'une part une étude génétique des combinaisons KIR/ligands dans les sens GvH et GvG et d'autre part proposé une explication mécanistique à nos résultats génétiques en étudiant le phénotype et la fonction des cellules NK KIR^+ de sang de cordon.

Cette étude rétrospective a été réalisée chez 50 patients atteints de pathologies myéloïdes (58%), de pathologies lymphoïdes (40%) et d'anémie aplastique (2%) ayant reçu une dUCBT, majoritairement après conditionnement d'intensité réduite (82%) entre Mars 2006 et Décembre 2011. Dans notre cohorte, nous confirmons que ni la dose de cellules nucléées et cellules $CD34^+$ totale ni la compatibilité HLA n'impacte la dominance d'une unité de sang de cordon.

La dominance d'un cordon en dUCBT a été corrélée à une réponse fonctionnelle des cellules T $CD8^+$ du cordon dominant contre le cordon non dominant (Gutman et al. 2010). L'analyse des incompatibilités HLA de classe I et HLA-DRB1 entre patients et UCB n'a toutefois pas mis en évidence de potentielle alloréactivité des cellules T dans cette cohorte.

En dUCBT, les incompatibilités HLA sont mieux tolérées entre le patient et les unités de sang de cordon, ce qui peut induire une alloréactivité des cellules NK. Une analyse détaillée du nombre et de la nature des ligands KIR (A3/A11, Bw4, C1 et C2) suggère une répartition différente des ligands KIR chez le patient et les cordons, avec notamment une augmentation des ligands Bw4 chez les patients et les cordons perdants bien que non significative. Une alloréactivité des cellules NK exprimant un KIR inhibiteur en absence du ligand correspondant HLA de classe I. D'autre part, une alloréactivité des cellules NK exprimant un KIR activateur est attendue en présence du ligand correspondant HLA de classe I. La potentielle alloréactivité des cellules NK KIR^+ a été évaluée en étudiant les combinaisons KIR/KIR ligand dans les sens GvH et GvG, en comparant cordon gagnant (Cg)/cordon

perdant (Cp) vs Cp/Cg, ou Cg/patient vs Cp/patient. Aucune corrélation entre les fréquences combinaisons médiées par les KIR inhibiteurs KIR2L1, KIR2DL2/L3 et KIR3DL2 en absence de leurs ligands respectifs (C2, C1 et A3/A11), ou les KIR activateurs KIR2S1/2/3/4/5 et KIR3DS1 en présence de leurs ligands connus (C2, C1, Bw4) ou présumés n'a été mise en évidence sur cette cohorte. Cependant, la combinaison Cp KIR3DL1^{+}/Cg Bw4^{-} est significativement augmentée, par rapport à la combinaison Cg KIR3DL1^{+}/Cp Bw4^{-} (26% vs 12%, p=0.020) dans le sens GvG suggérant un effet délétère des cellules NK KIR3DL1^{+} du cordon perdant envers les cellules Bw4^{-} du cordon gagnant. De plus, l'analyse du nombre et de la nature des incompatibilités KIR/ligand entre le patient et les deux UCB montre que les incompatibilités KIR3DL1^{+}/Bw4^{-} seules ou cumulées avec d'autres incompatibilités (KIR2DL1^{+}/C2^{-}, KIR2DL2/3^{+}/C1^{-}, KIR3DS1^{+}/Bw4^{+}, KIR2DS1^{+}/C2^{+}) sont prédominantes dans la direction GvG cordon perdant/ cordon gagnant. Ces données suggèrent ainsi que l'effet de cette incompatibilité KIR3DL1^{+}/Bw4^{-} est certainement modulé par d'autres alloréactivités NK KIR, notamment par les incompatibilités KIR2DL1^{+}/C2^{-} observées dans notre cohorte dans le sens Cg / Cp.

Les analyses statistiques ont été réalisées par le Dr Christelle Volteau (Plateforme de Biométrie, CHU Nantes) en considérant les principales variables des patients et UCB : la dose de cellules nucléées totales, la dose de cellules CD34^{+}, les compatibilités sexe et les compatibilités HLA. Les analyses univariées et multivariées réalisées utilisant le modèle de Cox ont confirmé que la présence du KIR3DL1 chez le cordon perdant en l'absence du ligand Bw4 chez le cordon gagnant est corrélée à la dominance d'un seul cordon après dUCBT (p=0.001), et ce quelle que soit la nature de la pathologie (myéloïde/lymphoïde) (HR=3.23, p=0.027), le conditionnement (réduit/myéloablatif) (HR=2.79, p=0.015) ou l'utilisation de TBI (HR=3.85, p=0.0008). De plus, les analyses de survie réalisées ont également montré que cette combinaison Cp KIR3DL1^{+}/Cg Bw4^{-} est significativement associée à un délai de

reconstitution des neutrophiles diminué par rapport aux dUCBT en présentant pas cette combinaison, mais également à une augmentation de l'incidence de rechute (HR=4.91, p=0.0134). Aucun effet sur la survie globale ou la GvHD n'a été mis en évidence dans ces analyses. Dans l'ensemble, la combinaison Cp KIR3DL1^{+}/Cg Bw4^{-} est la seule variable ayant un impact significatif sur le devenir de la dUCBT dans notre analyse multivariée.

Pour donner du sens aux résultats des analyses génétiques, nous avons étudié le phénotype des cellules de sang de cordon . Nous avons pu mettre en évidence une plus faible expression des molécules HLA de classe I chez les cellules de sang de cordon comparé à des PBMC provenant de donneurs de sang adultes, aussi bien sur cellules fraîches (MFI médiane=682 vs 1263, p=0.0002) que sur cellules décongelées (p=0.0003). Ces résultats mettent en évidence qu'une alloréactivité des cellules NK KIR^{+} serait privilégiée dans le sens GvG plutôt que dans le sens GvH, confortant nos données génétiques.

A ce stade, notre hypothèse de travail était que les cellules NK KIR3DL1^{+} de sang de cordon peuvent être précocement activées et alloréactives contre les cellules Bw4^{-} de l'autre unité. Nous avons par la suite voulu déterminer si des cellules de sang de cordon étaient capables de générer une alloréactivité des cellules NK. Nous avons d'une part confirmé que les cellules NK de sang de cordon peuvent être activées via la mise en évidence de l'expression intracellulaire des phosphoprotéines p38 (p=0.023). Nous avons également montré que les cellules NK de sang de cordon, bien qu'à priori immatures, présentent des capacités fonctionnelles. Des tests de dégranulation (CD107a) ont été réalisés à partir de cellules NK *ex vivo* et après amplification *in vitro*, en comparaison avec des cellules NK provenant de donneurs de sang adultes. Les résultats montrent une dégranulation diminuée des cellules NK KIR3DL1^{+} de sang de cordon vis-à-vis de la lignée 221 transfectée avec le ligand Bw4 comparé à la dégranulation vis-à-vis de la lignée 221 dépourvue de molécule HLA de classe I (p=0.002). De plus, les cellules NK KIR^{+} de sang de cordon présentent de fortes capacités de

dégranulation après amplification *in vitro* avec des lignées B-EBV, suggérant une alloréactivité des cellules NK de sang de cordon dans le contexte de la greffe. Enfin, nous avons évalué l'expression de l'annexin-V chez des cellules NK KIR3DL1^{+} amplifiées *in vitro* vis-à-vis de lignées 221 et 221-Bw4. Nous avons observé une expression d'annexin-V des cellules NK KIR3DL1^{+} et également des NK totales de sang de cordon ($p<0.0001$) suite à l'activation. Nous avons ainsi confirmé que l'activation de cellules NK KIR^{+} de sang de cordon peut induire leur mort par apoptose.

L'ensemble de ces résultats suggère que l'alloréactivité des cellules NK KIR^{+} entre les deux unités de sang de cordon est impliquée dans la dominance d'un seul cordon en double-greffe de sang de cordon. Récemment, Tarek et ses collaborateurs ont reporté l'absence d'une implication des génotypes HLA et KIR sur la dominance d'un cordon en dUCBT. Leur analyse génétique a ciblé la distribution des haplotypes KIR AA/Bx, des motifs centromériques et télomériques et du nombre de ligands KIR manquants chez les patients et les unités de sang de cordon (Tarek et al. 2014). Notre étude a évalué individuellement les combinaisons KIR/KIR ligand impliquant les KIR activateurs et inhibiteurs, dans les directions GvH et GvG. L'alloréactivité médiée par les NK KIR3DL1^{+} dans notre cohorte semble empêcher la reconstitution des cellules du cordon au bénéfice de l'autre unité injectée. L'implication des différentes combinaisons KIR/ligand sur la prise de greffe et la rechute leucémique devrait toutefois être évaluée sur une plus large cohorte de dUCBT afin de conforter ces résultats.

Impact of Graft-versus-Graft NK cell alloreactivity on single unit dominance after double umbilical cord blood transplantation

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Running title: KIR and cord blood dominance after dUCBT.

ABSTRACT

Full chimerism after double umbilical cord blood transplantation (dUCBT) is usually derived from only one of the two UCB units. Because UCB units and patients may be mismatched for HLA class I loci, we investigated the impact of the potential T cell and KIR^+ NK cell alloreactivities on one full UCB unit dominance in 50 dUCBT by analyzing KIR/HLA genetic incompatibilities. Cox multivariate analysis showed the effect of $KIR3DL1^+$ loser UCB unit/$Bw4^-$ winner UCB unit genetic combination on one full UCB unit dominance (HR=2.88[1.32-6.27], p=.0077) and relapse incidence (HR=4.91[1.39-17.3], p=.0134) after dUCBT. Surprisingly, cord blood cells exhibit extremely low HLA class I expression, which may account for the observed Graft-versus-Graft direction of this $KIR3DL1^+$ NK cell alloreactivity. Moreover, resting cord blood $KIR3DL1^+$ NK cells exhibited a basal alloreactivity against $Bw4^-$ target cells that increased upon activation and triggered death by apoptosis. We suggest that cord blood $KIR3DL1^+$ NK cells are stimulated as a result of GvG interactions, resulting in their self-destruction. Overall, our unicentric study suggests for the first time the significant impact of KIR^+ NK cell alloreactivity on one full UCB unit dominance in dUCBT context.

INTRODUCTION

Umbilical cord blood transplantation (UCBT) is an efficient alternative when fully HLA-matched related or unrelated donors are unavailable[1,2]. Compared to other hematopoietic stem cell sources, UCBT is associated with a lower incidence of acute Graft-versus-Host-Disease (GvHD) and relapse, but adversely with delayed engraftment and a lower overall probability of engraftment[3]. The choice of UCB unit(s) depends on the number of total nucleated cells (TNC) and $CD34^+$ stem cells, and on UCB unit/patient HLA-A, -B (low resolution) and -DRB1 (high resolution) matching[4-10]. Adult recipients require two UCB units (dUCBT) in order to increase the number of TNC and CD34+ stem cells infused[11, 12]. Interestingly, when full chimerism is obtained after dUCBT, it is usually derived from only one of the two UCB units infused. However, neither the total number of TNC or $CD34^+$ stem cells contained in the UCB unit, nor the level of HLA-A, -B and -DRB1 matching enable systematic prediction of which one of the two UCB units will engraft[5,13] although a significant association between non-dominant UCB unit TNC dose and engraftment has been recently reported after myeloablative dUCBT[14]. In this "ménage à trois", both T and/or NK cell alloreactivities between UCB units and patient in Graft-versus-Host (GvH) or Host-versus-Graft (HvG) directions and between the two UCB units in the Graft-versus-Graft (GvG) direction should be considered [15]. In this regard, a single UCB unit dominance after dUCBT was shown to correlate with a functional $CD8^+$ T cell response against the non-engrafted UCB unit [16]. In contrast to T lymphocytes, there is a rapid recovery of Natural Killer (NK) cells in both UCB-transplanted adults and children[17, 18]. These cells represent the only lymphoid cell population potentially able to control leukemic relapse in the months preceding T-cell reconstitution. Indeed, in extensively T-cell depleted HSCT, as is the case for haplo-identical grafts with HLA class I mismatched donor/recipient pairs, alloreactive NK cells can exert a

beneficial Graft-versus-Leukemia (GvL) effect[19]. The effector functions of NK cells are tuned by inhibitory and activating receptors such as the Killer cell Immunoglobulin-like Receptors (KIR), which are specific to allotypic determinants shared by different HLA-class I molecules (referred to as KIR ligands) [20]. In particular, HLA-Cw allotypes with Asn^{80} (C1 ligands) or with Lys^{80} (C2 ligands) are respectively recognized by KIR2DL2/2DL3 and KIR2DL1 although KIR2DL2/3 present a larger spectrum of HLA-C recognition than KIR2DL1[21-23]. HLA-A and -B allotypes with a Bw4 motif are targeted by KIR3DL1, and HLA-A3/A11 are recognized by KIR3DL2[24, 25]. Lack of inhibitory KIR engagement can trigger alloreactive KIR^+ NK cell cytotoxicity only within functionally competent NK cells, i.e. cells which have undergone "licensing" upon prior engagement of their inhibitory KIRs with self-HLA class I molecules during their maturation[26]. Although the ligands and functions of inhibitory KIRs are well documented, this is not the case for activating KIR, except for KIR2DS1 and KIR2DS2 which respectively recognize C2 and C1 ligands as their inhibitory counterparts[23, 25, 27].

T-cell and/or NK-cell alloreactivity are particularly favored in a dUCBT context in which UCB units and patient may be mismatched for HLA-A, -B, -C or -DRB1 loci that might in turn impact on dUCBT outcome. Furthermore, KIR ligand disparities in the GvH direction have been reported to be either beneficial[28] or deleterious[29] in the context of UCBT. However, in these studies, neither KIR genes - especially activating ones - nor KIR/KIR ligand combinations have been taken into account when evaluating the actual impact of NK alloreactivity. Because suitable tools to select the best UCB unit pair leading to improve both dUCBT engraftment and immune reconstitution are lacking, we propose to investigate the contribution of both HLA and KIR genetic disparities on one full UCB unit dominance in patients receiving dUCBT since we recently demonstrated in accordance with chimerism data that KIR^+ cord blood NK cells reconstitute the patient's hematopoiesis a soon as day+14 post-

dUCBT [30]. To make sense to our genetic results and to propose a mechanistic explanation, we also investigated cord blood cells at phenotypic and functional levels. Overall, our study suggests on a local dUCBT cohort that KIR/HLA combinations between both UCB units could be considered as a new tool to better select UCB units favoring both engraftment and lower relapse incidence.

MATERIALS AND METHODS

Patient, disease and transplant characteristics

This retrospective study included 50 patients who received dUCBT and engrafted with one full dominant UCB unit at Nantes CHU between March 2006 and December 2011 (Table S1). The patients were recruited at a single hematological department, thus limiting variability of clinical endpoint definition. All patients provided written informed consent in accordance with the Helsinki Declaration before UCBT. The present study obtained approval from the University of Nantes Institutional Review Board (IRB).

Clinical endpoint

The main clinical end-point was engraftment with one full dominant UCB unit[31]. Engraftment was defined as the incidence of neutrophil recovery ($\geq 0.5 \times 10^9$/L) in association with full chimerism by 42 days after transplantation. Full chimerism was defined as greater than 90% of donor cells[13, 32]. The predominant UCB unit and the non-predominant UCB unit in engrafted patients were called the "winner" and the "loser" UCB unit respectively. Acute GvHD was evaluated according to the modified Glucksberg grading system[33].

Evaluation of haematopoietic chimerism

Haematopoietic chimerism data was measured for the first three months after dUCBT and was evaluated using real-time quantitative PCR of genetic markers specific for the patient and for the UCB units as previously described[30, 34].

Cells (PBMCs, cord blood samples and cell lines)

For phenotypic and/or functional assays, peripheral blood mononuclear cells (PBMC) and cord blood cells were isolated from healthy adult volunteers and from umbilical cord

blood samples respectively. All blood donors were recruited at the Blood Transfusion Center (EFS, Nantes, France) and umbilical cord blood samples were obtained at the Nantes CHU maternity unit. Informed consent was obtained from all healthy individuals and mothers. HLA class-I deficient 721.221 lymphoblastoid EBV-B cells, referred to as 221 cells, Bw4 (B*15:13) - and Bw6 (B*39:01)-transfected 221 cells (respectively named 221-Bw4 and 221-Bw6) were used to assess NK cell degranulation. Two different EBV-B cell lines were used as target cells to amplify NK cells as previously described[27].

HLA genotyping and UCB unit selection

Intermediate (HLA-A, -B) or high (HLA-DRB1) resolution typing was performed by reverse SSO Luminex technology (One Lambda, Inc., Canoga Park, CA, USA) for all patients and UCB units. High resolution typing for HLA-C was carried out retrospectively on all UCB unit/patient pairs by Sequence Based Typing kit (Abbott Molecular Park, IL, USA). UCB units were required to be matched to the recipient for at least four out of the six HLA-A, -B, -DRB1 alleles based on antigen-level HLA-A and -B, and allele level HLA-DRB1 typing and to each other at four (or more) of the six HLA antigens, though not necessarily at the same locus.

KIR genotyping

All patients, UCB units, cord blood samples and healthy individuals were typed for KIR2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 3DL1, 3DL2, 3DL3, 2DS1, 2DS2, 2DS3, 2DS4/1D, 2DS5, 3DS1 using either a KIR genotyping PCR-SSP kit (Invitrogen, Compiègne, France) or a multiplex PCR-SSP method as previously described[35].

Phenotypic analysis by flow cytometry

Cord blood and adult NK-cell surface phenotype was determined by four-color flow cytometry using the following mouse anti-human mAbs: anti-CD3-PerCP (SK7), anti-CD56-APC (B159) (BD Biosciences), anti-KIR3DL1/S1-PE (Z27), (Beckman Coulter, Immunotech, Marseille, France), anti-KIR3DL1-FITC (DX9), (Beckman Coulter, Immunotech, Marseille, France), anti-NKG2A FITC (Z199), (Beckman Coulter and EFS Nantes), anti-KIR2DL1/S1-PE (EB6), anti-KIR2DL2/3/S2-PE (GL183) (Beckman Coulter), anti-KIR2DL1/2/3/2DS1/2 (1A6), anti-KIR2DL1/2/3/S2-FITC (8C11) and anti-KIR2DS2/L3-FITC (1F12) [36]. HLA class I expression was evaluated using anti-HLA class I FITC (NaM41-1E3, EFS-PL, Nantes). Cells were also stained with a corresponding isotype-matched control mAb (BD Pharmingen, Franklin Lakes, NJ). Data was collected using a FACSCalibur (BD Biosciences) and analyzed using Flowjo 7.6.1 software (TreeStar).

Phosphoflow experiments by flow cytometry

Phosphoflow experiments were used to evaluate adult and cord blood NK cell activation. Isolated thawed PBMC were first labelled with anti-CD3-PerCP (SK7), anti-CD56-APC (B159) (BD Biosciences). After 30 minutes with PMA/ionomycin stimulation, cells were fixed with PFA 1%. Then, cells were permeabilized in a PBS 0.1% saponine solution, and phosphorylated ERK and P38 intracellular expression was measured using anti-ERK1/2-AF488 (20A) and anti-P38 MAPK-PE (36/p38 pT180/pY182) (BD Biosciences).

CD107a mobilization assay detected by flow cytometry

Adult and cord blood NK cells were tested for their cytolytic potential with the CD107a mobilization assay after stimulation with 221, 221-Bw4 and 221-Bw6 transfected cell lines. NK cells pre-stained with CD107-PE-Cy5 (H4A3, BD Biosciences) from thawed PBMC were cultured with different target cells (221, 221-Bw4, 221-Bw6) for five hours at an E:T ratio of 1:1 with brefeldin A (Sigma) at 10 µg/mL for the last four hours. The cells were

surface stained with Z27-PE, NKG2A-FITC and NKp46-allophycocyanin (9E2, BD Biosciences).

Apoptosis evaluation measured by Annexin-V staining

Two weeks stimulated cord blood cells were incubated with 221-Bw4 negative or 221-Bw4 positive targets at an E:T ratio of 1:1 for 2h at 37°C. After incubation, cells were surface stained with anti-KIR3DL1/S1-PE (Z27) (Beckman Coulter) and anti-NKp46-APC (BD Biosciences). Then, cells were resuspended in BD Biosciences Apoptosis kit Buffer and Annexin-V expression was evaluated by staining with anti-Annexin-V-PE (BD Biosciences).

Statistical analysis

Data was analyzed from the SFGM database with the data existing as of January 2015. Characteristics of dUCBT were described and compared. Continuous variables (expressed as medians and range) were compared between groups with Wilcoxon tests. Categorical variables (expressed as numbers and percentages) were compared between groups with χ^2 tests, Fisher's exact test or Mac Nemar test where appropriate. To analyze the likelihood of sustained UCB unit dominance, we considered variables associated with the infused UCB units (TNC cell doses, $CD34^+$ cell doses, $CD34^+$ post-thaw viability of each UCB unit) and with UCB unit/recipient matching such as sex, HLA-ABDRB1, HLA-C, HLA-Bw4, KIR ligand and KIR/KIR ligand combinations. Delay of neutrophil recovery and overall survival were estimated by the Kaplan-Meier method. Univariate and multivariate Cox regressions were used to evaluate the impact of the $KIR3DL1^+$ loser UCB unit/$Bw4^-$ winner UCB unit genetic combination and other risk factors on one full UCB unit dominance and overall survival after dUCBT. Cumulative incidence was used to estimate relapse incidence and acute GvHD (death without relapse/aGvHD were considered as competing risks). Univariate and multivariate proportional hazard models of Fine and Gray were used to evaluate the impact of

the KIR3DL1^{+} loser UCB unit/Bw4^{-} winner UCB unit genetic combination and other risk factors on relapse and acute GvHD incidence. Candidate variable for the multivariate models were those associated with the outcome in univariate analyses with the $p<0.20$ criterion. P-values<0.05 were considered to be statistically significant in multivariate analyses. An adjustment to the P values (q value) was performed using False Discovery Rate (FDR) correction. Analyses were performed using SAS 9.2 statistical software (SAS Institute, Inc.) and the "cmprsk" package of the R statistical software developed by Gray.

The one-way analysis of variance (ANOVA) test was used to compare HLA class I expression in adult and cord blood samples, the phosphoproteins MFI in phosphoflow experiment, the CD107a^{+} NK cell and CD107a^{+} KIR3DL1^{+} NK cell frequencies in functional assay. P-values <0.05 were considered as statistically significant.

RESULTS

Analysis of graft parameters likely to impact on one full UCB unit dominance after dUCBT

Fifty patients engrafted with one full dominant UCB unit after dUCBT and still living at 42 days were included (Table S1). This retrospective study first included 58 consecutive patients who received dUCBT at Nantes CHU between March 2006 and December 2011 but eight patients had primary engraftment failure with autologous recovery and were therefore excluded. Neutrophil recovery occurred at a median of 19 days [range 8-60]. TNC dose, $CD34^+$ stem cell dose infused after thawing in each UCB unit and the level of HLA-A, HLA-B (low resolution), HLA-DRB1 (high resolution) matching between UCB units and patients had no significant impact on one full UCB unit dominance after dUCBT (Table S1).

No impact of potential T cell alloreactivity based on HLA mismatches on one full UCB unit dominance after dUCBT

Even though UCB units contain approximatively 1-log fewer T cells compared to adult HSC grafts, an impact of $CD8^+$ T cell alloreactivity on single-UCB unit dominance after dUCBT has been previously reported[16]. Through analysis of HLA class I and HLA-DRB1 mismatches between patient and UCB units, we tested whether alloreactive T lymphocytes could affect one full UCB unit dominance in engrafted patients through GvH and GvG directions as represented in Figure 1A. No significant correlation between one UCB unit dominance and the frequencies of HLA-A, -B, -C and -DRB1 mismatches in both GvH and GvG directions (Figure 1B) was observed in our series. However, we cannot exclude the presence of functional alloreactive $CD8^+$ T cells from winner UCB units against loser UCB units as described[16]. Since cumulated HLA class I mismatches in both GvH and GvG

directions were more frequent than isolated ones (Fig S1), the number and the nature of HLA class I molecules present in patients and UCB units were further documented.

Disparities of KIR ligand distribution between both UCB units and patient

NK cell alloreactivity potentially constitutes an important immune response targeting HLA class I molecules in dUCBT, in which numerous HLA class I mismatches are tolerated between UCB units and patients. Potential KIR NK cell alloreactivity is expected when NK cells in one UCB unit display a particular inhibitory KIR and the corresponding HLA class I ligand is absent (hereafter referred to as KIR/ligand mismatch) in the patient (GvH direction) or in the second UCB unit (GvG direction) (Figure 1A). Potential KIR NK cell alloreactivity could also be expected when NK cells in one UCB unit have activating KIR gene(s) directed against known or putative HLA class I ligands expressed by patient cells (in the GvH direction), or by the second UCB unit (GvG direction) (Figure 1A). We showed that the frequencies of different KIR ligands (A3/A11^{+}, HLA-A/B Bw4^{+}, C1^{+} and C2^{+}) were similar between winner UCB and loser UCB units as well as between patients and controls (Figure 1C). Of note, although not significant, the frequency of HLA-A/B Bw4+ was increased in loser UCB units and patients compared to controls (Figure 1C). Detailed analysis of the number (Figure 1D) and nature (Figure 1E) of KIR ligand present in patients and UCB units highlighted that half of the patients exhibited all 3 KIR ligands (i.e C1^{+}C2^{+}Bw4^{+}) whereas UCB units were predominantly characterized by the presence of 2 KIR ligands (60% and 62% in winner UCB and loser UCB units respectively, Figure 1D). Moreover, the presence of only one KIR ligand such as C1C1^{+}Bw4^{-} is increased in winner UCB units compared to loser ones (12% vs 4%, Figure 1D). Loser UCB units present a dominance of C1^{+}Bw4^{+} ligands compared to winner UCB and patients (38% vs 22%, Figure 1E). Although not significant, KIR ligand distribution show differences in all 3 groups studied suggesting a potential alloreactivity mediated by KIR^{+} NK cells.

Potential alloreactivity of KIR3DL1$^+$ NK cells from the loser UCB toward the Bw4$^-$ winner UCB unit

In order to evaluate a potential NK cell alloreactivity mediated by KIR, we focused our study on main KIR/KIR ligand incompatibilities. Both inhibitory and activating KIR gene frequencies were similar in winner *vs* loser UCB units and in patient *vs* controls (data not shown). Moreover, there was no significant correlation between one full UCB unit dominance and inhibitory KIR/KIR ligand mismatches involving KIR2DL1/2/3 and KIR3DL2 or activating KIR/KIR ligand matches involving KIR2DS1/2/3/4/5 and KIR3DS1 genes and their respective or putative ligands in either the GvH or GvG directions (Figure 2A and data not shown). Since the KIR3DL1 allelic polymorphism is large and impacts on both KIR3DL1 phenotype and function[37], the KIR3DL1$^+$/Bw4$^-$ genetic combination was investigated taking into account only expressed KIR3DL1 alleles. Moreover, the absence of the Bw4 ligand was determined taking into account both HLA-A and HLA-B molecules. Interestingly, the KIR3DL1$^+$ loser UCB/Bw4$^-$ winner UCB unit genetic combination (i.e the loser UCB unit expresses KIR3DL1 and the winner UCB unit does not have the corresponding Bw4 ligand) was significantly higher in the GvG direction (26% vs 12%, p=0.020, n=13 vs n=6, Figure 2B), suggesting a deleterious KIR3DL1$^+$ NK cell alloreactivity from the loser UCB unit against the Bw4$^-$ winner UCB cells. Detailed analysis of the nature (i.e KIR2DL1$^+$/C2$^-$, KIR2DL2/L3$^+$/C1$^-$, 2DS1$^+$/C2$^+$ and KIR3DL1$^+$/Bw4$^-$, Fig. 2C) and the number (none, 1, 2, Figure 2D) of KIR/KIR ligand incompatibilities reveal similar patterns in the GvH direction, but some differences in the GvG direction although not significant. For example, concordant to previous KIR ligand distribution in UCB units, the KIR2DL1$^+$/C2$^-$ incompatibilities either alone or cumulated with other KIR/KIR ligand mismatches are predominant in the winner/loser UCB unit direction compared to the loser/winner UCB unit direction (41% alone and 2% cumulated with KIR3DL1+/Bw4- vs 25% alone and 8% cumulated with

KIR3DL1+/Bw4-, Figure 2C) whereas KIR3DL1^{+}/Bw4^{-} mismatches either alone or cumulated with other ones are predominant in the loser/winner UCB unit direction compared to the winner/loser UCB unit direction (14% alone, 8% cumulated with KIR2DL1+/C2- and 4% cumulated with KIR2DS1+/C2+ vs 8% alone, 2% cumulated with KIR2DL1+/C2- and 2% cumulated with KIR2DS1+/C2+ respectively, Figure 2C). Nonetheless, two KIR/KIR ligand incompatibilities are more frequent in the loser/winner UCB unit direction compared to the winner/loser UCB unit direction (22% vs 6%, Figure 2D). Taking together, these data suggest that the effect of KIR3DL1^{+}/Bw4^{-} incompatibilities may be modulated by other ones such as KIR2DL1^{+}/C2^{-} as observed in our cohort.

Only the TNC dose of winner UCB unit was significantly different between dUCBT presenting the expressed KIR3DL1^{+} loser/Bw4^{-} winner UCB genetic combination (n=13, median 1.99[1.16-2.49] x 10^{7}/kg) compared to other dUCBT without this genetic combination (n=37, 2.49[1.00-7.20] x 10^{7}/kg, p=0.008, Table S2). In both univariate and multivariate analyses using the Cox model, we confirmed that expressed KIR3DL1^{+} loser UCB/Bw4^{-} winner UCB genetic combination was associated with one full UCB unit dominance (Table 1). To limit the heterogeneity of the cohort, we stratified our population and performed Cox multivariate analyses restricted on patients with high risk diseases (74%), patients with myeloid diseases (58%), patients having received a total body irradiation (TBI) (96%), patients having not received ATG (74%) and patients having received a reduced intensity regimen (82%). Overall, the genetic combination "KIR3DL1^{+} loser UCB unit/Bw4^{-} winner UCB unit" remains significantly associated with neutrophil recovery with one full UCB unit dominance in all subgroups considered (Table S3). This combination remains also significantly associated with relapse incidence taking into account patients with myeloid diseases, patients having received a total body irradiation or patients that received a reduced intensity conditioning regiment prior dUCBT (Table S3). In 9 out of the 13 dUCBT with

KIR3DL1$^+$ loser/Bw4$^-$ winner UCB genetic combination, Bw4 environment was brought by the losing UCB unit alone (n=2), by the patient alone (n=2) or by both (n=5). In the remaining 4 dUCBT, KIR3DL1$^+$ loser UCB NK cells evolve in Bw4- environment suggesting that cord blood KIR3DL1$^+$ NK cells may be functional in the absence of licensing by the Bw4 molecule and may acquire functional capacities via other inhibitory NK receptors such as KIR2DL and NKG2A[38] and/or thanks to cytokine production in graft context which contribute to bypass a lack of KIR/HLA mediated licensing[39]. Altogether, our results suggest that alloreactive KIR3DL1$^+$ NK cells from loser UCB units are potentially deleterious to its engraftment in favor of the Bw4$^-$ UCB unit.

KIR3DL1$^+$ loser UCB/Bw4$^-$ winner UCB genetic combination impacts on UCB dominance and relapse incidence after dUCBT

We then evaluated the impact of this combination on the time to neutrophil recovery, relapse, overall survival and aGvHD incidence. Interestingly, the time to neutrophil recovery of KIR3DL1$^+$ loser UCB/Bw4$^-$ winner UCB pairs was significantly shortened compared to other dUCBT without this genetic combination (median 15[9-27] vs 24[6-60] days, p=0.001, Figure 3A). The KIR3DL1$^+$ loser UCB/Bw4$^-$ winner UCB genetic combination was significantly associated with an increased leukemia relapse incidence (p=0.017, Figure 3B). This deleterious effect on relapse incidence was confirmed by multivariate Cox analysis (HR=4.91[1.39-17.3], p=0.0134, data not shown). The KIR3DL1$^+$ loser UCB/Bw4$^-$ winner UCB genetic combination has no significant effect on overall survival (Figure 3C) and aGvHD incidence (Figure 3D).

Low expression of HLA class I molecules on cord blood cells

To make sense to our genetic data, we investigate the phenotype of cord blood cells. HLA-A and B molecule expression is down regulated during pregnancy, but HLA-C

expression is maintained to control NK cell recognition [40]. Along this line, we tested whether GvG and not GvH alloreactivity, suggested by our genetic results, could be due to lower HLA class I level expression on cord blood than adult cells. Indeed, HLA class I expression was significantly decreased on freshly isolated cord blood cells (MFI median=682) compared to adult PBMC (MFI median=1263; p=0.0002, Figure 4A). Since dUCBT are performed using thawed UCB units, this difference was also confirmed on thawed cord blood cells (p=0.0003, Figure 4A). Therefore, the lower expression of HLA class I molecules on cord blood cells argue that cord blood NK cell alloreactivity is privileged in the GvG direction rather than the GvH direction in a dUCBT setting.

Cord blood NK cells are activated by P38 pathway

We further evaluated if cord blood NK cells could be activated as in the context of dUCBT. Trotta *et al.* highlighted the role of ERK and P38 MAPK kinases but not JNK MAPK in NK-cell mediated cytotoxicity[41]. Thus, phosphoflow experiments were used to evaluate if cord blood NK cells can also be activated, as adult NK cell counterpart. Intracellular expression of phosphorylated ERK and P38 phosphoproteins was measured on cord blood NK cells using PMA/ionomycin stimulation for 30 minutes as a mechanism of activation and compared to adult NK cells. The protocol was first validated on cord blood and adult T cells (data not shown). ERK1/2 pathway is similarly activated in cord blood and adult NK cells for the highest dose of PMA/ionomycin (data not shown). As illustrated in Figure 4B, p38 phosphorylation is high in cord blood NK cells (p=0.032 and p=0.023, Figure 4B), underlying the susceptibility of cord blood NK cells to be activated by the P38 pathway. This difference can be explained in part by the fact that $CD56^{bright}$ NK cells expressed higher level of p38 than $CD56^{dim}$ NK cells after stimulation and that $CD56^{bright}$ NK cells were more represented in cord blood than in adult PBMC (data not shown).

Cord blood units exhibit a KIR3DL1$^+$ NK cell alloreactivity against Bw4$^-$ target cells.

Our genetic analysis suggested alloreactivity of loser UCB KIR3DL1$^+$ NK cells against Bw4$^-$ winner UCB cells. We hypothesized that cord blood KIR3DL1$^+$ NK cells, present in injected UCB units, are early activated and alloreactive against Bw4$^-$ UCB cells. Thus, to evaluate this hypothesis, we first determined the potential alloreactivity of resting and activated cord blood NK cells *in vitro*. We focused on the functional abilities of NK cells to recognize missing-self based on experiments realized from freshly isolated and activated KIR3DL1$^+$ cord blood NK cells compared to adult NK cells toward different target cells expressing or not Bw4 ligand in a degranulation assay. The degranulation CD107a profile of unmanipulated KIR3DL1$^+$ NKG2A$^-$ cord blood NK cells indicated a decreased degranulation of KIR3DL1$^+$ NK cells incubated with Bw4$^+$ compared to 221 cell line ($p=0.002$, Figure 4C). No KIR3DL1$^+$ NK cell alloreactivity was observed for the single Bw4$^-$ cord blood sample studied (data not shown). The low alloreactivity of resting cord blood KIR3DL1$^+$ NK cells compared to adult counterparts could be due to their immature status as reported[42]. Thus, we hypothesized that cord blood NK cell alloreactivity should be strongly triggered in a dUCBT. We further evaluated the degranulation of KIR3DL1$^+$ NK cells from 2-week stimulated cord blood PBMC in an *in vitro* model as previously described[27] in order to mimic the strong NK cell expansion observed early after dUCBT. We highlighted the alloreactivity of activated NK cells against 221 cell line in a degranulation assay ($p=2.79.10^{-18}$, Figure 4D). Activated cord blood KIR3DL1$^+$ NK cells showed strong alloreactivity against the 221 cell line compared to the 221-Bw4 cell line as measured by CD107a upregulation ($p=1.48.10^{-21}$ and $p=1.70.10^{-17}$, Figure 4E). Altogether, our results suggest that cord blood KIR3DL1$^+$ NK cells may exhibit a strong alloreactivity in a dUCBT context.

Death of activated cord blood NK cells by apoptosis

Finally, we investigated if activation of cord blood NK cells can result in premature death by apoptosis. We therefore evaluated Annexin V expression of 2 weeks stimulated KIR3DL1^{+} cord blood NK cells against 221-Bw4^{+} or 221-Bw4^{-} targets. Interestingly, we observed a high Annexin-V expression of KIR3DL1^{+} NK cells against 221-targets as all NK cells (data not shown) and a protection against 221-Bw4^{+} targets ($p<0.0001$) (Figure 4F). Our results suggest a conceivable death of cord blood NK cells after activation in dUCBT context.

DISCUSSION

In this study, we investigated for the first time the potential impact of both T cell and NK cell alloreactivities on one full UCB unit dominance after dUCBT by studying in particular the extent of KIR/KIR ligand genetic incompatibilities between UCB units and patients in both GvH and GvG directions, correlated with the phenotype and the function of cord blood KIR^+ NK cells. In this local cohort of dUCBT, excluding patients with mixed chimerism and engraftment failure, only the presence of the KIR3DL1 gene in the loser UCB unit and the absence of the corresponding Bw4 ligand in the winner UCB unit was significantly associated with a reduced delay of neutrophil recovery and a higher incidence of leukemia relapse. Supporting our KIR/KIR ligand genetic data, we reported a lower expression of HLA class I molecules on cord blood cells compared to adult PBMC that could favor a cord blood KIR^+ NK cell alloreactivity of one UCB unit against the second one rather than against the patient. Moreover, the absence of Bw4 ligand on winner UCB cells determines the direction of this NK cell alloreactivity between both UCB units.

Our genetic data are partly in agreement with previous studies that showed no significant impact of KIR ligand mismatches in the GvH direction on UCBT engraftment[28, 29, 43]. The data from Willemze et al.[28], including patients undergoing sUCBT, showed that inhibitory KIR-ligand incompatibilities in the GvH direction are associated with a decreased relapse incidence but had no significant impact on engraftment. In contrast, Brunstein et al. [29] reported a detrimental effect of KIR-ligand incompatibility on relapse incidence in recipients of sUCBT or dUCBT following RIC regimen but the impact of these KIR ligand incompatibilities on engraftment was not evaluated. Lastly, Garfall et al. reported no significant impact of KIR ligand incompatibility in the GvH direction on dUCBT engraftment[43]. However, our results extend the findings of these studies, as we highlight the

impact of KIR/KIR ligand incompatibilities on engraftment, especially in the GvG direction. Recently, Tarek et al. [44] reported no identifiable role of HLA and KIR genotypes in single unit dominance after dUCBT. Their genetic analysis was focused on the distribution of AA/Bx KIR haplotypes, centromeric/telomeric motifs and the number of missing KIR ligands within UCB units and patient. Contributing to novel information for the field, our study evaluated individual inhibitory and activating KIR/KIR ligand combinations in both the GvH and GvG directions.

In our series, the majority of dUCBT evolve in a Bw4+ environment. Of note, cord blood KIR3DL1$^+$ NK cells can acquire functional capacities firstly via Bw4+ cells from UCB units or patients and secondly via other inhibitory NK receptors[38] or a massive cytokine production and cell contacts[39] in the absence of Bw4 ligands. Indeed, NK cells with inhibitory KIR for non-self HLA class I can lyse target cells lacking cognate ligand after T-cell depleted HLA identical HSCT suggesting a breaking tolerance to self rapidly after bone marrow and peripheral stem cell transplantation [45]. However, NK cells become progressively tolerized to self thereafter the first three months post-HSCT[46]. Our results also suggest that activated cord blood KIR3DL1$^+$ NK cells exhibit a strong alloreactivity in a dUCBT context leading to potential death by apopotosis. Overall, both our genetic and cellular data suggest that KIR$^+$ NK-cell mediated immune interactions between both UCB units rather than T-cell and/or intrinsic properties of each infused UCB unit may explain single unit dominance observed after dUCBT. These results are also corroborated with data from Eldjerou et al. [47] who showed that UCB unit dominance is an *in vivo* phenomenon associated with a GvG immune interaction mediated with CD34$^-$ cells. No significant correlation between one UCB unit dominance and the frequencies of HLA-A, -B, -C and -DRB1 mismatches in GvH and GvG directions was observed in our series. However, we cannot exclude the presence of functional alloreactive CD8$^+$ T cells from winner UCB units against loser UCB units as described[16].

In our cohort, only the KIR3DL1^{+} loser UCB/Bw4^{-} winner UCB unit genetic combination was significantly associated with one full UCB unit dominance after dUCBT although some other KIR/KIR ligand incompatibilities differ between winner and loser UCB units. The KIR3DL1^{+} loser/Bw4^{-} winner UCB unit combination was particularly underlined in our study notably due to the attenuated effects mediated by other KIR2DL receptors which shared HLA-C ligands[38] and by the frequency of HLA-Bw4+ in loser UCB units and patients which is particularly high in our cohort compared to winner UCB units. Interestingly, among the 8 excluded patients with autologous recovery, no Bw4 mismatches were observed between both KIR3DL1^{+} UCB units. Moreover, the ligands of KIR3DL1 are clearly identified in contrast to KIR2DL2/2DL3 which interact with a larger spectrum of HLA-C molecules[23]. Overall, one could expect that others KIR/KIR ligand incompatibilities may impact on dUCBT outcome depending on graft parameters such as the level of HLA class I matching between both UCB units and patient, the disease and the ethnicity of the patient and UCB units. Of note, KIR gene/genotype and HLA class I allele frequencies differ between populations as observed for the distribution of C1, C2 and Bw4 ligands between Japanese and Caucasian populations[48]. Lastly, the large KIR allelic polymorphism such as reported for KIR3DL1 gene[37] may also impact on both the phenotype and the function of cord blood KIR^{+} NK cells. Thus, KIR allelic polymorphism, contribution of other KIR genes and corresponding KIR ligands should be further investigated on a larger cohort in order to determine the hierarchy between all KIR/KIR ligand combinations functionally implicated in dUCBT outcome.

In addition to one full UCB unit dominance, the KIR3DL1^{+}/Bw4^{-} winner UCB genetic combination was also significantly associated with a higher incidence of relapse in our series. This may reflect a poor NK cell alloreactivity of the winner UCB unit which needs to

be further investigated since functional NK cells able to kill AML blasts after UCBT have been reported[49].

Based on our results showing the significant impact of KIR3DL1^{+} loser UCB/Bw4^{-} winner UCB genetic combination on both the delay of neutrophil recovery and relapse incidence, we suggest that a strong alloreactivity of cord blood NK cells hampers their own engraftment, and favors engraftment of the UCB unit which may exhibit a poor anti-leukemic NK cell alloreactivity. Taking together, we suggest that the winner UCB unit displays KIR/HLA features favoring its engraftment such as low KIR/HLA incompatibilities in the GvG direction and low NK cell activation in contrast to the loser UCB unit, characterized by high KIR/HLA incompatibilities and subsequent high NK cell activation possibly leading to death (Figure 4G). Overall, if confirmed on a larger dUCBT cohort, our results suggest that it may prove advantageous to include KIR gene/KIR ligand content in the selection of UCB units, in addition to conventional tools such as UCB cell doses and HLA matching, to find the perfect UCB unit tandem which can both improve engraftment and decrease relapse incidence after dUCBT[50].

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DISCLOSURES

The authors declare no financial or commercial conflict of interest.

AUTHORSHIP CONTRIBUTION

PR performed KIR genotyping, phenotype and functional studies, interpretation of data and contributed to writing the manuscript.

CW performed phenotype and functional studies.

CV performed statistical analysis.

NL performed KIR genotyping

PC provided clinical data of dUCBT and commented on the manuscript.

LL collected and interpreted hematopoietic chimerism data.

JE provided cord blood samples.

AC collected HLA typing and commented on the manuscript.

MB provided advices on the study setting and edited the manuscript.

PM commented on the manuscript.

DS provided KIR multiplex primers and commented on the manuscript.

CR designed the study, analyzed and interpreted cellular data, commented on the manuscript and contributed to writing the manuscript.

KG designed the study, analyzed and interpreted immunogenetic data and wrote the paper.

All the authors have approved the manuscript for publication.

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FIGURE LEGENDS

Figure 1: HLA mismatches and KIR ligand distribution among patients, loser and winner UCB units A) Potential T cell and NK cell alloreactivity on one full UCB unit dominance after dUCBT. Potential T cell alloreactivity was evaluated depending on HLA-A, -B, -C and HLA-DRB1 mismatches between winner Umbilical Cord Blood (UCB) unit or loser UCB units against the Patient in the Graft-versus-Host direction (GvH), between patients having received a reduced intensity conditioning (RIC) before UCBT against winner or loser UCB units in the Host-versus-Graft direction (HvG), and between both UCB units in the Graft-versus-Graft direction (GvG). Potential NK cell alloreactivity was evaluated depending on the presence of a specific inhibitory KIR gene and the absence of the corresponding KIR ligand or on the presence of a specific activating KIR gene and the presence of the corresponding KIR ligand in the GvH, HvG and GvG directions. B) HLA class I and HLA-DRB1 mismatches have been estimated from HLA-A, HLA-B, HLA-C (intermediate resolution), and HLA-DRB1 (high resolution) typing between winner UCB units (n=50), loser UCB units (n=50) and patients (n=50) in both GvH and GvG directions. C) HLA-A3, A11+ (KIR3DL2), HLA-A and HLA-B Bw4+ (KIR3DL1), HLA-C1+ (KIR2DL2/L3/S2) and HLA-C2+ (KIR2DL1/S1) KIR ligand frequencies have been estimated from HLA-A, HLA-B and HLA-C typing on winner UCB units (n=50), loser UCB units (n=50), patients (n=50) and healthy blood donors (n=87). Charts representing the number (D) and the nature (E) of $Bw4^+$, $C1^+$, $C2^+$ KIR ligands expressed in percentages found in winner UCB units (n=50), loser UCB units (n=50) and in patients (n=50). Numbers and percentages were compared between groups with χ^2 tests or Fisher's exact test when appropriate.

Figure 2: Impact of KIR/KIR ligand genetic combinations on one full UCB unit dominance after dUCBT. A) Potential $KIR2DL1^+$, $KIR2DL2/L3^+$ and $KIR2DS1^+$ NK cell

alloreactivity was evaluated in dUCBT depending on the presence of the KIR2DL1, KIR2DL2/L3 or KIR2DS1 gene and the absence of C2 ligand (2DL1), absence of C1 ligand (2DL2/L3) or presence of C2 ligand (2DS1) respectively. The number of $KIR2DL1^{+}/C2^{-}$, $KIR2DL2/L3^{+}/C1^{-}$ and $KIR2DS1^{+}/C2^{+}$ genetic combinations between winner UCB unit/patient pairs (n=50) and loser UCB unit/patient pairs (n=50) in the GvH direction, and between winner UCB unit/loser UCB unit pairs (n=50) and loser UCB unit/winner UCB unit pairs (n=50) in the GvG direction was compared. B) Potential $KIR3DL1^{+}$ NK cell alloreactivity was evaluated in dUCBT depending on the presence of the expressed KIR3DL1 gene and absence of Bw4 ligand respectively. The number of $KIR3DL1^{+}/Bw4^{-}$ genetic combinations between winner UCB unit/patient pairs (n=50) and loser UCB unit/patient pairs (n=50) in the GvH direction, and between winner UCB unit/loser UCB unit pairs (n=50) and loser UCB unit/winner UCB unit pairs (n=50) in the GvG direction was compared. C) Charts representing the detailed KIR/KIR ligand combinations taking into account KIR2DL1, KIR2DL2/L3, KIR3DL1 and KIR2DS1 genes in both the Graft-versus-Host (GvH) and Graft-versus-Graft (GvG) directions. Presence of KIR2DL1, KIR2DL2/L3, KIR3DL1 or KIR2DS1 gene in winner UCB units (n=50) and in loser UCB units (n=50) was evaluated by multiplex KIR PCR-SSP. Absence of corresponding KIR ligand for 2DL1 (C2), 2DL2/L3 (C1), 3DL1 (Bw4) in patients (n=50, GvH direction), in loser UCB units, or in winner UCB units (n=50, GvG direction) was established from HLA class I typing. Presence of C2 ligand for KIR2DS1 in patients (n=50, GvH direction), in loser and winner UCB units (n=50, GvG direction) was established from HLA-C typing. D) Charts representing the number of KIR.KIR ligand combinations in both the Graft-versus-Host (GvH) and Graft-versus-Graft (GvG) directions taking into account isolated KIR2DL1+/C2-, KIR2DL2/L3+/C1-, KIR3DL1+/Bw4- and KIR2DS1+/C2+ incompatibilities and cumulated ones. Numbers and percentages were compared between groups with χ^2 tests or Fisher's exact test when appropriate.

Figure 3: KIR3DL1$^+$ loser UCB/Bw4$^-$ winner UCB genetic combination is associated with shortened neutrophil recovery but increased relapse incidence after dUCBT. Time to neutrophil recovery (A), cumulative incidence of relapse (B), cumulative incidence of overall survival (C) and cumulative incidence of acute GvHD (D) in dUCBT with expressed KIR3DL1$^+$ loser UCB/Bw4$^-$ winner UCB unit genetic combination and without this genetic combination for all patients.

Figure 4: Umbilical cord blood cells are characterized by a low HLA class I expression and cord blood KIR3DL1$^+$ NK cells are stimulated as a result of Graft-versus-Graft interactions, resulting in their self-destruction (A) Scatter plots representing the MFI of HLA class I expression on freshly isolated (n=15) and thawed (n=8) cord blood cells, and freshly isolated (n=11) and thawed (n=18) adult PBMC. Results are expressed as the subtraction of mean intensity fluorescence of IgG control to mean intensity fluorescence of HLA class I molecules. Statistical significance between two groups was determined using the one-way analysis of variance (ANOVA) test. **(**B) Representation of pP38 mean intensity fluorescence on cord blood (n=4, dotted line) and adult (n=6, full line) NK cells by titration experiment with PMA/ionomycin for 30 minutes. Histograms illustrating the MFI of pP38 phosphoproteins on cord blood and adult NK cells after stimulation with PMA/ionomycin for 30 minutes (black line) compared to medium (grey filled line) are shown for one representing experiment. Statistical significance between two groups was determined using the one-way analysis of variance (ANOVA) test. (C) Summary box and whisker plot summarizing the percentages of CD107a$^+$ KIR3DL1$^+$ NKG2A$^-$ NK cells after 5h degranulation assay at an effector: target ratio of 1:1 in different stimulation conditions: medium, 221, 221-Bw4 and 221-Bw6 cell lines for experiments performed from 7 Bw4$^+$ cord blood samples. Top and bottom whiskers represent values of the top and bottom 25% of cases respectively; boxed area, interquartile range. (D) Summary box and whisker plot summarizing the percentages of

$CD107a^+$ NK and (E) $CD107a^+$ $KIR3DL1^+$ NK cells for experiments performed from *in vitro* amplified cord blood cells (n=16) at day 17 and determined after 5h degranulation assay in different stimulation conditions: medium, 221 and 221-Bw4 cell lines at an NK:target ratio of 1:1. Statistical significance between two groups was determined using the one-way analysis of variance (ANOVA) test. (F) Scatter plots representing annexin-V expression of $KIR3DL1^+$ cord blood NK cells against 221 and 221-Bw4 cell lines after 2 weeks *in vitro* amplification of cord blood cells with allogeneic EBV-B cell lines. Statistical significance between two groups was determined using the one-way analysis of variance (ANOVA) test. **(**G) Model representing the characteristics of both the winner and the loser UCB units based on KIR/HLA incompatibilities in the GvG direction. Engraftment of the UCB unit (winner) is associated with lower KIR/HLA incompatibilities resulting in lower NK cell activation, in contrast to the loser UCB unit which does not expand probably due to higher KIR/HLA incompatibilities leading to NK cell activation and probably death.

Table 1: Analysis of risk factors influencing neutrophil recovery after dUCBT (n=50) with one full UCB unit dominance using Cox model

Variables	Median[min-max] n (%)	Hazard ratio [CI 95%]	p value	FDR qvalue
Univariate analysis				
TNC x 10^7/kg winner UCB unit infused	2.14[1.00-7.20]	1.01[0.79-1.28]	0.95	0.95
TNC x 10^7/kg loser UCB unit infused	1.89[1.18-5.26]	0.94[0.69-1.29]	0.69	0.71
$CD34^+$ x 10^5/kg winner UCB unit infused	0.42[0.10-7.90]	1.09[0.89-1.33]	0.40	0.71
$CD34^+$ x 10^5/kg loser UCB unit infused	0.42[0.00-3.33]	0.90[0.62-1.33]	0.61	0.71
$CD34^+$ post-thaw viability winner UCB unit	91.75[12.40-100.00]	1.00[0.99-1.02]	0.92	0.92
$CD34^+$ post-thaw viability loser UCB unit	86.50[47.00-100.00]	0.98[0.96-1.01]	0.14	0.63
$CD34^+$ post-thaw viability < 75% winner UCB unit	9 (18.00 %)	1.22[0.59-2.53]	0.60	0.71
$CD34^+$ post-thaw viability < 75% loser UCB unit	13 (26.53 %)	1.80[0.93-3.48]	0.08	0.56
Winner UCB /patient sex matched	23 (46.00 %)	0.70[0.39-1.23]	0.21	0.63
Winner UCB /patient HLA-A, -B, -DR matched	29 (58.00 %)	0.68[0.38-1.22]	0.20	0.63
Winner UCB /patient HLA-C matched	8 (16.00 %)	0.82[0.38-1.77]	0.62	0.71
Winner UCB /patient KIR ligand matched	27 (54.00 %)	0.88[0.50-1.55]	0.66	0.71
Loser UCB /patient sex matched	29 (58.00 %)	1.49[0.83-2.68]	0.18	0.63
Loser UCB /patient HLA-A, -B, -DR matched	31 (62.00 %)	0.52[0.29-0.94]	0.03	0.32
Loser UCB /patient HLA-C matched	9 (18.00 %)	1.12[0.54-2.33]	0.75	0.75
Loser UCB /patient KIR ligand matched	20 (40.00 %)	1.26[0.71-2.24]	0.44	0.71
$KIR2DS1^+$ loser UCB /$C2^+$ patient	18 (36.00 %)	0.88[0.48-1.59]	0.66	0.71
Loser UCB /winner UCB unit sex matched	24 (48.00 %)	1.24[0.71-2.18]	0.45	0.71
Loser UCB /winner UCB unit HLA-A, B, DR matched	31 (62.00 %)	1.01[0.55-1.84]	0.98	0.98
Loser UCB /winner UCB unit HLA-C matched	11 (22.00 %)	1.38[0.70-2.73]	0.35	0.72
Loser UCB /winner UCB unit KIR ligand matched	23 (46.00 %)	1.26[0.71-2.23]	0.43	0.71
Bw4+ loser UCB /Bw4- winner UCB unit	11 (22.00 %)	0.85[0.42-1.71]	0.65	0.71
$KIR2DL1^+$ loser UCB/$C2^-$ winner UCB unit	15 (30.00 %)	0.89[0.48-1.66]	0.72	0.72
$KIR2DL2/L3^+$ loser UCB/$C1^-$ winner UCB unit	7 (14.00 %)	0.83[0.37-1.86]	0.66	0.71
$KIR3DL1^+$ loser UCB/$Bw4^-$ winner UCB unit	13 (26.00 %)	**2.98[1.48-6.00]**	**0.002**	**0.048**
Multivariate analysis				
Winner UCB /patient HLA-A, -B, -DR matched	28 (57.14 %)	0.55[0.27-1.12]	0.10	
$CD34^+$ post-thaw viability < 75% loser UCB unit	13 (26.53 %)	1.97[0.92-4.22]	0.079	
Loser UCB /patient sex matched	28 (57.14 %)	1.65[0.90-3.03]	0.11	
Loser UCB /patient HLA-A, -B, -DR matched	30 (61.22 %)	0.67[0.34-1.29]	0.23	
$KIR3DL1^+$ loser UCB /$Bw4^-$ winner UCB unit	12 (24.49 %)	**2.88[1.32-6.27]**	**0.0077**	

Abbreviations: TBI: total body irradiation; TNC: total nucleated cell; KIR: killer immunoglobulin-like receptor; UCB: umbilical cord blood; UCBT: umbilical cord blood transplantation; CI: confidence interval.

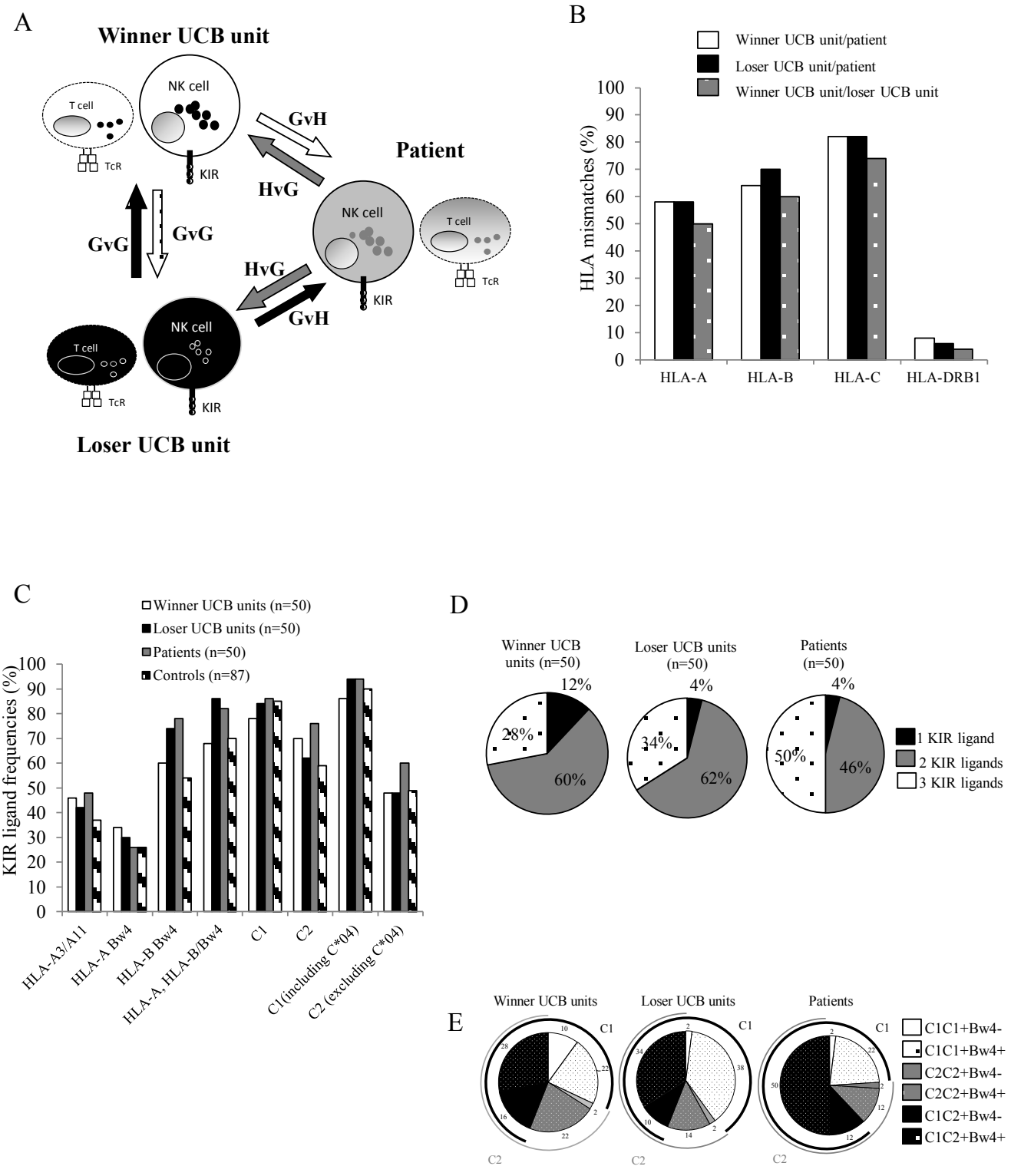

A
Winner UCB unit
T cell
NK cell
TcR
KIR
GvH
HvG
Patient
GvG
GvG
HvG
GvH
NK cell
T cell
TcR
KIR
NK cell
T cell
TcR
KIR
Loser UCB unit
B
Winner UCB unit/patient
Loser UCB unit/patient
Winner UCB unit/loser UCB unit
HLA mismatches (%)
100
90
80
70
60
50
40
30
20
10
0
HLA-A
HLA-B
HLA-C
HLA-DRB1
C
Winner UCB units (n=50)
Loser UCB units (n=50)
Patients (n=50)
Controls (n=87)
KIR ligand frequencies (%)
100
90
80
70
60
50
40
30
20
10
0
HLA-A3/A11
HLA-A Bw4
HLA-B Bw4
HLA-A, HLA-B/Bw4
C1
C2
C1(including C*04)
C2 (excluding C*04)
D
Winner UCB units (n=50)
Loser UCB units (n=50)
Patients (n=50)
12%
60%
28%
4%
62%
34%
4%
46%
50%
1 KIR ligand
2 KIR ligands
3 KIR ligands
E
Winner UCB units
Loser UCB units
Patients
C1
C2
C1C1+Bw4-
C1C1+Bw4+
C2C2+Bw4-
C2C2+Bw4+
C1C2+Bw4-
C1C2+Bw4+

Figure 1

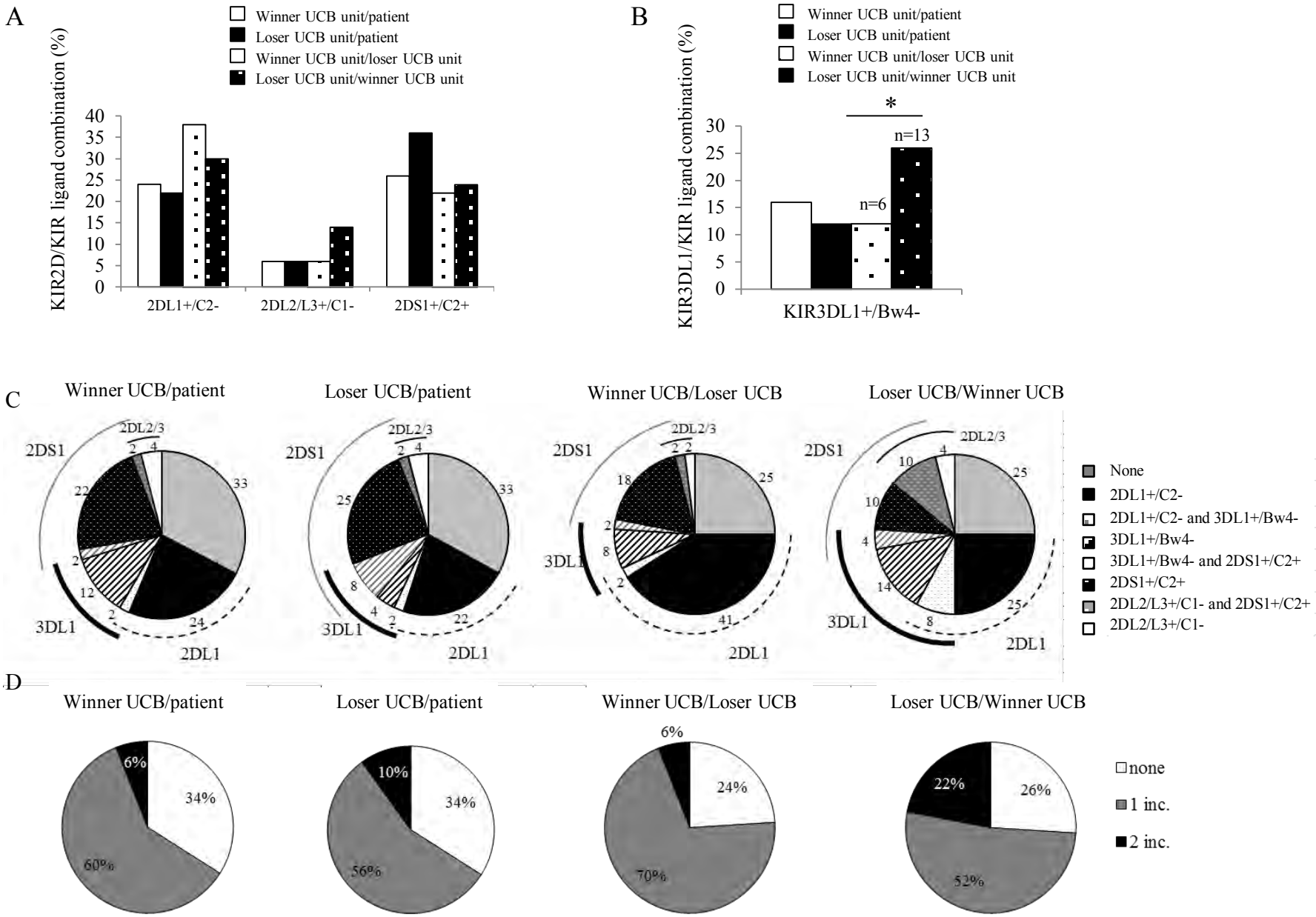
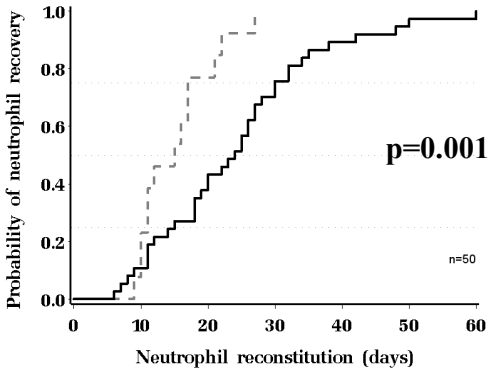

A
Winner UCB unit/patient
Loser UCB unit/patient
Winner UCB unit/loser UCB unit
Loser UCB unit/winner UCB unit
KIR2D/KIR ligand combination (%)
40
35
30
25
20
15
10
5
0
2DL1+/C2-
2DL2/L3+/C1-
2DS1+/C2+
B
Winner UCB unit/patient
Loser UCB unit/patient
Winner UCB unit/loser UCB unit
Loser UCB unit/winner UCB unit
KIR3DL1/KIR ligand combination (%)
*
n=13
n=6
30
25
20
15
10
5
0
KIR3DL1+/Bw4-
C
Winner UCB/patient
Loser UCB/patient
Winner UCB/Loser UCB
Loser UCB/Winner UCB
2DS1
2DL2/3
3DL1
2DL1
None
2DL1+/C2-
2DL1+/C2- and 3DL1+/Bw4-
3DL1+/Bw4-
3DL1+/Bw4- and 2DS1+/C2+
2DS1+/C2+
2DL2/L3+/C1- and 2DS1+/C2+
2DL2/L3+/C1-
D
Winner UCB/patient
Loser UCB/patient
Winner UCB/Loser UCB
Loser UCB/Winner UCB
6%
34%
60%
10%
34%
56%
6%
24%
70%
22%
26%
52%
none
1 inc.
2 inc.

Figure 2

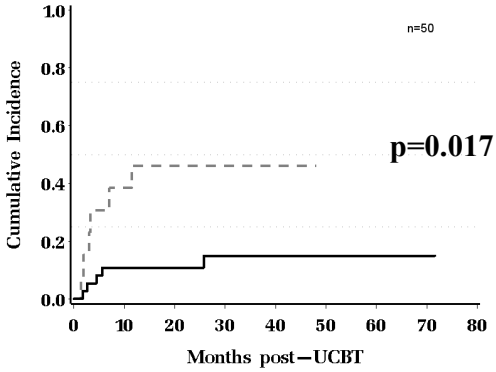
A



KIR3DL1+ loser UCB unit/
Bw4- winner UCB unit (n=13)

Others (n=37)

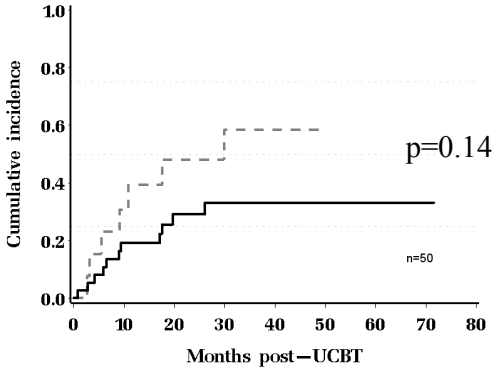
B



KIR3DL1+ loser UCB unit/
Bw4- winner UCB unit (n=13)

Others (n=37)

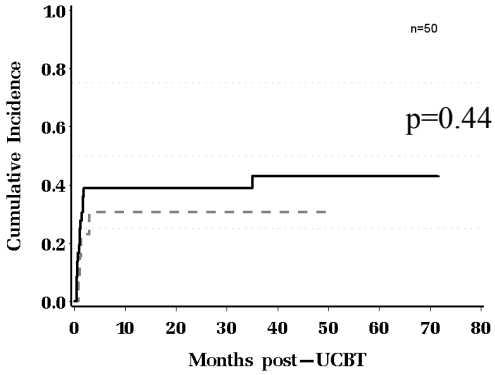
C



KIR3DL1+ loser UCB unit/
Bw4- winner UCB unit (n=13)

Others (n=37)

D



KIR3DL1+ loser UCB unit/
Bw4- winner UCB unit (n=13)

Others (n=37)

Figure 3

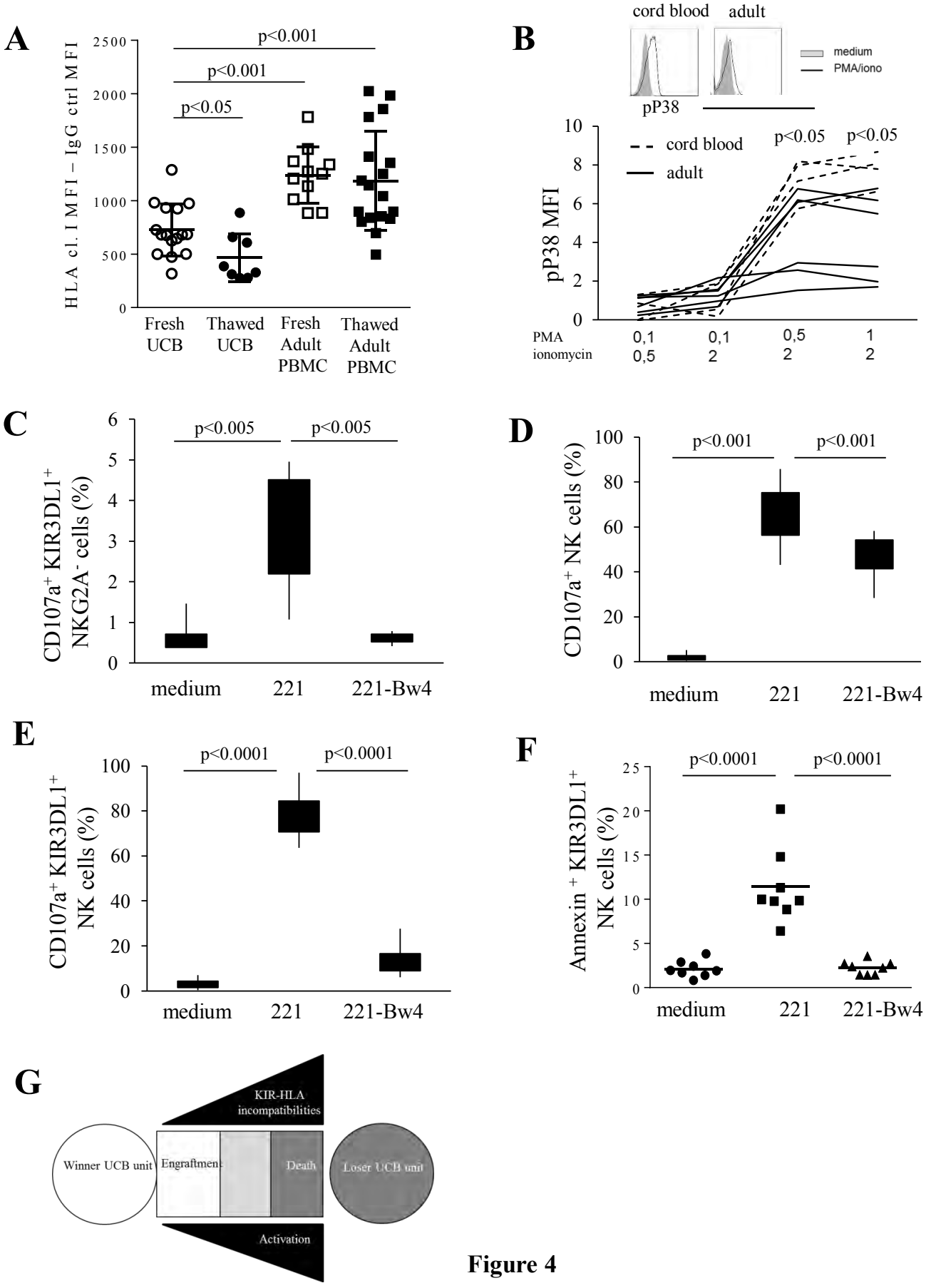

A
HLA cl. I MFI – IgG ctrl MFI
p<0.001
p<0.001
p<0.05
Fresh UCB
Thawed UCB
Fresh Adult PBMC
Thawed Adult PBMC
B
cord blood
adult
medium
PMA/iono
pP38
pP38 MFI
cord blood
adult
p<0.05
p<0.05
PMA
ionomycin
C
CD107a+ KIR3DL1+ NKG2A- cells (%)
p<0.005
p<0.005
medium
221
221-Bw4
D
CD107a+ NK cells (%)
p<0.001
p<0.001
medium
221
221-Bw4
E
CD107a+ KIR3DL1+ NK cells (%)
p<0.0001
p<0.0001
medium
221
221-Bw4
F
Annexin + KIR3DL1+ NK cells (%)
p<0.0001
p<0.0001
medium
221
221-Bw4
G
KIR-HLA incompatibilities
Winner UCB unit
Engraftment
Death
Loser UCB unit
Activation

Figure 4

RESULTATS

ARTICLE N°2 : Etude rétrospective et multicentrique des combinaisons génétiques KIR/HLA sur la dominance d'un seul cordon après double-greffe de sang de cordon

Is there any impact of KIR/HLA genetic combinations after double umbilical cord blood transplantation ? Results of a French multicentric retrospective study.

Pauline Rettman, Florent Malard, Nolwenn Legrand, Odile Avinens, Jean-François Eliaou, Christophe Picard, Anne Dormoy, Xavier Lafarge, Muriel de Matteis, Pascale Perrier, Pascale Loiseau, Anne Devys, Léna Absi, Marylise Fort, Dominique Masson, Fabienne Quainon, Ioannis Theodorou, Agnès Batho, Anne Parissiadis, Florent Delbos, Mireille Drouet, David Senitzer, Evelyne Marry, Nicole Raus, Ibrahim Yakoub-Agha, Anne Cesbron, Christelle Retière and Katia Gagne on behalf of the Société Française de Greffe de Moelle et de Thérapie Cellulaire (SFGM-TC) and the Société Francophone d'Histocompatibilité et d'Immunogénétique (SFHI).

Dans le cadre du premier projet, nous avions mené une étude rétrospective de l'impact d'une potentielle alloréactivité des cellules NK KIR$^+$ sur la dominance d'un seul cordon sur un effectif local de double-greffes de sang de cordon (dUCBT) (n=50). L'analyse des combinaisons génétiques KIR/KIR ligand, conforté par des données phénotypiques et fonctionnelles avait montré que l'alloréactivité des cellules NK KIR3DL1$^+$ de sang de cordon dans le sens Graft-versus-Graft (GvG) favoriser la prise de greffe du sang de cordon Bw4$^-$ mais augmente défavorablement le taux de rechute après dUCBT (Rettman et al soumis). Quelques études unicentriques et multicentriques ont rapporté l'effet des disparités des ligands KIR sur le devenir des dUCBT, mais sans toutefois prendre en compte les gènes KIR, et donc les combinaisons KIR/ligands KIR, et présentent également des résultats discordants (Willemze et al. 2009; Brunstein et al. 2009; Tanaka et al. 2013; Garfall et al. 2013). De plus, une seule étude a rapporté l'absence d'effet des combinaisons KIR/HLA sur la dominance d'un sang de cordon (Tarek et al. 2014).

Afin d'évaluer l'implication des combinaisons KIR/HLA sur le devenir des dUCBT, nous avons mené une étude multicentrique de l'impact des incompatibilités des ligands KIR (HLA de classe I), des gènes KIR et des combinaisons KIR/KIR ligand sur la dominance d'un seul cordon après dUCBT à partir d'un large effectif (n=293), en collaboration avec la Société Française de Greffe de Moelle et de Thérapie Cellulaire (SFGM-TC, Pr I. Yakoub-Agha), le Registre de France Greffe de Moelle (RFGM, Dr E. Marry) et la Société Francophone d'Histocompatibilité et d'Immunogénétique (SFHI, Pr JF. Eliaou), incluant 19 laboratoires HLA et centres greffeurs associés. Les typages des 14 gènes KIR fonctionnels ont été réalisés par PCR multiplex à l'aide des groupes d'amorces KIR définies par le groupe de D. Senitzer (Sun et al. 2004). Les données génotypiques, biologiques et cliniques ont été confrontées dans des analyses statistiques multivariées conduites par la Société Méthodomics (Dr PA. Gourraud, Dr C. Le Gall, Mortagne sur Sèvre).

A partir de l'effectif total, nous avons sélectionné uniquement les dUCBT avec prise de greffe et données cliniques/de chimérisme renseignées (n=225) et nous avons comparé les profils cliniques et immunogénétiques des cordons dits gagnants (Cg) et des cordons dits perdants (Cp). Aucune différence statistique significative n'a été observée en termes de statut CMV, de sexe, de dose de cellules nucléées totales et cellules $CD34^+$ injectées par cordon, de niveau de compatibilité HLA-A, -B, -DRB1 ou de fréquence de gènes KIR et des ligands KIR. L'analyse des fréquences des différentes combinaisons KIR/KIR ligand (ligands connus ou putatifs) dans le sens Graft-versus-Host (GvH) (entre chaque cordon et le patient) et dans le sens Graft-versus-Graft (GvG) (entre les deux cordons) n'a montré aucune corrélation avec la prise d'un seul cordon. En particulier, la fréquence de la combinaison Cp $KIR3DL1^+$/Cg $Bw4^-$ n'est pas statistiquement associée à la dominance d'un seul cordon sur cet effectif contrairement à ce que nous avions observé sur la cohorte locale. L'analyse détaillée de la distribution des combinaisons KIR/KIR ligands, seules ou cumulées, ne diffère pas statistiquement dans les sens GvH et GvG.

Nous avons émis l'hypothèse que l'absence de corrélation significative entre la combinaison Cp $KIR3DL1^+$/Cg $Bw4^-$ et la dominance d'un seul cordon pouvait être liée aux politiques de greffes variables selon les centres et à l'hétérogénéité des paramètres liés aux patients ou aux greffons. Nous avons évalué un possible « effet centre » en comparant les caractéristiques des dUCBT des 8 centres greffeurs avec au moins 10 dUCBT par centre. Nous avons ainsi confirmé que de nombreux paramètres cliniques et biologiques propres aux patients et/ou aux cordons injectés différent statistiquement entre les centres greffeurs, en particulier le diagnostic initial avant greffe, le régime de conditionnement, l'utilisation et la dose d'ATG, la prophylaxie de la GvHD, l'intervalle de reconstitution en neutrophiles et les doses de cellules nucléées totales et de cellules $CD34^+$ injectées par cordon. Ces différences attestent de la grande hétérogénéité des pratiques selon les centres greffeurs. De plus, les

fréquences de 3 combinaisons KIR/KIR ligands différent cependant statistiquement entre les centres (Cp 2DL2/L3^{+}/P C1^{-}, Cp 2DL2/L3^{+}/Cg C1^{-} et Cg 3DS1^{+}/P Bw4^{+}). Au vue de cette hétérogénéité, une cohorte réduite plus homogène a été construite (n)121), en réalisant une analyse de correspondance multivariée (CAM) excluant notamment les dUCBT atypiques et celles présentant beaucoup de données manquantes. L'analyse des combinaisons KIR/KIR ligand sur la dominance d'un seul cordon a ensuite été reproduite mais, aucune différence significative n'a été observée entre les fréquences des combinaisons KIR/KIR ligands dans les sens GvH ou GvG entre les patients et les cordons gagnants et perdants dans cette cohorte réduite.

Il a précédemment été rapporté que l'impact des disparités des ligands KIR et/ou des combinaisons KIR /KIR ligands sur le devenir des dUCBT peut varier selon les pathologies, le régime de conditionnement et/ou l'utilisation de l'ATG (Willemze et al. 2009; Brunstein et al. 2009; Tanaka et al. 2013; Garfall et al. 2013). Nous avons donc comparé les données démographiques et cliniques entre la cohorte globale (n=225) et la cohorte réduite (n=121) de dUCBT ce qui a révélé des différences significatives en termes de pathologies (lymphoïdes vs myéloïdes), de régime de conditionnement (myéloablatif vs non-myéloablatif), et d'utilisation/de dose de TBI. Nous avons donc stratifié les dUCBT en fonction des pathologies (myéloïdes/lymphoïdes), du conditionnement (myéloablatif/non-myéloablatif) et de l'utilisation ou non d'ATG, ces 3 paramètres cliniques impactant sur la dominance d'un seul cordon après dUCBT. D'une part, la fréquence de la combinaison Cp KIR3DL2^{+}/A3,A11^{-} Cg diffère statistiquement selon la pathologie (lymphoïdes vs myéloïdes) dans la cohorte globale. D'autre part, les fréquences des combinaisons Cp KIR2DL2/3^{+}/C1^{-} Cg et Cp KIR3DS1^{+}/Bw4^{-} Cg diffèrent statistiquement selon la nature du conditionnement (myéloablatif/non-myéloablatif) dans la cohorte réduite. La présence d'incompatibilités KIR/KIR ligand semble impacter la prise de greffe en particulier pour les patients atteints de

pathologies myéloïdes, ce qui est en accord avec la mise en évidence de l'alloréactivité des cellules NK vis-à-vis de lignées leucémiques myéloïdes plutôt que lymphoïdes (Ruggeri et al. 2002).

Pour évaluer l'impact de ces combinaisons KIR/ligand KIR sur le devenir des dUCBT au cours du temps, nous avons par la suite réalisé des analyses de survie sur différents paramètres cliniques : la sortie d'aplasie/délai de reconstitution en neutrophiles, la survie globale (OS), la survie sans progression (PFS), l'incidence de rechute, la mortalité non liée à la rechute (NRM), l'incidence de GvH aigue et chronique sur les cohortes de dUCBT globale (n=225) et réduite (n=121). Les résultats des analyses multivariées de Cox ne rapportent aucune corrélation significative entre les combinaisons KIR/KIR ligand et l'OS, la PFS ou l'incidence de rechute. Contrairement à ce que nous avions précédemment montré, la combinaison Cp KIR3DL1^{+}/Cg Bw4^{-} n'impacte ni le délai de reconstitution en neutrophiles, ni aucun paramètre clinique. Enfin, sur la cohorte globale, 2 combinaisons KIR/KIR ligand (Cg 2DS1^{+}/Cp C2^{+} et Cg 2DL2/L3^{+}/P C1^{-}) impactent la sortie d'aplasie, assurant une reconstitution de neutrophiles plus rapide par rapport aux dUCBT dépourvues de ces combinaisons.

Contrairement aux résultats obtenus dans notre cohorte locale dans laquelle les cordons gagnants étaient caractérisés par une faible fréquence en Bw4, nous n'observons aucun impact des combinaisons génétiques KIR/ligands sur la dominance d'un sang de cordon après dUCBT dans cette étude multicentrique. L'implication de nombreux paramètres cliniques peut expliquer la moindre importance des combinaisons génétiques KIR/ligands dans ce contexte de dUCBT. Nous avons cependant mis en évidence le potentiel bénéfique des combinaisons KIR/KIR ligand en particulier dans le sens GvH sur la reconstitution des neutrophiles. Il a précédemment été montré que la forte dose en cellules NK contenue par un greffon de moelle osseuse est corrélée à une augmentation de la reconstitution des

neutrophiles (Larghero et al. 2007). Il est donc possible que la prolifération de cellules NK KIR^+ alloréactives du cordon gagnant favorise la reconstitution des neutrophiles. Toutefois, d'autres études multicentriques de dUCBT sont nécessaires afin de confirmer nos résultats et déterminer si les génotypes KIR/ligands KIR devraient être inclus dans la sélection d'une unité de sang de cordon afin d'améliorer le devenir des dUCBT.

Is there any impact of KIR/HLA genetic combinations on double umbilical cord blood transplantation outcome ? Results of a French multicentric retrospective study.

Pauline Rettman[1], Florent Malard[2,3], Nolwenn Legrand[1], Odile Avinens[4], Jean-François Eliaou[4,5], Christophe Picard[6], Anne Dormoy[7], Xavier Lafarge[8,] Muriel de Matteis[9,] Anne Kennel[10], Pascale Loiseau[11,12], Anne Devys[2,13], Léna Absi[14], Marylise Fort[15], Dominique Masson[16], Fabienne Quainon[17,] Ioannis Theodorou[18,] Agnès Batho[19,] Anne Parissiadis[20], Florent Delbos[21], Mireille Drouet[22], David Senitzer[23], Evelyne Marry[24], Nicole Raus[25], Ibrahim Yakoub-Agha[25], Anne Cesbron[2,12], Christelle Retière[1,26] and Katia Gagne[1,2,12,26,27] on behalf of the Société Française de Greffe de Moelle et de Thérapie Cellulaire (SFGM-TC) and the Société Francophone d'Histocompatibilité et d'Immunogénétique (SFHI).

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Key words: double umbilical cord blood transplantation, NK cell, KIR, HLA, cord blood dominance

Full chimerism after double umbilical cord blood transplantation (dUCBT) is usually derived from only one of the two UCB units injected. Because UCB units and patients may be mismatched for HLA class I loci, T and/or Natural Killer (NK) cell alloreactivities between UCB units and patient in the Graft-versus-Host (GvH) direction, and between the two UCB units in the Graft-versus-Graft (GvG) direction should be considered [1]. By contrast to T lymphocytes, there is a rapid recovery of NK cells after UCBT [2], representing the only lymphoid cell population potentially able to control leukemic relapse in the months preceding T-cell reconstitution [3]. The effector functions of NK cells are tuned by inhibitory and activating receptors such as the Killer cell Immunoglobulin-like Receptors (KIR), which are specific to allotypic determinants shared by different HLA-class I molecules (referred to as KIR ligands) [4]. Lack of inhibitory KIR engagement can trigger alloreactive KIR^+ NK cell cytotoxicity only within functionally competent NK cells [5]. Although the ligands and functions of inhibitory KIR are well documented, this is not the case for activating KIR, except for KIR2DS1 [6] and KIR2DS2 [7]. We recently demonstrated that KIR^+ cord blood NK cells reconstitute the patient hematopoiesis as soon as day+14 after dUCBT [8]. Few studies reported the effect of KIR ligand disparities on UCBT outcome, with discordant results depending on the clinical endpoint or graft parameters [9-12]. Furthermore, none of these studies take into account KIR genes especially activating ones. The impact of KIR/KIR ligand combinations on UCB dominance especially in the GvG direction was only evaluated in an unicentric study by Tarek et al. [13] who reported no identifiable role of KIR/HLA genotypes on UCB dominance after dUCBT. Contributing to novel information for the field, we recently evaluated the impact of inhibitory and activating KIR/KIR ligand combinations in both GvH and GvG directions on UCB dominance after dUCBT. The analysis of genetic KIR/KIR ligand combinations on a local dUCBT cohort (n=50) showed that only the $KIR3DL1^+$ loser UCB/$Bw4^-$ winner UCB combination, significantly increased in the GvG

direction, impacted on neutrophil recovery and increased relapse incidence post-dUCBT [14]. Here, we propose to investigate the strength of this KIR/KIR ligand combination on dUCBT outcome in a large cohort of dUCBT. In this multicentric dUCBT study, we retrospectively studied for the first time the distribution of KIR ligands, KIR genes and KIR/KIR ligand genetic combinations by comparing the characteristics of winner vs loser UCB units.

This study first included 293 patients who received dUCBT between July 2005 and December 2011 in 19 different French graft centers (Suppl. Table 1). Only 253 patients engrafted, of which 227 with complete hematopoietic chimerism data including winner UCB unit identity were selected in this study (Suppl. Table 1). No significant differences in terms of sex matching, CMV status, HLA-A, -B, -DRB1 matching, total nucleated cell and $CD34^+$ cell doses infused per UCB unit were observed between patient/winner UCB units and patient/loser UCB units. As reported by Tarek et al. [13], the frequencies of KIR ligands, KIR genes and KIR genotypes were not statistically different between patients, winner and loser UCB units in our cohort (Suppl. Table 2). Analysis of KIR/KIR ligand genetic combinations in either GvH or GvG directions showed no significant correlation with one full UCB unit dominance (Figure 1A). In particular, the $KIR3DL1^+$ loser/$Bw4^-$ winner UCB unit genetic combination was not significantly higher in the GvG direction (22% vs 17%, p=0.23) in contrast to what we observed on a local cohort of 50 dUCBT [14]. The detailed distribution of both isolated and cumulative KIR/KIR ligand combinations did not differ statistically neither in the GvH nor in the GvG direction (Figure 1B). We hypothesized that the absence of significance of $KIR3DL1^+$ loser/$Bw4^-$ winner UCB unit genetic combination on this larger cohort could be related to the transplant policy of each graft center and/or, the heterogeneity of graft parameters. The comparison of graft characteristics of 8 centers with at least 10 dUCBT per center included in this study, highlighted the significant difference of many parameters specific to patients and/or infused UCB such as the initial diagnosis before

transplant, the conditioning regimen intensity and the TBI dose, the use of ATG, GvHD prophylaxis, TNC and $CD34^+$ cell dose infused (data not shown). In addition, the number of KIR/KIR ligand incompatibilities in GvG and GvH directions differed depending on each graft center. To illustrate this point, the proportion of KIR/KIR ligand incompatibilities (none, one, more than one) in the loser UCB/winner UCB direction was represented in each graft center taking into account the size of each center in the studied cohort (Figure 1C-1D). This observation can explain that the high frequency of $Bw4^-$ winner UCB units observed in our previous local dUCBT cohort[14], referred in this study as graft center#6 was not confirmed in this large multicentric cohort (data not shown). Overall, given the heterogeneity of this multicentric dUCBT cohort, a more homogeneous cohort of 121 dUCBT (Suppl. Table 1) was built excluding "atypical" dUCBT using Multivariate Correspondence Analysis (Figure 1E). In this case, grafts with specific patterns on some clinical characteristics, not representative of all the grafts, were removed to reduce the data heterogeneity. However, no significant difference between KIR/KIR ligand genetic combination frequencies (Suppl. Figure 1A) and detailed distribution of isolated an cumulative KIR/KIR ligand combinations (Suppl. Figure 1B) in the GvH or GvG direction on UCB unit dominance was observed between patients, winner and loser UCB unit in this restricted dUCBT cohort. It has been reported that the impact of KIR ligand disparities and/or KIR/KIR ligand combinations after HLA non-identical HSCT may depend on diseases, conditioning intensity, and/or use of ATG [3,9-13]. Comparison of all demographic/clinical data revealed that only the myeloablative conditioning regimen statistically differ between the entire (n=227) and the restricted (n=121) dUCBT cohorts (Suppl. Table 1, p=0.00415). Thus, KIR/KIR ligand genetic combinations were further re-evaluated depending on conditioning regimen intensity, diseases, and the use of ATG. In the entire dUCBT cohort, the frequency of the $KIR3DL2^+$ loser/A3, $A11^-$ winner UCB combination was statistically different according to diseases

(lymphoid 50% vs myeloid 67.6%, p=0.0153, Figure 1F). In the restricted dUCBT cohort, the frequencies of the KIR2DL2/3^{+} loser/C1^{-} winner UCB and KIR3DS1^{+} winner/Bw4^{+} loser UCB genetic combinations were statistically different between myeloablative versus reduced intensity conditioning regimen (6.5% vs. 26.7%, p=0.047, 26.9% vs. 56.2%, p=0.0381 respectively, data noty shown). No significant difference of KIR/KIR ligand frequency was observed depending on the use of ATG in both the entire and restricted dUCBT cohort (data not shown). Overall, some KIR/KIR ligand genetic frequencies differ between lymphoid and myeloid diseases arguing the effectiveness of donor alloreactive KIR^{+} NK cells reported only on AML patients and not on ALL patients after T depleted HLA haplo-identical HSCT [3].

In order to verify whether KIR2DL1^{+}/C2^{-}, KIR2DL2/L3^{+}/C1^{-}, KIR3DL1^{+}/Bw4^{-}, KIR3DL2^{+}/A3,A11^{-}, KIR2DS1^{+}/C2^{+}, KIR2DS2^{+}/C1^{+} and KIR3DS1^{+}/Bw4^{+} genetic combinations in the GvH and GvG direction may impact over the time on dUCBT outcome, multivariate survival analyses were conducted on neutrophil recovery, overall survival (OS), progression-free survival (PFS), incidence of relapse, non-relapse related mortality (NRM), incidence of acute and chronic GvHD on the entire (n=225) and the restricted dUCBT cohort (n=121). Multivariate survival analyses of the KIR2DL1^{+}/C2^{-}, KIR2DL2/L3^{+}/C1^{-}, KIR3DL2^{+}/A3,A11^{-}, KIR2DS1^{+}/C2^{+}, KIR2DS2^{+}/C1^{+} and KIR3DS1^{+}/Bw4^{+} combinations in GvH or GvG direction reported no significant result on OS, PFS and the incidence of relapse post-dUCBT (data not shown). The combination KIR2DL2/L3^{+} winner/C1^{-} loser UCB significantly increased NRM (Table 1). Moreover, the KIR2DL2/L3^{+} winner/C1^{-} patient genetic combination increased the aGvHD incidence while both the KIR3DS1^{+} winner/Bw4^{+} loser UCB and the KIR2DL1^{+} loser/C2^{-} winner UCB combinations decreased aGvHD incidence (Table 1). Lastly, the KIR2DL2/2DL3^{+} winner/C1^{-} loser UCB genetic combination and the patient-winner UCB unit HLA-A, -B, -C, -DRB1 high resolution matching significantly increased the cGvH incidence (Table 1). In the entire dUCBT, 2 KIR/KIR ligand

genetic combinations promoted neutrophil recovery namely KIR2DL2/L3^{+} winner/C1^{-} patient (Figure 2A), and KIR2DS1^{+} winner/C2^{+} loser UCB (Figure 2B). Multivariate analyses confirmed that these 2 KIR/KIR ligand combinations significantly impact on neutrophil recovery (Table 1).

In contrast to our previous study on a local cohort of 50 dUCBT marked by a high frequency of Bw4^{-} winner UCB units [14], we cannot observe an impact of KIR/KIR ligand genetic combinations on cord blood dominance from a large dUCBT cohort regrouping different French graft centers. This result can be explained by the multiparametric characteristics of dUCBT suggesting the weak impact of KIR/KIR ligand genetic combinations on cord blood dominance. However, we highlight the potential benefit of some KIR/KIR ligand genetic combinations on neutrophil recovery. Based on a previous report showing that a higher NK cell dose in graft was associated with an increased speed of neutrophil recovery [15], it is conceivable that the proliferation of alloreactive KIR^{+} NK cells from winner UCB unit may contribute to favor a faster neutrophil recovery. Overall, our results suggest that KIR/KIR ligand genetic combinations between UCB units and patients should be looked for to improve the speed of neutrophil recovery which remains a main difficulty after dUCBT. Nonetheless, extended genetic analysis should be investigated on other multicentric dUCBT cohorts to confirm this observation. Finally, mechanistic studies are further needed to explain this beneficial effect.

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AUTHORSHIP CONTRIBUTION

P.R. performed KIR genotyping, interpretation of data and contributed to writing the manuscript.

F.M. provided clinical data of dUCBT and reviewed the manuscript.

N.L performed KIR genotyping.

O.A., J.F.E., C.P., A.Do., X.L., M.d.M., A.K., P.L., A.D., L.A., M.F., D.M., F.Q., I.T., A.B., A.P., F.D., M.D. and A.C. provided DNA cord blood samples, collected HLA typing and reviewed the manuscript.

D.S. provided KIR multiplex primers and reviewed the manuscript.

E.M. provided HLA typing from French Registry.

N.R. provided clinical data from SFGMT-TC database.

I.Y.A. provided clinical data from SFGMT-TC database and reviewed the manuscript.

C.R. designed the study, reviewed the manuscript and contributed to writing the manuscript.

K.G. designed the study, analyzed and interpreted immunogenetic data and wrote the paper.

All the authors have approved the manuscript for publication.

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FIGURE LEGENDS

Figure 1: Frequencies and distribution of KIR/KIR ligand genetic combinations in Graft-versus-Host (GvH) and Graft-versus-Graft (GvG) direction after dUCBT. A) Potential KIR2DL1$^+$, KIR2DL2/L3$^+$ KIR3DL1$^+$, KIR3DL2$^+$ NK cell alloreactivity was evaluated in 225 dUCBT depending on the presence of the KIR2DL1, KIR2DL2/L3, KIR3DL1 or KIR3DL2 gene and the absence of respective C2, C1, Bw4 or A3/A11 ligands. Potential KIR2DS1$^+$, KIR2DS2$^+$, KIR3DS1$^+$ NK cell alloreactivity was evaluated in 225 dUCBT depending on the presence of the KIR2DS1, KIR2DS2, KIR3DS1 gene and the presence of corresponding C2, C1 or putative Bw4 ligand respectively. The frequencies of KIR2DL1$^+$/C2$^-$, KIR2DL2/L3$^+$/C1$^-$, KIR3DL1$^+$/Bw4$^-$, KIR3DL2$^+$/A3,A11$^-$, KIR2DS1$^+$/C2$^+$, KIR2DS2$^+$/C1$^+$, and KIR3DS1$^+$/Bw4$^+$ genetic combinations between winner UCB/patient and loser UCB/patient pairs in the Graft-versus-Host (GvH) direction, and between winner UCB/loser UCB and loser UCB/winner UCB unit pairs in the Graft-versus-Graft (GvG) direction were compared. Presence of KIR genes in winner and in loser UCB units was evaluated by multiplex KIR PCR-SSP. Absence or presence of corresponding KIR ligands were deduced from HLA-A, -B, -C typing. B) Charts representing the detailed KIR2DL1$^+$/C2, KIR2DL2/L3$^+$/C1$^-$, KIR3DL1$^+$/Bw4$^-$ and KIR2DS1$^+$/C2$^+$ ligand genetic combinations either isolated or cumulated in 225 dUCBT in both GvH and GvG directions. C) Charts representing the number of KIR2DL2/L3$^+$/C1$^-$ and KIR3DS1$^+$/Bw4$^+$ genetic combinations depending on the presence of KIR2DL2/L3 gene in loser UCB units with the absence of corresponding C1 ligand in patients (left panel) or winner UCB units (center panel) and on the presence of KIR3DS1 gene in winner UCB units and the presence of corresponding Bw4 ligand in patients (right panel) in 8 graft centers included in this multicentric study. D) Chart representing the selection of the homogeneous cohort by Multiple Correspondence Analysis

(MCA). This dimensional reduction technique is widely used with large dataset including a lot of parameters to have a global overview of the data in order to detect patterns and exclude outliers. In this case, after removing outliers, only grafts within an ellipse centered on the origin of the MCA individual graph, including a 75% percentage of grafts and whose form was proportional to the variance explained by each axis (dimension) from the plan 1-2 were kept. E) Potential KIR3DL2^{+} NK cell alloreactivity in the absence of corresponding A3/A11 ligand was evaluated in lymphoid and myeloid diseases in the entire dUCBT cohort (n=225). The frequencies of KIR3DL2^{+}/A3,A11^{-} genetic combinations between winner UCB/patient and loser UCB/patient pairs in the GvH direction, and between winner UCB/loser UCB and loser UCB/winner UCB unit pairs in the GvG direction were compared. F) Potential KIR2DL2/3^{+} NK cell alloreactivity in the absence of corresponding C1 ligand and G) potential KIR3DS1^{+} NK cell alloreactivity in the presence of the KIR3DS1 gene and the presence of putative Bw4 ligand were evaluated in the restricted dUCBT cohort (n=121) with either a non-myeloablative (non-MA) or myeloablative (MA) conditioning regimen. The frequencies of KIR2DL2/3^{+}/C1^{-} (F) and KIR3DS1^{+}/Bw4^{+} (G) genetic combinations between winner UCB/patient and loser UCB/patient pairs in the GvH direction, and between winner UCB/loser UCB and loser UCB/winner UCB unit pairs in the GvG direction were compared. Numbers and percentages were compared between groups with χ^2 tests. P-values <0.05 were considered as statistically significant.

Figure 2: Some KIR2D/HLA-C ligand genetic combination significantly impact on the delay of neutrophil recovery on dUCBT outcome (N=225). A) Cumulative incidence of neutrophil recovery of dUCBT with (dotted) and without (full line) the KIR2DL2/3^{+} winner/C1^{-} patient genetic combination; B) Cumulative incidence of neutrophil recovery of dUCBT with (dotted) and without (full line) the KIR2DL2/3^{+} loser/C1^{-} winner UCB genetic combination. C) Cumulative incidence of neutrophil recovery of dUCBT with (dotted) and

without (full line) the KIR2DS1^{+} winner/C2^{+} loser UCB genetic combination. P-values <0.05 were considered as statistically significant; D) Proposed model representing the characteristics of the winner, the loser UCB units and the patients based on significant KIR2D/HLA-C genetic combinations found in the Graft-versus-Host (GvH) and Graft-versus-Graft (GvG) direction significantly improving neutrophil recovery after dUCBT.

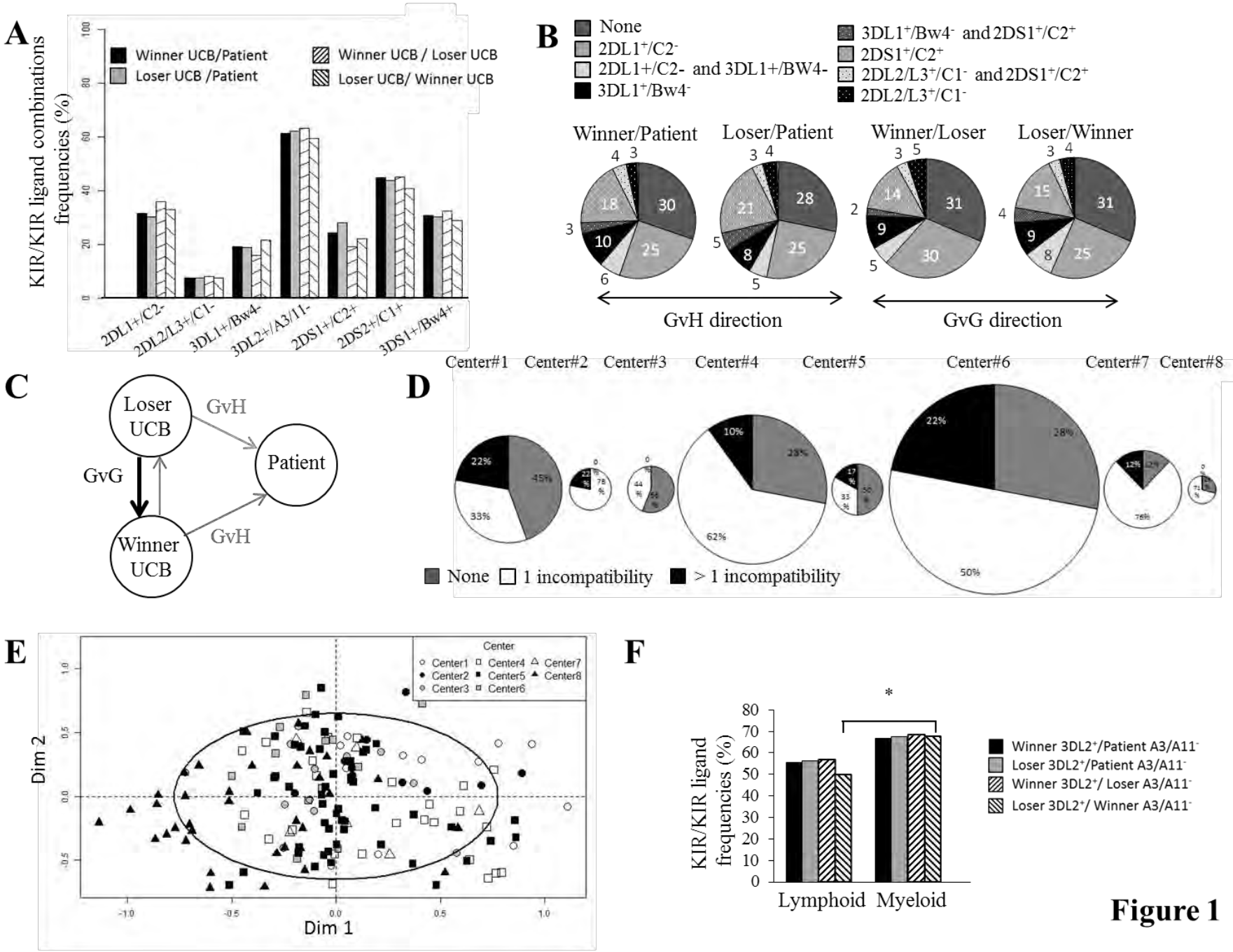

A
KIR/KIR ligand combinations frequencies (%)
Winner UCB/Patient
Loser UCB /Patient
Winner UCB / Loser UCB
Loser UCB/ Winner UCB
2DL1+/C2-
2DL2/L3+/C1-
3DL1+/Bw4-
3DL2+/A3/11-
2DS1+/C2+
2DS2+/C1+
3DS1+/Bw4+
B
None
2DL1+/C2-
2DL1+/C2- and 3DL1+/BW4-
3DL1+/Bw4-
3DL1+/Bw4- and 2DS1+/C2+
2DS1+/C2+
2DL2/L3+/C1- and 2DS1+/C2+
2DL2/L3+/C1-
Winner/Patient
Loser/Patient
Winner/Loser
Loser/Winner
GvH direction
GvG direction
C
Loser UCB
Patient
Winner UCB
GvH
GvG
GvH
D
Center#1
Center#2
Center#3
Center#4
Center#5
Center#6
Center#7
Center#8
None
1 incompatibility
> 1 incompatibility
E
Center
Center1
Center2
Center3
Center4
Center5
Center6
Center7
Center8
Dim 1
Dim 2
F
KIR/KIR ligand frequencies (%)
Lymphoid
Myeloid
Winner 3DL2+/Patient A3/A11-
Loser 3DL2+/Patient A3/A11-
Winner 3DL2+/ Loser A3/A11-
Loser 3DL2+/ Winner A3/A11-

Figure 1

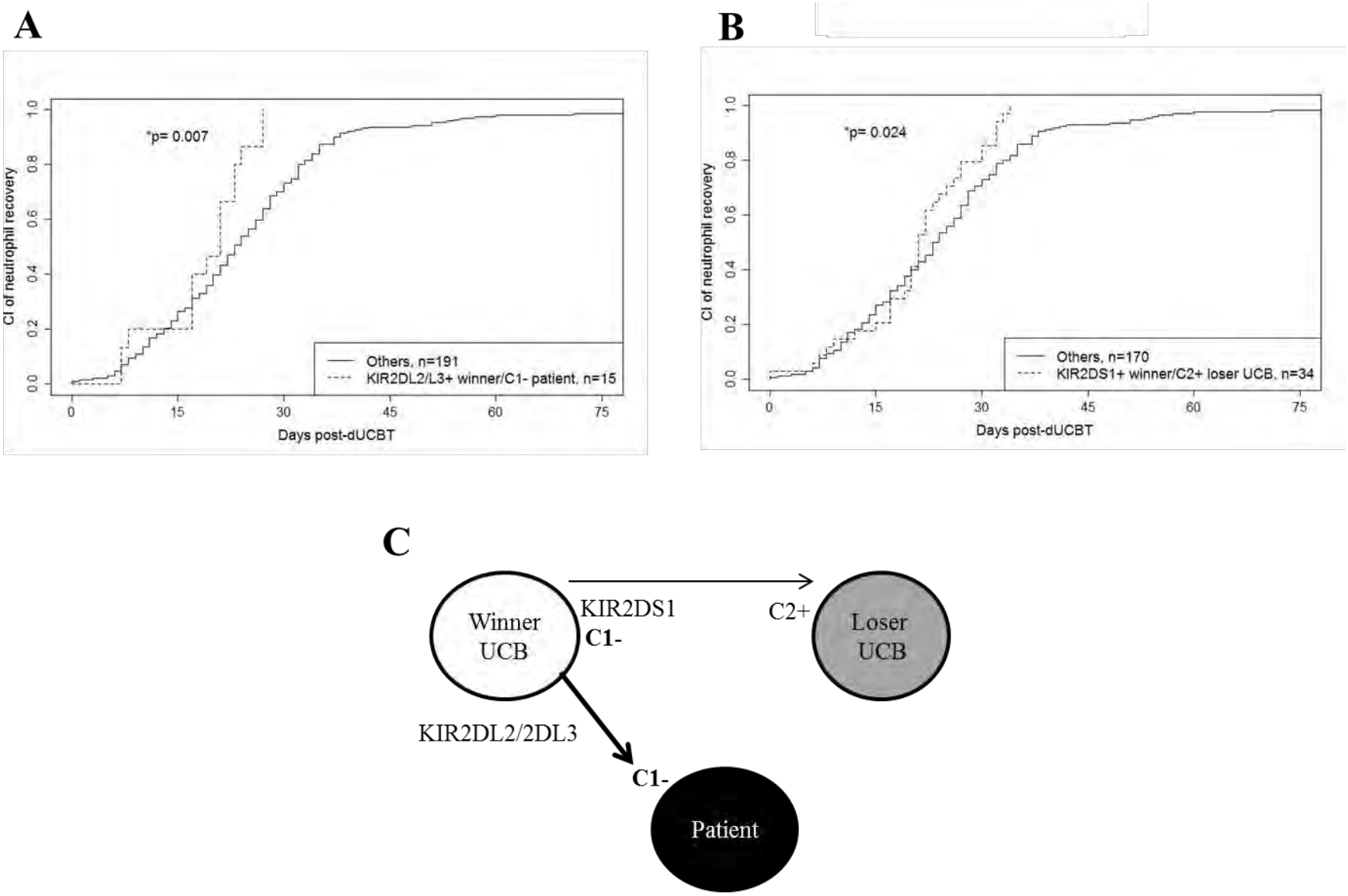
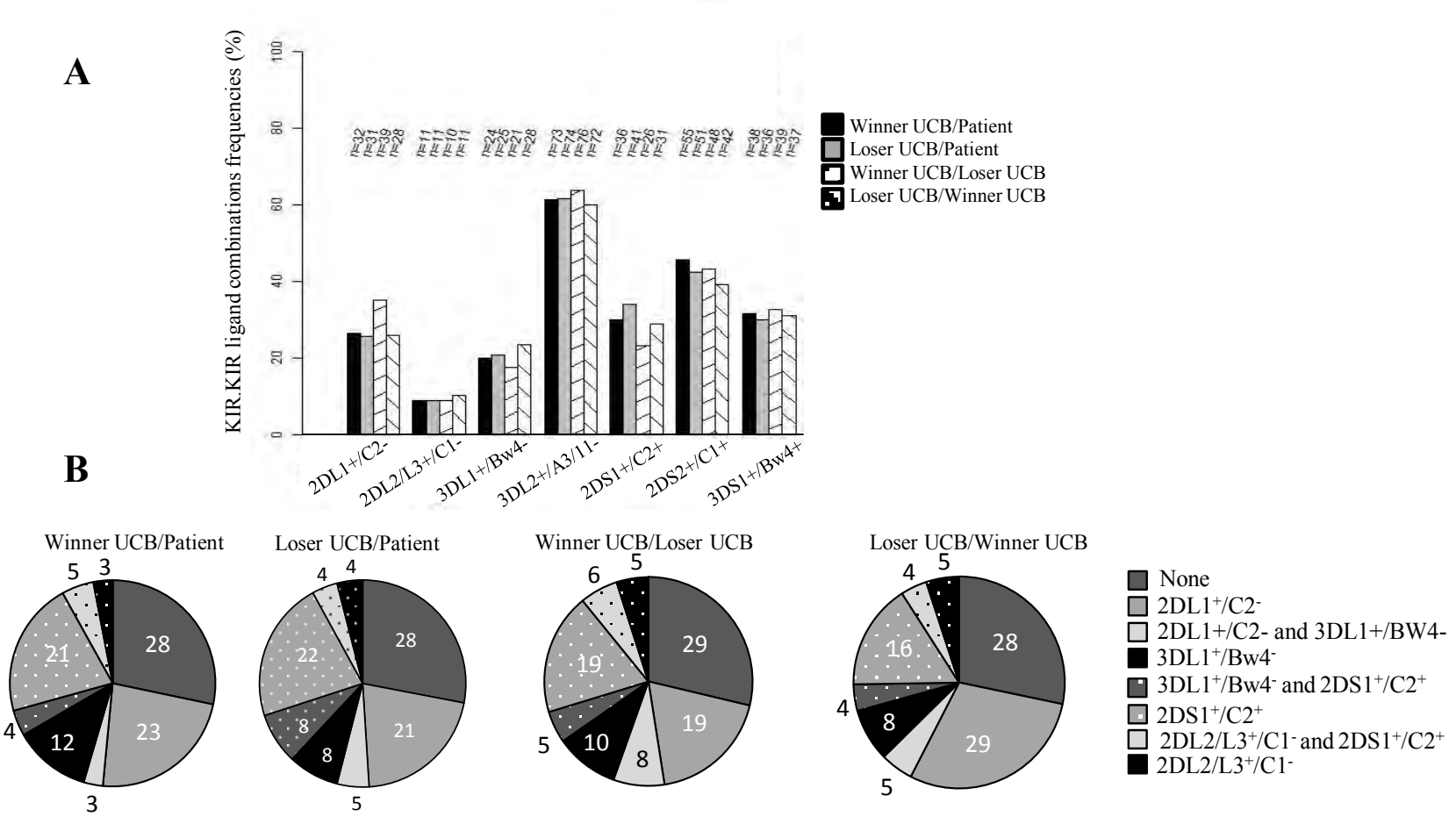

A
*p= 0.007
CI of neutrophil recovery
Days post-dUCBT
Others, n=191
KIR2DL2/L3+ winner/C1- patient, n=15
B
*p= 0.024
CI of neutrophil recovery
Days post-dUCBT
Others, n=170
KIR2DS1+ winner/C2+ loser UCB, n=34
C
Winner UCB
KIR2DS1
C1-
C2+
Loser UCB
KIR2DL2/2DL3
C1-
Patient

Figure 2



Suppl. Figure 1 : Frequencies (A) and detailed distribution (B) of KIR.KIR ligand genetic combinations depending on UCB unit dominance observed in the restricted dUCBT cohort. Potential KIR2DL1$^+$, KIR2DL2/L3$^+$ KIR3DL1$^+$, KIR3DL2$^+$ NK cell alloreactivity was evaluated in 121 dUCBT depending on the presence of the KIR2DL1, KIR2DL2/L3, KIR3DL1 or KIR3DL2 gene the absence of corresponding C2 (2DL1), C1 (2DL2/L3), Bw4 (KIR3DL1) or A3/A11 (KIR3DL2) ligand respectively. Potential KIR2DS1$^+$, KIR2DS2$^+$, KIR3DS1$^+$ NK cell alloreactivity was evaluated in 121 dUCBT depending on the presence of the KIR2DS1, KIR2DS2, KIR3DS1 gene and the presence of corresponding C2 (2DS1), C1 (2DS2) or putative Bw4 (3DS1) ligand respectively. The frequencies of KIR2DL1$^+$/C2$^-$, KIR2DL2/L3$^+$/C1$^-$, KIR3DL1$^+$/Bw4$^-$, KIR3DL2$^+$/A3/A11$^-$, KIR2DS1$^+$/C2$^+$, KIR2DS2$^+$/C1$^+$, and KIR3DS1$^+$/Bw4$^+$ genetic combinations between winner UCB/patient and loser UCB/patient pairs in the Graft-versus-Host (GvH) direction, and between winner UCB/loser UCB and loser UCB/winner UCB unit pairs in the Graft-versus-Graft (GvG) direction was compared. B) Charts representing the detailed KIR.KIR ligand genetic combinations taking into account KIR2DL1, KIR2DL2/L3, KIR3DL1 and KIR2DS1 genes in both the GvH and GvG directions. Presence of KIR2DL1, KIR2DL2/L3, KIR3DL1 or KIR2DS1 gene in winner UCB units (n=121) and in loser UCB units (n=121) was evaluated by multiplex KIR PCR-SSP. Absence of corresponding KIR ligand for 2DL1 (C2), 2DL2/L3 (C1), 3DL1 (Bw4) in patients (n=121, GvH direction), in loser UCB units, or in winner UCB units (n=121, GvG direction) was established from HLA class I typing. Presence of C2 ligand for KIR2DS1 in patients (n=121, GvH direction), in loser and winner UCB units (n=121, GvG direction) was established from HLA-C typing.

RESULTATS

ARTICLE N°3 : Caractérisation phénotypique et fonctionnelle des cellules Natural Killer de sang de cordon

New insights on Natural Killer cells repertoire from a thorough analysis of cord blood cells

Pauline Rettman, Catherine Willem, Gaëlle David, Raphaëlle Riou, Nolwenn Legrand, Julie Esbelin, Anne Cesbron, David Senitzer, Katia Gagne and Christelle Retière

La mise en évidence du rôle de l'alloréactivité des cellules NK KIR^+ dans la dominance d'un seul cordon après double-greffes de sang de cordon sur un effectif local (**article n°1**) soulève la nécessité de mieux comprendre la biologie des cellules NK de sang de cordon. Il a été mis en évidence la rapide maturation de cellules NK de sang de cordon après dUCBT, assurant un effet anti-leucémique contre des blastes de leucémie aigüe myéloïde (Beziat et al. 2009). Ces cellules présentent un statut immature mais semblent néanmoins acquérir très rapidement des fonctionnalités impactant le suivi clinique du patient. Contrairement au répertoire adulte, le phénotype des cellules NK de sang de cordon a été rapporté par peu de groupes (Le Garff-Tavernier et al. 2010; Schönberg et al. 2011a), sans prise en compte du contenu en gènes KIR et/ou la discrimination entre les récepteurs KIR inhibiteurs et KIR activateurs.

Les fonctions effectrices des cellules NK sont régulées par un ensemble de récepteurs activateurs et inhibiteurs dont les récepteurs spécifiques des molécules HLA de classe de classe I comme les récepteurs KIR, ILT2, ainsi que les hétérodimères CD94/récepteur « lectin-like » de type C (CD94/NKG2A, CD94/NKG2C) et des récepteurs assurant l'activation des cellules NK, dont l'homodimère NKG2D, DNAM-1 et les récepteurs de cytotoxicité naturelle (NKp30, NKp44, NKp46).

Pour approfondir la caractérisation des cellules NK issues de sangs de cordon (SC), nous avons réalisé une étude phénotypique de l'ensemble de ces récepteurs à partir de 73 prélèvements de SC en collaboration avec la maternité du CHU de Nantes (Dr J. Esbelin). Le phénotype des cellules NK issues de SC a été mis en lien avec l'étude phénotypique du répertoire des cellules NK de 158 individus sains adultes (donneurs de sang de l'EFS) (David et al. 2013). Nous avons confirmé d'une part que les fréquences des populations NK ($CD3^-$ $CD56^+$) et $CD56^{bright}$ de SC sont similaires à celles observées chez l'adulte, mais ces populations sont augmentées en nombre absolu, comme précédemment observé (Le Garff-

Tavernier et al. 2010). D'autre part, les fréquences des cellules NK KIR2D$^+$ (KIR2DL1, 2DL2/3, 2DS1, 2DS2) sont plus faibles que chez l'adulte (Schönberg et al. 2011a). Nous avons précédemment mis en évidence que le répertoire des cellules NK provenant d'individus adultes est plus impacté par le contenu en gènes KIR que par l'environnement HLA de classe I en termes de ligands KIR (David et al. 2013). Des analyses similaires ont été réalisées sur cellules de SC et ont montré 1) l'absence d'influence de l'environnement HLA-C1 sur la fréquence des sous-populations NK KIR2DL2/3/S2$^+$ et NK KIR2DL2$^+$ KIR2DL3/S2$^-$ et 2) l'impact respectif de la présence des gènes KIR2DL2 et KIR2DL3, comme observé chez l'adulte. De la même façon, la fréquence des cellules NK KIR2DL1/S1$^+$ est similaire chez les SC HLA-C2$^+$ et C2$^-$ et diminuée en présence du gène KIR2DS1. Par ailleurs, la fréquence des cellules NK KIR2DL1$^+$ KIR2DL2/L3/S1/S2$^-$ de SC est significativement augmentée chez les SC de génotype KIR AA, et le gène KIR2DL2 diminue en particulier la fréquence de cette population, indépendamment de l'environnement autologue HLA-C2, comme observé chez l'adulte. Nous avons également étudié la sous-population NK KIR3DL1$^+$, ce récepteur présentant un très large polymorphisme allélique qui impacte le phénotype et la fonction de ces cellules chez les individus adultes (Gardiner et al. 2001; O'Connor et al. 2007). La fréquence des cellules NK KIR3DL1$^+$ de SC n'est pas impactée par l'environnement HLA-Bw4 mais diminuée en présence du gène KIR3DS1, KIR3DL1 et KIR3DS1 étant deux allèles du même gène, comme précédemment rapporté par notre équipe par l'étude du répertoire KIR3DL1 d'individus adultes (Gagne et al. 2013). Nous avons également montré que 50% des cellules NK KIR3DL1$^+$ expriment les récepteurs KIR2D (KIR2DL1/L2/L3, KIR2DS1/S2) comme observé chez l'adulte, suggérant une expression consécutive des récepteurs KIR2D sur les cellules NK KIR3DL1$^+$ très précocement. D'autre part, les différentes combinaisons KIR3DL1/KIR3DS1 en fonction de l'intensité moyenne de fluorescence (high, low) obtenues avec des anticorps spécifiques anti-KIR3DL1/3DS1

observées chez l'adulte (Gagne et al. 2013) sont représentées dans notre cohorte de SC. Nous confirmons une plus faible fréquence et une plus forte MFI des sous-populations NK KIR3DL1^{high} et NK KIR3DL1^{low} de SC comparé à l'adulte quel que soit l'environnement HLA-Bw4. Dans l'ensemble, nos résultats montrent que le répertoire NK KIR de sang de cordon est similaire à l'adulte et semble déjà structuré, indépendamment de l'environnement autologue HLA de classe I.

Nous avons complété l'analyse phénotypique des cellules de SC en évaluant l'expression des récepteurs CD94/NKG2A, CD94/NKG2C, des NCR et du CD57. Conformément aux études préalables, nous observons une plus forte expression du récepteur NKG2A comparé à l'adulte (Le Garff-Tavernier et al. 2010; Schönberg et al. 2011a) avec une expression préférentielle des cellules NKG2A^{+}KIR^{-}. Nous avons également observé une corrélation négative entre la fréquence des NK NKG2A^{+} et NK KIR^{+} d'une part, et entre les cellules NK NKG2A^{+}KIR^{-} et KIR^{+}NKG2A^{-} d'autre part, comme observé chez l'adulte.

Bien que l'expression du récepteur NKG2C soit associée à l'infection à cytomégalovirus (CMV) chez l'adulte (Gumá et al. 2004; Béziat et al. 2013a), nous avons mis en évidence une expression du récepteur NKG2C chez les cellules NK de SC, avec des fréquences supérieures à celles observée chez des individus adultes, co-exprimant ou non les récepteurs KIR. L'expression homogène du récepteur NKG2C en absence de sérologie CMV élimine le possible lien avec une infection à CMV maternofétale. Nous avons enfin mis en évidence la présence d'une sous-population NK NKG2C^{+} de SC co-exprimant le NKG2A, contrairement à ce qui est observé chez l'adulte.

L'étude des autres récepteurs NK a montré que les cellules NK de SC présentent une plus forte expression du récepteur NKp30 en termes de MFI, et confirmé la très forte fréquence des récepteurs CD94, NKG2D et NKp46 mais une fréquence plus faible du récepteur NKp44

comparé à l'adulte. Enfin, comme précédemment reporté, les cellules NK de SC expriment très faiblement le récepteur CD57 contrairement à l'adulte.

Au vue du phénotype observé des cellules NK de SC, nous avons ensuite étudié leur fonctionnalité, en comparant leur capacité de dégranulation (CD107a) et de production d'IFNγ. Dans l'ensemble, les cellules NK de SC sont capables de dégranuler et de sécréter l'IFNγ vis-à-vis d'une lignée 221 dépourvue de molécule HLA de classe I mais de façon plus faible que ce qui est observé chez l'adulte. Les études portant sur l'éducation des cellules NK par les molécules HLA de classe I du soi suggèrent que plus un individu possède de ligands KIR, plus le pool de cellules NK dites éduquées sera important (Anfossi et al. 2006). Nous avons observé que plus les cellules NK d'individus adultes expriment de récepteurs KIR, plus la lyse spontanée de ces cellules est importante. Ceci n'est toutefois pas observé au niveau de cellules NK de SC. Nous avons ensuite évalué les capacités fonctionnelles des sous-populations NK KIR^+ de SC de génotype KIR AA, n'exprimant donc aucun KIR activateur à l'exception du récepteur KIR2DS4. Schönberg et ses collaborateurs ont précédemment mis en évidence l'éducation fonctionnelle des cellules NK KIR de SC par les molécules HLA de classe I du soi (Schönberg et al. 2011b). Nous avons confirmé ces résultats et mis en évidence l'inhibition de la dégranulation et de la production d'IFNγ des sous-populations NK $KIR2DL1^+$, $KIR2DL3^+$ et $KIR3DL1^+$ de SC vis-à-vis des lignées 221-C2, 221-C1 et 221-Bw4 respectivement, et ce quel que soit l'environnement autologue HLA de classe I.

Afin d'évaluer le lien entre le profil phénotypique des cellules NK de SC et de prendre en compte l'expression des récepteurs activateurs dans la modulation de la fonctionnalité de ces cellules, nous avons par la suite évalué l'expression des récepteurs DNAM-1, 2B4, CD16 et CD161 en comparant les profils des cellules NK de SC présentant une faible ou une forte dégranulation à ceux obtenus chez un individu adulte. Nous avons montré une corrélation entre l'expression du récepteur CD161 et le potentiel de dégranulation des cellules NK de SC

ainsi qu'une tendance similaire, bien que non significative, avec l'expression des récepteurs DNAM-1, CD16 et 2B4, suggérant une contribution des récepteurs activateurs sur la fonctionnalité des cellules NK de SC.

Suite à la mise en évidence de l'expression du récepteur NKG2C sur les cellules NK de SC, nous avons évalué les capacités de prolifération et de dégranulation des cellules NK NKG2C$^+$ de SC vis-à-vis de lignées 221 et 221-HLA-E, ligand connu du NKG2C. Nous avons observé une forte augmentation de la fréquence des cellules NK NKG2C$^+$ après 14 jours de stimulation *in vitro* avec la lignée 221-HLA-E. De plus, la dégranulation cellules NK NKG2C$^+$est augmentée vis-à-vis de la lignée 221-HLA-E à un ratio E :C 50 :1 comparé à l'adulte. Au contraire, la dégranulation des cellules NK NKG2A$^+$ de SC est significativement inhibée vis-à-vis de la lignée 221-HLA-E à un ration E :C 1 :1 contrairement à l'adulte. Ces résultats montrent donc la forte inhibition des cellules NK NKG2A de SC et la fonctionnalité des cellules NK NKG2C de SC. Nous avons ensuite évalué la co-expression du récepteur NKG2C avec différents récepteurs dont les récepteurs ILT2, DNAM-1, CD161, 2B4 et CD16 chez des cellules NK de SC comparé à des cellules provenant d'individus adultes CMV$^+$ présentant une expansion NKG2C. Les cellules NK NKG2C de SC co-expriment les récepteurs DNAM-1, CD161, NB4 et CD16 avec de plus faibles MFI que ce qui est observé chez l'adulte. Cependant, nous n'avons pas observé de population co-exprimant le récepteur NKG2C avec le récepteur ILT2 sur les cellules NK de SC contrairement à ce qui est observé chez l'adulte. Nous avons également rapporté la co-expression du NKG2C avec le NKG2A chez les cellules NK de SC. Les résultats de ces études fonctionnelles suggèrent que la forte inhibition des cellules NKG2A limite l'activation des cellules NK par le NKG2C.

Dans l'ensemble, cette étude approfondie du répertoire NK des cellules de SC à partir d'un large effectif nous a permis de réévaluer la structuration et la fonctionnalité du répertoire NK. Nous avons ainsi confirmé que la formation du répertoire NK est indépendante de

l'environnement autologue HLA de classe I et influencée par le contenu en gènes KIR. Le répertoire NK semble déjà structuré à ce stade précoce. Différentes études suggèrent que l'histoire immunologique des individus, et en particulier les infections virales, vont structurer le répertoire NK de façon HLA-dépendante (Béziat et al. 2013a; Azzi et al. 2014) et ainsi évoluer au cours de la vie en fonction de l'âge et des facteurs environnementaux (Lutz et al. 2005; Lu et al. 2007; Mian et al. 2008a; Le Garff-Tavernier et al. 2010). Les cellules NK de SC présentent donc un réel potentiel clinique. L'expression du NKG2C et la fonctionnalité de ces cellules suggère le potentiel anti-viral des cellules NK de SC en particulier dans le contrôle de l'infection à CMV. Il faut noter qu'une expansion de cellules NK NKG2C^{+} est observée après réactivation du CMV. De plus, une forte expansion des cellules NK est observée après dUCBT et leur efficacité vis-à-vis de blastes leucémiques a été rapportée (Beziat et al. 2009). Ces cellules ont également de fortes capacités de prolifération *in vitro* (Tanaka et al. 2012) et présentent un répertoire non biaisé par l'histoire immunologique de l'individu. Les cellules NK de SC présentent donc un réel potentiel clinique et pourraient être utilisées en immunothérapie.

New insights on Natural Killer cells repertoire from a thorough analysis of cord blood cells

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ABSTRACT

Although cord blood NK cells are characterized as immature lymphocytes, their impressive expansion and efficient GvL response have been highlighted early after UCBT. In order to better evaluate their potential interest as source of effective NK cells, we revisited the study of NK cell repertoire from a large cohort of cord blood. Our study showed that cord blood NK cell repertoire appear to be early structured, depending on KIR gene content but not on autologous HLA environment. NKG2A was expressed on a wide frequency of cord blood NK cells which was inversely correlated to KIR^+ NK cell frequency. Self-HLA class I molecule educated cord blood KIR^+ NK cells present a lower spontaneous lysis compared with adult counterparts, probably related to the low expression of activating NK receptors as DNAM-1. Interestingly, we described for the first time a proliferative and cytotoxic $NKG2C^+$ NK cell subset representing more than 10% of cord blood NK cells. NKG2A strongly inhibited cord blood NK cell degranulation and its co-expression on $NKG2C^+$ NK cells may contribute to limit their activation. The effect of HLA class I environment on NK repertoire formation seems to be at last observable on educated KIR^+ NK cells, particularly after viral infection that lead to specific and long-lasting expansion of NK cell clones. Overall, cord blood NK cell repertoire is early structured and harbor numerous functional abilities shared by adult NK cells. In addition, their naïve viral status and their fast expansion confer to cord blood NK cells numerous advantages in immunotherapy.

INTRODUCTION

Natural Killer cells are the first line of defense against virus infection and tumors, given their ability to discriminate self from non self. These innate lymphocytes express different receptors specific to HLA class I molecules, present on almost all cells leading to self-recognition. In viral and tumoral processes, HLA class I processing and cell surface expression are frequently targeted to escape adaptive cellular immunity. This new phenotype marked by the absence or reduced expression of HLA class I molecules trigger NK cell cytotoxicity. The absence of inhibitory receptor engagement with cognate HLA ligand leads NK cells to recognition of missing self[1,2]. NK cells effector functions are regulated following a fine-tuning orchestrated by a broad range of inhibitory and activating receptors. HLA specific NK receptors include the Killer cell Immunoglobulin-like Receptors (KIR), heterodimer CD94/C-type lectin receptors (CD94/NKG2A, CD94/NKG2C) and ILT2. Inhibitory signals are not enough to trigger NK cell cytotoxicity and have to be supplemented by activating signals transmitted by different activating receptors as such NKG2D, DNAM-1, 2B4 and Natural Cytotoxicity Receptors (NCR) for whom the ligands are less documented.

KIR receptors recognize specific allotypic determinants shared by different HLA-class I molecules, referred to as KIR ligands. Group C1 and C2 HLA-Cw allotypes are recognized by KIR2DL2/2DL3 and KIR2DL1 respectively, although KIR2DL2/3 present a larger spectrum of HLA-C recognition than KIR2DL1[3,4], and HLA-A and -B allotypes with a Bw4 motif are targeted by KIR3DL1. Activating KIR specificities are less described, except KIR2DS1 and KIR2DS2 which respectively recognize C2 and C1 ligands [4–6]. CD94/NKG2C and CD94/NKG2A recognize HLA-E molecules and are respectively activating and inhibitory receptors. ILT2 is an inhibitory receptor which can bound to HLA class I molecules and non-classical HLA-G and HLA-E molecules[7]. The homodimer NKG2D binding with MIC-A,

MIC-B and ULBP ligands activate NK cell effector functions. NKp30 and NKp44 recognize respectively surface proteins of the B7-H6 family [8] and Proliferating Cell Nuclear Antigen [9] and most of NCR ligands are express on tumoral cells. Of note, these receptors also recognize viral hemaglutinin. Finally, CD112 and nectin-like CD155 are identified ligands of DNAM-1 [10], also frequently expressed on virally-infected and tumoral cells. Overall, NK cells express a broad range of activating and inhibitory receptors regulating NK cell anti-viral and anti-leukemic activities.

Due to their ability to recognize missing-self, NK cells are alloreactive in HLA class I mismatched context. The role of KIR^+ NK cell alloreactivity has been reported, in particular after HLA haploidentical hematopoietic stem cell transplantation (HSCT). Moreover, NK cells are the first population to reconstitute the hematopoiesis after HSCT. In HLA class I mismatched donor/recipient pairs, alloreactive KIR^+ NK cells can exert a beneficial Graft-versus-Leukemia (GvL) effect [11]. When fully HLA-matched related or unrelated donors are unavailable, double umbilical cord blood transplantation (dUCBT) is an efficient alternative [12,13]. We have recently shown that KIR/HLA incompatibilities may contribute to the dominance of one cord blood (CB) unit following dUCBT (Rettman submitted) and highlighted the importance of KIR genes to early assess the chimerism status after dUCBT [14]. It has been shown that CB NK cells mature rapidly after UCBT and harbor strong cytotoxic activity against blast cells [15]. Recent deep investigations brought new insights into the shaping of NK cell repertoire by persistant viral infection and aging [16–19]. Despite a thorough analysis of adult NK cell repertoire, few studies have been devoted to CB NK cell biology [19,20]. In this study, we propose an-in-dept monitoring of CB NK cell repertoire from cord blood samples (n=73) in comparison to healthy adult individuals (n=158) to reevaluate its structural formation and functionality in light of new insights in the NK cell field.

MATERIAL AND METHOD

Cells (PBMCs, cord blood samples and cell lines)

For phenotypic and functional assays, peripheral blood mononuclear cells (PBMC) and CB cells were isolated from healthy adult volunteers and from umbilical CB samples respectively. All blood donors were recruited at the Blood Transfusion Center (EFS, Nantes, France) and umbilical CB samples were obtained at the Nantes CHU maternity unit. Informed consent was obtained from all healthy individuals and mothers. HLA class-I deficient 721.221 lymphoblastoid EBV-B cells, referred to as 221 cells were used as positive control to assess NK cell degranulation and IFNγ secretion. HLA-B*15:13 (Bw4), B*39:01 (Bw6), C*03:04 (C1) and C*02:02 (C2) transfected 221 cells were used to evaluate degranulation and IFNγ secretion of different KIR^+ NK cell subsets.

KIR and HLA genotyping

Genomic DNA from healthy adult donors and CB were provided by the HLA laboratory (Dr A. Cesbron EFS Nantes, France). All DNAs were typed for KIR2DL1, 2DL2, 2DL3, 2DL4, 2DL5A/B, 3DL1, 3DL2, 3DL3, 2DS1, 2DS2, 2DS3, 2DS4/1D, 2DS5, 3DS1 using a KIR multiplex PCR-SSP method as previously described [21]. High resolution typing for HLA-A, HLA-B and HLA-C loci was carried out on all healthy adult donors and CB by a Sequence Based Typing kit (Abbott Molecular Park, IL, USA). KIR genotypes were determined based on the presence or absence of activating KIR, KIR AA genotype presenting only KIR2DS4 as activating KIR, and KIR B^+ genotype presenting several activating KIR. KIR ligand C1, C2, Bw4 and Bw6 was defined based on allelic HLA class I typing.

Phenotypic analysis by flow cytometry

NK cell–surface phenotype from freshly isolated CB cells and adult PBMC was determined by four-color flow cytometry using the following mouse anti-human mAbs: anti–KIR2DL1-FITC (143211; R&D Systems), anti–KIR2DL1/2DS1-PE (EB6), anti–KIR2DL2/2DL3/2DS2-PE (GL183), anti-KIR2DS4 (FES172), anti-KIR3DL1/3DS1 (Z27), anti-KIR3DL1-FITC (DX9), (Beckman Coulter Immunotech, Marseille, France), anti–CD3-PerCP (SK7), anti–CD56-allophycocyanin (B159; BD Biosciences), anti-NKp30-PE (Z25; Beckman Coulter) anti-NKp44-PE (Z231; Beckman Coulter), anti-NKG2D-PE (ID11; BD Biosciences), anti-NKG2C-PE (R&D System), anti-NKG2A-PE (Z199; Beckman Coulter), anti-CD85j-FITC (BD Pharmigen), anti-CD226-FITC (BD Pharmigen), anti-CD244-FITC (BD Pharmigen), anti-CD161-FITC (BD Pharmigen), anti-CD16-FITC (BD Biosciences), anti–KIR2DL1/2/3/2DS2-FITC (8C11), anti-KIR2DL3/2DS2-FITC (1F12), anti–KIR2DL2/2DL3/2DS2-FITC (4A8) and anti–KIR2DL1/2/3/2DS1/2-FITC (1A6), generated and characterized in our laboratory and previously described [22]. Data were collected using a FACSCalibur (BD Biosciences), and analyzed using Flowjo 7.6.1 software (TreeStar).

CD107a mobilization assay and IFNγ secretion assay detected by flow cytometry

Adult and CB NK cells from AA KIR genotype were tested for their cytolytic potential with a CD107a mobilization assay after stimulation with 221, 221-C1, 221-C2, 221-Bw4 and 221-Bw6 transfected cell lines. Thawed PBMC pre-stained with CD107a (H4A3, BD Biosciences) were cultured with different target cells (221, 221-C1, 221-C2, 221-Bw4, 221-Bw6) for five hours at an E:T ratio of 1:1 or 50:1 with brefeldin A (Sigma) at 10 µg/mL for the last four hours. The cells were surface stained with anti–KIR2DL1/2DS1-PE (EB6, Beckman Coulter), anti–KIR2DL2/2DL3/2DS2-APC (GL183, Beckman Coulter) and CD3-PerCP (9E2, BD Biosciences).

For the IFNγ secretion assay, after five hours culture with target cells, adult and CB cells were surface stained with anti–KIR2DL1/2DS1-FITC (EB6, Miltenyi), anti–KIR2DL2/2DL3/2DS2-APC (GL183, Beckman Coulter) and anti-CD3-PerCP (9E2, BD Biosciences) at an E:T ratio of 1:1 and fixed with PFA 4%. Then, cells were permeabilized in a PBS 0.1% saponine solution and IFNγ intracellular expression was measured using anti-IFNγ (BD Biosciences) after 1h incubation at room temperature.

All flow cytometry data were collected using a FACSCalibur (BD Biosciences), and analyzed using Flowjo 7.6.1 software (TreeStar).

In vitro amplification of cord blood NK cells

Thawed CB cells were cultured with 221 or 221-HLA-E cells at an E:T ratio of 10:1 for 2 weeks at 37°C in an *in vitro* model as previously described [5]. After 14 days of co-culture, $NKG2C^+$ and $NKG2A^+$ CB NK cells were targeted in a CD107a mobilization assay against 221 and 221-HLA-E target cells as described using CD107a-PeCy5, CD3-PerCP (9E2, BD Biosciences), NKG2A-FITC (Beckman Coulter), NKG2C-PE (R&D system).

Statistical analyses

Comparisons of NK cell frequencies between different series of individuals were performed using ANOVA test on GraphPadPrism software. The p values < 0.05 were considered to be statistically significant.

RESULTS

Impact of KIR genotype but not HLA class I autologous environment on cord blood KIR NK cell repertoire

In the current study, 73 CB and 158 healthy adult samples were included in an in-depth phenotypic NK cell analysis. As starting point to explore CB NK cell repertoire, we confirmed that the frequency of NK cells ($CD56^+CD3^-$) as well as $CD56^{bright}$ NK cell subset was similar in CB cells and adult PBMC but that the absolute number of NK cells was higher in CB samples in comparison with adult counterpart (data not shown) as previously reported [19].

In a previous study performed from the cohort of healthy adults included in this report, we have shown that the adult NK cell repertoire is more impacted by the KIR gene content than the HLA class I environment in terms of KIR ligands [4]. We focused our phenotypic analysis on inhibitory (KIR2DL1, KIR2DL2/3 and KIR3DL1) and activating KIR (KIR2DS1, KIR2DS2) for which KIR ligands are well characterized and those known to contribute into the functional education of NK cells. Thus, we observed that the frequency of the $KIR2D^+$ (KIR2DL1, 2DL2/3, 2DS1, 2DS2) NK cells was lower in CB in comparison to adult counterpart (respective mean of CB is 33.1 and adult is 39.5, p=0.0004) (Figure 1A) as previously described [19,23]. Next, we dissected KIR2D CB NK cell subsets, in focusing on $KIR2DL2/3/2DS2^+$ and $KIR2DL1/S1^+$ NK cells and different single positive KIR NK cell populations as $KIR2DL1^+$ and $KIR2DL2^+$ NK cells. We targeted only NK cells expressing C1-specific KIR2DL2/L3/S2 receptors using KIR2DL2/3/2DS2 specific mAb as illustrated in Figure 1B. The frequency of $KIR2DL2/L3/S2^+$ CB NK cells was significantly lower in comparison with adult NK cells in HLA-$C1^+$ individuals (mean 24.05 vs 32.35 $p<0.0001$) (Figure 1C), but $KIR2DL2/L3/S2^+$ CB NK cell frequency was not influenced by HLA-C

environment. However, the presence of KIR2DL2 gene decreased the frequency of KIR2DL2/L3/S2^{+} NK cells frequency in both adult ($p<0.0001$) and CB NK cells ($p=0.0042$) (Figure 1C). To refine our investigation, we analyzed the KIR2DL2^{+} KIR2DL3/2DS2^{-} NK cells thanks to a combination of specific antibodies as illustrated in Figure 1B. We observed a significant lower frequency of single positive KIR2DL2^{+} NK cells in CB compared with adult cells in C1^{+} individuals (mean= 6.3 vs mean= 9.1, p= 0.0004) and a trend toward a decrease in C1^{-} individuals due probably to the few number of C1^{-} CB samples (Figure 1D). In accordance with the fact that KIR2DL2 and KIR2DL3 segregate as alleles [24], the presence of KIR2DL3 gene significantly decreased the frequency of KIR2DL2^{+} NK cells in adults (mean 11.9 vs mean 8.2 , $p<0.0001$). Even not significant, KIR2DL2^{+} NK cells frequency tend to be higher in KIR2DL3^{-} CB compared to KIR2DL3^{+} CB (Figure 1D).

We next targeted KIR2DL1/S1^{+} NK cells using specific mAbs as illustrated in Figure 1E. We observed similar frequency of KIR2DL1^{+}/KIR2DS1^{+} NK cells expression in CB and adult samples, in both C2^{+} and C2^{-} environments (Figure 1F). Interestingly, the presence of KIR2DS1 gene significantly decreased the frequency of KIR2DL1/KIR2DS1^{+} CB NK cells (mean 24.3 vs 17.9 $p=0.0068$) as observed in adults (26.7 vs 19.5 $p<0.0001$) (Figure 1F). Using a combination of KIR2DL2/L3/S2 specific mAb and KIR2DL1/2/3/S2-specific mAb generated and characterized in our laboratory [22], we studied KIR2DL1^{+} KIR2DL2/3/2DS1/2^{-} NK cells (Figure 1G). Single-positive KIR2DL1^{+} NK cell frequency was significantly higher in CB with AA KIR genotype compared with CB with AB/BB KIR genotype (called B^{+}) (mean=12.9, mean=8.5, $p<0.0001$) (Figure 1H) and similar in regard of C2 environment (Figure 1I). The presence of both KIR2DS1 and KIR2DL2 genes significantly decreased single-positive KIR2DL1^{+} NK cell frequency in adults (mean=18.8 vs 11.2, $p<0.0001$ and mean=12.4 vs 7.7, $p<0.0001$ respectively). In contrast, only KIR2DL2 gene significantly decreased single-positive KIR2DL1^{+} NK cell frequency in CB samples (mean 11.7 vs 8.4,

p=0.0079) (Figure 1I). Overall, as we previously reported in adults, more than HLA-C environment, the presence of KIR2DL2 and KIR2DS1 genes limit KIR2DL1 acquisition on NK cells at early stage of NK cell differentiation.

There is no influence of Bw4 ligand on KIR3DL1$^+$ cord blood NK cell frequency

In parallel to KIR2D NK cell subset, we investigated NK cells expressing Bw4 specific KIR3DL1. KIR3DL1 receptor presents an extensive allelic polymorphism, which directly impacts NK cell phenotype and function[25–27]. We previously reported the influence of KIR3DL1 alleles and KIR3DL1/KIR3DS1 allele combination on adult KIR3DL1$^+$ NK cell repertoire[28]. Here, we investigated KIR3DL1$^+$ and KIR3DS1$^+$ CB NK cells, using a combination of specific mAbs to discriminate KIR3DL1 and KIR3DS1 as illustrated in Figure 2A. Of note, KIR3DL1$^+$ NK cell frequency is lower in CB compared to adult cells in KIR3DS1$^-$ individuals (data not shown). A similar KIR3DL1$^+$ NK cell frequency in Bw4$^+$ and Bw4$^-$ environments in CB (Figure 2B), as previously described in adult NK cells [20,28]. Moreover, there was no impact of the number of Bw4 motif on KIR3DL1$^+$ NK cell frequency in CB (data not shown). In accordance with the fact that KIR3DL1 and KIR3DS1 segregate as alleles, there is a higher KIR3DL1$^+$ CB NK cell frequency in KIR3DS1$^-$ compared to KIR3DS1$^+$ samples (mean=14.4 vs 8.3, p=0.0006) (Figure 2C). From only KIR3DS1$^-$ CB, we observed similar KIR3DL1$^+$ NK cell frequency, whatever Bw4 autologous environment (Figure 2D).

Interestingly, a positive correlation was observed between KIR3DL1$^+$ CB NK cell frequency and KIR3DL1$^+$KIR2D$^+$ CB NK cell frequency (r=0.9677, p<0.0001) (Figure 2E). We highlighted here that nearly 50% of KIR3DL1$^+$ NK cells express KIR2D receptors in early life. These data suggest a consecutive expression of KIR2D (KIR2DL1/L2/L3, KIR2DS1/S2) on KIR3DL1$^+$ CB NK cells as we have previously reported in adult NK cell repertoire[28]. The

different KIR3DL1/S1 combinations according to the mean intensity of fluorescence (MFI) ($3DL1^{high}$, $3DL1^{low}$) and the presence of KIR3DS1 were represented in our CB cohort, as illustrated in Figure 2F. There was a significantly higher $KIR3DL1^{+}$ NK cell frequency in $KIR3DL1^{high/low}$ CB samples compared to $KIR3DL1^{high}$/KIR3DS1 (mean=16.9 vs 8.7, p=0.0134) and $KIR3DL1^{low}$/KIR3DS1 individuals (mean=16.9 vs 7.9 p=0.0054), highlighting the impact of KIR3DS1 on KIR3DL1 NK cell frequency (Figure 2G) independently of Bw4 environment. We also observed a higher $KIR3DL1^{+}$ NK cell frequency in $KIR3DL1^{high}$ compared to $KIR3DL1^{low}$/KIR3DS1 CB samples (mean=14.6 vs 7.9, p=0.0486). As we previously showed in a smaller CB cohort [28], there is a lower $KIR3DL1^{high}$ (mean=10.8 vs 18.2) and $KIR3DL1^{low}$ (mean=7.8 vs 11.6) CB NK cell frequency compared to adult counterparts (p=0.0003 and p=0.0110 respectively) (Figure 2H). Moreover, we confirmed that there is a higher $KIR3DL1^{high}$ MFI on $KIR3DL1^{+}$ CB NK cells compared to adult counterpart (mean=937 vs 679, p<0.0001) (Figure 2I). As $KIR3DL1^{+}$ CB NK cells seems to present a lower frequency but a higher MFI [28], we evaluated KIR3DL1 expression level according to KIR3DL1 combinations and showed that KIR3DL1 MFI was sharply higher in $KIR3DL1^{high}$ CB versus $KIR3DL1^{high/low}$ (mean= 974 vs 632, p=0.0082) or $KIR3DL1^{low}$ (mean=974 vs 298, p<0.0001), independently of Bw4 environment (Figure 2I) as observed in adults. A similar profile was observed in presence of KIR3DS1 gene with a high MFI of $KIR3DL1^{high}$/3DS1 compared to $KIR3DL1^{low}$/3DS1 (mean=803 vs 276, p=0.0020) (Figure 2J). Our results show a consecutive expression of KIR2D receptors on $KIR3DL1^{+}$ CB NK cells with a lower $KIR3DL1^{+}$ NK cell frequency but a higher KIR3DL1 MFI associated with all known combinations of KIR3DL1/S1 alleles, irrespectively of Bw4 environment. Overall, although a lower frequency of KIR^{+} NK cells in CB, the NK repertoire profile is similar to adult NK repertoire and appears to be early structured.

Extended phenotype of cord blood NK cells

To complete the CB phenotypic analysis, we further investigated other NK receptor expression as CD94/NKG2A and CD94/NKG2C heterodimers, NCR and CD57 on CB NK cells. In accordance with previous studies[19,23], CB NK cells express more NKG2A than adult counterparts as illustrated in Figure 3A for representative individuals, and presented for all studied samples in Figure 3B (CB mean 74.9, adults mean 54.9, $p<0.0001$). CB NK cell phenotype was significantly marked by a preferential representation of $NKG2A^+$ KIR^- NK cell subsets in higher frequencies than in adult counterparts (mean=54.3 in CB, mean=44.1 in adults, $p<0.0001$ and mean=20.8 in CB, mean=10.9 in adults, $p<0.0001$ respectively) and a lower frequency of NK cells expressing KIR without NKG2A (mean=9.3 in CB, mean=23 in adults, $p<0.0001$). Interestingly, as shown in adult NK cells ($r=-0.4845$, $p=0.0057$), there is a negative correlation between $NKG2A^+$ and KIR^+ CB NK cells ($r=-0.5243$, $p=0.0006$) (Figure 3C). Similarly, we highlighted a negative correlation between KIR^+NKG2A^- NK cells and $NKG2A^+KIR^-$ NK cells, in CB cells ($r=-0.5830$, $p=0.0001$), also observed in adults ($r=-0.8252$, $p<0.0001$) as previously reported [29].

Although NKG2C was commonly associated to cytomegalovirus (CMV) infection[16,30–32], we evaluated NKG2C expression on CB and CMV negative adult NK cells using a combination of NKG2C and KIR (KIR2DL1/2/3/2DS1/2)-specific mAbs as illustrated in figure 3D. Interestingly, there is a much higher frequency of overall $NKG2C^+$ and $NKG2C^+KIR^-$ NK cell subsets in CB compared to adults (mean=13.7 vs 6.8, $p<0.0001$ and mean=8.7 vs 4.2, $p<0.0001$ respectively) and a trend toward highly $NKG2C^+KIR^+$ NK cell frequency although not significant (Figure 3E). Of note, we did not report NKG2C expression of CB T $CD3^+$ cells (data not shown). In absence of CMV serology, the homogenous NKG2C expression in all studied CB samples discard a possible link with a maternofetal CMV infection. We observed no correlation between NKG2A and NKG2C expression on CB NK

cells in contrast to adult CMV^+ NK cells (r=0.5848, p=0.0007) (Figure 3F). Of note, CB NK cells present a double positive $NKG2A^+NKG2C^+$ population which is not observed in adults as illustrated in representative density plots (figure 3F). There was no correlation between NKG2C and KIR expression on CB NK cells (data not shown).

Next, we evaluated NKp30 expression using anti-NKp30 and anti-KIR (KIR2DL1/2/3/2DS1/2)-specific mAbs as illustrated on Figure 3G. CB NK cells presented a higher NKp30 MFI compared to adult counterparts (mean in CB=31.2, mean in adults=19.1, p=0.0001) (Figure 3H). We confirmed a low expression of NKp44 but a very high expression of CD94, NKG2D and NKp46 on CB NK cells (data not shown), as previously reported [19]. As expected, we finally confirmed the sharply lower frequency of CD57, a late stage marker of differentiation on CB versus adult NK cells (mean in CB=3.4, mean in adults=41.8, p<0.0001, Figure 3I) highlighting their early stage of CB NK cell differentiation according to previous studies[33]. Overall, NKG2A is expressed on a wide frequency of CB NK cells which is inversely correlated to KIR^+ NK cell frequency and surprisingly we highlighted the questionable expression of NKG2C on CB NK cells.

Cord blood NK cells have a weaker spontaneous lysis than adult counterpart but are functionally competent

In accordance with the phenotype of CB NK cells, we further investigated their functional potential, comparing the degranulation (CD107a) and IFNγ responses from CB and PBMC against the standard HLA class I-negative target cell line, 721.221 (221). Even though CB NK cells degranulated against 221 cells (p=0.0004), they presented a significant lower response against 221 compared with adult NK cells (p=0.0461) (Figure 4A). Moreover IFNγ response is comparable in CB and adult NK cell (figure 4B). Previous studies of NK cell education by self-HLA class I molecules suggested that the NK cell pool of a given individual would be more educated as the number of KIR ligand is important[2]. Interestingly, we highlighted a

positive correlation between NK cell degranulation ($CD107a^+$ $CD3^-$ NK cells) and the overall KIR NK cell frequency in adults (r=0.5153, p=0.0084) but not in CB (Figure 4C). The functional education of KIR^+ CB NK cells by self-HLA class I molecules has been previously highlighted, showing that $KIR2DL1^+$ NK cells from C2C2 donors exhibited higher effector responses than $KIR2DL2/L3^+$ NK cells from the same donor as $KIR2DL2/L3^+$ NK cells from C1C1 donor [20]. Our analysis on 10 CB with AA KIR genotype confirmed these observations (data not shown). We then compared the degranulation (CD107a) and IFNγ responses of $KIR2DL1^+$, $KIR2DL3^+$ and $KIR3DL1^+$ single-positive NK cells from AA haplotype CB samples (n=10) to evaluate the capacities of inhibition of each KIR^+ NK cell subsets against 221 targets transfected with their specific KIR ligands C2, C1 and Bw4 respectively (Figure 4D, 4E). We observed an inhibition of $KIR2DL1^+$ CB NK cells against 221-C2 target cells in term of CD107a degranulation (p=0.0187, Figure 4D), which is observed from both $C2^+$ and $C2^-$ CB samples (data not shown). We showed a greater inhibition of $KIR2DL3^+$ CB NK cells degranulation against 221-C1 target cells (p<0.0001, Figure 4D), irrespectively of autologous HLA class I environment (data not shown). Moreover, we observed a strong inhibition of $KIR3DL1^+$ CB NK cells degranulation against 221-Bw4 target cells (p<0.0001, Figure 4D), from both $Bw4^+$ and $Bw4^-$ CB samples (data not shown). Similarly, we showed an inhibition of $KIR2DL1^+$ and $KIR2DL3^+$ CB NK IFNγ production against 221-C2 and 221-C1 target cells respectively (p=0.0126 and 0.003 respectively, Figure 4E).

It is now well admitted that functional education of NK cells is mediated not only by the interactions between HLA class I molecules and inhibitory receptors as KIR but only modulated by activating NK cell receptor engagement. Thus, in attempt to investigate the link between the phenotypic profile and the function, we analyzed the expression of the activating NK receptors as DNAM-1, 2B4, CD16 and CD161 of a CB presenting a weak degranulation (UCB 2, light grey), a stronger degranulation (UCB 1, dark grey) in comparison with adult

counterparts (black) (Figure 4F). CB NK cells presenting a weak degranulation (UCB 2, light grey) expressed a lower level of DNAM-1, 2B4, CD16 and CD161 in comparison to the CB which presented a stronger degranulation (UCB 1, dark grey) (Figure 4I). We also observed a positive correlation between CD161 expression and the degranulation of CB NK cells ($r=0.6154$, $p=0.0043$) and a trend toward a positive correlation between DNAM-1, NB4 and CD16 expression and CB NK cell degranulation, although not significant due to our limited cohort (data not shown). Overall, our results suggest that the acquisition of activating NK receptors appear to contribute to mount the functional potential of CB NK cells at early stages of differentiation.

Full responsiveness of NKG2C$^+$ cord blood NK cells

Given the expression of NKG2C highlighted by the phenotypic analyses, we investigated the functionality of NKG2C$^+$ CB NK cells and further evaluated their proliferation and degranulation potential. Thawed CB cells were co-cultured with 221 cell lines transfected or not to express HLA-E, known ligand of NKG2C, for two weeks and we then assessed NKG2C$^+$ NK cell frequency (Figure 5A). In this *in vitro* model of expansion[5], NK cell absolute number was strongly increased whatever the nature of target cells (221 or 221-HLA-E) (data not shown). As illustrated for a representative CB sample, NKG2C$^+$ NK cells mostly expanded after 14 days of stimulation with 221-HLA-E cell line (10% at day 0 to 37% at day 14) and less with 221 cell line (10% at day 0 to 11 % at day 14). The sharp increase of NKG2C$^+$ NK cell frequency from day 0 to day 14 after 221-HLA-E stimulation was confirmed for 5 experiments (Figure 5B) ($p=0.0007$). We then evaluated the *ex vivo* degranulation profile of thawed NKG2C$^+$NKG2A$^-$ CB NK cells in parallel to NKG2A$^+$NKG2C$^-$ CB NK cells against 221 and 221-HLA-E targets (Figure 5C, 5D). At ratio E:T 1:1, the degranulation of CB and adult NKG2C$^+$ NKG2A$^-$ NK cells was not enhanced by HLA-E expression on 221 target cells (Figure 5C, left panel) and in opposite, we observed a

higher degranulation of CB NKG2A$^+$ NKG2C$^-$ against 221 target (mean CB=31.12, mean adult =10.31 $p<0.0001$) in contrast to adult counterpart (Figure 5C, right panel), inhibited with 221-HLA-E (mean 221=31.1, mean 221 HLA-E =14, $p<0.0001$) . Interestingly, at ratio E:T 50:1, both NKG2C$^+$ NKG2A$^-$ and NKG2A$^+$ NKG2C$^-$ NK cell subets weakly degranulated against 221 target cells leading to underline a strong triggered degranulation of NKG2C$^+$ NKG2A$^-$ NK cell subet against 221-HLA-E target cells in comparison to 221 target cells for CB and adult NK cells ($p<0.0001$ and $p=0.0116$ respectively) (Figure 5D, left panel). Altogether, these results highlight a strong inhibition of NKG2A$^+$ CB NK cells and the full responsiveness of NKG2C$^+$ CB NK cells in presence of HLA-E on target cells.

We next studied the phenotype of CB NKG2C$^+$ NK cell subset focusing on ILT2, DNAM-1, CD161, 2B4 and CD16 in comparison with CMV seropositive adult with NKG2C$^+$ NK cell amplification (Figure 5E). CB NKG2C$^+$ NK cells expressed DNAM-1, CD161, 2B4 and CD16 with a lower MFI as observed in adult counterpart, as illustrated for one representative experiment over 6. However, we did not observe a co-expression of ILT2 with NKG2C on CB NK cells as observed on adult NK cell counterparts, , accordingly with the absence of ILT2 expression on CB NK cells as previously reported on all CB NK cells[19]. Of note, CB NKG2C$^+$ NK cells co-expressed inhibitory NKG2A receptors in contrast to adult counterparts as previously reported in Figure 3F. Thus, it is possible that the strong inhibition mediated by NKG2A limits the triggering of NK cell by NKG2C. These functional results underline a potential role of NKG2A on CB NK cells to limit the reactivity at early stages of their development.

DISCUSSION

Different groups investigated the formation of KIR^+ NK cell repertoire and the impact of cognate HLA class I ligand on NK cell repertoire is still debated. Some studies supported either a model with sequential and random acquisition of KIRs in the absence of selection independently of HLA class I molecules[34] or a model of sequential acquisition of KIR expression influenced by HLA class I environment[20,35]. Although NK cells from adult individuals were well studied, CB NK cells are less characterized and their immaturity status is still debated. Schönberg et *al* investigated for the first time the role of HLA class I on CB NK cell repertoire formation and functional education. They concluded that initial KIR CB NK repertoire was not biased toward HLA class I molecules but observed that HLA class I molecules had an effect on the functional education of KIR^+ NK cells [20].

As previously done from a large cohort of adult healthy cohort [4], we performed a deep investigation of phenotype and functions of CB NK cell repertoire from a large cohort of CB samples to reevaluate its structural formation and functionality in light of new insights in the NK cell field. We confirmed that KIR^+ CB NK cell repertoire formation was independent of autologous HLA class I environment as previously reported [23]. Interestingly, we showed that CB NK cell repertoire was influenced by KIR gene content as observed in adults [4]. In particular, $KIR2DL1^+$ NK cell frequency was particularly impacted by the presence of both KIR2DL2 and KIR2DS1 genes. We showed a consecutive expression of KIR2D receptors on $KIR3DL1^+$ CB NK cells with a lower $KIR3DL1^+$ NK cell frequency but a higher KIR3DL1 MFI associated with all known combinations of KIR3DL1/S1 alleles, irrespectively of autologous Bw4 environment. Although a lower frequency of KIR^+ NK cells in CB, the NK repertoire profile was similar to adult NK repertoire and appeared to be early structured. NKG2A was expressed on a wide frequency of CB NK cells which was inversely correlated

to KIR^+ NK cell frequency. Adult NK cell repertoire was shaped toward increased clonal frequencies of KIR for self-HLA class I molecules, associated with a decreased frequency of $NKG2A^+$ NK cells. The effect of HLA class I environment on NK repertoire formation was finally observable on educated KIR^+ NK cells, particularly after viral infection that lead to specific and long-lasting expansion of NK cell clones. Taking into account this notion, the structuration of CB NK repertoire appears completed at this stage. We hypothesize that the individual infection history will next shape the NK cell repertoire in HLA dependent way following the fact that educated KIR^+ NK cells are preferentially expanded. Of note, virus infections differently impact NK cell repertoire as we observed a $NKG2C^+$ NK cell expansion [16,32] or the induction of adaptative NK cell subsets[18] after CMV infection, and $CD56^{dim}$ $NKG2A^+$ KIR^- NK cell expansion after B-EBV infection[17]. On a larger perspective, NK cell repertoire will evolve over the life span related to environmental factors such as viruses [32,36], toxic agents (e.i. smoke[37,38], pollution and aging[19,39,40]), privileging the expansion of experimented NK cell clones.

We confirmed the functional education of KIR^+ CB NK cells by self-HLA class I molecules that was nuanced by a lower spontaneous lysis of CB NK cells compared with adults as previously described [19,23,41,42]. This observation may be related to the low expression of activating NK receptors as notably DNAM-1 that appear to contribute to increase the functional potential of CB NK cells [43]. Of note, we underlined a low expression of CD16 on CB NK cells that corroborate the poor ADCC of CB NK cells, previously reported [15].

Recent studies described a $NKG2C^+$ NK cell expansion in CMV seropositive individuals [16,31,32,44] and other viral infections as chikungunya [45] and HCV[30] but always with a co-infection with CMV. In contrast, this $NKG2C^+$ NK cell expansion was not associated to EBV infection, another herpes virus [46]. However, CMV naïve adult individuals express very low level of NKG2C on NK cells. In this study, we investigated for the first time the NKG2C

expression on a large cohort of CB samples leading to highlight its questionable expression on CB NK cells. Surprisingly, we showed that CB NKG2C$^+$ NKG2A$^-$ NK cells could significantly expand *in vitro* after 14 days of stimulation with HLA-E$^+$ 221 cells and present a strong degranulation activity against 221-HLA-E target cells in comparison to 221 target cells at a high E:T ratio. The co-expression of NKG2A on a main part of CB NKG2C$^+$ NK cells may participate to limit CB NKG2C$^+$ NK cell activation. Moreover, HLA-E expression on 221 target cells inhibited efficiently NKG2A$^+$ CB NK cell degranulation. In contrast to adult NKG2C$^+$ NK cells expanded in CMV$^+$ individuals, CB NKG2C$^+$ NK cells did not express ILT2. Interestingly, it has been reported that CB NK cells can rapidly become fully mature after dUCBT, with a structured and functional repertoire, thus expressing all receptors observed in adult counterparts, including DNAM-1 and ILT2[15]. Of note, it has been shown that CB NKG2C$^+$ NK cell expansion can also be promoted after CMV reactivation [47]. Thus, there is a clinical potential of CB NKG2C$^+$ NK cells to control CMV reactivation. CB NK cells are functional and their NK repertoire has not yet shaped by immunological background. These cells can highly expand *in vitro* [48,49] and their efficacy against AML blasts has been highlighted[15]. Overall, CB NK cells present numerous interests to be used in immunotherapy.

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FIGURE LEGENDS

FIGURE 1. The nature of KIR genes impacts the structure of CB KIR$^+$ NK cell repertoire. **(A)** Scatter plots represent overall KIR2D$^+$ (KIR2DL1, 2DL2/3, 2DS1, 2DS2) NK cell frequency in adult (black circle, n=148) and cord blood (CB) (open circle, n=49) samples. Anti-KIR2DL1/S1 (EB6) and anti-KIR2DL2/L3/S2 (4A8) antibodies were used to evaluate KIR2D$^+$ frequency as the sum of EB6$^+$4A8$^-$, EB6$^+$4A8$^+$ and EB6$^-$4A8$^+$ populations **(B)** Density plot illustrating KIR2DL2/L3/S2$^+$ and KIR2DL2$^+$ KIR2DL3/S2$^-$ NK cells gating strategy for one representative CB sample **(C)** Scatter plots representing KIR2DL2/L3/S2$^+$ (GL183$^+$) NK cells frequencies in adult (black circle) and CB (open circle) cells according to the presence (+) of HLA-C ligand (C1$^+$) (adult n=121, CB n=66) and the absence (-) of HLA-C ligand (C1$^-$) (adults n=36, CB n=7) or the presence (+) of KIR2DL2 gene (adults n=86, CB n=33) and the absence (-) of KIR2DL2 gene (adults n=71, CB n=35) **(D)** Scatter plots represent KIR2DL2$^+$ KIR2DL3/S2$^-$ NK (1F12$^-$ GL183$^+$) cells frequencies in adult (black circle) and CB (open circle) cells according to the presence (+) of HLA-C1 ligand (adult n=61, CB n=27) and the absence (-) of HLA-C1 ligand (adults n=20, CB n=5) or the presence (+) of KIR2DL3 gene, (adults n=62, CB n=32) and the absence (-) of KIR2DL3 gene (adults n=18, CB n=5) **(E)** Density plot illustrating KIR2DL1/S1$^+$ NK cells gating strategy using anti-KIR2DL1/S1 (EB6) and anti-KIR2DL2/L3/S2 (4A8) antibodies for one representative CB sample **(F)** Scatter plots representing KIR2DL1/S1$^+$ NK cells frequencies compared in adult (black circle) and CB (open circle) cells according to the presence of HLA-C2 ligand, (adult n=89, CB n=45) and the absence of HLA-C2 ligand (adults n=57, CB n=28) or the presence (+) of KIR2DS1 gene (adults n=54, CB n=29) and the absence (-) of KIR2DS1 gene (adults n=88, CB n=40) **(G)** Density plot illustrating KIR2DL1$^+$ KIR2DL2/L3/S2$^-$ NK cells gating strategy using anti- KIR2DL2/L3/S2 (GL183) and anti-KIR2DL1/L2/L3/S2 (8C11) antibodies for one representative CB sample **(H)** Scatter plots represent KIR2DL1$^+$

KIR2DL2/L3/S2$^-$ NK cells frequencies in CB samples with AA KIR genotype (n=26) and B$^+$ KIR genotype (n=49) **(I)** Scatter plots representing KIR2DL1$^+$ KIR2DL2/L3/S2$^-$ NK cells frequencies compared in adult (black circle) and CB (open circle) cells according to the presence (+) of HLA-C2 ligand (adult n=93, CB n=45) and the absence (-) of HLA-C2 ligand (adults n=55, CB n=30), or the presence (+) of KIR2DS1 gene (adults n=79, CB n=42) and the absence (-) of KIR2DS1 gene (adults n=35, CB n=28) or the presence (+) of KIR2DL2 gene (adults n=66, CB n=37) and the absence (-) of KIR2DL2 gene (adults n=81, CB n=32). Statistical significance (*p<0.05, **p<0.001, ***p<0.0005, ****p<0.0001) between more than two groups was determined using the one-way ANOVA test. HLA-C ligand (C1, C2) were determined by HLA-C typing. KIR2DL2, KIR2DL3, KIR2DS1 genes presence was determined by PCR-SSP Multiplex

FIGURE 2. There is no influence of Bw4 ligand on KIR3DL1$^+$ CB NK cell frequency

(A) Density plot illustrating KIR3DL1$^+$ and KIR3DS1$^+$ NK cells gating strategy using anti-KIR3DL1 (DX9) and anti-KIR3DL1/S1 (Z27) antibodies for one representative CB sample. **(B)** Scatter plots representing KIR3DL1$^+$ CB NK cells frequencies Bw4$^-$ (n=25) and Bw4$^+$ (n=44) CB samples. The presence or absence of Bw4 ligand was deduced from HLA-A and HLA-B typing **(C)** Scatter plots representing the significantly higher KIR3DL1$^+$ CB NK cells frequencies in KIR3DS1$^-$ (n=45) versus KIR3DS1$^+$ (n=23) CB samples (p=0.0006). The presence of absence of KIR3DS1 gene was determined by PCR-SSP Multiplex **(D)** Scatter plots representing KIR3DL1$^+$ /KIR3DS1$^-$ CB NK cells frequencies in Bw4$^-$ (n=19) and Bw4$^+$ (n=27) CB samples. **(E)** Dot plots representing the correlation between the frequency of CB KIR3DL1$^+$KIR2D$^+$ NK cells and KIR3DL1$^+$ NK cells for 47 CB samples (p<0.0001, r=0.9677) **(F)** Density plots illustrating KIR3DL1/KIR3DS1 combinations (high/high, high/low; low, high/3DS1, low/3DS1) using a combination of anti-KIR3DL1/KIR3DS1 (Z27) and anti-KIR2D (1A6) mAbs for several representative CB. **(G)** Scatter plots of KIR3DL1$^+$

CB NK cell frequency according to the KIR3DL1 mean expression level or KIR3DS1 presence : KIR3DL1^{high} (n=19), KIR3DL1$^{high/low}$ (n=15), KIR3DL1^{low} (n=13), KIR3DL1^{high}/3DS1 (n=13) and KIR3DL1^{low}/3DS1 (n=12) in Bw4^{+} (black circle) and Bw4^{-} (open circle) samples. **(H)** Scatter plots of KIR3DL1^{+} NK cell frequency discriminating KIR3DL1^{high} individuals (adult n=75, CB n=46) from KIR3DL1^{low} individuals (adult n=29, CB n=40) **(I)** Scatter plots of KIR3DL1^{+} NK cell mean expression level discriminating KIR3DL1^{high} individuals (adult n=75, CB n=47) from KIR3DL1^{low} individuals (adult n=28), CB n=40) **(J)** Scatter plots of KIR3DL1 CB NK cell mean expression level according to the KIR3DL1 mean expression level or the presence/absence of KIR3DS1 : KIR3DL1^{high} (n=19), KIR3DL1$^{high/low}$ (n=15), KIR3DL1^{low} (n=13), KIR3DL1^{high}/3DS1 (n=13) and KIR3DL1^{low}/3DS1 (n=12) in Bw4^{+} (black circle) and Bw4^{-} (open circle) CB samples. Statistical significance (*p<0.05, **p<0.001, ***p<0.0005, ****p<0.0001) between more than two groups was determined using the one-way ANOVA test

FIGURE 3. Evaluation of other receptors expression on cord blood NK cells (A) Density plot illustrating NKG2A^{+} NK cell expression for one representative adult and one representative CB sample using anti-NKG2A and anti-KIR2D (1A6) antibodies **(B)** Scatter plots representing NK cells frequencies in adult (black circle) and CB (open circle) cells according to the presence of NKG2A and KIR receptors in adult (n=31) and CB (n=41) samples **(C)** Dot plots representing the correlation between the frequency of NKG2A^{+} NK cells and KIR^{+} NK cells on the left in CB (open circle, n=39, r= -0.5243 p=0.0006) and adults (black circle, n=31, r= -0.4845, p=0.0057) and between the frequency of KIR^{+}NKG2A^{-} NK cells and KIR^{-}NKG2A^{+} NK cells on the right in CB (open circle, n=38, r= -0.5830, p=0.0001) and adults (black circle, n=27, r= -0.8252, p<0.0001) samples **(D)** Density plot illustrating NKG2C^{+} NK cells expression for one representative adult and one representative CB sample using anti-NKG2C and anti-KIR2D (1A6) antibodies **(E)** Scatter plots representing NKG2C^{+}

NK cell frequencies in adult (black circle) and CB (open circle) cells according to the presence of KIR receptors in adult (n=30) and CB (n=41) samples **(F)** On the left, dot plots representing the correlation between the frequencies of $NKG2A^+$ NK cells and $NKG2C^+$ NK cells in CB (open circle, n=39) and CMV^+ adults (black circle, n=30, r=0.5848, p=0.0007) samples. On the right, illustrative density plots of cord blood NKG2C and NKG2A expression using anti-NKG2A and anti-NKG2C antibodies **(G)** Density plot illustrating $NKp30^+$ NK cell expression for one representative adult and one representative CB sample **(H)** Scatter plots representing $NKp30^+$ NK cell frequencies in adult (black circle, n=90) and CB (open circle, n=58) samples using anti-NKp30 and anti-KIR2D (1A6) antibodies **(I)** Scatter plots representing $CD57^+$ NK cell frequencies in adult (black circle, n=79) and CB (open circle, n=39) cells samples. Statistical significance (*p<0.05, **p<0.001, ***p<0.0005, ****p<0.0001) between more than two groups was determined using the one-way ANOVA test

FIGURE 4. Cord blood NK cells have a weaker spontaneous lysis than adult but are functionally competent (A) Bars representing the degranulation of adult (black circle, n=11) and CB (open circle, n=10) $CD3^-$ cells in medium and 221 conditions **(B)** Bars representing the IFNγ production of adult (black circle, n=11) and CB (open circle, n=10) $CD3^-$ cells in medium and 221 conditions **(C)** Dot plots representing the correlation between the frequency of $CD107a^+$ $CD3^-$ cells and KIR^+ $CD3^-$ cells in adults (black circle, n=25, r=0.5153, p=0.0084) and CB (open circle, n=10) samples **(D)** Dot plots representing the degranulation of CB $CD3^-$ $KIR2DL1^+$ cells in different culture conditions : medium, with 221-C*03:04 ($C2^-$) and 221-C*02:02 ($C2^+$) transfected cell lines (left panel, n=8), $CD3^-$ $KIR2DL3^+$ cells in medium, 221-C*02:02 ($C1^-$), 221-C*03:04 ($C1^+$) conditions (center panel, n=10) and of $CD3^-$ $KIR3DL1^+$ cells in medium, 221-B*39:01 ($Bw4^-$), 221-B*15:13 ($Bw4^+$) conditions (right panel, n=9). **(E)** Dot plots representing IFNγ production of CB $CD3^-$ $KIR2DL1^+$ cells in

medium, 221-C*03:04 ($C2^-$), 221-C*02:02 ($C2^+$) conditions (left panel, n=8) and $CD3^-$ $KIR2DL3^+$ cells in medium, 221-C*02:02 ($C1^-$), 221-C*03:04 ($C1^+$) conditions (center panel, n=10) **(F)** Histograms illustrating the expression of DNAM-1, 2B4, CD16 and Cd161 receptors for one representative adult (black), one cord blood presenting a strong degranulation (dark grey) and one CB presenting a weak degranulation (light grey). Dot plots representing the correlation between CB CD161 mean fluorescence intensity and the frequency of $CD107a^+CD3^-$ frequency is represented in the right panel (n=11, r=0.6154, p=0.0043). Statistical significance (*p<0.05, **p<0.001, ***p<0.0005, ****p<0.0001) between more than two groups was determined using the one-way ANOVA test

FIGURE 5. Full responsiveness of $NKG2C^+$ cord blood NK cells (A) Density plot illustrating cord blood $CD3^-$ $NKG2C^+$ and $NKG2A^+$ cell expression using specific anti-NKG2C and anti-NKG2A mAbs at day 0 and after 8 or 14 days of co-culture with 221 or transfected 221-HLA-E cell lines for one representative CB sample **(B)** Bars representing the frequency of CB $NKG2C^+$ NK cell at day 0 and day 14 after co-culture at ratio E:C 10:1 with 221 (grey) or 221-HLA-E (black) cell lines for 4 CB samples **(C)** Dot plots representing $CD107a^+$ $NKG2C^+$ $NKG2A^-$ $CD3^-$ cell frequency (left panel) and $CD107a^+$ $NKG2A^+$ $NKG2C^-$ $CD3^-$ cell frequency at E:C 1:1 ratio in CB (open n=16) and adult (black n=7) in different culture conditions : medium (circle), 221 (square), 221-HLA-E (triangle) **(D)** Dot plots representing $CD107a^+$ $NKG2C^+$ $NKG2A^-$ $CD3^-$ cell frequency (left panel) and $CD107a^+$ $NKG2A^+$ $NKG2C^-$ $CD3^-$ cell frequency at E:C 1:1 ratio in CB (open, n=5) and adult (black, n=5) at E:C 50:1 ratio in different culture conditions : medium (circle), 221 (square), 221-HLA-E (triangle) **(E)** Density plot illustrating adult CMV+ and cord blood NKG2C co-expression with ILT2; DNAM-1, CD161, NB4 and CD16 receptors using combinations of specific mAbs. Statistical significance (*p<0.05, **p<0.001, ***p<0.0005, ****p<0.0001) between more than two groups was determined using the one-way ANOVA test

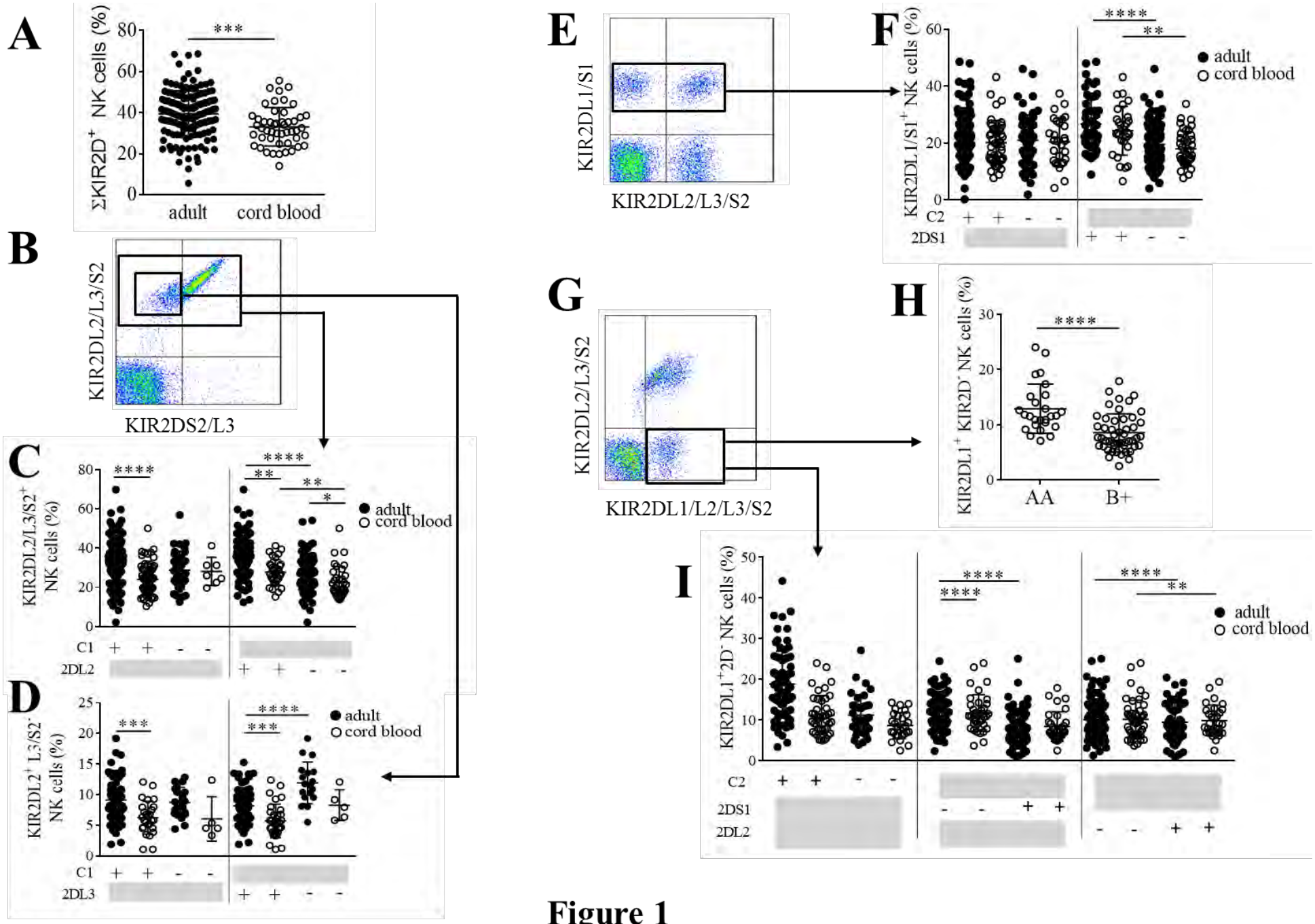

A
ΣKIR2D+ NK cells (%)
adult
cord blood
B
KIR2DL2/L3/S2
KIR2DS2/L3
C
KIR2DL2/L3/S2+ NK cells (%)
C1
2DL2
adult
cord blood
D
KIR2DL2+ L3/S2- NK cells (%)
C1
2DL3
E
KIR2DL1/S1
KIR2DL2/L3/S2
F
KIR2DL1/S1+ NK cells (%)
C2
2DS1
G
KIR2DL2/L3/S2
KIR2DL1/L2/L3/S2
H
KIR2DL1+ KIR2D- NK cells (%)
AA
B+
I
KIR2DL1+2D- NK cells (%)
C2
2DS1
2DL2

Figure 1

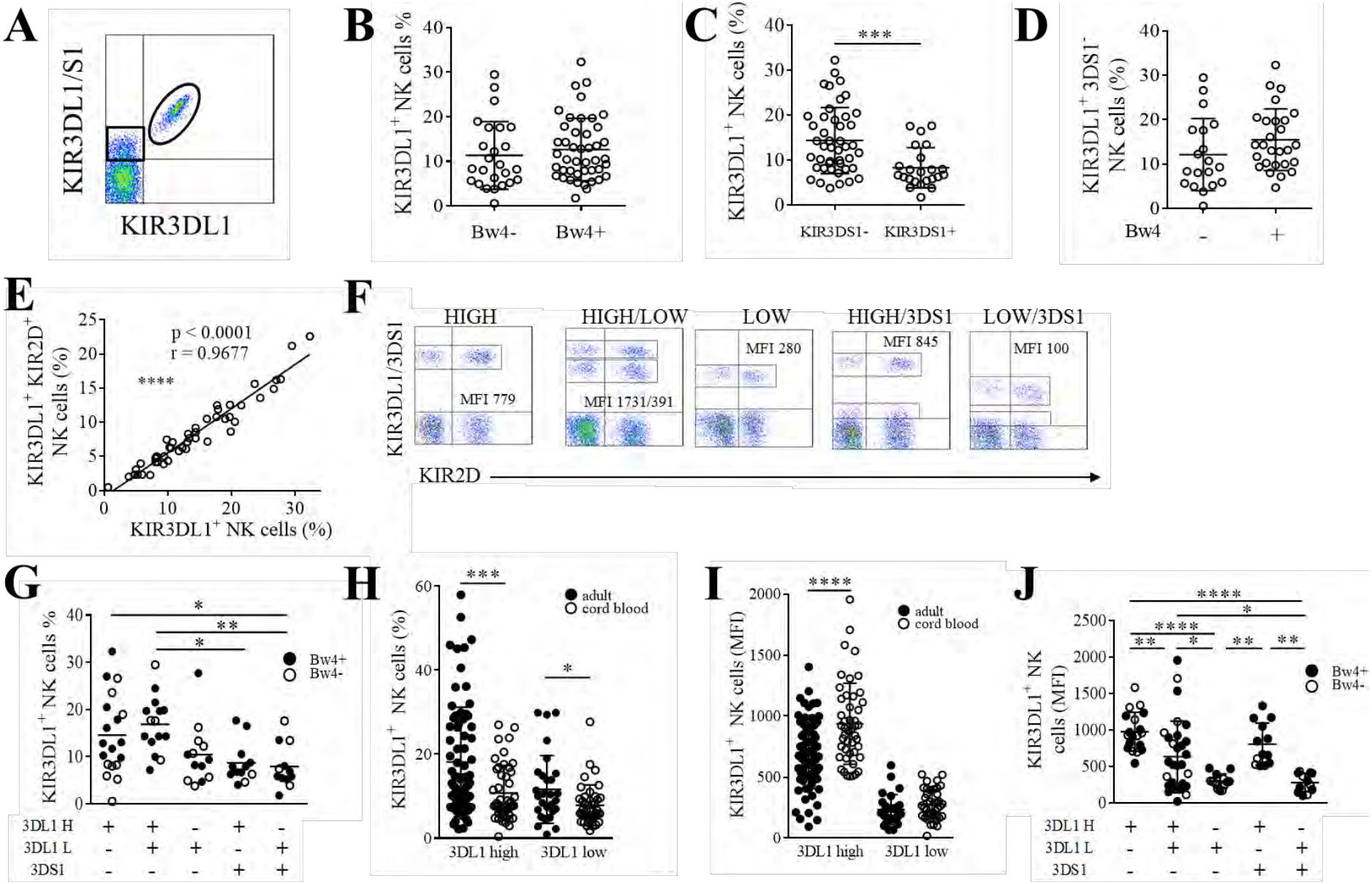

A
KIR3DL1/S1
KIR3DL1
B
KIR3DL1+ NK cells %
Bw4-
Bw4+
C
KIR3DL1+ NK cells (%)
***
KIR3DS1-
KIR3DS1+
D
KIR3DL1+ 3DS1- NK cells (%)
Bw4
E
KIR3DL1+ KIR2D+ NK cells (%)
p < 0.0001
r = 0.9677
****
KIR3DL1+ NK cells (%)
F
KIR3DL1/3DS1
HIGH
HIGH/LOW
LOW
HIGH/3DS1
LOW/3DS1
MFI 779
MFI 1731/391
MFI 280
MFI 845
MFI 100
KIR2D
G
KIR3DL1+ NK cells %
Bw4+
Bw4-
3DL1 H
3DL1 L
3DS1
H
KIR3DL1+ NK cells (%)
adult
cord blood
3DL1 high
3DL1 low
I
KIR3DL1+ NK cells (MFI)
adult
cord blood
3DL1 high
3DL1 low
J
KIR3DL1+ NK cells (MFI)
Bw4+
Bw4-
3DL1 H
3DL1 L
3DS1

Figure 2

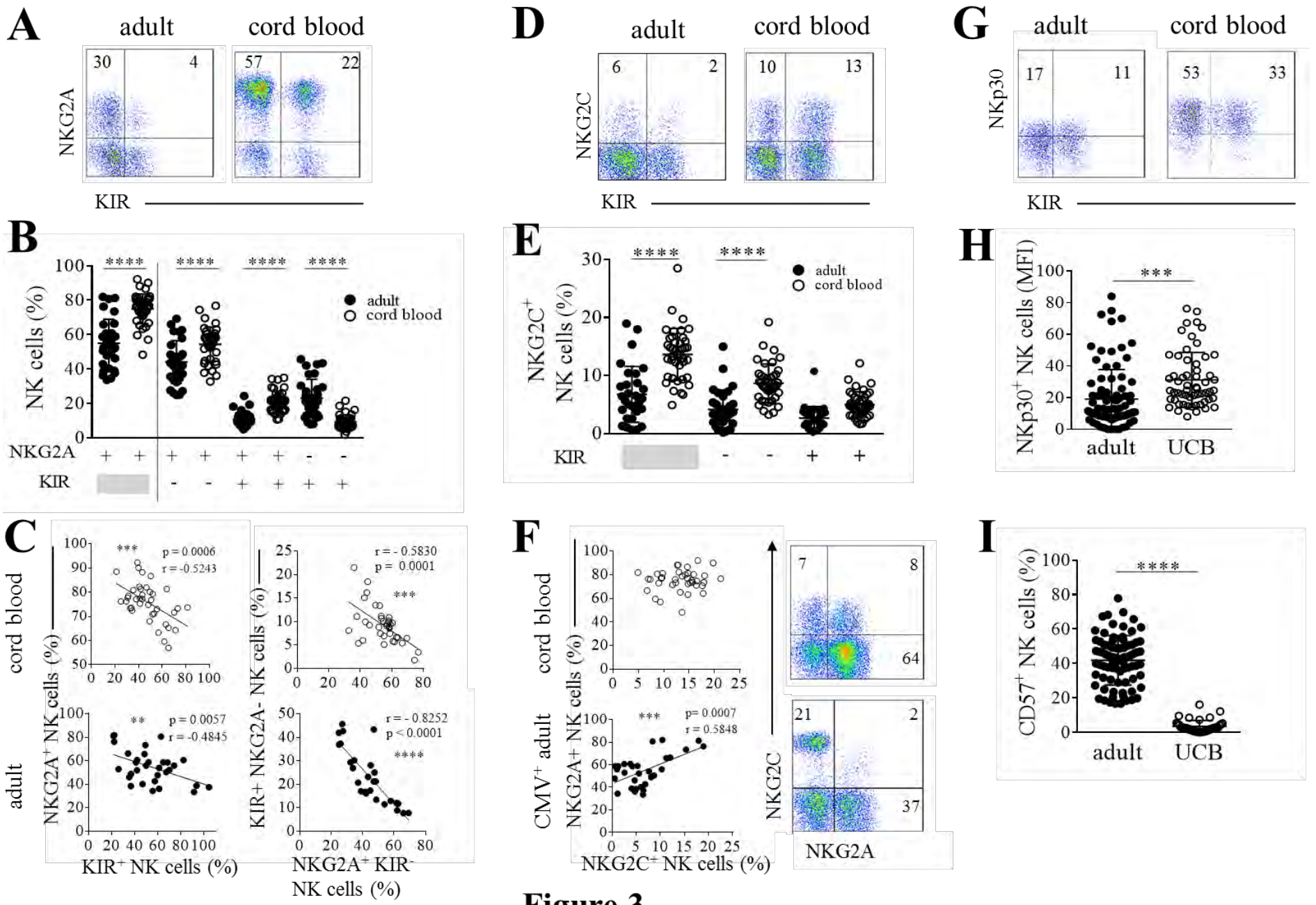

A
adult
cord blood
30
4
57
22
NKG2A
KIR
D
adult
cord blood
6
2
10
13
NKG2C
KIR
G
adult
cord blood
17
11
53
33
NKp30
KIR
B
NK cells (%)
adult
cord blood
NKG2A
KIR
E
NKG2C+ NK cells (%)
adult
cord blood
KIR
H
NKp30+ NK cells (MFI)
adult
UCB
C
cord blood
adult
NKG2A+ NK cells (%)
KIR+ NKG2A- NK cells (%)
p = 0.0006
r = -0.5243
r = - 0.5830
p = 0.0001
p = 0.0057
r = -0.4845
r = - 0.8252
p < 0.0001
KIR+ NK cells (%)
NKG2A+ KIR- NK cells (%)
F
cord blood
CMV+ adult
NKG2A+ NK cells (%)
p= 0.0007
r = 0.5848
NKG2C+ NK cells (%)
NKG2C
NKG2A
7
8
64
21
2
37
I
CD57+ NK cells (%)
adult
UCB

Figure 3

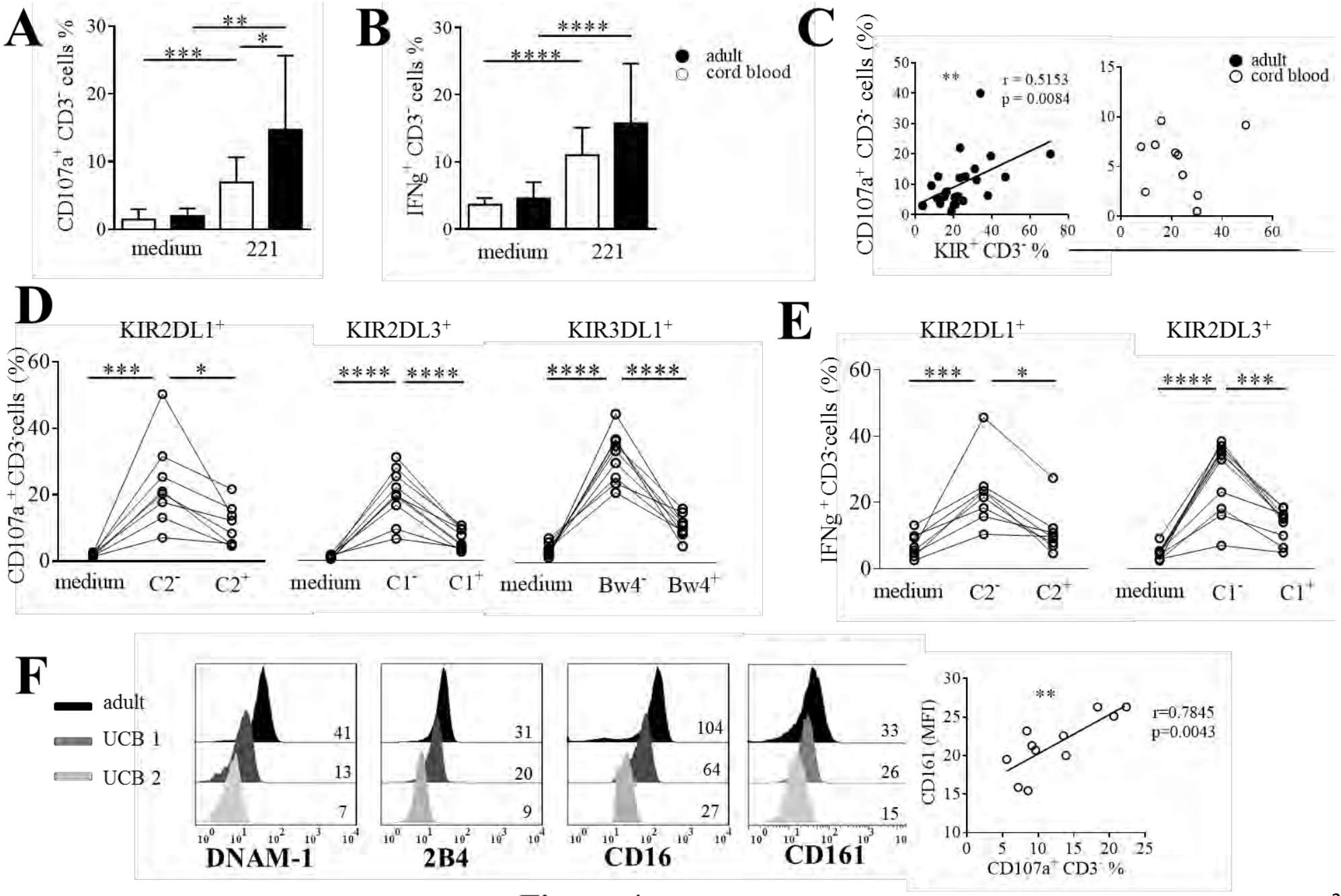

A
CD107a+ CD3- cells %
medium
221
B
IFNg+ CD3- cells %
adult
cord blood
C
CD107a+ CD3- cells (%)
KIR+ CD3- %
r = 0.5153
p = 0.0084
D
KIR2DL1+
KIR2DL3+
KIR3DL1+
CD107a+CD3-cells (%)
medium
C2-
C2+
C1-
C1+
Bw4-
Bw4+
E
IFNg+CD3-cells (%)
F
adult
UCB 1
UCB 2
DNAM-1
2B4
CD16
CD161
CD161 (MFI)
r=0.7845
p=0.0043

Figure 4

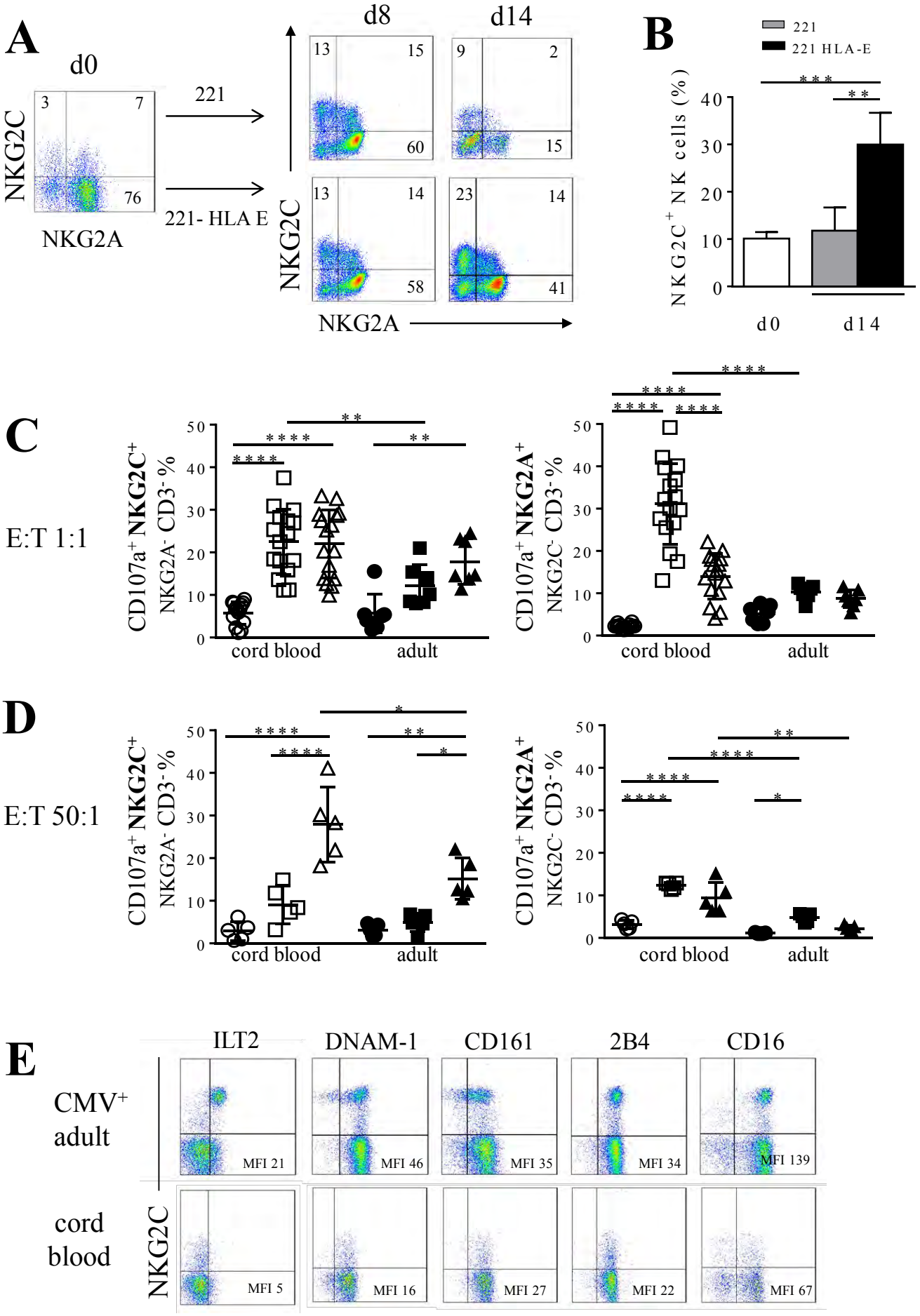

A
d0
d8
d14
NKG2C
NKG2A
221
221- HLA E
3
7
76
13
15
60
9
2
15
13
14
58
23
14
41
B
221
221 HLA-E
NKG2C+ NK cells (%)
d0
d14
C
E:T 1:1
CD107a+ NKG2C+ NKG2A- CD3- %
CD107a+ NKG2A+ NKG2C- CD3- %
cord blood
adult
D
E:T 50:1
E
CMV+ adult
cord blood
ILT2
DNAM-1
CD161
2B4
CD16
MFI 21
MFI 46
MFI 35
MFI 34
MFI 139
MFI 5
MFI 16
MFI 27
MFI 22
MFI 67

Figure 5

RESULTATS

ARTICLE N°4 **:** Utilisation des gènes KIR comme marqueurs précoces du chimérisme hématopoïétique après double-greffe de sang de cordon

Use of Killer cell Immunoglobulin-like Receptor genes as early markers of hematopoietic chimerism after double-umbilical cord blood transplantation

Pauline Rettman, Nolwenn Legrand, Catherine Willem, Laurence Lodé, Patrice Chevallier, Anne Cesbron , David Senitzer, Christelle Retière, Katia Gagne

Haematologica 2015 (sous presse)

La détermination du chimérisme hématopoïétique après double greffe de sang de cordon est établie pour détecter l'échec de la prise de greffe et également pour prédire la rechute, le rejet du greffon et la GvHD aiguë (Thomson et al. 2000; Lamba et al. 2004; Peterlin et al. 2015). Les techniques d'amplification de marqueurs polymorphiques comme les STR (Short Tandem Repeat) ou les SNP (Single Nucleotid Polymorphism) par PCR constituent les méthodes standards d'analyse du chimérisme (Lion et al. 2001; Chalandon et al. 2003). A ce jour, la PCR quantitative en temps réel basée sur l'amplification des SNP est la méthode la plus sensible permettant d'identifier la prise de greffe et de détecter un éventuel chimérisme mixte ou la non-prise (Alizadeh et al. 2002; Koldehoff et al. 2006; Kletzel et al. 2013). Toutefois, cette technique peut être limitée à cause de l'absence de marqueurs SNP discriminants entre les 2 sangs de cordon et le patient, et n'est pas forcément toujours fiable pour déterminer un chimérisme précoce après double greffe de sang de cordon (dUCBT). Dans ce contexte, de nouvelles approches sont nécessaires.

Les cellules NK sont la première population qui reconstitue le système hématopoïétique après greffe de cellules souches. Nous avons précédemment corrélé l'alloréactivité des cellules NK KIR^+ de sang de cordon avec la dominance d'un seul sang de cordon après dUCBT. Le contenu en gènes KIR varie d'un individu à l'autre et les gènes KIR définissent différents génotypes. Nous proposons ainsi dans cette étude d'utiliser les gènes KIR comme marqueurs additionnels pour la détermination précoce du chimérisme après dUCBT.

Cette étude est réalisée à partir d'une cohorte locale de 40 dUCBT provenant du service d'hématologie du CHU de Nantes (Dr P. Chevallier, Pr P. Moreau, Dr L. Lodé) ayant été traité entre Mars 2006 et Décembre 2011. Afin de déterminer quelle unité de sang de cordon (UCB) est dominante après dUCBT, nous avons comparé les génotypages KIR du patient avant dUCBT, des deux unités de sang de cordon injectées et du receveur post-dUCBT (J+90) sur l'effectif total de 40 dUCBT à partir des marqueurs KIR comparativement aux marqueurs

conventionnels utilisés au laboratoire d'hématologie biologique du CHU de Nantes. La présence ou l'absence de chacun des 14 gènes KIR a pu être déterminée par PCR Multiplex grâce aux amorces fournies par D.Senitzer (Sun et al. 2004) et nous a permis de déterminer l'identité des cellules reconstituant l'hématopoïèse du patient qu'il s'agisse d'une prise avec chimérisme mixte entre les cellules du patient et d'une UCB ou entre deux UCB, d'un chimérisme total à partir d'une UCB («full donor») ou d'une non prise («patient reconstitution»). L'analyse du phénotype des cellules NK KIR avant et après dUCBT à J+60 nous a également permis de confirmer les résultats des génotypes KIR. Un logiciel de «clustering» a été utilisé afin de déterminer l'utilité de chacun des gènes KIR dans la détermination du chimérisme, mettant en évidence l'implication majeure de 3 gènes KIR inhibiteurs (KIR2DL2, KIR2DL3 et KIR2DL5) et de l'ensemble des gènes KIR activateurs (KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, KIR3DS1).

Dans l'ensemble l'analyse des génotypages KIR nous a permis d'identifier 60% de chimérisme total, 20% de chimérisme mixte et 7,5% de non prise dans cette cohorte. Toutefois, le génotypage KIR n'a pas été suffisant pour identifier le statut du chimérisme dans 5 greffes que nous avons appelé «indéterminées». En effet, dans ces 5 dUCBT, nous observons un profil génotypique KIR identique entre les patients et les UCB, rendant impossible leur discrimination les uns des autres. A l'aide des génotypages KIR, nous avons également identifié 5 autres dUCBT avec un chimérisme mixte contrairement aux résultats obtenus par l'étude conventionnelle des marqueurs SNP, identifiées comme chimérisme total (n=4) ou non prise (n=1). Dans l'ensemble, l'analyse du chimérisme post dUCBT par les génotypages KIR est concordante aux résultats obtenus avec la méthode conventionnelle basée sur les marqueurs SNP réalisée au service d'hématologie du CHU de Nantes (Dr L. Lodé) pour 31 dUCBT sur 40. Lorsque nous disposions des ADN, nous avons complémenté les génotypages KIR par des typages HLA de classe I alléliques afin de ré-évaluer les dUCBT

pour lesquelles nous obtenions résultats discordants (n=1) ou indéterminés (n=5). Les gènes HLA de classe I présentent en effet un large polymorphisme ce qui permet de les utiliser comme marqueurs secondaires afin de complémenter les génotypages KIR lorsque non discriminants. Cependant, notons que les typages HLA de classe I ne peuvent pas être utilisés comme uniques marqueurs du chimérisme étant donné qu'il n'y a souvent qu'un seul allèle HLA de classe discriminant le patient des deux unités de sang de cordon dans cette cohorte. Nous avons ainsi pu déterminer le statut du chimérisme post dUCBT par l'analyse des génotypages KIR et avec quelques typages HLA de classe I secondaires, et obtenir des résultats concordants à 100% avec ceux obtenus par l'analyse de marqueurs SNP conventionnels.

Nous avons évalué la cinétique du chimérisme après dUCBT chez certains patients pour lesquels différents échantillons d'ADN nous ont été fournis par le laboratoire d'hématologie du CHU de Nantes. Nous avons ainsi évalué la nature du chimérisme précocement, dès J+20 après dUCBT. Le profil génotypique KIR observé dès J+20 reste stable au cours du temps pour les 10 patients étudiés. Nous avons également confirmé que le génotypage KIR réalisé très tôt après dUCBT est lié à la reconstitution d'une seule unité de sang de cordon en déterminant le phénotype des cellules NK KIR par cytométrie en flux à J+14, J+28 et J+60 après dUCBT. Les résultats de génotypages KIR et de phénotypages des cellules NK KIR^+ mettent en évidence que le répertoire NK KIR est constitué à partir d'une seule unité de sang de cordon dès J+14 après dUCBT.

Nous avons également mis en évidence que les cellules NK KIR^+ apparaissent dès J14 post-dUCBT et permettent d'identifier s'il y a eu prise de greffe et, si oui, la nature du cordon dominant. Nous avons confirmé l'intérêt des gènes KIR comme marqueurs additionnels du chimérisme pour déterminer quelle unité de sang de cordon reconstitue le système immunitaire du patient après dUCBT. Il est important de préciser que l'analyse des marqueurs

KIR est une technique qualitative et non quantitative qui est n'est pas proposée ici pour remplacer les techniques conventionnelles basées sur l'amplification par qPCR de marqueurs polymorphes mais pour permettre une détermination qualitative précoce du statut du chimérisme après dUCBT. Cette méthode permet de détecter la présence ou l'absence de différents gènes KIR conférant ainsi de multiples marqueurs discriminants pouvant être phénotypiquement et fonctionnellement évalué très tôt après dUCBT, et permet de détecter précocement aussi bien le chimérisme total que la non-prise de greffe.

Dans cette étude, 3 gènes KIR inhibiteurs sont particulièrement discriminants pour déterminer la nature du chimérisme post dUCBT, dont le KIR2DL5 pour lequel nous avons une information semi-allélique (KIR2DL5A et KIR2DL5B). Les gènes KIR présentent un important polymorphisme allélique, notamment le gène KIR3DL1 dont le polymorphisme allélique impacte le phénotype et la fonction des cellules NK. La nature des allèles KIR de chaque cordon pourrait être identifiée par séquençage nouvelle génération, actuellement en cours de développement au laboratoire, ce qui permettra d'apporter d'autres informations pour déterminer encore plus précisément le statut du chimérisme hématopoïétique post dUCBT. Le chimérisme mixte après dUCBT a précédemment été corrélé avec une augmentation de l'incidence de la rechute (Reshef et al. 2014). Nous n'avons toutefois pas pu vérifier cette association dans notre cohorte puisque seulement 3 dUCBT avec chimérisme mixte étaient inclues. Néanmoins, il serait intéressant d'évaluer sur une plus large cohorte si la détection du chimérisme mixte par génotypage KIR précocement après dUCBT pourrait être utilisée comme marqueur prédictif et pronostique de la rechute. La détermination du chimérisme très précocement dès J+14 après dUCBT, contre J+21 par les techniques conventionnelles, constitue un véritable atout pour les cliniciens qui peuvent ainsi adapter le traitement selon chaque patient en modulant par exemple le traitement immunosuppresseur afin de limiter la neutropénie et gérer la rechute. Les gènes KIR et HLA étant situés sur des

chromosomes différents, la détermination du chimérisme hématopoïétique par les gènes KIR peut être appliquée à d'autres types de greffes notamment en greffes HLA compatibles et HLA haploidentiques.

Use of killer cell immunoglobulin-like receptor genes as early markers of hematopoietic chimerism after double-umbilical cord blood transplantation

It has been established that hematopoietic chimerism after double umbilical cord blood transplantation (dUCBT) can predict disease relapse and detect potential engraftment failure.[1-3] The use of a precise method to evaluate residual host haematopoiesis is imperative because it has been reported that the risk of relapse is higher in patients with mixed chimerism.[4] Up to now, real-time quantitative polymerase chain reaction (PCR) of single nucleotide polymorphism (SNP) markers remains the best method to accurately assess and quantify chimerism.[5,6] However, this method may be limited by the lack of available in-house SNP discriminant markers between both umbilical cord blood (UCB) units and patients and may not be reliable to assess early chimerism status after dUCBT. In this context, additional approaches are needed. Natural killer (NK) cells are the first population to reconstitute the patient's hematopoietic system following hematopoietic stem cell transplantation, before T lymphocytes[7] and we previously reported that cord blood NK cells express killer cell immunoglobulin-like receptors (KIR).[8] Here, we investigated KIR genes as additional markers for early chimerism assessment after dUCBT. KIR gene content varies between individuals who can exhibit seven to 14 inhibitory and activating KIR genes, defining multiple KIR genotypes. This broad KIR gene polymorphism should be useful as chimerism markers in the context of dUCBT.

In order to determinate which UCB unit dominates engraftment, KIR genotyping was performed on 40 patients who underwent dUCBT at Nantes CHU, using a multiplex sequence-specific primer-PCR method[9] on day 90 after UCBT and was compared to KIR genotyping in patients and in UCB units prior to dUCBT. The presence or absence of each KIR gene among 14 studied in patients, UCB units and recipients led us to determine the identity of cells reconstituting recipient hematopoiesis. As illustrated in Figure 1A, recipient #11 was characterized by the absence of *KIR2DL2* and *KIR2DS2* genes, which were present in this patient before dUCBT. Interestingly, *KIR2DL2* and *KIR2DS2* were absent in UCB1, but present in UCB2, highlighting that hematopoietic reconstitution resulted from only one UCB unit (unit 1) in this patient (Figure 1A). Phenotypic analysis of cord blood KIR^+ NK cells before dUCBT and recipient KIR+ NK cells at day 60 after dUCBT using available anti-KIR monoclonal antibodies allowed discrimination of inhibitory and activating KIR expression between the two UCB units (Figure 1B) and confirmed KIR genotyping results. In particular, UCB1 NK cells did not express KIR2DS1 and KIR2DL2, unlike NK cells from UCB2. Moreover, KIR3DL1 was only expressed on NK cells from UCB1. We observed that the recipient's KIR^+ NK cells on day 60 after dUCBT had the same KIR expression profile as NK cells from UCB1, with expression of KIR3DL1 and absence of KIR2DS1 and KIR2DL2, confirming engraftment of patient #11 with one full dominant UCB1.

Analysis of KIR genotyping also allows mixed chimerism to be detected, as illustrated in Figure 1C for patient #13 who presented the KIR2DL5A allele and the deleted non-expressed KIR2DS4 allele (i.e. 1D) before dUCBT. Both UCB units had the KIR2DL5B allele and expressed KIR2DS4. Recipient #13 had both A and B KIR2DL5 alleles and KIR2DS4, in particular, highlighting a mixed chimerism between the patient and one UCB unit. Although rare,10 mixed chimerism between both UCB units may also be detected using KIR genotyping (*Online Supplementary Figure S1*). In several other cases, analysis of KIR genotype after dUCBT highlighted engraftment failure and autologous patient reconstitution, as illustrated in recipient #33 (Figure 1D).

KIR genotyping was performed in all recipients and was focused on discriminating KIR genes that allowed us to assess hematopoietic chimerism status by their presence or absence (*Online Supplementary Table S1*). Based on the calculated frequencies of each discriminating KIR gene, we highlight the major implication of three inhibitory and all activating KIR genes (Figure 2A). Overall, qualitative chimerism analysis at day 90 after dUCBT using KIR markers indicated full UCB unit reconstitution (60%), mixed patient/UCB unit reconstitution (20%) and an autologous recovery (7.5%) in the recipients included in this study (Figure 2B). However, KIR genotyping did not allow chimerism status to be assigned in the five remaining dUCBT, which were therefore called "indeterminate" (Figure 2B). Although KIR genotyping is a reproducible and reliable method as initially reported,[9] even using a small amount of cord blood DNA (*Online Supplementary Figure S2*), the patients, UCB units and recipients had the same KIR genotype in these five cases, thus preventing any chimerism assessment using KIR markers. Moreover, KIR genotyping analysis of five other dUCBT revealed mixed chimerism, which was discordant with the conventional SNP analysis, being evaluated as "full donor chimerism" (n=4) or "patient reconstitution" (n=1) as illustrated for patient #15 (Figure 2C). Overall, 31 out of 40 dUCBT (3 dUCBT with patient reconstitution, 3 dUCBT with mixed chimerism and 25 dUCBT with full donor engraftment) had concordant results with the conventional SNP-based analysis (Figure 2D).

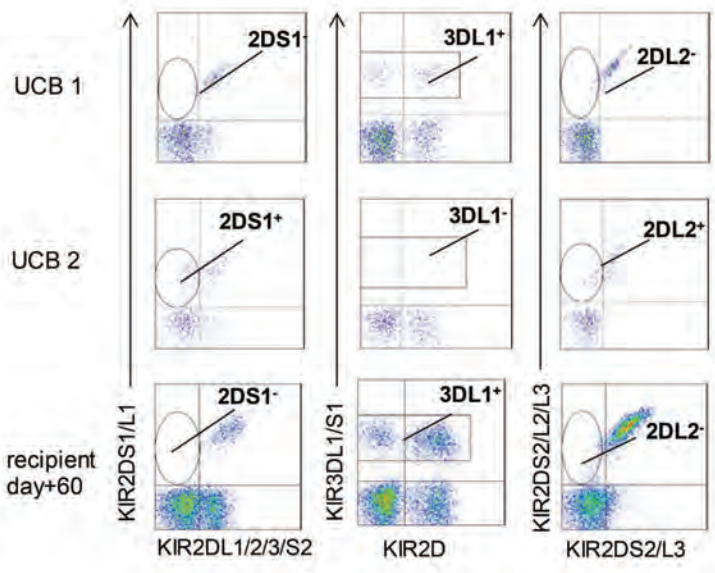
For some dUCBT with available DNA (n=6), we complemented the KIR genotyping with HLA class I allele typing to provide additional information since numerous HLA class I incompatibilities between UCB units and patients are frequently encountered in the context of dUCBT. Of note, HLA class I genes presenting large allelic polymorphism are useful markers to re-assess the "indeterminate" dUCBT and the few discordant results between KIR genotyping and the SNP–based method. Our analysis based on KIR genotyping, with secondary complementary HLA class I allele typing, applied in a few cases, allowed chimerism status to be accurately determined in 100% of patients in full concordance with conventional approaches (Figure 2E). Indeed, as illustrated in Figure 2F, KIR genotyping of recipient #39 on day 90 did not allow chimerism status to be assigned. However, using HLA-B typing on day 90 this recipient was typed as HLA-B*35:03, thus demonstrating full UCB1 engraftment, which correlated with the data from the conventional SNP method. In our dUCBT cohort, HLA class I typing was not appropriate as a single method for assessing chimerism because there is often only one discriminating HLA class I allele between both UCB units and the patient (*Online Supplementary Table S2*). Importantly, HLA class I DNA typing combined with other techniques has already been reported in haploidentical hematopoietic stem cell transplantation to determine chimerism status.[12] More broadly, allelic KIR typing could also be a complementary tool to compare both UCB units and the recipient's genotypes. Indeed, inhibitory KIR present a high allelic polymorphism such as KIR3DL1 which also affects

A

KIR genotyping

	2DL1	2DL2	2DL3	2DL4	2DL5	3DL1	3DL2	3DL3	2DS1	2DS2	2DS3	2DS4	1D	2DS5	3DS1
Patient # 11	+	+	+	+	-	+	+	+	-	+	-	-	+	-	-
UCB 1	+	-	+	+	-	+	+	+	-	-	-	-	+	-	-
UCB 2	+	+	+	+	+	NULL	+	+	+	+	+	-	+	-	+
Recipient # 11 d+90	+	-	+	+	-	+	+	+	-	-	-	-	+	-	-

B



C

KIR genotyping

	2DL1	2DL2	2DL3	2DL4	2DL5	3DL1	3DL2	3DL3	2DS1	2DS2	2DS3	2DS4	1D	2DS5	3DS1
Patient # 13	+	+	+	+	A	+	+	+	+	+	+	-	+	+	+
UCB 1	+	+	+	+	B	+	+	+	-	+	+	+	+	-	-
UCB 2	+	+	+	+	B	+	+	+	-	+	+	+	+	-	-
Recipient # 13 d+90	+	+	+	+	AB	+	+	+	+	+	+	+	+	+	+

D

KIR genotyping

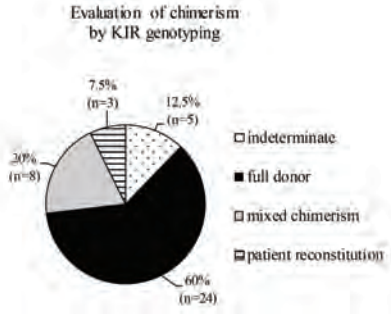
	2DL1	2DL2	2DL3	2DL4	2DL5	3DL1	3DL2	3DL3	2DS1	2DS2	2DS3	2DS4	1D	2DS5	3DS1
Patient # 33	+	+	+	+	AB	+	+	+	+	+	+	+	-	-	+
UCB 1	+	+	+	+	-	+	+	+	-	+	-	-	+	-	-
UCB 2	+	-	+	+	-	+	+	+	-	-	-	-	+	-	-
Recipient # 33 d+90	+	+	+	+	AB	+	+	+	+	+	+	+	-	-	+

Figure 1. Evaluation of hematopoietic chimerism status using KIR genotyping. (A) KIR genotyping performed for patient #11 before and after-dUCBT (day 90) and on the corresponding UCB units. Genomic DNA from patients, UCB units, and recipients at different times after dUCBT were provided by the HLA laboratory (Dr A. Cesbron EFS Nantes, France) and the Hematology Department of Nantes CHU hospital (Dr L. Lodé). The presence or absence of inhibitory and activating KIR genes was determined using a KIR multiplex PCR-sequence-specific primer method as previously described[9]. For some dUCBT, KIR genotyping with an INVITROGEN kit was performed to allow the assessment of KIR2DL5 alleles and KIR3DL1 alleles were assigned as already reported[8]. KIR genes involved in the evaluation of chimerism status are highlighted in black boxes. Results indicate full donor chimerism from UCB 1. (B) Representative flow cytometry density plots leading to target KIR2DS1 using anti-KIR2DL1/S1 (anti-KIR2DL1, R&Dsystems) with anti-KIR2DL1/2/3/S2 (8C11,[16]), KIR3DL1 using -KIR3DL1/S1 (Z27, Beckman Coulter) with KIR2D (EB6 + GL183, Beckman Coulter) and KIR2DL2 expression on NK cells using anti-KIR2DL2/L3/S2 (GL183, Beckman Coulter) with anti-KIR2DL3/S2 (1F12[16]) for UCB1 and UCB2 units and the recipient#11 at day 60 after dUCBT. Data were collected using a FACSCalibur (BD Biosciences), and analyzed using Flowjo 7.6.1 software (TreeStar). (C) KIR genotyping performed on patient #13 before and after-dUCBT (day 90) and the corresponding UCB units. Data are indicative of mixed chimerism. (D) KIR genotyping performed on patient #33 before and after-dUCBT (day 90) and the corresponding UCB units before dUCBT. Data are indicative of patient reconstitution.

A

	gene	number	frequency
inhibitory KIR	2DL5	28	70
	2DL2	15	37.5
	2DL3	8	20
	3DL1	4	10
	2DL1	3	7.5
activating KIR	2DS5	24	60
	2DS1	20	50
	3DS1	18	45
	2DS4	15	37.5
	2DS3	15	37.5
	2DS2	13	32.5
	1D	8	20

B



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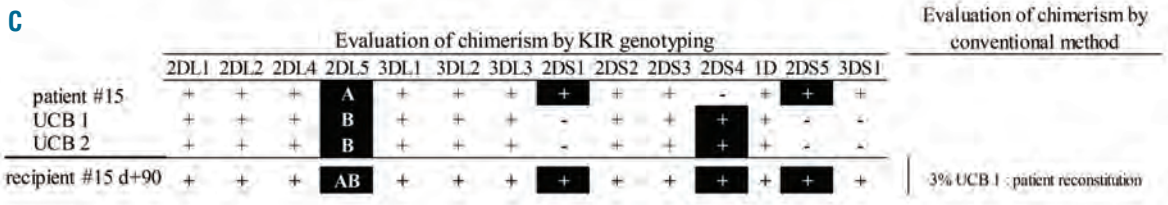

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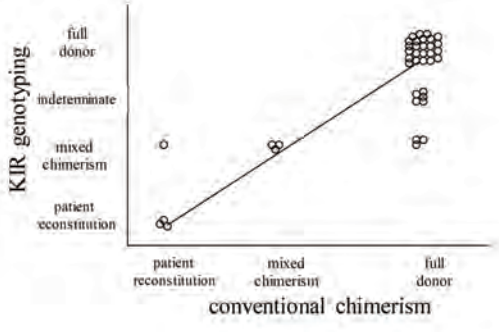

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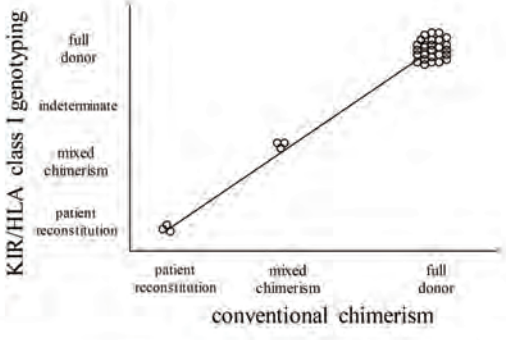

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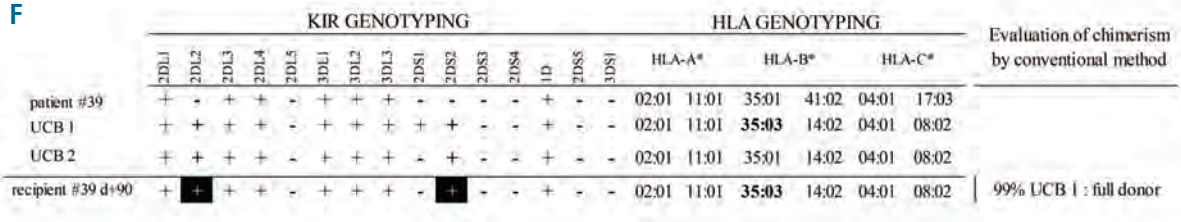

Figure 2. KIR/HLA genotyping analysis is concordant with conventional chimerism evaluation by SNP markers. (A) Number and frequency of each inhibitory and activating KIR gene implicated in the assessment of hematopoietic chimerism using KIR genotyping. (B) Pie chart illustrating the proportions of full donor (black), mixed chimerism (gray), patient reconstitution (shaded) and indeterminate reconstitution (dotted) after evaluation of hematopoietic chimerism status using KIR genotyping in 40 recipients at day 90 after dUCBT. (C) Representative KIR genotyping performed for patient #15 before and after dUCBT (day 90) and on the corresponding UCB units illustrating discordant results between KIR genotyping analysis and conventional SNP analysis. Hematopoietic chimerism data were available for the first 3 months after dUCBT and were obtained using informative SNP markers for both patients and UCB units. Serial peripheral blood samples were separated into neutrophil and mononuclear lymphoid fractions, and provided the total white cell count. Hematopoietic chimerism after-dUCBT was evaluated on whole peripheral blood cells (n=17) and/or sorted $CD3^+$ T cells (n=23). Genomic DNA from UCB units and from the recipient after-dUCBT were amplified with multiple PCR primer sets to identify markers capable of distinguishing UCB units from patient alleles. SNP analysis was performed by quantitative real-time PCR (qPCR) as described by Alizadeh *et al.*[13] on a Rotorgene Q (Qiagen). Calibration curves for each SNP marker were generated following normalization to the albumin housekeeping gene. This allowed each SNP to be quantified, and to determine the percentage from UCB units or patients. Full donor chimerism was defined as the presence of more than 95% of donor cells, mixed chimerism if more than 5% and less than 95% were donor cells, and autologous recovery if less than 5% of cells were donor cells. Discriminating KIR underlined in black boxes highlight mixed chimerism based on KIR2DL5, KIR2DS1, KIR2DS4 and KIR2DS5. (D) Correlation between KIR genotyping and conventional SNP chimerism analysis from 40 dUCBT. (E) Correlation between KIR/HLA genotyping and conventional SNP chimerism analysis from 37 dUCBT on available DNA samples. (F) Representative KIR and HLA class I genotyping for patient #39, both UCB units and recipient #39 with day 90 post-dUCBT. Discriminating KIR are shown by black boxes. Discriminating HLA class I alleles are shown in bold. High resolution typing for HLA-A, HLA-B and HLA-C loci was carried out using a Sequence Based Typing kit (Abbott Molecular Park, LL, USA) prospectively on all patients and UCB units before dUCBT and retrospectively on some recipients after-dUCBT.

KIR3DL1 expression.[8] The study of allele polymorphism of all KIR genes by next-generation sequencing should reveal the extreme diversity of KIR genes, as recently reported,[13] and could provide the missing information needed to determine hematopoietic chimerism precisely after dUCBT, thus discriminating both UCB units and patient at KIR allele level. To evaluate KIR as chimerism markers early after dUCBT (from days 14-20), DNA samples were provided at multiple time points after dUCBT and used to evaluate the kinetics of hematopoietic chimerism for some recipients. As illustrated in Figure 3A, the absence of *KIR2DL3*, *1D*, and *KIR2DS5* genes and the presence of the *KIR2DS4* gene demonstrated UCB unit 1 reconstitution as early as day 20 after dUCBT in recipient #5. This reconstitution remained stable at days 30 and 75 after dUCBT, in accordance with the evaluation of hematopoietic chimerism by SNP-PCR established in that case only on day 75 post-dUCBT (Figure 3A).

KIR genotyping enabled chimerism status to be evaluated as early as day 20 after dUCBT in ten other recipients (*data not shown*). To confirm that KIR genotyping performed early after dUCBT is linked to the reconstitution of only one UCB unit, KIR$^+$ NK cell phenotype was determined by flow cytometry from day 14 to 60 after dUCBT, as illustrated for recipient #31 (Figure 3B). Full UCB1 reconstitution was highlighted by the absence of *KIR2DL2* and *KIR2DS2* genes as illustrated in the KIR amplification patterns. The KIR$^+$ NK cell phenotype marked by the absence of KIR2DL2 on day 14 remained

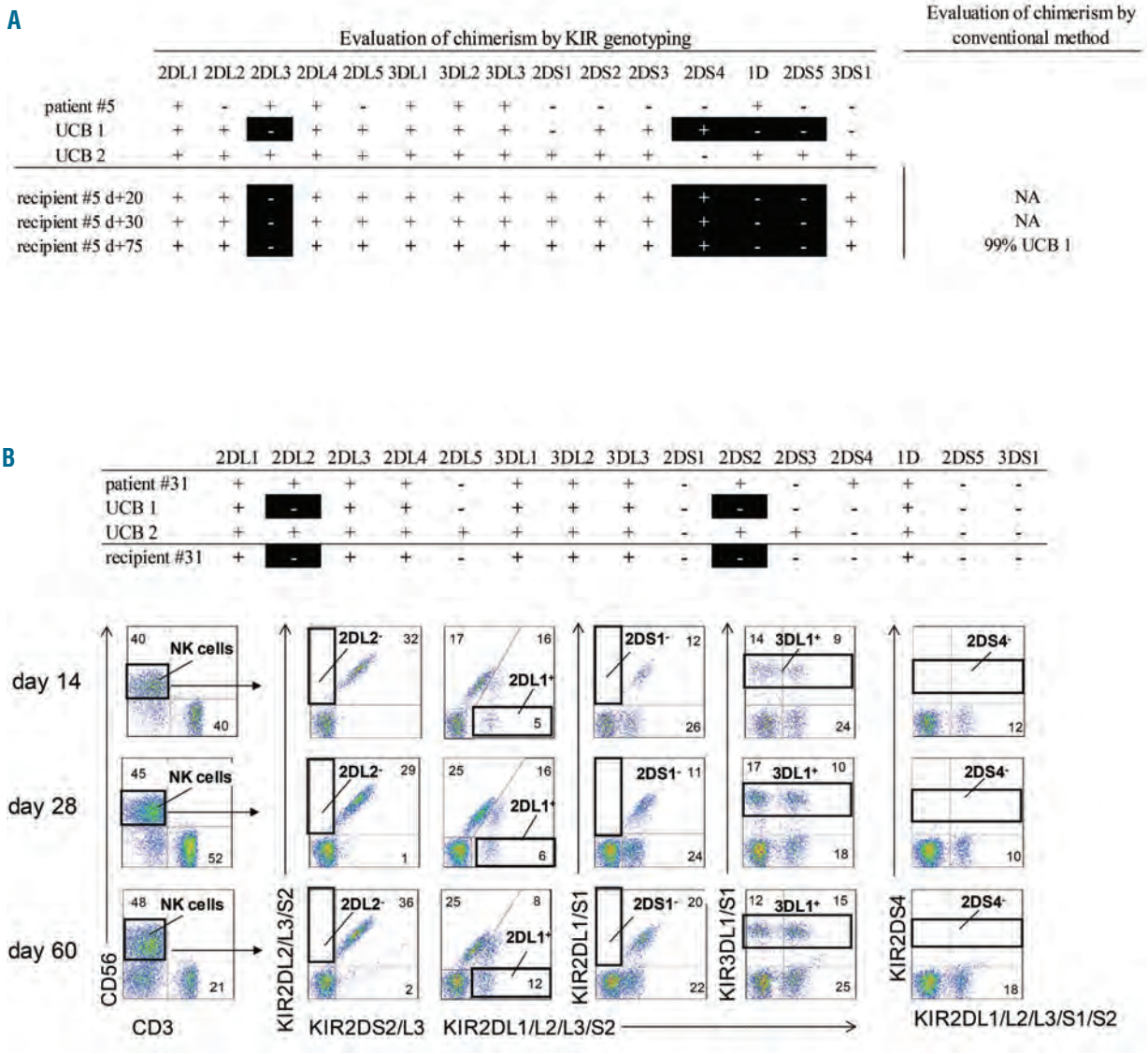

Figure 3. Early determination of chimerism status after dUCBT by KIR genotyping analysis compared to SNP markers. (A) KIR genotyping performed on patient #5 and the corresponding UCB units before dUCBT and on recipient #5 on days 20, 30 and 75 after dUCBT with conventional SNP chimerism data available on day 75 after dUCBT, illustrating the early and stable full donor engraftment. NA: Not available. (B) KIR genotyping performed for patient#31 before and after dUCBT (day 90) and on the corresponding UCB units. Representative flow cytometry density plots illustrate the early KIR$^+$ NK cell reconstitution stained with anti-KIR2DL2/L3/S2 (GL183, Beckman Coulter, Immunotech, Marseille, France), anti-KIR3DL1/S1 (Z27, Beckman Coulter), anti-KIR2DS4 (FES172, Beckman Coulter), anti-KIR2DL1/S1 (EB6, R&D systems), anti-KIR2DL3/S2 (1F1216), and anti-KIR2DL1/2/3/S2 (8C1116) for patient #31 on days 14, 28 and 60 after dUCBT. Data were collected using a FACSCalibur (BD Biosciences), and analyzed using Flowjo 7.6.1 software (TreeStar).

stable on days 28 and 60 after dUCBT. Interestingly, genotypic and phenotypic results showed that the KIR NK cell repertoire is constituted from only one UCB unit as early as day 14 after dUCBT.

Overall, our data support that KIR genes can be considered as additional markers to assess hematopoietic chimerism status as early as day 14 after dUCBT. Donor chimerism determined on bone marrow samples by semi-quantitative PCR of short tandem repeats on day 21 was predictive of subsequent neutrophil recovery.[14] However, the impact of early mixed chimerism on relapse incidence was not investigated in this study which was restricted to assessment of UCB engraftment alone. Correlation of early mixed chimerism with relapse incidence, as recently reported in allogeneic hematopoietic stem cell transplantation with reduced intensity conditioning,[15] was not possible in our cohort of limited size since only three dUCBT with early mixed chimerism were included. However, this should be prospectively investigated in a larger dUCBT cohort to determine whether detection of mixed chimerism using KIR genotypes as early as day 14 after dUCBT could be used as early predictive and prognostic markers of relapse.

Using KIR genotypes with supplemental HLA class I typing for some dUCBT with available DNA, we detected both engraftment with either full or mixed chimerism or graft failure after dUCBT, in concordance with conventional SNP-quantitative PCR method. Determination of hematopoietic chimerism based on KIR genotyping can also be applied to HLA matched unrelated and haploidentical hematopoietic stem cell transplant recipients since KIR and HLA genes are located on different chromosomes. From a clinical point of view, the determination of chimerism status after dUCBT with KIR markers as early as day +14 will allow physicians to adapt treatment therapy promptly, for example by modulating immunosuppression to manage, in particular, leukemic relapse.

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This letter has a Supplementary Appendix.

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DISCUSSION ET PERSPECTIVES

Dans le contexte des double-greffes de sang de cordon (dUCBT), seule l'une des deux unités reconstitue l'hématopoïèse du patient et aucun critère clinique ou biologique ne permet à ce jour de prédire systématiquement quelle unité de sang de cordon sera dominante. La dominance d'un cordon pourrait être expliquée par les incompatibilités HLA de classe I qui sont fréquentes en dUCBT et pouvant potentiellement induire une alloréactivité des cellules NK médiée par les récepteurs KIR. L'objectif de ces travaux de thèse était donc de trouver une réponse à cette première interrogation par l'étude en parallèle des incompatibilités KIR et HLA de classe I et de la biologie des cellules NK de sang de cordon.

Les études précédemment réalisées portant uniquement sur les incompatibilités HLA de classe I en termes de ligands KIR et présentant des résultats contradictoires (Willemze et al. 2009; Brunstein et al. 2009; Garfall et al. 2013; Tarek et al. 2014), nous avons mené une étude génétique rétrospective de l'impact des ligands KIR, des gènes KIR et des combinaisons KIR/KIR ligand sur la dominance d'un cordon après dUCBT sur une cohorte locale. Nous n'avons pas mis en évidence de différences de profils des cordons dominants et non dominants au niveau génétique (fréquences des KIR, des ligands KIR) ou biologique (dose de cellules $CD34^+$, de cellules nucléées totales infusées après décongélation). Cette première étude confirme donc que les différents paramètres cliniques ne sont pas impliqués dans la dominance d'une seule unité de sang de cordon (SC). L'analyse génétique des combinaisons KIR/HLA a corrélé la présence de la combinaison $KIR3DL1^+/Bw4^-$ dans le sens GvG 1) à la dominance d'un cordon, 2) à reconstitution des neutrophiles plus rapide et 3) à une augmentation de l'incidence de la rechute après dUCBT. Il semblerait donc que l'alloréactivité des cellules NK $KIR3DL1^+$ soit délétère à la reconstitution des cellules de ce cordon au profit de la reconstitution des cellules du cordon $Bw4^-$, qui présente toutefois un pool plus réduit de cellules alloréactives, assurant un effet anti-leucémique limité. Ces résultats étaient surprenants puisqu'il aurait été plus attendu d'observer une alloréactivité des

cellules NK du cordon dominant dirigée contre le cordon non dominant. Afin de donner un sens mécanistique à ces données génétiques, des études cellulaires phénotypiques et fonctionnelles ont été menées à partir de cellules de SC. Nous avons d'une part mis en évidence l'expression de molécules HLA de classe I significativement plus faible à la surface des cellules de SC comparée à l'adulte, ce qui corrobore l'effet GvG observé dans notre cohorte. L'expression diminuée des molécules HLA-A et B à la surface des cellules du trophoblaste a précédemment été rapportée, étant considérée comme l'un des mécanismes permettant au foetus d'échapper au système immunitaire de la mère au cours de la grossesse (Goodfellow et al. 1976; Trowsdale and Betz 2006). D'autre part, des études fonctionnelles de dégranulation et d'activation ont confirmé que les cellules NK de SC, bien qu'à priori immatures, présentent des capacités fonctionnelles *ex vivo*. Dans un contexte d'amplification *in vitro*, les cellules NK de SC peuvent même être aussi fonctionnelles que les cellules NK d'individus adultes.

Afin de confirmer ces résultats et d'évaluer la potentielle implication des combinaisons KIR/KIR ligand sur le devenir des dUCBT, nous avons mené une large étude multicentrique des gènes KIR, des gènes HLA et des combinaisons KIR/HLA en collaboration avec les principaux centres greffeurs français. Cette étude a d'une part mis en évidence l'hétérogénéité des pratiques de greffes des centres impliqués, notamment en termes de pathologies (myéloïde ou lymphoïde), d'intensité de conditionnement (myéloablatif ou réduit), de doses de cellules $CD34^+$ et cellules nucléées totales injectées et d'utilisation de l'ATG. Ces données mettent en évidence la difficulté d'étudier l'impact des combinaisons génétiques KIR/HLA sur le devenir des dUCBT à partir de cohortes multicentriques, regroupant des greffes hétérogènes en termes de paramètres cliniques et biologiques. Nous avons également réalisé cette même analyse sur une cohorte multicentrique réduite plus homogène mais n'avons pas mis en évidence de corrélation entre la combinaison

KIR3DL1^{+}/Bw4^{-} ni aucune autre combinaison KIR/KIR ligand et la dominance d'un seul cordon après dUCBT. L'absence de corrélation de la combinaison KIR3DL1^{+}/Bw4^{-} avec le devenir des dUCBT sur un plus large effectif peut notamment être expliquée par la variation de facteurs génétiques comme la fréquence des ligands Bw4 qui était particulièrement faible chez les cordons dominants sur la cohorte locale. Dans un contexte mettant en jeu de nombreux paramètres cliniques, ces données suggèrent donc la faible implication des combinaisons KIR/KIR ligand et donc de l'alloréactivité NK sur la dominance d'un seul sang de cordon après dUCBT. Toutefois, les analyses multivariées ont mis en évidence l'impact bénéfique de certaines combinaisons génétiques KIR/HLA sur le délai de reconstitution des neutrophiles suggérant qu'une potentielle alloréactivité NK KIR^{+} favoriserait une sortie d'aplasie plus rapide après dUCBT. Une précédente étude avait également rapporté que la forte dose de cellules NK présentes dans un greffon était associée à une reconstitution des neutrophiles plus rapide (Larghero et al. 2007). Dans l'ensemble, nos données ont confirmé qu'il n'est pas nécessaire de sélectionner des unités de SC sur des bases génétiques KIR/HLA pour assurer la dominance d'un seul SC. Cependant, l'effet d'une potentielle alloréactivité NK KIR^{+} sur la reconstitution des neutrophiles doit être confirmé par d'autres études multicentriques afin de dégager les mécanismes cellulaires impliqués et pourrait être un facteur à prendre en compte dans la sélection d'un SC afin d'accélérer la sortie d'aplasie après dUCBT.

Au-delà des combinaisons génétiques KIR/HLA, il serait important de prendre en compte la nature de l'allèle KIR et également la nature du peptide présenté par les molécules HLA de classe I aux récepteurs KIR. En effet les récepteurs KIR présentent d'une part un important polymorphisme allélique. Plus particulièrement, le polymorphisme allélique du récepteur KIR3DL1 impacte le phénotype et la fonction des cellules NK, et des profils de

forte, faible et non expression (null) peuvent être observés. D'autre part, l'équipe de S.Khakoo a montré que la nature du peptide présenté par les molécules HLA de classe I impacte la force des interactions entre les récepteurs KIR et les complexes HLA/peptide et ainsi le potentiel d'inhibition des cellules NK (Fadda et al. 2010 ; Borhis et al. 2013). De la même façon, la nature de l'allèle KIR impacte la force de l'interaction KIR/KIR ligand et donc les capacités d'inhibition de la cellule NK. Ainsi, la hiérarchie dans les capacités des cellules NK KIR2DL1^{+} à inhiber la lyse de cibles exprimant le ligand HLA-C2 serait dûe à la présence d'une arginine au lieu d'une cystéine conférant une plus forte capacité d'inhibition au récepteur codé par l'allèle KIR2DL1*010 comparé au récepteur codé par l'allèle KIR2DL1*004 respectivement (Bari et al. 2009). Le polymorphisme allélique KIR3DL1 affecte également la force de l'interaction du récepteur KIR3DL1 avec le ligand Bw4. Pour étudier le polymorphisme allélique de tous les gènes KIR, de nouvelles technologies devraient être développées. Ainsi, le séquençage nouvelle génération (NGS), une technologie émergente récemment mise au point pour le génotypage allélique HLA (Lind et al. 2010), et actuellement en cours de développement au laboratoire pour le typage allélique KIR , devrait permettre le typage simultané de tous les gènes KIR de plusieurs dizaines d'échantillons en même temps. La nature des allèles KIR impliqués dans l'étude de l'impact des combinaisons KIR/ligand sur le devenir des dUCBT pourrait ainsi également être prise en compte afin d'apporter des informations supplémentaires sur l'impact des gènes KIR au niveau allélique.

L'étude de l'implication de l'alloréactivité des cellules NK KIR sur le devenir des dUCBT nous a mené à considérer les gènes KIR comme marqueurs du chimérisme hématopoïétique après dUCBT. En effet, les techniques habituellement utilisées pour établir le statut du chimérisme après greffes de CSH présentent quelques limites notamment pour la détermination précoce du chimérisme. Afin de déterminer le statut du chimérisme, les profils génétiques KIR des deux UCB, du patient avant dUCBT et des receveurs à J90 après dUCBT

ont été comparés à l'analyse réalisée par la méthode conventionnelle d'amplification des marqueurs polymorphes SNP. Confortés par des données phénotypiques des cellules NK KIR après dUCBT, nos résultats ont mis en évidence l'utilité des gènes KIR comme marqueurs additionnels précoces du chimérisme hématopoïétique, permettant la détection de la non prise ou de la prise de greffe avec chimérisme mixte ou prise d'un seul cordon, ainsi que l'identité du cordon dominant. Nos résultats ont également montré que le répertoire NK KIR est constitué à partir d'une seule unité de sang de cordon très tôt dès J14 après dUCBT (Figure 21).

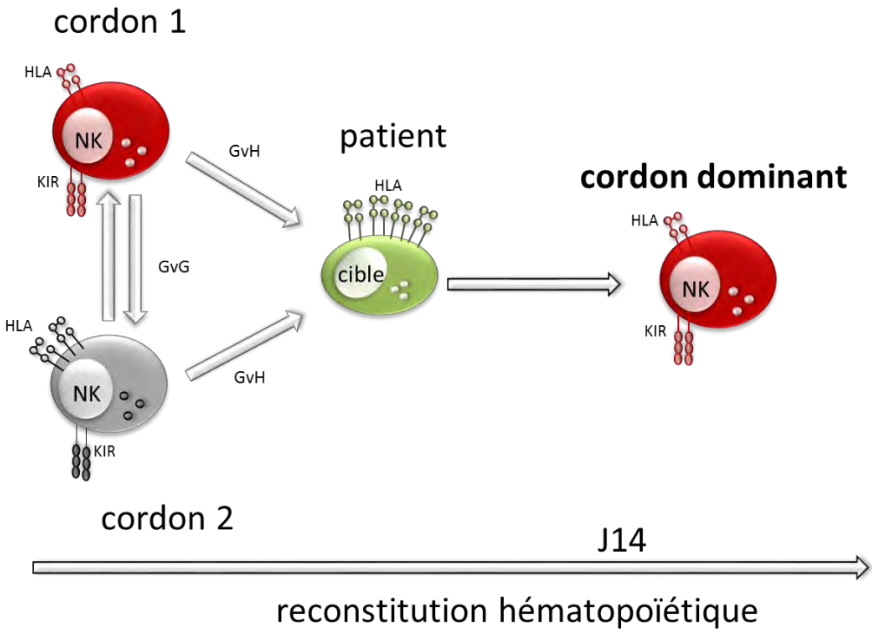

Figure 1 : Reconstitution des cellules NK après dUCBT
Après co-injection de deux unités de sang de cordon, les cellules NK KIR^{+} du cordon dominant peuvent être détectées dès J14 après dUCBT.

Un chimérisme mixte a été corrélé à une augmentation de l'incidence de la rechute leucémique après greffes de CSH (Reshef et al. 2014). Il n'a pas été possible de réaliser cette analyse sur notre cohorte, ne comportant que 3 dUCBT avec chimérisme mixte. Bien qu'il soit nécessaire de le confirmer sur une plus large cohorte, les gènes KIR pourraient également être utilisés comme marqueurs prédictifs précoces et pronostiques de la rechute leucémique. Précédemment, une étude réalisée à partir d'échantillon de moelle osseuse avait permis de

prédire la reconstitution des neutrophiles par l'amplification de marqueurs conventionnels à J21 après dUCBT (Avery et al. 2012). D'un point de vue clinique, la possibilité de détecter le statut du chimérisme 7 jours plus tôt permettrait donc aux cliniciens de prévoir un greffon de secours en cas de non prise de la greffe et également d'adapter les traitements immunosuppresseurs pour gérer en particulier la rechute leucémique. Les gènes KIR et HLA étant localisés sur des chromosomes différents, cette technique peut également être utilisée dans d'autres types de greffes comme les greffes HLA identiques ou HLA haploidentiques.

La potentielle alloréactivité des cellules T sur le devenir des dUCBT a été évaluée dans notre cohorte en étudiant les fréquences des incompatibilités HLA-A, -B, -C et -DRB1 entre patients et SC. Les incompatibilités HLA de classe I et DRB1 n'ont pas été corrélées à la dominance d'un sang de cordon suggérant l'absence d'impact d'une potentielle alloréactivité des cellules T sur la prise de greffe dans notre cohorte. Après dUCBT, la reconstitution des LT est retardée et la thymopoïèse est altérée. En effet, seuls des LT naïfs n'ayant pas rencontré l'antigène sont présents dans les premiers mois après dUCBT et l'apparition de cellules T au répertoire complet n'est parfois observée qu'au 6ème mois voire un an après dUCBT (Garderet et al. 1998; Politikos and Boussiotis 2014). La reconstitution d'un compartiment T fonctionnel avec un répertoire spécifique de l'antigène dépend de la production de novo de LT naïfs par le thymus par les receveurs comme montré après greffe de moelle osseuse (Roux et al. 2000). La reconstitution du répertoire T dépendante du thymus est également impactée par les incompatibilités HLA entre donneur et receveur, perturbant notamment la sélection thymique (Godthelp et al. 1999). Toutefois, nous ne pouvons pas exclure la présence de lymphocytes T CD8^{+} fonctionnels et alloréactifs et leur implication dans la prise de greffe. Gutman et ses collaborateurs ont en effet rapporté la présence de LT

$CD8^+$ du cordon dominant alloréactifs vis-à-vis du cordon non dominant (Gutman et al. 2010).

D'autres populations cellulaires peuvent également être impliquées dans la dominance d'un sang de cordon ou la reconstitution des neutrophiles après dUCBT. Ces dernières années, de nombreux travaux ont montré l'existence de différentes populations de cellules lymphoïdes innées (ILC) qui constituent des cellules effectrices et régulatrices importantes de l'immunité innée et qui réagissent rapidement suite à des signaux de stress. De récentes observations ont notamment corrélé la présence des ILC, et en particulier la population ILC3 productrice d'IL22, à une diminution de l'incidence de la GvHD aigüe après greffes de CSH suggérant un rôle protecteur des ILC dans ce contexte (Hanash et al. 2012; Munneke et al. 2014). Bien que leur reconstitution soit lente après greffes de CSH, d'autres études sont nécessaires afin d'évaluer la potentielle implication de ces cellules sur le devenir des dUCBT.

L'étude des incompatibilités KIR et HLA en dUCBT nous a amenés à étudier l'alloréactivité des cellules NK KIR^+ sur un plan cellulaire et à nous intéresser à la biologie des cellules NK issues de sang de cordon sous un angle nouveau, en prenant en compte les données récentes de la littérature. Contrairement au répertoire NK adulte, le phénotype des cellules NK de sang de cordon a été peu étudié (Dalle et al. 2005; Le Garff-Tavernier et al. 2010; Schonberg et al. 2011). D'autre part, l'étude individuelle des sous populations NK KIR^+ n'a pas toujours été réalisée en absence d'anticorps discriminants les KIR activateurs des KIR inhibiteurs dont nous disposons au laboratoire (David et al. 2009). L'étude phénotypique réalisée sur un large panel de sangs de cordon a confirmé que les cellules NK de sang de cordon présentent un répertoire NK KIR complet, structuré et fonctionnel. La formation du répertoire NK semble indépendante de l'environnement HLA de classe I autologue en accord

avec les précédentes études (Schönberg et al. 2011, Sleiman et al. 2014) mais est clairement impactée par le contenu en gènes KIR.

Dans l'ensemble, nos résultats confirment un modèle d'acquisition séquentielle des récepteurs KIR en accord avec les études réalisées par les équipes de Schönberg et Sleiman (Schönberg et al. 2011b; Sleiman et al. 2014). Il faut noter que certaines études sont en désaccord avec ce modèle (Andersson et al. 2009; Sternberg-Simon et al. 2013). Toutefois, elles ont été réalisées à partir de cellules NK d'individus adultes dont le répertoire a pu être biaisé par l'histoire immunologique de l'individu et les facteurs environnementaux (Lu et al. 2007; Le Garff-Tavernier et al. 2010; Béziat et al. 2013). Notre étude montre que les cellules NK de sang de cordon constituent un modèle d'étude idéal de la formation du répertoire NK permettant d'éviter tout biais induit par l'environnement ou les infections virales et bactériennes.

Bien que l'immaturité des cellules NK de sang de cordon soit débattue, cette étude a confirmé la formation très précoce du répertoire NK KIR et nous a également permis de mieux appréhender l'alloréactivité des cellules NK de sang de cordon. Schönberg et ses collaborateurs ont précédemment mis en évidence l'éducation fonctionnelle des cellules NK de sang de cordon par les molécules HLA de classe I du soi (Schönberg et al. 2011a). Nous avons confirmé ces résultats et mis en évidence les capacités d'inhibition de la dégranulation et de la production d'IFNγ des sous-populations NK KIR^+ (KIR2DL1, KIR2DL3, KIR3DL1) vis-à-vis de lignées 221 transfectées avec leurs ligands respectifs (HLA-C2, HLA-C1, HLA-Bw4), quel que soit l'environnement HLA autologue. Les études de l'éducation des cellules NK par les molécules HLA de classe I du soi ont suggéré que plus un individus présente de ligands pour les récepteurs KIR, plus le pool de cellules NK éduquées est important (Anfossi et al. 2006). Nous avons pour la première fois montré que plus les cellules NK adultes expriment de récepteurs KIR plus leur capacité de dégranulation vis-à-vis d'une lignée dépourvue de ligands HLA est importante. De façon intéressante, ceci n'a pas été observé

chez les cellules NK de sang de cordon. Il faut envisager que l'absence d'expression de certains récepteurs activateurs (NCR, DNAM-1 ou le 2B4) à ce stade impacte la fonctionnalité des cellules NK de sang de cordon. Cette étude a ainsi confirmé que les cellules NK de sang de cordon présentent un phénotype proche de celui observé chez l'adulte avec une expression de l'ensemble des récepteurs KIR dans des fréquences plus faibles et également d'autres récepteurs comme les NCR, NKG2D, DNAM-1, 2B4 ou encore le CD161. Les cellules NK de sang de cordon sont particulièrement caractérisées par une expression plus élevée du récepteur NKG2A et l'absence de l'ILT2. Ces récepteurs modulent l'activation des cellules NK de sang de cordon dont nous avons confirmé les capacités de dégranulation et de production d'IFNγ, dans des proportions plus faibles que celles observées chez l'adulte (Figure 22).

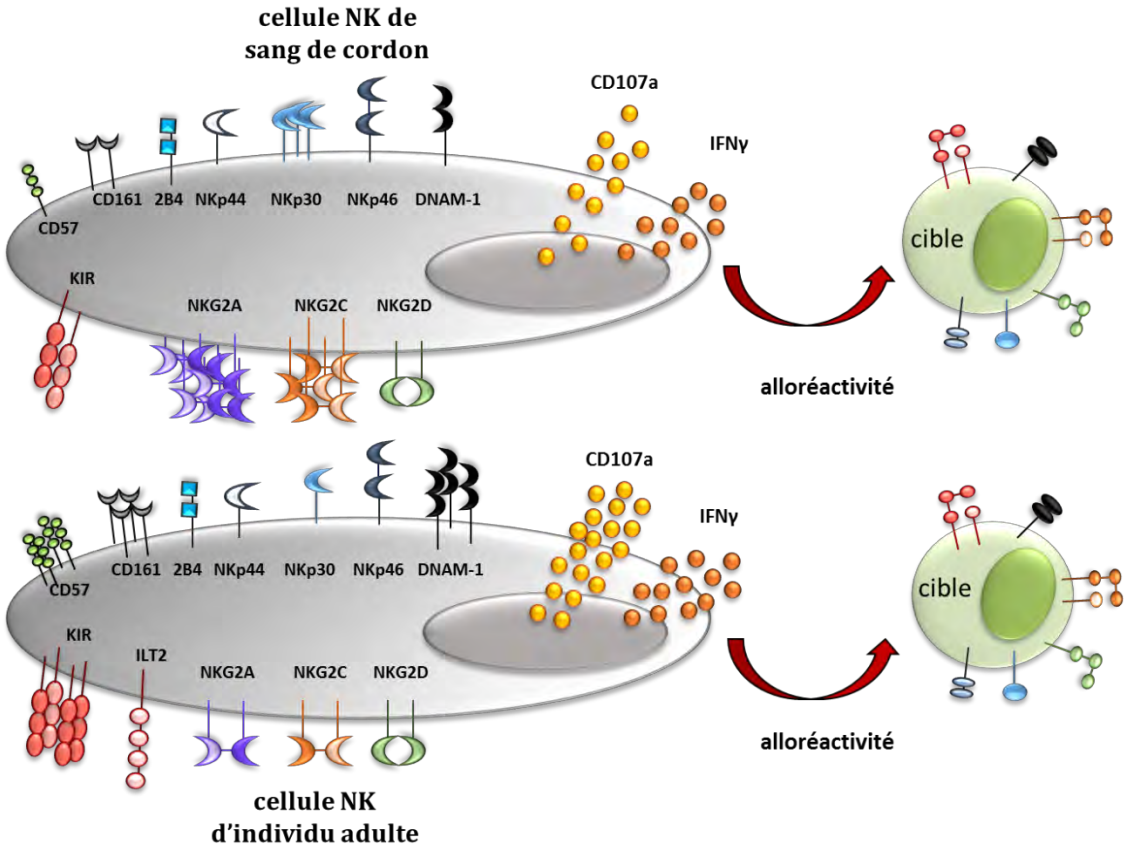

Figure 2 : Comparaison du phénotype et de la fonction des cellules NK issues de sang de cordon et d'individus adultes
Les cellules NK de SC expriment l'ensemble des récepteurs NK observés chez les cellules d'individus adultes à l'exception de l'ILT2 et présentent une expression particulièrement

élevée du récepteur NKG2A. Ces cellules sont fonctionnelles bien que leurs capacités de dégranulation et de production d'IFNγ, soient plus faibles que celles observées chez les cellules NK d'individus adultes

Bien qu'une expansion de cellules NK NKG2C$^+$ soit corrélée à l'infection à CMV chez les individus adultes (Gumá et al. 2004; Béziat et al. 2013), nous avons mis en évidence l'expression du récepteur NKG2C pour tous les sangs de cordon étudiés, ce qui écarte le lien avec une possible infection maternofétale. Nous avons étudié la fonctionnalité de ces cellules et confirmé d'une part leur capacité de prolifération *in vitro* en présence de la lignée 221-HLA-E, et d'autre part leur capacité de dégranulation vis-à-vis de cette lignée *ex vivo*, bien que plus faible par rapport à ce qui est observé chez l'adulte. Nous avons montré une absence de sous-population co-exprimant NKG2C et ILT2 contrairement à ce qui est observé chez l'adulte. Toutefois, l'acquisition rapide du récepteur ILT2 par les cellules NK NKG2C$^+$ après dUCBT a été mise en évidence (Beziat et al. 2009), confirmant la pleine fonctionnalité de ces cellules après maturation.

Dans l'ensemble, la mise en évidence de l'expression du récepteur NKG2C à la surface de cellules naïves dont le répertoire n'a pas été biaisé par l'histoire immunologique de l'individu, et en particulier les infections virales, soulève différentes questions. D'autres études sont nécessaires afin de déterminer si, comme pour les récepteurs KIR, on observe des fréquences plus faibles mais une intensité de fluorescence plus élevée du récepteur NKG2C. Il est également possible que l'expression de certains marqueurs soit plus marquée dans les premiers stades de développement des cellules NK pour ensuite être plus faiblement exprimés à des stades tardifs, comme observé chez l'adulte. Bien que les dUCBT soient caractérisées par une réactivation au virus HHV6 (Chevallier et al. 2010; Hill et al. 2012), il est envisageable que les cellules NK de SC exprimant le récepteur NKG2C soient capables de contrôler l'infection à CMV suite à une réactivation du virus, une amplification de cellules NK NKG2C$^+$ pouvant être observée après dUCBT (Beziat et al. 2009; Chiesa et al. 2014).

L'étude phénotypique des cellules NK de sang de cordon a confirmé l'expression d'un panel complet de récepteurs dont les NCR (NKp46, NKp30 et NKp44), DNAM-1 et l'expression particulièrement élevée des récepteurs NKG2A et NKG2D (Figure 22).

Les récepteurs NCR, DNAM-1 et NKG2D ont un rôle majeur dans l'effet anti-leucémique médié par les cellules NK (Pende et al. 2001; El-Sherbiny et al. 2007; Pogge von Strandmann et al. 2007; Brandt et al. 2009). L'échappement des blastes leucémiques LAM à la lyse NK a notamment été corrélé à la faible d'expression des NCR sur les cellules NK ou à l'absence d'expression de leurs ligands à la surface des lignées leucémiques (Costello et al. 2002). Dans le contexte tumoral, la diminution de l'expression de molécules HLA de classe I d'une part (Urlacher et al. 1987) et l'induction de ligands de stress tels que les ULBP ou les molécules MIC-A/B d'autre part (Kim et al. 2006; Kato et al. 2007) rend les cellules tumorales sensibles à la lyse par les cellules NK KIR^+ et les cellules NK $NKG2D^+$ dans ce cas de figure. Nous avons observé une plus faible dégranulation des cellules NK de SC vis-à-vis de la lignée 221 comparé à l'adulte, ce qui soulève la question du potentiel anti-leucémique des cellules NK de sang de cordon.

Pour répondre à cette question, notre équipe réalise actuellement une étude de l'alloréactivité des populations NK vis-à-vis d'un panel de lignées leucémiques standards (LAM, LAL, lymphome de Burkitt) et de lignées primaires (Figure 23). Cette étude est réalisée en parallèle chez des individus adultes afin de déterminer si les réponses médiées par les cellules NK de SC et d'individus adultes sont similaires. L'objectif est d'identifier les sous-populations NK les plus impliquées dans l'effet anti-leucémique. La caractérisation phénotypique des lignées leucémiques et en particulier l'évaluation de l'expression des ligands pour les récepteurs des cellules NK est nécessaire pour permettre une meilleure compréhension des populations

potentiellement impliquées dans l'effet anti-leucémique selon la nature de la lignée. Certaines leucémies semblent en effet plus résistantes à la lyse NK comme les leucémies LAL. Un fort effet GvL a en effet été observé chez des patients LAM mais pas chez les patients LAL après greffes de CSH haploidentiques (Ruggeri et al. 2002), potentiellement corrélé à une sous-expression des ligands de DNAM-1 (Pende et al. 2005). Au contraire les lignées LAM présentent une forte expression des ligands de DNAM-1 (Salih et al. 2003) et du NKG2D (Pende et al. 2005) les rendant particulièrement susceptibles à la lyse NK.

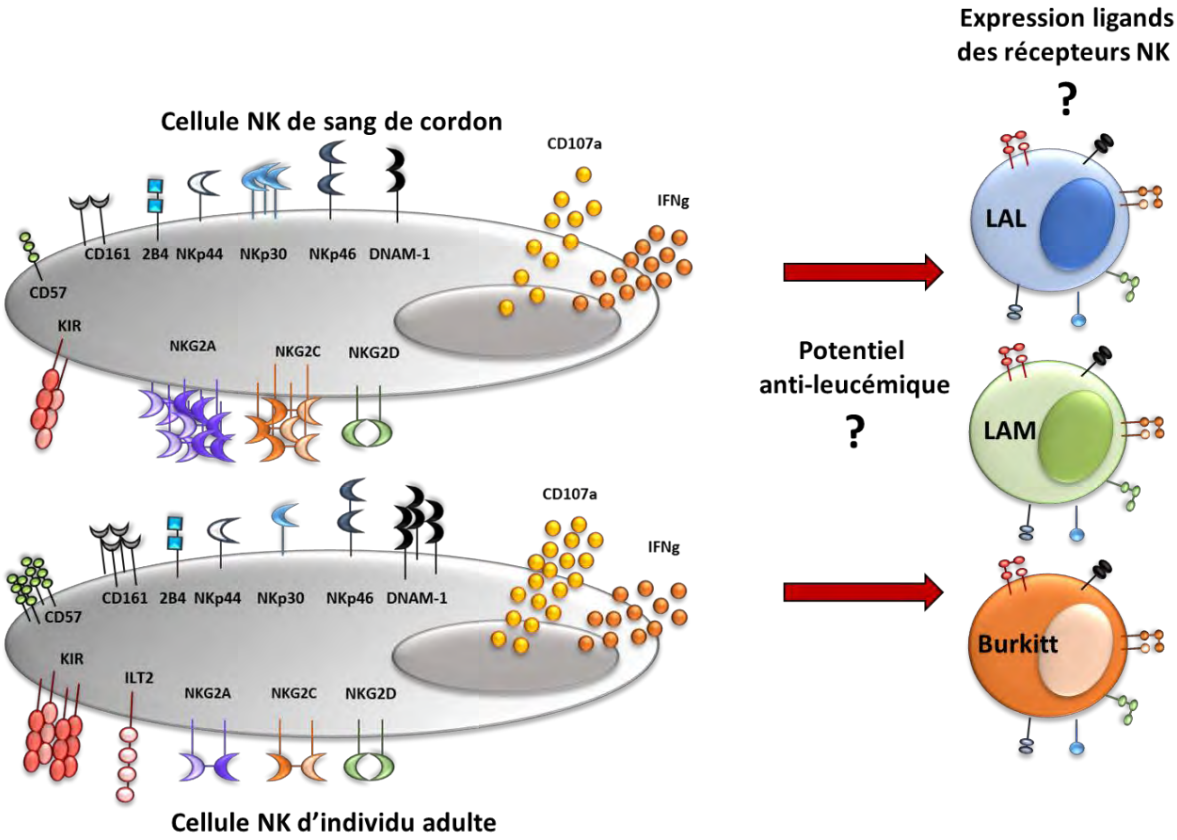

Figure 3 : Etude du potentiel anti-leucémique des cellules NK
L'étude du potentiel anti-leucémique des cellules NK issues de SC et d'individus adultes est évaluée par les capacités de dégranulation (CD107a) et de production d'IFNγ des sous-populations NK vis-à-vis d'un panel de lignées leucémiques dont les lignées LAL, LAM et les lymphomes de burkitt. L'étude phénotypique des lignées leucémiques est réalisée en parallèle afin d'évaluer l'expression des ligands des récepteurs NK.

Les résultats préliminaires de notre étude montrent un réel potentiel anti-leucémique des cellules NK de sang de cordon avec une dégranulation et une production d'IFNγ vis-à-vis des lignées leucémiques tant LAM que LAL. Nos données suggèrent non seulement un rôle des sous-populations NK KIR^+ mais également d'autres sous-populations NK en particulier les cellules NK $NKG2D^+$ et $DNAM\text{-}1^+$, dont le potentiel anti-leucémique a été mis en évidence chez l'adulte (Salih et al. 2003; Pende et al. 2005).

Bien que les greffes de sang de cordon constituent une alternative de choix en l'absence d'un donneur HLA identique, les cliniciens reconsidèrent à l'heure actuelle l'utilisation des greffes de CSH haploidentiques, grâce à la mise en place de nouvelles stratégies comme la non-déplétion des lymphocytes T associée à de fortes doses de cyclophosphamide après greffe. Ceci permet notamment de préserver les cellules effectrices contenues dans le greffon tout en réduisant l'incidence et la sévérité des réactions du greffon contre l'hôte (Bolaños-Meade et al. 2012; Martelli et al. 2014). Ainsi, bien que les cellules de sang de cordon puissent être moins utilisées à l'avenir comme sources de CSH, l'ensemble des données présentées dans ces travaux de thèse ont mis en évidence le réel potentiel clinique des cellules NK de sang de cordon et leur utilisation en immunothérapies.

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ANNEXES

The Journal of Immunology

Large Spectrum of HLA-C Recognition by Killer Ig–like Receptor (KIR)2DL2 and KIR2DL3 and Restricted C1 Specificity of KIR2DS2: Dominant Impact of KIR2DL2/KIR2DS2 on KIR2D NK Cell Repertoire Formation

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Large Spectrum of HLA-C Recognition by Killer Ig–like Receptor (KIR)2DL2 and KIR2DL3 and Restricted C1 Specificity of KIR2DS2: Dominant Impact of KIR2DL2/KIR2DS2 on KIR2D NK Cell Repertoire Formation

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The interactions of killer Ig–like receptor 2D (KIR2D) with HLA-C ligands contribute to functional NK cell education and regulate NK cell functions. Although simple alloreactive rules have been established for inhibitory KIR2DL, those governing activating KIR2DS function are still undefined, and those governing the formation of the KIR2D repertoire are still debated. In this study, we investigated the specificity of KIR2DL1/2/3 and KIR2DS1/2, dissected each KIR2D function, and assessed the impact of revisited specificities on the KIR2D NK cell repertoire formation from a large cohort of 159 KIR and HLA genotyped individuals. We report that KIR2DL2$^+$ and KIR2DL3$^+$ NK cells reacted similarly against HLA-C$^+$ target cells, irrespective of C1 or C2 allele expression. In contrast, KIR2DL1$^+$ NK cells specifically reacted against C2 alleles, suggesting a larger spectrum of HLA-C recognition by KIR2DL2 and KIR2DL3 than KIR2DL1. KIR2DS2$^+$ KIR2DL2$^-$ NK cell clones were C1-reactive irrespective of their HLA-C environment. However, when KIR2DS2 and KIR2DL2 were coexpressed, NK cell inhibition via KIR2DL2 overrode NK cell activation via KIR2DS2. In contrast, KIR2DL1 and KIR2DS2 had an additive enhancing effect on NK cell responses against C1C1 target cells. KIR2DL2/3/S2 NK cells predominated within the KIR repertoire in KIR2DL2/S2$^+$ individuals. In contrast, the KIR2DL1/S1 NK cell compartment is dominant in C2C2 KIR2DL2/S2$^-$ individuals. Moreover, our results suggest that together with KIR2DL2, activating KIR2DS1 and KIR2DS2 expression limits KIR2DL1 acquisition on NK cells. Altogether, our results suggest that the NK cell repertoire is remolded by the activating and inhibitory KIR2D and their cognate ligands. ***The Journal of Immunology*****, 2013, 191: 4778–4788.**

Natural killer cells constitute the first line of defense against viral infections and tumor cells. These effectors of innate immunity discriminate self and nonself via inhibitory receptors that recognize HLA class I molecules in an allelic fashion. The absence or default of HLA class I expression on altered cells is a well-established characteristic of virally transformed and tumor cells, and leads to enhanced NK cell proliferation, cytotoxicity, and cytokine production (1). These functional abilities confer to alloreactive NK cells a preponderant contribution to cell responses in allogeneic hematopoietic stem cell transplantation (HSCT) (2, 3). Similarly, a semiallogeneic context observed in pregnancy constitutes a new HLA environment that requires a complete modulation of self HLA molecules to avoid activation of NK cells via inhibitory receptors. Among inhibitory receptors, killer Ig–like receptors (KIRs) recognize mainly HLA-C molecules. The nonclassical HLA-E molecule presents a peptide from the signal peptide of HLA class I molecules and constitutes a second line of self-presentation. This molecule is recognized by the inhibitory heterodimer CD94/NKG2A, which is acquired before KIRs on NK cells during development (4). Finally, ILT-2 recognizes all HLA class I molecules by engaging the conserved β2-microglobulin and α3 domain of HLA class I molecules. KIRs are clonally expressed (5) on NK cells, leading to large combinations of KIR expression in different proportions of each KIR NK cell subset (6). During development, NK cells acquire a functional potential that is, in part, determined by the capacities of each NK cell to engage its inhibitory receptors with self HLA class I molecules (7). In addition, the number of KIR gene copies contributes to increased NK cell responsiveness (8). Different theories have been proposed to explain the formation of the KIR NK cell repertoire. Some groups have proposed a model following a sequential acquisition of KIR that is dependent on the HLA environment (9). In parallel, based on combinatorial analysis of KIR NK cells from haplotype AA individuals, Malmberg's group (10) has proposed that the variegated

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Abbreviations used in this article: HSCT, hematopoietic stem cell transplantation; KIR, killer Ig–like receptor.

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repertoires are generated through sequential and random acquisition of KIRs in a manner that is independent of the presence or absence of cognate HLA class I molecules. Thus, the impact of self HLA class I molecules on the frequency of all KIR NK subsets is still debated. Even though NK receptor–specific genes are not located in chromosome 6 like the HLA gene complex, the HLA environment appears nevertheless an important determinant to the formation and function of the NK cell repertoire.

The KIR gene family comprises 16 genes and is located on chromosome 19. KIR2DL1, KIR2DL2, and KIR2DL3 recognize HLA-C molecules depending on the nature of amino acid at position 80 and leads to two groups of ligands: C1 (Asn^{80}) and C2 (Lys^{80}). Thus, KIR2DL1 recognizes HLA-C molecules of C2 group, and KIR2DL2 and KIR2DL3, which are allelic genes, recognize HLA-C molecules of C1group (11). KIR2DL2 presents a weaker affinity to C2 ligand (12) with the N77K80 motif (13). Activating KIR specificity is less well documented even though KIR2DS1 recognizes HLA-C of group C2 as its inhibitory counterpart (14, 15). KIR2DS3 is not expressed at the cell surface (16), and its function is questionable especially because the gene is missing from some populations such as the Yucpa group studied by Parham's group (17). If the alloreactive rules of C2 specific $KIR2DS1^+$ NK cells are well understood, those involving KIR2DS2 are less documented. This is largely explained by the absence of Abs distinguishing the activating KIR2DS2 and inhibitory KIR2DL2 forms. Studies to date have mainly relied on the use of Abs specific for KIR2DL2/3 and KIR2DS2 as the GL183, which does not allow differentiation between inhibitory and activating forms. This may induce a possible bias in the interpretation of results involving two functional receptors with opposite signals but sharing potentially the same ligands, most likely because of the high homology of the extracellular portion of KIR2DL2 and KIR2DS2. Thus, we dissected the main inhibitory KIR2DL1/2/3 and activating KIR2DS1/2 reactivity from primary NK cells from HLA and KIR genotyped individuals. Our experimental approach has been validated in studying alloreactive KIR2DL1 and KIR2DS1 NK cell subsets against a wide range of target cell lines expressing different HLA-C ligands from C1 and C2 groups. Taking into account all revisited specificities of the main inhibitory KIR2DL1/2/3, activating KIR2DS1/2 and the alloreactive rules, we analyzed phenotypically the KIR NK cell repertoire from a large cohort of 159 KIR and HLA genotyped individuals to evaluate the influence of KIR ligand expression on NK cell repertoire formation.

Materials and Methods

Cells (PBMCs and cell lines)

PBMCs were isolated as previously described (15, 18). All blood donors were recruited at the Blood Transfusion Center (Etablissement Français du Sang, Nantes, France), and informed consent was given by all donors. HLA class I–deficient 721.221 lymphoblastoid cells, referred to as 221 cells, were used as positive control to assess NK cell degranulation. HLA-C*03:04 (C1), HLA-C*08:02 (C1), HLA-C*04:01 (C2), HLA-C*02:02 (C2), HLA-C*06:02 (C2), HLA-C*07:01 (C1), and HLA-C*15:01 (C2) transfected 221 cells were used to evaluate NK cell degranulation of different KIR2D NK cell subsets. HLA-C*02:02, HLA-C*03:04, HLA-C*04:03, and HLA-C*07:01 transfected 221 cells were provided by Prof. P. Parham. HLA-C*08:02, HLA-C*02:02, HLA-C*06:02, and HLA-C*15:01 transfected 221 cells were obtained by stable transfection of 221 cell line by electroporation (BioRad) using, respectively, pcINeo–HLA-C*08:02 (provided by Dr. Agnès Moreau, INSERM Unite Mixte de Recherche 892, Nantes, France), pcINeo–HLA-C*06:02 (provided by Dr. Agnès Moreau, INSERM Unite Mixte de Recherche 892), and HLA-C*15:01 (provided by Dr. Frédérique Triebel, Immutep S.A., Chatenay-Malabry, France). All HLA-C cDNA encoded sequences were verified by DNA sequencing as described: HLA-C polymorphic exons were amplified using the HLA-C.exon 1.68G (5′-GGCCCTGACCGAGACCTG-3′) forward primer and the HLA-C.exon 4.661C (5′-CCTCAGGGTGGCCTCATG-3′) reverse primer. Amplification of exons 2 and 3 of the HLA-C locus was performed in 50 μl using 80 ng DNA, 0.2 mM dNTP (Invitrogen), 1 U Taq Gold polymerase (Applied Biosystems), 1× Taq Gold buffer (Applied), 2.5 mM $MgCl_2$ Gold (Applied), and 1 μM of each primer. Amplification presequencing was performed in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystem). The PCR amplification program included one denaturation step of 95°C for 10 min, 36 cycles of 96°C for 20 s, 60°C for 30 s, and 72°C for 3 min, followed by a final elongation step of 72°C for 10 min. Products were electrophoresed on 2% agarose gels. HLA-C cDNA sequencing was performed by the Sequencing Core Facility (SFR François Bonamy, Nantes, France) using an ABI 3730 automatic system (Applied). HLA-C cDNA sequence data files were analyzed using Assign software (Conexio Genomics, Applecross, Australia) with IPD-HLA database version 3.9.0 (July 2012). The 221 cell line and HLA-C–transfected 221 cell lines were cultured in RPMI 1640 medium (Life Technologies, Paisley, U.K.) containing glutamine (Life Technologies) and penicillin-streptomycin (Life Technologies), and supplemented with 10% FBS (Life Technologies). *Mycoplasma* tests performed by PCR were negative for all cell lines.

HLA and KIR genotyping

HLA class I allele assignment and KIR gene content typing were performed as previously described (15, 18). KIR haplotypes were determined as previously described (6). The A haplotypes correspond to seven KIR genes, including KIR2DS4 as the only activating KIR gene. In contrast, B haplotypes are characterized by the presence of more than one activating KIR gene and the absence of the KIR2DS4 gene.

KIR NK cell line and clone isolation and amplification

PBMCs were stained with anti-CD3 mAb (X35, a gift from Dr. Franck Vérité, Etablissement Français du Sang, Rennes), and $CD3^+$ cells were depleted using murine IgG coupled magnetic Dynabeads according to the manufacturer's instructions (Dynal, Oslo, Norway). Then cells were labeled with appropriate mAb combinations, and the cells were sorted using a FACSCalibur equipped with a fluidic sort module (BD Biosciences). Sorted cells were amplified after in vitro stimulation with irradiated allogeneic PBMCs and HLA-C*03:04 (C1) and HLA-C*04:01 (C2) transfected 221 cell lines used as feeders following the PBMC/EBV-B cells ratio at 10:1. $KIR2DS2^+$ NK cells were cloned at 0.3 cell/well from the selected and amplified lines under limiting conditions in 96-well U-bottom microtiter plates with 50,000 irradiated autologous lymphocytes and 5000 irradiated HLA-C*03:04 (C1) and HLA-C*04:01 (C2) transfected 221 cell lines in a final volume of 100 μl. Growing colonies with a probability of monoclonality of 95% were kept for further analysis. NK cell lines and clones were cultured in RPMI 1640 medium (Life Technologies, Paisley, U.K.) containing glutamine (Life Technologies) and penicillin-streptomycin (Life Technologies), and supplemented with 10% human serum (Etablissement Français du Sang, Nantes, France) and 200 U/ml IL-2 (Chiron, Suresnes, France). Cells were maintained for 3 wk and expanded in their culture medium containing IL-2 without restimulation with feeders before functional analysis to decrease their spontaneous degranulation.

KIR2DL2 RT-PCR

Total RNA was purified from isolated $KIR2DS2^+$ NK clones using NucleoSpin RNAXS (Macherey-Nagel). Qualitative KIR2DL2 RT-PCR was performed using a One-Step PrimeScript RT-PCR kit (TaKaRa, Japan) with KIR2DL2-specific primers from Thompson et al. (19).

Phenotypic analysis by flow cytometry

The NK cell–surface phenotype was determined by three- or four-color flow cytometry using the following mouse anti-human mAbs: anti–KIR2DL1-FITC (143211; R&D Systems), anti–KIR2DL1/2DS1-PE (EB6), anti–KIR2DL2/2DS2-PE (GL183; Beckman Coulter Immunotech, Marseille, France), anti–CD3-PerCP (SK7), anti–CD56-allophycocyanin (B159; BD Biosciences), anti–KIR2DL1/2/3/2DS2-FITC (8C11), anti-KIR2DL3/2DS2 (1F12), and anti–KIR2DL1/2/3/2DS1/2-FITC (1A6), generated and characterized in our laboratory and previously described (20). The $KIR2DS2^+$ NK cell clone phenotype was determined using the following mouse anti-human mAbs: anti-KIR2DS4 (FES172; Beckman Coulter), anti-KIR3DL1/3DS1 (Z27; Beckman Coulter), anti-NKp30 (Z25; Beckman Coulter) anti-NKp44 (Z231; Beckman Coulter), anti-NKG2D (ID11; BD Biosciences), anti-2B4 (2-69; BD Biosciences), anti-ILT2 (GHI/75; BD Biosciences), anti-NKG2A (Z199; Beckman Coulter), and anti-CD16 (NKP15; BD Biosciences).

CD107a mobilization assay detected by flow cytometry

NK cells were preincubated with anti-CD107a (H4A3; BD Biosciences). NK cell degranulation was assessed after incubation for 5 h alone (negative

control), or with different target cells (E:T ratio = 10:1) with brefeldin A (Sigma) at 10 μg/ml for the last 4 h. Cell-surface staining was performed using the anti-human KIR mAbs mentioned in the previous section and NKG2A-FITC or -PE (Z199; Beckman Coulter). All flow cytometry data were collected using a FACSCalibur (BD Biosciences) and analyzed using FlowJo 7.6.1 software (TreeStar).

Statistical analyses

Comparisons of NK cell frequencies between two different series of individuals were performed using ANOVA test. The p values < 0.05 were considered to be statistically significant. Top and bottom whiskers represent values of the top and bottom 25% of cases, respectively; boxed area represents interquartile range.

Results

$C1^-$ activated $KIR2DL2^+$ and $KIR2DL3^+$ NK cells present a similar degranulation potential to their $C1^+$ counterparts

As a first step, we studied degranulation of KIR2DL NK subpopulations expressing either KIR2DL1, KIR2DL2, or KIR2DL3 using appropriate combinations of KIR-specific Abs targeting each of these receptors as previously described (20) (Fig. 1A). Thus, $KIR2DL1^+$ $KIR2DL2/3/S1/2^-$ NK cells were sorted using a combination of KIR2DL1/2/3/S2-specific 8C11 and KIR2DL2/3/S2-specific GL183 Abs from $KIR2DS1^-$ genotyped individuals (Fig. 1A). $KIR2DL2^+$ $KIR2DL1/3/S1/2^-$ NK cells were sorted using a combination of a mix of KIR2DL3/S2-specific 1F12 with KIR2DL1-specific 143211 Abs and KIR2DL2/3/S2-specific GL183 from $KIR2DS1^-$ genotyped individuals (Fig. 1A). $KIR2DL3^+$ $KIR2DL1/2/S1/2^-$ NK cells were sorted using a combination of KIR2DL2/3/S2-specific GL183 and KIR2DL1/S1-specific EB6 Abs from $KIR2DL2/S2^-$ genotyped individuals (Fig. 1A). All sorted KIR2DL NK cells were expanded after in vitro stimulation. NK cells expressing inhibitory NKG2A receptors were systematically excluded from functional studies. KIR2DL1 recognized HLA-C*04:01 (i.e., a "C2 allele"), but not HLA-C*03:04 (i.e., a "C1 allele"; Fig. 1B). However, $KIR2DL2^+$ (Fig. 1C) and $KIR2DL3^+$ NK cells (Fig. 1D) similarly recognized both HLA-C*03:04 (C1) and HLA-C*04:01 (C2) molecules as shown by the CD107a upregulation assay. The same profile has been obtained for IFN-γ production with a lower frequency of positive cells (data not shown). Moreover, $KIR2DL2^+$ and $KIR2DL3^+$ NK cells from $C1^+$ individuals showed a similar degranulation potential to NK cells from $C1^-$ individuals, as assessed against the HLA class I–deficient 221 cell line (Fig. 1C, 1D). This result was also observed for $KIR2DL2^+$ and $KIR2DL3^+$ NK cells amplified with 221 cells as feeders, eliminating a possible education of these NK cells by HLA-C molecules expressed by feeders (data not shown). In contrast, $KIR2DL1^+$ NK cells showed efficient degranulation against the 221 cell line only in $C2^+$ individuals (Fig. 1B). Although variable from one individual to another, the frequency of $CD107a^+$ $KIR2DL2^+$ or $CD107a^+$ $KIR2DL3^+$ NK cells did not correlate with the presence or absence of particular C1 or C2 alleles. Altogether, these results suggest that the stringent C2 specificity of KIR2DL1 confers an NK education only in $C2^+$ individuals; in contrast, some HLA class I molecules previously identified as C2 ligands could be recognized by KIR2DL2 and KIR2DL3 receptors and could mediate, at least to some extent, education of KIR2DL2/3 NK cells. Thus, a preactivation of these KIR2DL NK cell subsets, notably by cytokine stimulation, seems sufficient to decrease their activation threshold.

Broad HLA-C specificity of $KIR2DL2^+$ and $KIR2DL3^+$ NK cells but restricted C2 specificity of $KIR2DL1^+$ NK cells

To investigate the range of C2 specificity of KIR2DL2 and KIR2DL3, we evaluated the ability of KIR2DL NK cell subsets to recognize different C1 and C2 ligands, when compared with control $KIR2DL1^+$ cells (Fig. 1E). All HLA-C transfected 221 cell lines similarly expressed HLA class I molecules except 221-C*15:02, which is less recognized by KIR2DL2 and KIR2DL3 NK cells (Fig. 1F). The C2 specificity of KIR2DL1 was confirmed toward four different HLA ligands: HLA-C*04:01, C*02:02, C*06:02, and C*15:01 with a hierarchy of recognition. Indeed, HLA-C*04:01 inhibited more efficiently KIR2DL1 NK cell degranulation than HLA-C*02:02 and HLA-C*15:01. HLA-C*06:02 was less but variably recognized by KIR2DL1 NK cells. Although the C1 allele HLA-C*03:04 was not recognized by KIR2DL1, another C1 allele HLA-C*08:02 (C1) partially inhibited KIR2DL1 NK cell degranulation. The same hierarchy of C2 allele-induced inhibition was also observed with KIR2DL2 and KIR2DL3 NK cells. Indeed, these cells, irrespective of their C1 molecule, were strongly inhibited by HLA-C*04:01, inhibited at an intermediate level by HLA-C*15:02, and less inhibited by HLA-C*02:02 and C*06:02. In contrast with KIR2DL1 cells, HLA-C*08:02 (C1) was less recognized than HLA-C*03:04 (C1) by $KIR2DL2^+$ and $KIR2DL3^+$ NK cell subsets. These results suggest broad HLA-C specificity of KIR2DL2 and KIR2DL3 cells, and a hierarchy of recognized HLA-C ligands within each C1 and C2 group.

Stringent C2 alloreactivity of $KIR2DS1^+$ NK cells

In parallel with KIR2DL, we investigated C2 specificity of KIR2DS1 NK cells to validate our experimental approach before investigating HLA-C specificity of KIR2DS2 in this setting. We previously showed that different functional capabilities (degranulation, IFN-γ secretion, and proliferation) of $KIR2DS1^+$ NK cell lines were triggered by 221-HLA-C*04:01$^+$ (C2) cells, only among $C2^-$ individuals (15). In this study, we confirmed degranulation of sorted and in vitro expanded $KIR2DS1^+$ $KIR2DL2/3/S2^-$ $NKG2A^-$ NK cells (Fig. 2A) by a broad panel of HLA-C1 transfected 221 cells (Fig. 2B), as illustrated for one representative individual (Fig. 2C). Interestingly, HLA-C*02:02 induced stronger CD107a expression than HLA-C*04:01 and HLA-C*06:02 by $KIR2DS1^+$ NK cells. We cloned $KIR2DS1^+$ $KIR2D^-$ NK cells and confirmed the absence of $KIR2DS1^+$ NK cell alloreactivity against $C2^+$ target cells in $C2^+$ individuals (Fig. 2D). In contrast, $C2^-$ $KIR2DS1^+$ NK cell clones showed strong alloresponsiveness against HLA-C*04:01- and HLA-C*02:02- (C2) target cells (Fig. 2D). The C2 specificity of KIR2DS1 was confirmed for four clones isolated from three $C2^-$ individuals. Our results suggest that KIR2DS1 shows stringent C2 specificity, like its inhibitory counterpart, KIR2DL1.

C1 specificity of KIR2DS2

To address the specificity of KIR2DS2 NK cells and reassess the rules governing activation of $KIR2DS2^+$ NK cells, we used $KIR2DS2^+$ $KIR2DL1/3/S1^-$ NK cell lines from $KIR2DL3^-$ genotyped individuals, obtained after sorting with a combination of the KIR2DL3/S2-specific 1F12 and KIR2DL1/S1-specific EB6 Abs (20), and subsequent in vitro expansion (Fig. 3A). Because the $KIR2DS2^+$ NK cell population can coexpress the inhibitory KIR2DL2, we cloned $KIR2DS2^+$ $KIR2DL1/S1^-$ NK cells and discriminated $KIR2DS2^+$ clones from $KIR2DS2^-$ ones by KIR2DL2 RT-PCR analysis (Fig. 3B). Fig. 3C illustrates the degranulation of two $KIR2DS2^+$ ($KIR2DL2^-$ and $KIR2DL2^+$) NK cell clones isolated from the same individual against 221, 221-C1 (HLA-C*03:04), and 221-C2 (HLA-C*04:01) cell lines. In accordance with their KIR2DL2 RNA profile, the degranulation of $KIR2DS2^+$ $KIR2DL2^-$ NK cell clones was strongly activated by the 221-C1 (HLA-C*03:04) cell line, but not by 221 or 221-C2 (HLA-C*04:01) cell lines. In contrast, degranulation of $KIR2DS2^+$ $KIR2DL2^+$ NK cell clones was strongly inhibited by both 221-C1 (HLA-C*03:04) and 221-C2 (HLA-C*04:01) cell lines. This observation confirms not only the broader specificity of KIR2DL2 NK cells (Fig. 1C), but also the

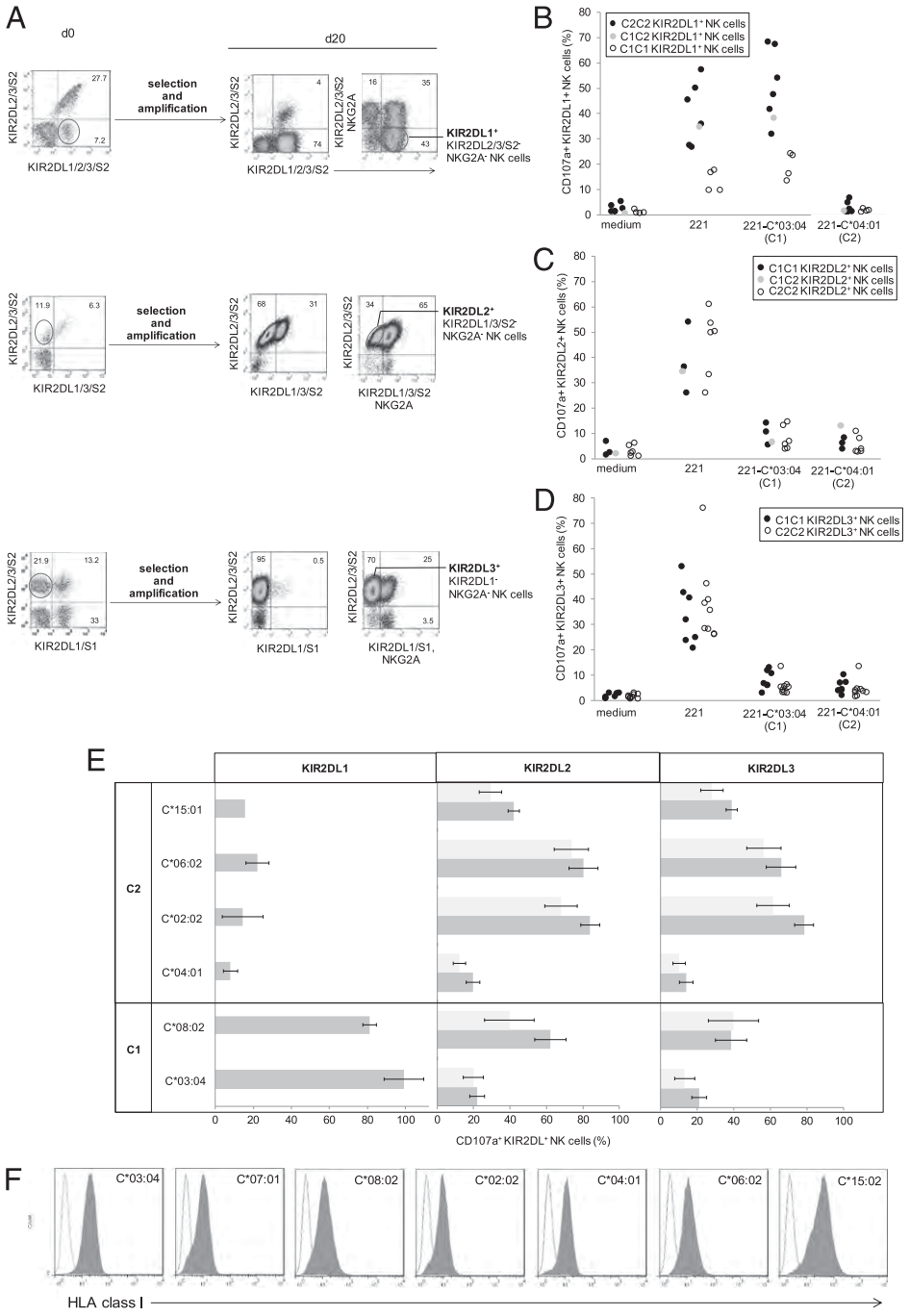

FIGURE 1. Comparable degranulation potential of $C1^-$ and $C1^+$ activated $KIR2DL2^+$ and $KIR2DL3^+$ NK cells because of a large HLA-C specificity. (**A**) After depletion of $CD3^+$ cells from PBMCs, $KIR2DL1^+$ $KIR2DL2/3/S1/2^-$ NK cells ($NKp46^+$ $8C11^+$ $GL183^-$) were cell sorted (7.2% of the population; day 0 [d0]) and amplified in vitro with irradiated allogeneic PBMCs and EBV-B cells as feeders. After 3 wk of stimulation (d20), the phenotype of these sorted and stimulated NK cells was assessed by flow cytometry and is illustrated for 1 representative $KIR2DL1^+$ genotyped individual out of 11 studied individuals. Seventy-four percent of these amplified cells are $KIR2DL1^+$ $KIR2DL2/3/S1/2^-$ NK cells ($NKp46^+$ $8C11^+$ $GL183^-$) and 43% are $NKG2A^-$. Following the same protocol, $KIR2DL2^+$ $KIR2DL1/3/S1/2^-$ NK cells ($NKp46^+$ $1F12^-$ 143211^-GL183^+) were cell sorted (11.9% of the population; d0) and amplified in vitro. After 3 wk of stimulation, the phenotype of these sorted and stimulated cells was assessed by flow cytometry and is illustrated for 1 representative $KIR2DL2^+$ $2DL3^-$ genotyped individual out of 10 studied individuals. Sixty-eight percent of these amplified cells are $KIR2DL2^+$ KIR2DL1/3/S1/2^- NK cells ($NKp46^+$ $1F12^-$ 143211^- $GL183^+$) and 34% are $NKG2A^-$. Finally, $KIR2DL3^+$ $KIR2DL1/2/S1/2^-$ NK cells ($NKp46^+$ $GL183^+$ $EB6^-$) were cell sorted (21.9% of the population; d0) and amplified in vitro. The phenotype of these sorted and stimulated cells is illustrated for 1 representative $KIR2DL3^+$ $KIR2DL2^-$ genotyped individual out of 15 studied individuals. Ninety-five percent of these amplified cells are $KIR2DL3^+$ $KIR2DL1/2/S1/2^-$ NK cells ($NKp46^+$ $GL183^+$ $EB6^-$) and 70% are $NKG2A^-$. Scatter plots displaying the $CD107a^+$ $KIR2DL^+$ NK cell frequency determined after 5-h degranulation assay at an E:T ratio of 1:1 in different stimulation conditions: medium, 221 cells, HLA-C*03:04 (C1) and HLA-C*04:01 (C2) transfected 221 cells (**B**) for selected and amplified $KIR2DL1^+$ $KIR2DL2/3/S1/2^-$ $NKG2A^-$ NK cells from $C2^+$ ($n = 7$) and $C2^-$ ($n = 4$) individuals; (**C**) for selected and amplified $KIR2DL2^+$ $KIR2DL1/3/S1/2^-$ $NKG2A^-$ NK cells from $C1^+$ ($n = 4$) and $C1^-$ ($n = 6$) individuals; and (**D**) for selected and amplified $KIR2DL3^+$ $KIR2DL1/2/S1/2^-$ $NKG2A^-$ NK cells from $C1^+$ ($n = 7$) and $C1^-$ ($n = 8$) individuals. (**E**) Bars represent the mean of $CD107a^+$ $KIR2DL^+$ NK cell percentages (± SD) evaluated with a larger panel of C1 (HLA-C*03:01 and C*08:02) and C2 (HLA-C*04:01, C*02:02, C*06:02, and C*15:01) transfected 221 cells from previously studied individuals in (B)–(D). Educated NK cells ($C1^+$ $KIR2DL2^+$, $C1^+$ $KIR2DL3^+$, and *(Figure legend continues)*

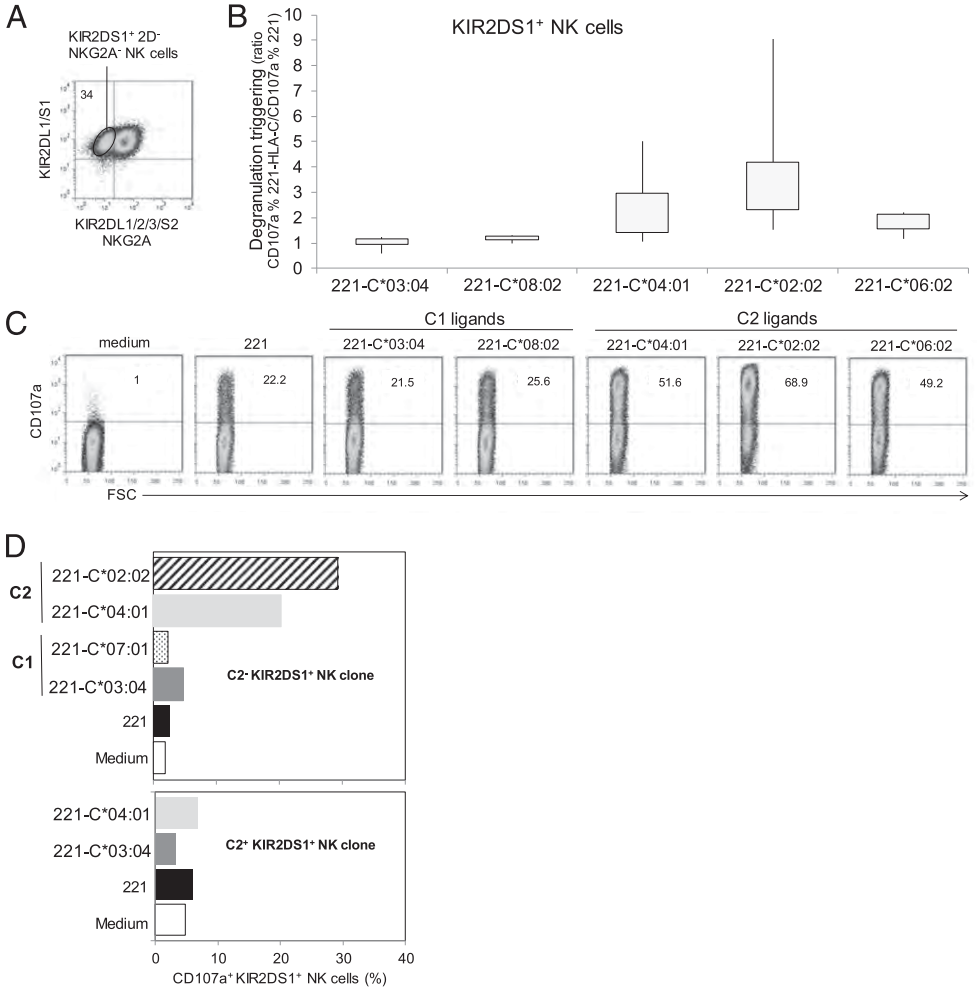

FIGURE 2. Stringent C2 alloreactivity of KIR2DS1$^+$ NK cells. (**A**) After depletion of CD3$^+$ cells from PBMCs, KIR2DS1$^+$ KIR2DL1/2/3/S2$^-$ NK cells (NKp46$^+$ EB6$^+$ 8C11$^-$) were cell sorted and amplified in vitro with irradiated allogeneic PBMCs and EBV-B cells as feeders. After 3 wk of stimulation, the phenotype of these sorted and stimulated cells was assessed by flow cytometry and is illustrated for one representative KIR2DS1$^+$ genotyped individual of eight studied individuals. Thirty-four percent of these amplified cells are KIR2DS1$^+$ KIR2DL1/2/3/S2$^-$ NKG2A$^-$ NK cells (NKp46$^+$ EB6$^+$ 8C11$^-$ NKG2A$^-$). (**B**) Summary box and whisker plot summarizing the percentages of CD107a$^+$ KIR2DS1$^+$ NK cells after 5-h incubation alone or in the presence of 221 cells as positive control, at an E:T ratio of 1:1, for eight experiments performed. To homogenize the data obtained from independent experiments, the data are presented as the ratio of the degranulation obtained with HLA-C transfected 221 cells to the degranulation obtained with untransfected 221 cells. (**C**) Representative density plots of KIR2DS1$^+$ KIR2DL1/2/3/S2$^-$ NKG2A$^-$ (NKp46$^+$ EB6$^+$ 8C11$^-$ NKG2A$^-$) NK cell degranulation observed in the different culture conditions. (**D**) Bars indicate the percentage of CD107a$^+$ KIR2DS1$^+$ NK cells from one C2$^-$ representative KIR2DS1$^+$ NK cell clone out of four clones isolated from three different C2$^-$ individuals and one C2$^+$ KIR2DS1$^+$ NK cell clone in a degranulation assay following the E:T ratio of 1:1 with different HLA-C transfected 221 target cells.

dominant effect of NKR-induced inhibitory over activating signals. To determine the impact of the HLA-C environment on C1 reactivity of KIR2DS2$^+$ NK cells, we evaluated the degranulation of 27 KIR2DS2$^+$ KIR2DL2$^-$ NK cell clones (Fig. 4A) and 14 KIR2DS2$^+$ KIR2DL2$^+$ NK cell clones (Fig. 4B) derived from 10 individuals (2 C1C1, 4 C1C2, and 4 C2C2) against 221, 221-C1 (HLA-C*03:04), and 221-C2 cells (HLA-C*04:01). Of note, we could not generate KIR2DS2$^+$ KIR2DL2$^+$ NK clones from C1C1 individuals. NK cell degranulation toward the 221 cell line varied from one KIR2DS2$^+$ NK cell clone to another. Because this variability could be linked to expression of other inhibitory or activating NKRs, we assessed expression of unengaged inhibitory receptors, like KIR3DL1 and ILT2, and activating receptors (e.g., KIR2DS4, NKp30, NKp44, NKG2D, 2B4, and CD16). For some clones, KIR3DL1 and NCR expression could explain a high reactivity toward 221 target cells of some clones, such as the 1F9 clone from D9 individual. However, this heterogeneity was most probably linked to previous in vitro stimulation of NK cell clones. A CD107a mobilization assay showed increased degranulation of KIR2DS2$^+$ NK cell clones with the 221-C1 (HLA-C*03:04) cell line in contrast with 221 and 221-C2 (HLA-C*04:01) counterparts, irrespective of the HLA-C background of individuals, even though few KIR2DS2$^+$ KIR2DL1/2/2DS1$^-$ clones were obtained from C1C1 individuals. Overall, the degranulation patterns of KIR2DS2$^+$ KIR2DL2$^-$ clones were similar in C1$^+$ (n = 11) versus C1$^-$ individuals (n = 8), because these clones were stimulated by the 221-C1 (HLA-C*03:04) cell line

C2$^+$ KIR2DL1$^+$) are indicated in light gray and uneducated NK cells (C1$^-$ KIR2DL2$^+$, C1$^-$ KIR2DL3$^+$, and C2$^-$ KIR2DL1$^+$) in dark gray. To homogenize the data obtained from independent experiments, we present the data as the degranulation obtained with HLA-C transfected 221 cells relative to the degranulation obtained with untransfected 221 cells at an E:T ratio of 1:1. (**F**) Histograms represent HLA class I expression of different HLA-C transfected 221 cell lines (filled) and 221 cell line as negative control (open).

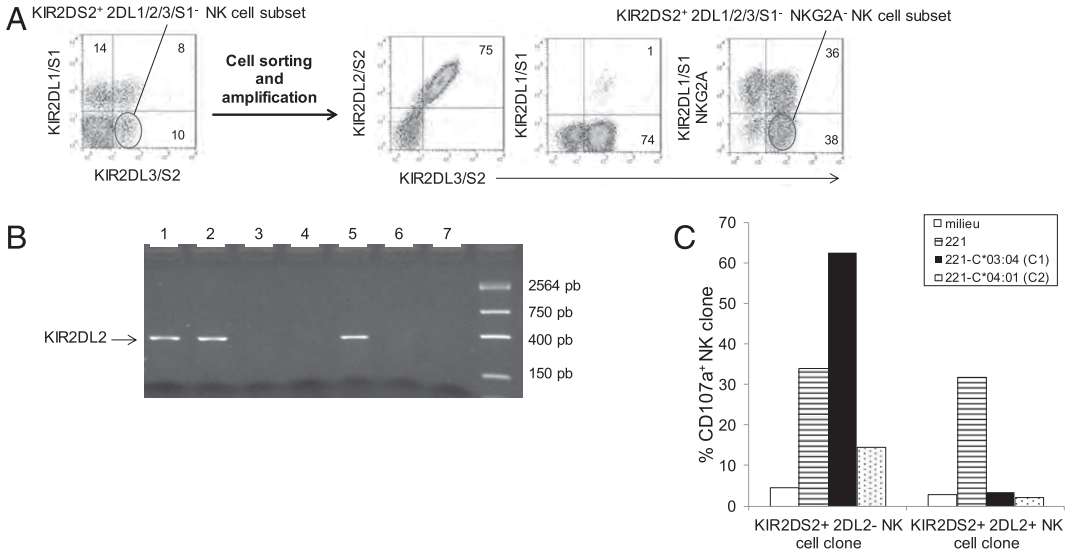

FIGURE 3. C1 specificity of KIR2DS2. (**A**) After depletion of $CD3^+$ cells from PBMCs, $KIR2DS2^+$ $KIR2DL1/3/2DS1^-$ NK cells ($NKp46^+$ $EB6^-$ $1F12^+$) were cell sorted (10% of the population; d0) from $KIR2DL3^-$ genotyped individuals and amplified in vitro with irradiated allogeneic PBMCs and EBV-B cells as feeders. After 3 wk of stimulation, the phenotype of these sorted and stimulated cells was assessed by flow cytometry and is illustrated for 1 representative $KIR2DS2^+$ genotyped individual of 10 studied individuals. Seventy-four percent of these amplified cells are $KIR2DS2^+$ $KIR2DL1/3/2DS1^-$ NK cells ($NKp46^+$ $EB6^-$ $1F12^+$) and 38% are $NKG2A^-$. (**B**) Visualization of specific KIR2DL2 RT-PCR products on a 2% agarose gel in 0.5× Tris Borate EDTA Buffer with Sequence Specific Primer Size Marker (One Lambda). RNA was extracted from $KIR2DS2^+$ $KIR2DL1/3/S1^-$ NK cell clones of interest and specific RT-PCR was then performed using KIR2DL2-specific PCR-SSP primers showing a specific RT-PCR product at 383 bp for some $KIR2DS2^+$ clones. (**C**) Histograms illustrating the degranulation of one $KIR2DS2^+$ $KIR2DL2^-$ NK cell clone and one representative $KIR2DS2^+$ $KIR2DL2^+$ NK cell clone in different culture conditions: medium, 221, HLA-C*03:04 (C1), and HLA-C*04:01 (C2) transfected 221 target cells at an ET ratio of 1:1.

(Fig. 4C), but not by 221 or 221-C2 (HLA-C*04:01) counterparts. These results suggest that, in contrast with KIR2DS1 NK cells, which are C2 alloreactive only from the $C2^-$ environment, KIR2DS2 NK cells are C1-reactive irrespective of their HLA-C haplotype. In parallel, degranulation of $KIR2DS2^+$ $KIR2DL2^+$ clones is inhibited by both 221-C1 (HLA-C*03:04) and 221-C2 (HLA-C*04:01), in line with results obtained with $KIR2DL2^+$ cell lines (Fig. 1), regardless of the HLA-C background and NK cell phenotype (Fig. 4B). The inhibitory C1-KIR2DL2 signaling bypassed the activating C1-KIR2DS2 signaling, highlighting the preponderant impact of KIR2DL2 on NK cell inhibition.

Additive effect of KIR2DL1 and KIR2DS2 against $C1^+$ target cells

Taking into account that activation of KIR2DS2 in the presence of a C1 ligand should have an additive effect on the activation mediated by the lack of engagement of KIR2DL1, we then evaluated the impact of the coexpression of KIR2DS2 and KIR2DL1 on recognition of $C1C1^+$ target cells. To this end, we sorted $KIR2DL1^+$ $KIR2DS2^+$ NK cells from KIR2DS1 and KIR2DL3 negative genotyped $C2^+$ individuals using anti-KIR2DL3/2DS2 (1F12)– and anti-KIR2DL1/S1 (EB6)–specific Abs (Fig. 5A). The sorted $KIR2DL1^+$ $KIR2DS2^+$ cells were cloned and phenotyped by KIR2DL2 RT-PCR as previously described (Fig. 3B), to discriminate $KIR2DL2^+$ NK cell clones. We observed a functionally additive effect of KIR2DL1 and KIR2DS2 on the recognition of the 221-HLA-C*03:04 (C1) cell line (Fig. 5B, 5C). C2 ligands, like HLA-C*02:02 and HLA-C*04:01, engaged KIR2DL1 receptors and inhibited $KIR2DS2^+$ $KIR2DL1^+$ NK cell degranulation.

The nature of KIR2D and HLA-C ligand affects the composition of the NK cell repertoire

To determine the functional impact of HLA-C–specific KIR2DL and KIR2DS receptor expression, we evaluated the phenotypic distribution of each KIR2D NK cell subset: $KIR2DL1/S1^+$ $KIR2DL2/3/S2^-$, $KIR2DL1/S1^-$ $KIR2DL2/3/S2^+$, and $KIR2DL1/S1^+$ $KIR2DL2/3/S2^+$ NK cell subsets within KIR2DL1/2/3/S1/2 NK cells, in a cohort of 159 KIR and HLA genotyped individuals (Supplemental Table I). Because almost all studied individuals were $KIR2DL1^+$ genotyped (96%), this analysis was performed according to KIR2DL2, KIR2DL3, and KIR2DS2 genotype and HLA-C environment (Fig. 6A). In this cohort, all $KIR2DL2^+$ individuals were $KIR2DS2^+$ genotyped; thus, we assigned the individuals in one of three groups determined by the presence of KIR2DL3, KIR2DL2/3/S2, and KIR2DL2/S2 genes. All studied populations were similarly represented, regardless of the HLA-C (C1C1, C1C2, or C2C2) environment (data not shown). However, $KIR2DL1/S1^+$ $KIR2DL2/3/S2^-$ NK cell frequency was significantly decreased in $KIR2DL2/S2^+$ individuals with a $C2^+$ haplotype (Fig. 6A, Supplemental Table II). The double-stained population frequency was not significantly different in the studied groups. The $KIR2DL2/3/S2^+$ $KIR2DL1/S1^-$ NK cell frequency was significantly higher in $KIR2DL2/S2^+$ compared with $KIR2DL3^+$ individuals (Fig. 6A, Supplemental Table II) with all HLA-C molecules, and in $KIR2DL2/3/S2^+$ compared with $KIR2DL3^+$ individuals with C1C1 and C2C2 molecules. Finally, we analyzed the distribution of each KIR2D NK cell subset, as defined by their mean frequencies in the pool of KIR2DL1/2/3/S1/2 NK cells (Fig. 6B), according to KIR2DL2, KIR2DL3, and KIR2DS2 genotypes and expression of either C1 or C2 molecules. The $KIR2DL1/S1^+$ NK cell subset predominated in $KIR2DL3^+$ individuals, and its mean frequency increased with the number of C2 alleles (mean frequencies are 36.1, 38.4, and 47.2%, respectively, in C1C1, C1C2, and C2C2 environments). In contrast, the presence of KIR2DL2 and KIR2DS2 correlated with increased mean frequencies of $KIR2DL2/3/S2^+$ NK cells, particularly in $KIR2DL2/S2^+$ individuals. Although KIR2DL2 and KIR2DL3 recognized a broad spectrum of HLA-C ligands, the higher affinity of KIR2DL2 than KIR2DL3 toward HLA-C ligands could account for decreased frequency of the $KIR2DL1^+$ NK cell pool in the former individuals. The six individuals with a $KIR2DL1^-$

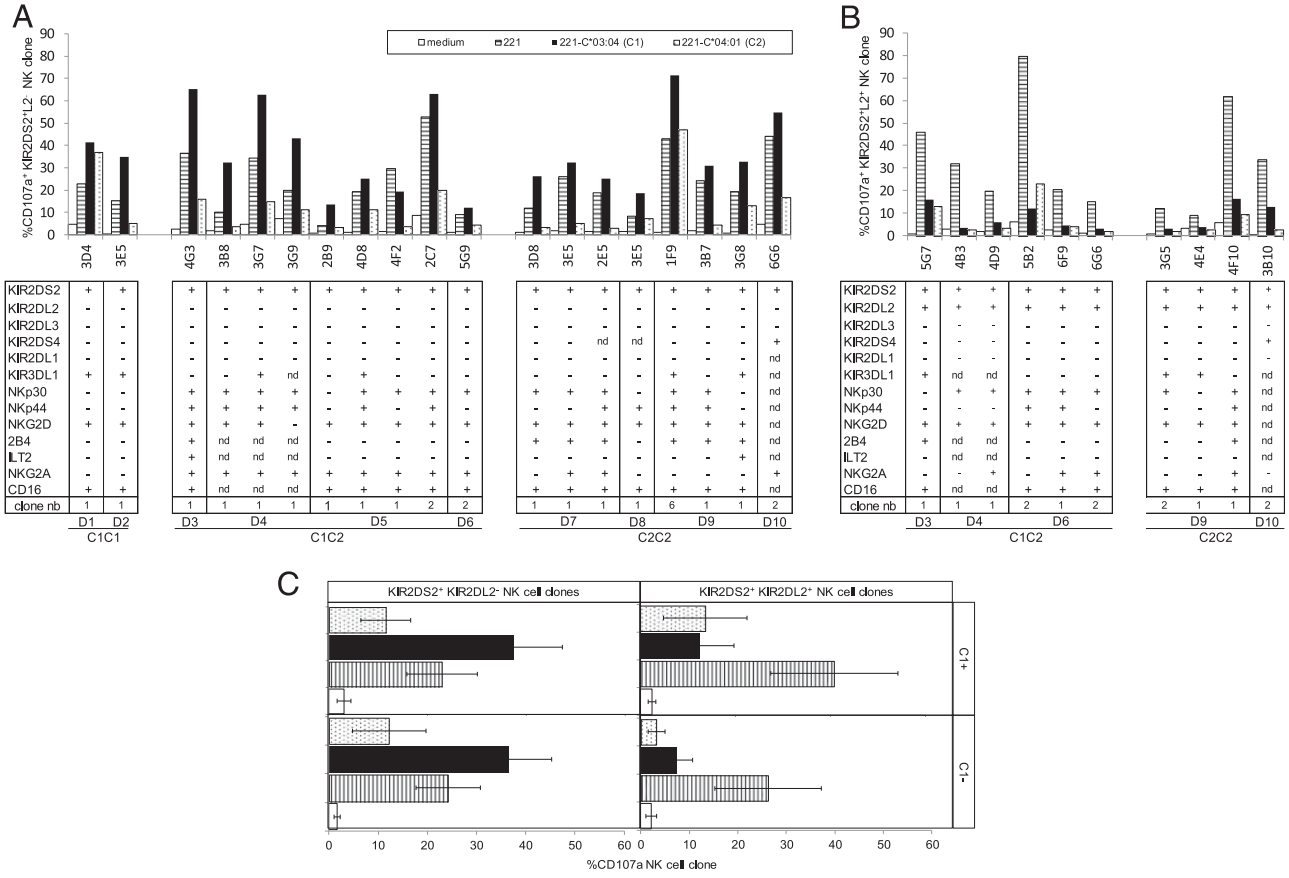

FIGURE 4. C1 reactivity of KIR2DS2$^+$ KIR2DL2$^-$ NK cell clones regardless of the HLA-C background. Histograms represent the percentage of (**A**) CD107a$^+$ KIR2DS2$^+$ KIR2DL2$^-$ NK cell clones and (**B**) KIR2DS2$^+$ KIR2DL2$^+$ NK cell clones isolated from C1C1, C1C2, and C2C2 individuals in different conditions of degranulation assay: medium, 221, HLA-C*03:04 (C1), and HLA-C*04:01 (C2) transfected 221 target cells at an ET ratio of 1:1. (**C**) Bars indicate the mean of the percentage of CD107a$^+$ KIR2DS2$^+$ KIR2DL2$^-$ NK cell clones (±SD) and CD107a$^+$ KIR2DS2$^+$ KIR2DL2$^+$ NK cell clones (±SD), grouped following C1$^+$ and C1$^-$ background, in different culture conditions: medium, 221, HLA-C*03:04 (C1), and HLA-C*04:01 (C2) transfected 221 target cells at an E:T ratio of 1:1.

genotype in our cohort were KIR2DL2/S2$^+$ KIR2DL3$^-$. We observed a significantly higher KIR2DL2/3/S2$^+$ NK cell frequency (mean = 36.1 ± 5) in these individuals when compared with KIR2DL1$^+$ KIR2DL2/S2$^+$ 2DL3$^-$ individuals (mean = 20.3 ± 5, p = 0.005; Fig. 6C), possibly as a result of compensatory KIR2DL2/S2 expression in the absence of KIR2DL1 expression. Thus, KIR2DL1 substitution by KIR2DL2 is phenotypically marked in the KIR2D repertoire and possibly allows maintenance of self C2 recognition.

Significant impact of KIR2DS1 and KIR2DS2 expression on KIR2D NK cell repertoire formation

To determine the impact of KIR2DS1 gene on the KIR2DL1/S1 NK cell compartment, we assessed the frequency of KIR2DL1$^+$ 2DS1$^-$, KIR2DL1$^+$ 2DS1$^+$, and KIR2DL1$^-$ 2DS1$^+$ NK cell subsets (Fig. 7A). Taking into account only the HLA-C environment, no difference in frequency was observed. However, a significantly decreased frequency of KIR2DL1$^+$ 2DS1$^-$ NK cells was observed particularly in B$^+$ haplotypes regardless of the HLA-C molecules, linked directly to KIR2DS1 expression (Fig. 7A, Supplemental Table II). The KIR2DL1$^-$ KIR2DS1$^+$ NK cell subset is significantly more represented in BB than AB haplotypes because of the increased number of KIR2DS1 allele copies. KIR2DS1 expression contributed to a significantly increased KIR2DL1/S1 NK cell pool, expressed with or without KIR2DL1 on NK cells (Fig.7C, 7D). Using a 1F12/GL183 Ab combination (20), we investigated the frequency of KIR2DL2$^+$ 2DL3/S2$^-$ and KIR2DL3/S2$^+$ NK cell subsets according to KIR2DL2, KIR2DL3, and KIR2DS2 genes and HLA-C environment (Fig. 7B, 7E). In accordance with the fact that KIR2DL2 and KIR2DL3 segregate as alleles (21), we observed that KIR2DL2$^+$ KIR2DL3/S2$^-$ NK cell frequency is significantly higher in KIR2DL2/S2$^+$ than KIR2DL2/3/S2$^+$ genotyped individuals with a C2C2 haplotype (Fig. 7B). However, KIR2DL2$^+$ KIR2DL3/S2$^-$ NK cell frequency was not twice in KIR2DL2/S2$^+$ genotyped individuals that was observed in KIR2DL2/3/S2$^+$ individuals with only one KIR2DL2 allele (Fig. 7E), which suggests either coexpression of KIR2DL2 with KIR2DS2, or decreased expression of KIR2DL2. Thus, the expression of the KIR2DS1 contributes to broaden significantly the pool of KIR2DL1/S1 NK cells even though it significantly limits the KIR2DL1$^+$ 2DS1$^-$ cell subset frequency. In addition, C1 reactive KIR2DS2 expression seems to function to enlarge the KIR2DL2/3/S2 NK cell pool, and it is conceivable that its additive effect with KIR2DL1 may contribute to limit KIR2DL1 expression on NK cells. In summary, our results suggest that together with KIR2DL2, activating KIR2DS1 and KIR2DS2 expression limits KIR2DL1 acquisition on NK cells.

Discussion

In this study, we revisited the HLA-C specificity of the main KIR2DL and determined C1 reactivity of KIR2DS2 from selected KIR2DL$^+$ or KIR2DS$^+$ NK cell lines and clones, and investigated these specificities on KIR2D NK cell formation. Our results are in accordance with numerous studies (9, 12, 13, 17, 22–28), and notably

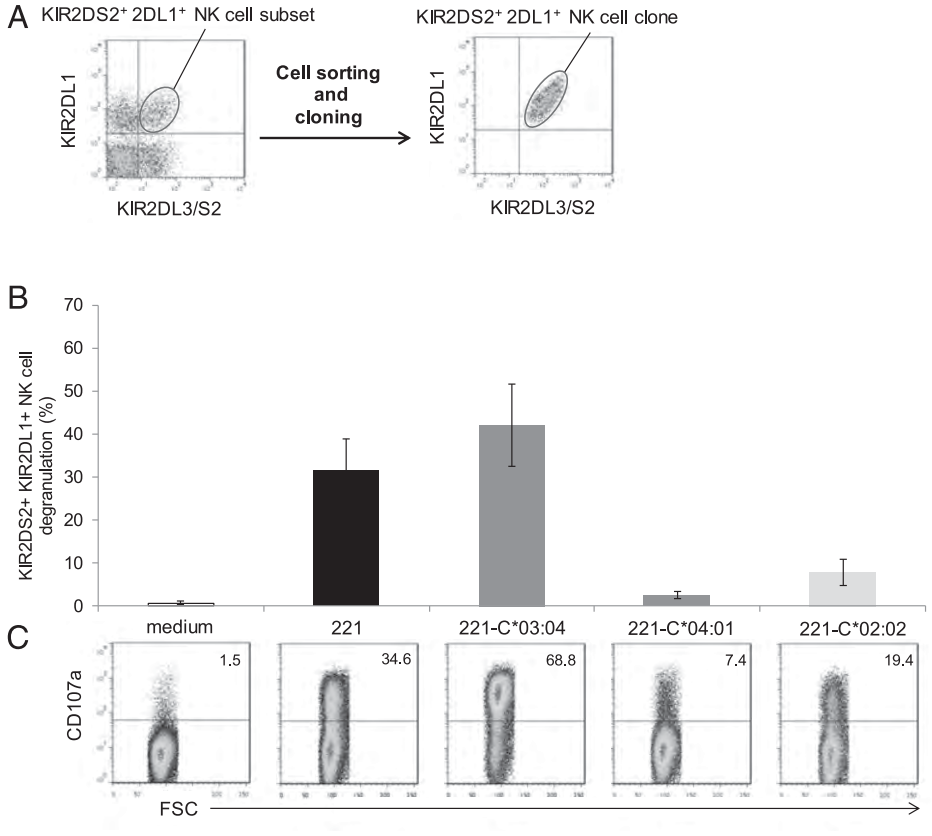

FIGURE 5. Additive effect of KIR2DL1 and KIR2DS2 against $C1^+$ target cells. (**A**) After depletion of $CD3^+$ cells from PBMCs, $KIR2DS2^+$ $KIR2DL1^+$ $KIR2DL2/3/S1^-$ NK cells ($NKp46^+$ $EB6^+$ $1F12^+$) were cell sorted from $C2^+$ $KIR2DL3/S1^-$ genotyped individuals and amplified in vitro with irradiated allogeneic PBMCs and EBV-B cells as feeders. After 3 wk of stimulation, NK cells were cloned and only $KIR2DL2^-$ NK cell clones verified by RT-PCR have been selected. The phenotype of one representative $KIR2DS2^+$ $KIR2DL1^+$ $KIR2DL2/3/S1^-$ NK cell clone is shown. (**B**) CD107a mobilization assay was performed on four $KIR2DS2^+$ $KIR2DL1^+$ $KIR2DL2/3/S1^-$ NK cell clones isolated from one $C2^+$ individual. These results were confirmed for eight $KIR2DS2^+$ $KIR2DL1^+$ $KIR2DL2/3/S1^-$ NK cell clones isolated from two different $C2^+$ individuals. Bars indicate the mean of $CD107^+$ NK cell percentages (± SD) in different culture conditions: medium, 221, HLA-C*03:04 (C1), and HLA-C*04:01 (C2) transfected 221 target cells at an E:T ratio of 10:1. (**C**) Representative density plots of $KIR2DS2^+$ $KIR2DL1^+$ $KIR2DL2/3/S1^-$ ($NKp46^+$ $EB6^+$ $1F12^+$) NK cell degranulation observed in the different culture conditions.

those obtained by Winter et al. (13) showing that KIR2DL2 and KIR2DL3 recognize C2 allotypes using NK92 infected with Vac-KIR2DL2 or -KIR2DL3. More recently, Moesta et al. (12) have shown that KIR2DL2 is a stronger receptor for HLA-C ligand than KIR2DL3. However, among C2 allotypes, we showed that HLA-C*04:01 ligand is better recognized than the other evaluated ligands (HLA-C*02:02, -C*06:02, and -C*15:01). These results are in line with a recent observation drawn from an analysis of

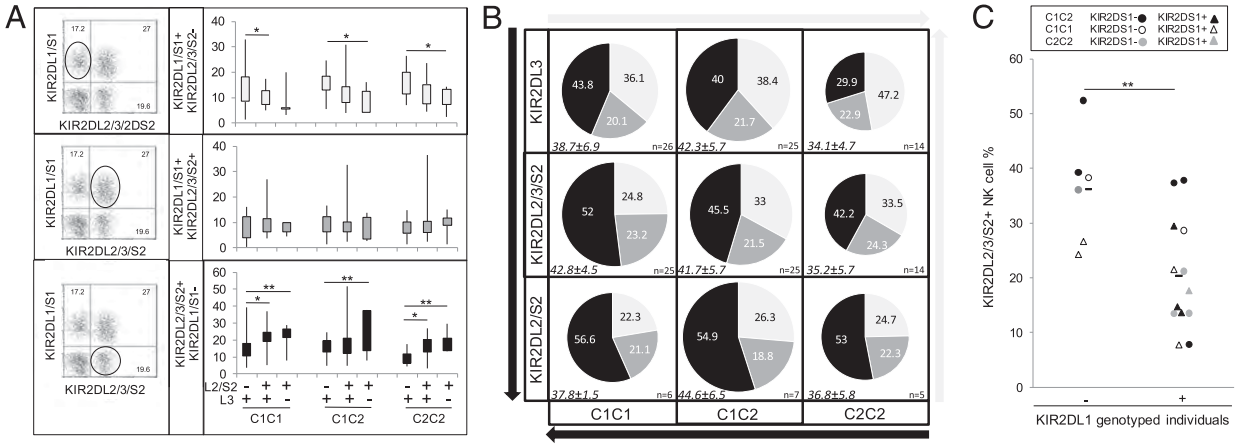

FIGURE 6. The nature of KIR2D and HLA-C ligands directs the structure of the NK cell repertoire. (**A**) Representative density plot illustrating the different KIR2D NK cell subsets in the large cohort of HLA and KIR genotyped individuals (Supplemental Table I) grouped according to the presence of KIR2DL2/3 and 2DS2 genes ($KIR2DL3^+$, $KIR2DL2/3/S2^+$, and $KIR2DL2/S2^+$) and HLA-C haplotype (C1C1, C1C2, and C2C2). $KIR2DL1/S1^+$ $KIR2DL2/3/S2^-$ ($EB6^+$ $GL183^-$) NK cell subset is indicated in white, $KIR2DL1/S1^+$ $KIR2DL2/3/S2^+$ ($EB6^+$ $GL183^+$) NK cell subset is indicated in gray, and $KIR2DL1/S1^-$ $KIR2DL2/3/S2^+$ ($EB6^-$ $GL183^+$) NK cell subset is indicated in black. Box and whisker plot summarizing the frequency of each KIR2D NK cell subset out of all NK cells. (**B**) Pie charts depict the pattern of KIR2D composition in nine groups of individuals distributed following the presence of KIR2DL2/3 and 2DS2 genes ($KIR2DL3^+$, $KIR2DL2/3/S2^+$, and $KIR2DL2/S2^+$) and HLA-C molecules (C1C1, C1C2, and C2C2). We summed $KIR2DL1/S1^+$ $KIR2DL2/3/S2^-$, $KIR2DL1/S1^-$ $KIR2DL2/3/S2^+$, and $KIR2DL1/S1^+$ $KIR2DL2/3/S2^+$ NK cell subsets, weighting them according to their frequency indicated in the pie chart. The size of the pie chart is proportional to the frequency of KIR2D NK cells (KIR2DL1/2/3/2DS1/2), and it is indicated (±SD) in the *bottom left* of each group in italics. The number of studied individuals in each group is indicated in the *bottom right*. (**C**) Scatter plots represent $KIR2DL2/3/2DS2^+$ NK cell frequency in $KIR2DL1^-$ ($n = 6$) and $KIR2DL1^+$ ($n = 13$) genotyped individuals. The mean frequency is indicated in each group by a black feature. Individuals have been identified following KIR2DS1 genotype and HLA-C molecules (C1C1, C1C2, and C2C2). Statistical significance ($^*p < 0.05$, $^{**}p \leq 0.001$) between two groups was determined using the one-way ANOVA test (Supplemental Table II).

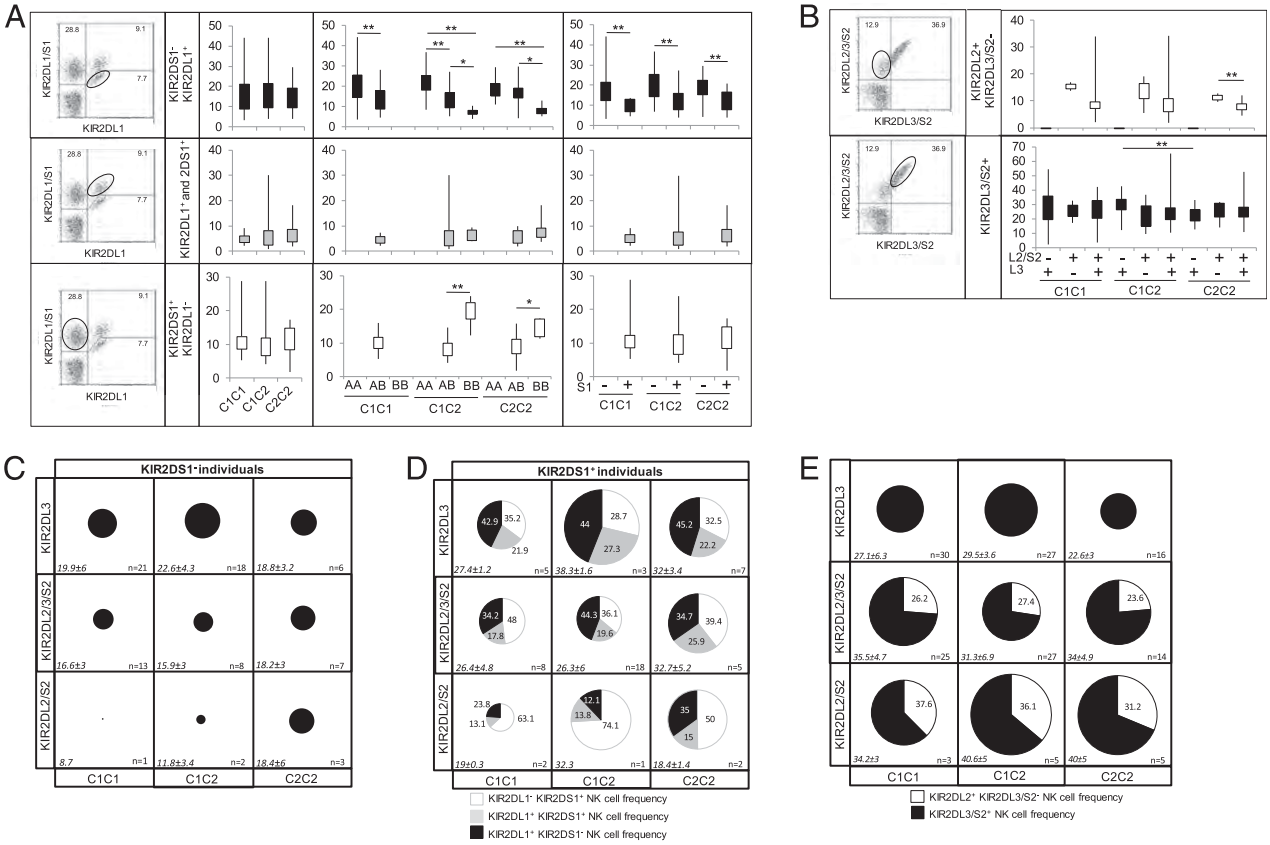

FIGURE 7. Significant impact of KIR2DS1 and KIR2DS2 expression on KIR2D NK cell repertoire formation. Representative density plot illustrating the different NK cell subsets expressing (**A**) KIR2DL1 or/and KIR2DS1, studied in the large cohort of HLA and KIR genotyped individuals (Supplemental Table I) grouped according to the HLA-C molecules (C1C1, C1C2, and C2C2), KIR haplotype (AA, AB, and BB), and presence or absence of the KIR2DS1 gene. KIR2DL1$^+$S1$^-$ (EB6^{low} 143211$^+$) NK cell subset is indicated in black, KIR2DL1$^+$S1$^+$ (EB6$^+$ 143211$^+$) NK cell subset is indicated in gray, and KIR2DL1$^-$S1$^+$ (EB6$^+$ 143211$^-$) NK cell subset is indicated in white. (**B**) Representative density plot illustrating the different NK cell subsets expressing KIR2DL2 and/or KIR2DL2/3/S2 studied in individuals grouped in function of the presence of KIR2DL2/3 and 2DS2 genes (KIR2DL3$^+$, KIR2DL2/3/S2$^+$, and KIR2DL2/S2$^+$) and HLA-C molecules (C1C1, C1C2, and C2C2). KIR2DL2$^+$ 2DL3/2DS2$^-$ (1F12$^-$ GL183$^+$) NK cell subset is indicated in white; KIR2DL2$^-$ 2DL3/2DS2$^+$ (1F12$^+$ GL183$^+$) NK cell subset is indicated in black. Box and whisker plot summarizing the frequency of each KIR2D NK cell subset out of all NK cells. Pie charts show the KIR2DL1 NK cell subset in nine groups of (**C**) KIR2DS1$^-$ genotyped individuals and (**D**) KIR2DS1$^+$ genotyped individuals distributed following the presence of KIR2DL2/3 and 2DS2 genes (KIR2DL3$^+$, KIR2DL2/3/S2$^+$, and KIR2DL2/S2$^+$) and HLA-C molecules (C1C1, C1C2, and C2C2). We summed KIR2DL1$^+$ S1$^-$, KIR2DL1$^+$ S1$^+$, and KIR2DL1$^-$ S1$^+$ NK cell subsets, weighting them according to their frequency indicated in the pie chart. (**E**) Pie charts depict the pattern of KIR2D composition in the KIR2DL2/3/2DS2 NK cell pool in nine groups of individuals distributed following the presence of KIR2DL2/3 and 2DS2 genes (KIR2DL3$^+$, KIR2DL2/3/S2$^+$, and KIR2DL2/S2$^+$) and HLA-C molecules (C1C1, C1C2, and C2C2). We summed KIR2DL2$^+$ 2DL3/S2$^-$ and KIR2DL2$^-$ 2DL3/S2$^+$ NK cell subsets, weighting them according to their frequency indicated in the pie chart. The size of the pie chart is proportional to the frequency of KIR NK cell pool and it is indicated (±SD) in the *bottom left* of each group in italics. The number of studied individuals in each group is indicated in the *bottom right*. Statistical significance (*$p < 0.05$, **$p \leq 0.001$) between two groups was determined using the one-way ANOVA test (Supplemental Table II).

a large panel of single-HLA class I molecule beads based on Luminex, which showed a broader pattern of HLA-C recognition by KIR2DL2-Fc fusion protein, even using unloaded HLA class I molecules (12). Recently, we have shown that KIR2DL3$^+$ NK cell lines equally recognized either C1C1 or C2C2 immature dendritic cells (29). The low affinity of the KIR2DL2/3 to HLA-C ligand is likely reinforced and stabilized within the immune synapse by other receptor–ligand interactions, thus allowing functional KIR-HLA interactions. Besides the wide spectrum of HLA-C recognition by KIR2DL2 and KIR2DL3, another important message of our study is the ability of activated KIR2DL2$^+$ and KIR2DL3$^+$ NK cells to recognize the "missing-self" irrespective of their HLA-C background. Our results are in agreement with those of Anfossi et al. (7), who suggested education of KIR2DL3$^+$ NK cells mainly from a C1$^+$ environment. Indeed, we observed that resting C1$^-$ KIR2DL3$^+$ NK cells are less receptive to missing-self activation (data not shown); however, KIR2DL3 engagement with C2 ligands during NK development or cytokine stimulation could enhance the response to missing-self after a preactivation, resulting in a decrease of their activation threshold. This point is essential in particular pathological contexts, such as viral infection, tumor processes, and HSCT, where activation could increase the missing-self response of poorly educated cells such as C1$^-$ KIR2DL3$^+$ NK cells or KIR2DL2$^+$ NK cells.

In contrast with other approaches based on soluble KIR-Ig proteins, KIR2D transfected cells, or a vaccinia expression system, our results were drawn from NK cells purified from different individuals with a physiological KIR2D expression. The more sensitive cellular model probably allows assessment of low-avidity interactions between of KIR2D and HLA-C, which could explain discordant results with those obtained with binding assays using KIR2D-Ig fusion proteins (13). This is typically the case for soluble KIR2DS receptors, which did not bind to any of the HLA class I molecules expressed on a large panel of transfected cells (13). In our cellular model, we showed that KIR2DS2 recognizes only C1 ligands, in contrast with its inhibitory KIR2DL2 form, in

all HLA-C environments. Failed C1/KIR2DS2 interactions using soluble KIR2DS2-Ig fusion proteins or KIR2DS2 tetramers (30) would suggest weak affinities. Thus, the few substitutions between the extracellular parts of both receptors could explain these apparent discrepancies. KIR2DS1 and KIR2DS2 show a lower affinity than their inhibitory counterparts with overlapping specificity, ensuring that inhibition signals override activation signals. However, in the absence of inhibitory KIR signals, the engagement of activating KIR2DS is sufficient to trigger NK cell responses.

The broader specificity of KIR2DL2 and KIR2DL3 toward HLA-C alleles also recognized by KIR2DL1 would explain the predominant expression of this compartment within the KIR2D NK cell repertoire. KIR2DL1 is predominantly represented in a C2C2 environment in the presence of KIR2DL3, but its representation is decreased in the presence of KIR2DL2 and KIR2DS2 genes. One possible explanation for this is that KIR2DL2 is a stronger competitor than KIR2DL3 for C2 ligands, explaining its predominant representation in the KIR2D NK cell pool in a C2C2 environment. Moreover, the high proportion of KIR2DS1$^+$ NK cells in the KIR2DL1/S1 pool could contribute to the decreased frequency of KIR2DL1$^+$ 2DS1$^-$ NK cells. Thus, all inhibitory and activating KIR2D are functional, but the strength of HLA-C affinity is different, ensuring a hierarchy between these KIR2D, and explains, in part, the formation of the NK cell repertoire depending on the HLA-C environment. In this regard, we observed in KIR2DL1$^-$ genotyped individuals that KIR2DL2/3/2DS2 expression was significantly increased compared with KIR2DL1$^+$ genotyped counterparts, suggesting an adjustment of the KIR2D repertoire formation after the distribution of each pool. This observation may not be compatible with a sequential acquisition of KIR with KIR2DL1 expression after KIR2DL2 and KIR2DL3 as proposed by Uhrberg's group (9). It is likely that the adjustment is more continuous and that the KIR2D expression can be readjusted according to other KIR2D expressed, as previously proposed (10).

The C2-expressing fetuses carried by group A KIR homozygous mothers constitute a risk factor for pre-eclampsia (31) and recurrent miscarriage (32) during pregnancy. In contrast, fetal C1 homozygosity and maternal group B KIR haplotype are protective (31, 32). Thus, it is conceivable that strong inhibitory KIR2DL2 maintains proper inhibition of NK cell responses against C1 fetal cells, whereas the weak inhibitor KIR2DL3, which is potentially efficient in C2 individuals on activated NK cells, may not properly inhibit NK cell response against allogeneic C2 ligands, resulting in pre-eclampsia or miscarriage. Moreover, although KIR2DL2 and KIR2DL3 segregate as alleles, only KIR2DL3 was associated with resistance to hepatitis C virus (33). The weaker interaction between KIR2DL3 and C1 ligand could be reinforced by particular peptides of viral or endogenous origin (34). In an HSCT context, it has been documented that KIR2DL2/3 and KIR2DS2 are predominantly expressed during hematopoietic reconstitution (35). In this activated context, KIR2DL2$^+$ and KIR2DL3$^+$ NK cells from C2C2 donors should ensure the missing-self recognition to compensate the default of functional KIR2DL1$^+$ NK cell recovery.

A previous study has defined five types of NK cell repertoire based on KIR dominant or NKG2A dominant expression, receptor-null expression, single-receptor expression, KIR-KIR coexpression, and NKG2A-KIR coexpression, and revealed a balance of KIR/NKG2A (36). This study was performed mainly from AA haplotype individuals and it would be interesting to investigate these types of repertoires taking into account the presence or absence of KIR2DL2/S2 and KIR2DS1 genes. Moreover, different affinities for the same HLA-C ligand have been shown for different KIR2DL3 allele products (12, 17), and this affinity can be modulated by the nature of loaded peptide (23). Thus, the formation of the KIR2D NK repertoire is probably finely adjusted depending on the KIR2DL polymorphism and on the other NK cell subsets. In this regard, we have recently shown the impact of KIR3DL1 allelic nature on KIR3DL1 NK repertoire formation (37). These recent investigations suggest primary expression of KIR3DL1 followed by KIR2D acquisition. The impact of allelic KIR3DL1 and a Bw4 environment on KIR2D NK cell repertoire should be studied in a larger cohort. Altogether, our results suggest that the nature of the HLA-C–specific activating and inhibitory KIR2D expressed and the ligand environment directs the structure of NK cell repertoire.

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Disclosures

The authors have no financial conflicts of interest.

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Both the nature of KIR3DL1 alleles and the KIR3DL1/S1 allele combination affect the KIR3DL1 NK-cell repertoire in the French population

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NK-cell functions are regulated by many activating and inhibitory receptors including KIR3DL1. Extensive allelic polymorphism and variability in expression can directly alter NK-cell phenotype and functions. Here we investigated the KIR3DL1$^+$ NK-cell repertoire, taking into account the allelic KIR3DL1/S1 polymorphism, KIR3DL1 phenotype, and function. All 109 studied individuals possessed at least one KIR3DL1 allele, with weak KIR3DL1*054, or null alleles being frequently present. In KIR3DL1$^{high/null}$ individuals, we observed a bimodal distribution of KIR3DL1$^+$ NK cells identified by a different KIR3DL1 expression level and cell frequency regardless of a similar amount of both KIR3DL1 transcripts, HLA background, or KIR2D expression. However, this bimodal distribution can be explained by a functional selection following a hierarchy of KIR3DL1 receptors. The higher expression of KIR3DL1 observed on cord blood NK cells suggests the expression of the functional KIR3DL1*004 receptors. Thus, the low amplification of KIR3DL1^{high}, KIR3DL1*004 NK-cell subsets during development may be due to extensive signaling via these two receptors. Albeit in a nonexclusive manner, individual immunological experience may contribute to shaping the KIR3DL1 NK-cell repertoire. Together, this study provides new insight into the mechanisms regulating the KIR3DL1 NK-cell repertoire.

Keywords: Allelic polymorphism · French population · KIR3DL1 · KIR3DL1/S1 allele combinations · NK-cell repertoire

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Introduction

The effector functions of NK cells are regulated by inhibitory and activating receptors, e.g. killer cell immunoglobulin-like receptors (KIR), which are specific for allotypic determinants shared by different HLA-class I molecules [1]. In particular, HLA-Cw allotypes with Asn^{80} (C1 ligands) or Lys^{80} (C2 ligands) are, respectively, recognized by KIR2DL2/2DL3 and KIR2DL1. HLA-A and HLA-B allotypes with a Bw4 motif are recognized by KIR3DL1 whereas HLA-A3/A11 are recognized by KIR3DL2 [2,3]. Lack of inhibitory KIR engagement can trigger alloreactive KIR NK-cell cytotoxicity only within functionally competent NK cells [4]. Although the ligands and functions of inhibitory KIR receptors are well documented, this is not the case for activating KIR receptors and their ligands, except for KIR2DS1 which recognizes C2 ligands only in C2-adult individuals [5]. Moreover, the activating receptor KIR3DS1, which segregates as an allele of KIR3DL1 [6], shares more than 97% sequence homology in its extracellular domain with the KIR3DL1 receptor. However, a functional interaction with HLA-A or HLA-B allotypes sharing the Bw4 public epitope has not been demonstrated in vitro [7, 8] despite a significantly higher frequency of KIR3DS1$^+$ NK cells observed in Bw4$^+$ than in Bw4$^-$ individuals [9].

KIR genes are located on chromosome 19q13.4. To date, 14 functional KIR genes have been characterized [10]. Within the human population, genomic diversity of the KIR region is achieved on several levels. KIR gene content varies between individuals who can exhibit 7–14 inhibitory and activating KIR genes [11]. Population studies have demonstrated two major KIR haplotypes: A and B [12]. The A haplotypes correspond to 7 KIR genes, including KIR2DS4 as the only activating KIR gene. In contrast, B haplotypes are more diverse and are characterized by the presence of more than one activating KIR gene and the absence of the KIR2DS4 gene [13, 14]. KIR gene polymorphism is the largest contributor to KIR region diversity, with multiple alleles defined [15]. While polymorphism is limited in the KIR2DL1 and KIR2DL2/L3 genes, it is much broader for KIR3DL1 and can even alter NK-cell functions [16,17]. Indeed, depending on the KIR3DL1 allele present in a given individual, the level of KIR3DL1 expression differs on the NK-cell surface, i.e. null for KIR3DL1*004, low for KIR3DL1*005 and *007, and high for KIR3DL1*001, *002, *01502, and *008 alleles [18–20]. Two KIR3DL1 alleles can be transcribed in one NK-cell clone, and the proportion of clones expressing both alleles is high in comparison to clones expressing only one KIR3DL1 allele. However, based on the phenotypic pattern on all NK cells, only the nature of each allele is marked [19].

In addition to allelic polymorphism, the variability in expression levels of KIR3DL1 molecules on the NK-cell surface also varies depending on the presence or absence of autologous Bw4 molecules, promoter polymorphism, and DNA methylation [21–23]. Functional interaction between KIR3DL1 receptors and their HLA-Bw4 ligands are modulated by KIR3DL1 allotypes [24, 25] and the peptide content of HLA class I molecules [26]. Moreover, KIR3DL1$^+$ NK-cell cytotoxicity differs depending on KIR3DL1 allotypes and/or HLA-A, HLA-B Bw4$^+$ targets [3,27,28]. From a clinical standpoint, KIR3DL1 allelic polymorphism has been correlated with HIV progression and viral load [29]. We have previously shown that KIR3DL1/3DS1 gene disparities of donor/recipient pairs in hematopoietic stem cell transplantation have a significant impact on hematopoietic stem cell transplantation outcome [30].

Until now, the impact of KIR3DL1/3DS1 allele combinations on NK-cell phenotype and function has only been studied in one Japanese cohort not representative of Caucasians [31]. However, the mechanisms shaping the KIR3DL1 repertoire remain ill-defined. The link between KIR3DL1 allelic polymorphism, levels of KIR3DL1 expression at the NK-cell surface, and frequency of KIR3DL1$^+$ NK-cell subsets has yet to be clarified. This is necessary in order to better understand the mechanisms that shape the KIR3DL1$^+$ NK-cell repertoire. In this study, we hypothesize that the high KIR3DL1 allelic polymorphism, including frequent null alleles observed in Caucasians, might impact the formation of the KIR3DL1 NK-cell repertoire. We therefore analyzed the KIR3DL1 NK-cell repertoire taking into account the KIR3DL1 allelic polymorphism, KIR3DL1/KIR3DS1 allele combinations, and phenotypic patterns such as frequency, the mean level of all KIR3DL1$^+$ NK-cell subsets, the Bw4 environment, and the functional potential of these KIR3DL1$^+$ NK-cell subsets.

Results

High proportion of KIR3DL1^{high} and KIR3DL1^{null} alleles in French individuals

KIR3DL1/3DS1 allele combinations were investigated in AA ($n = 34$), AB ($n = 67$), or BB ($n = 8$) KIR genotyped individuals. Overall, 33 different KIR3DL1/3DS1 allele combinations were observed (Fig. 1A, central panel). AA and AB KIR genotyped individuals exhibited a higher KIR3DL1 allelic variability compared with BB KIR genotyped individuals. The KIR3DL1*001, *004 allele combination was the most frequent in AA KIR genotyped individuals (Fig. 1A). KIR3DL1*001, *002, *004, *005, *007, *008, *015 known alleles were the most frequent (Fig. 1A, right panel). In AA KIR genotyped individuals, the frequencies of KIR3DL1 alleles ranged from 1.61 (KIR3DL1*008, *015, *005, *009, *019) to 32.26% (KIR3DL1*004; Fig. 1A, right panel). In AB KIR genotyped individuals, the frequencies of KIR3DL1 alleles ranged from 0.71 (KIR3DL1*017, *009) to 17.73% (KIR3DL1*001; Fig. 1A, right panel). In our cohort, the highly expressed KIR3DL1*001 and null KIR3DL1*004 alleles were the most frequent, being observed at one or two doses in 39 (35%) and 32 (29%) KIR genotyped individuals, respectively (Fig. 1A, right panel). The presence of autologous HLA-A and/or HLA-B Bw4$^+$ molecules did not affect both the KIR3DL1 allele and KIR3DL1/3DS1 allele combination frequencies (data not shown).

The frequency of KIR3DL1$^+$ NK-cell populations and the KIR3DL1 expression level were further assessed using the KIR3DL1- and KIR3DS1-specific Z27 specific monoclonal antibody in 109 KIR3DL1 allele-typed individuals (Fig. 1B).

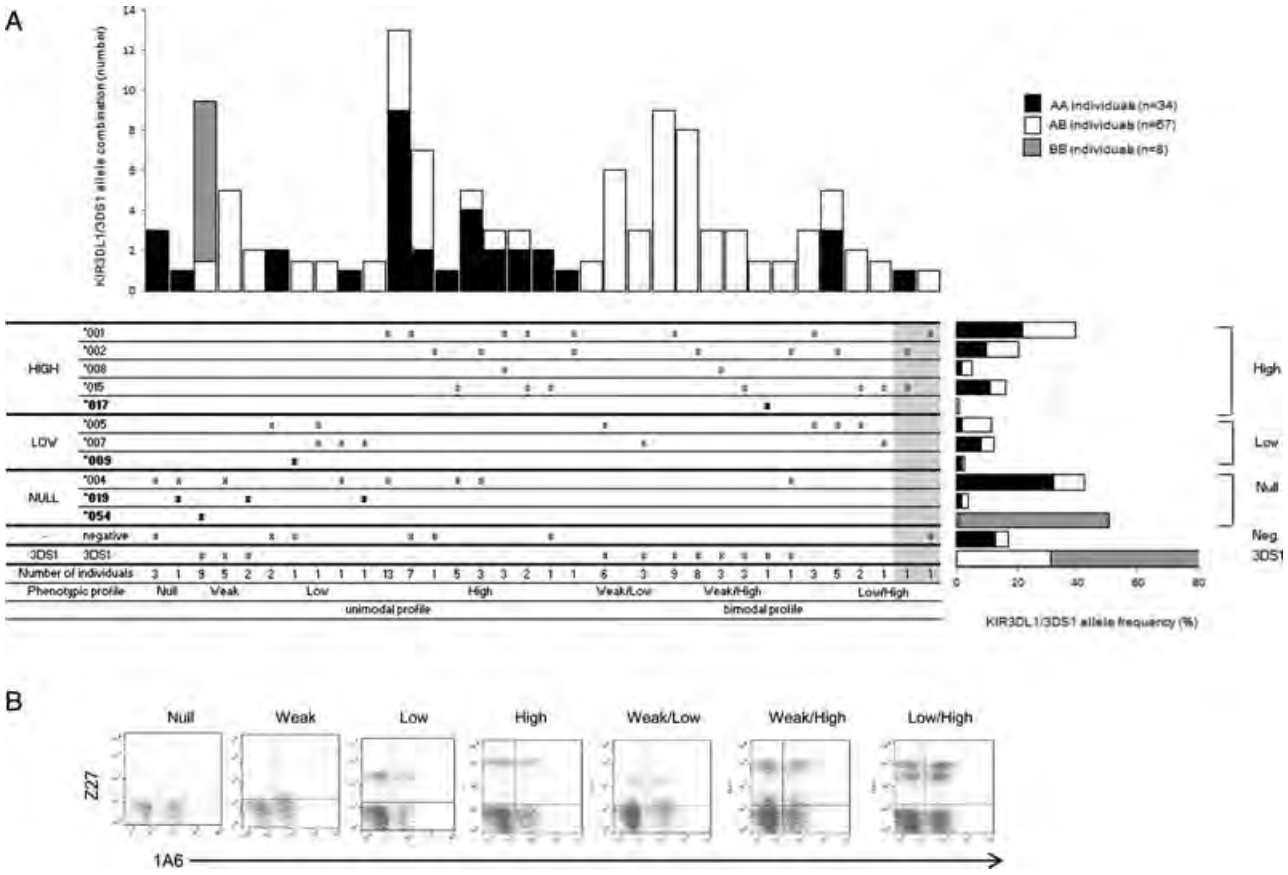

Figure 1. High proportion of KIR3DL1^{high} and KIR3DL1^{null} alleles in French individuals. (A) The number of KIR3DL1/3DS1 allele combinations and corresponding KIR3DL1/3DS1 allele frequency were established combining both allele-group–specific PCR-SSP and sequencing of polymorphic exons in AA (n = 34), AB (n = 67), and BB (n = 8) KIR genotyped individuals as described in the Materials and Methods (top). Distribution of KIR3DL1/3DS1 allele combinations and corresponding number of individuals are shown for each allele according to level of expression intensity on the NK-cell surface (i.e. null, low, high) and KIR3DS1 (weak) using the KIR3DL1/3DS1-specific Z27 mAb (bottom). New KIR3DL1 phenotypes are indicated in bold type and unexpected KIR3DL1 profiles are highlighted with gray shading. (B) Representative flow cytometry density plots of KIR3DL1$^+$ and KIR3DS1$^+$ NK cells for different KIR3DL1/S1 allele combinations identified from 109 studied individuals are shown. The staining was done using the NKp46-specific mAb to target NK cells, KIR2D-specific 1A6 mAb, and KIR3DL1/3DS1-specific Z27 mAb to evaluate KIR3DL1/3DS1$^+$ NK cells with or without KIR2D receptor expression. Data shown are representative of 109 individuals.

To examine the potential effect of KIR3DL1/3DS1 allele combinations on NK KIR3DL1$^+$ phenotype, individuals were divided into different groups based on null, low, and high KIR3DL1 expression, and weak expression of KIR3DS1. Seven different phenotypic patterns were identified: no binding pattern (KIR3DL1^{null},$^{null/null}$ genotypes), a unimodal pattern with weak intensity (KIR3DL1^{null}/KIR3DS1), a unimodal pattern with low intensity (KIR3DL1$^{low/low}$ or KIR3DL1$^{low/null}$), a unimodal pattern with high intensity (KIR3DL1$^{high/high}$ or KIR3DL1$^{high/null}$), a bimodal pattern with weak and low intensities (KIR3DS1/KIR3DL1^{low}), a bimodal pattern with weak and high intensities (KIR3DS1/KIR3DL1^{high}), and a bimodal pattern with low and high intensities (KIR3DL1$^{low/high}$). The bimodal expression patterns observed in KIR3DL1$^{low/high}$ and KIR3DS1/KIR3DL1^{high} individuals indicates the presence of NK-cell subsets expressing each of the expected KIR3DL1 alleles, and are in agreement with the reported mutually exclusive expression of KIR3DL1 and KIR3DS1 [6]. All combinations including KIR3DL1^{high}, KIR3DL1^{low}, KIR3DL1^{null}, or KIR3DS1 were observed. The in-depth analysis of KIR3DL1 alleles and NK-cell phenotypes revealed the KIR3DL1 phenotypic profile for KIR3DL1*009 and KIR3DL1*01702 alleles which are, respectively, expressed at low and high levels on the NK-cell surface and highlight unusual KIR3DL1 phenotypic profiles in a low proportion of individuals (Fig. 1A). The distribution of all KIR3DL1/3DS1 allele combinations shows that KIR3DL1^{high} alleles were abundant, and mainly associated with KIR3DL1^{null} or KIR3DS1 (Fig. 1A and B).

The KIR3DL1*054 allele is frequently present and mainly associated with KIR3DS1

All KIR3DS1$^+$ individuals for whom no KIR3DL1 allele was identified at the genotypic level had the KIR3DL1*054 allele [32], as illustrated in Figure 2A for two individuals and two well-characterized cell lines (13th International Workshop; WT51 and

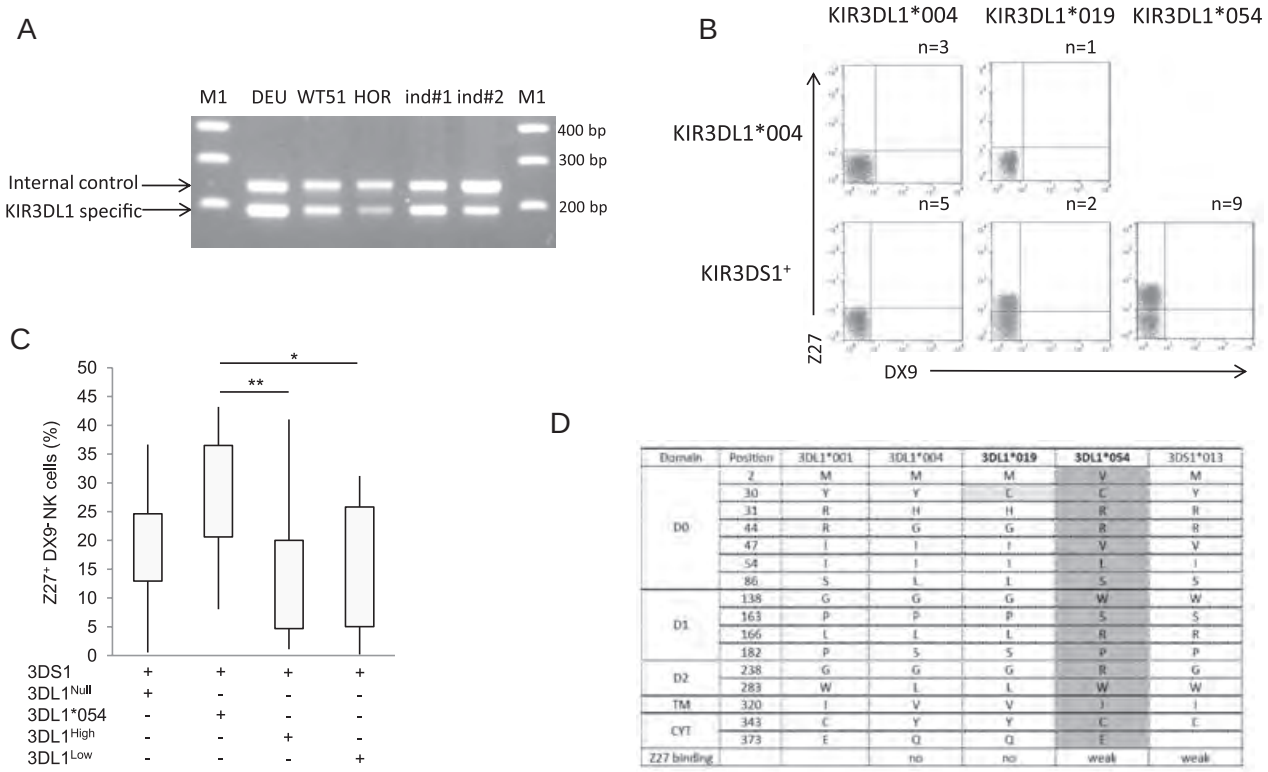

Figure 2. The KIR3DL1*054 allele is frequently present and mainly associated to KIR3DS1. (A) Visualization of KIR3DL1 PCR amplification products in a 2% Seakem® (Lonza) agarose gel in 0.5X TBE buffer with a 100 bp Ext. ladder marker (Lonza, M1). The figure shows one KIR3DL1$^+$ control Workshop DNA (DEU), two KIR3DL1$^-$ control Workshop DNAs (WT51, HOR) and two individuals previously determined as KIR3DL1$^-$ using the KIR genotyping SSP kit from Invitrogen. Both an internal control band at 256 bp and a specific PCR product amplifying exon 3 at 197 bp using homemade KIR3DL1 primers [53] can be observed. (B) Representative flow cytometry density plots of KIR3DL1$^+$ and KIR3DS1$^+$ NK cells for different KIR3DL1/S1 allele combinations integrating KIR3DL1*004, KIR3DL1*019, or KIR3DL1*054 with the KIR3DS1 or KIR3DL1*004 allele are shown. Density plots are gated on CD3$^-$ CD56$^+$ NK cells. KIR3DL1$^+$ and KIR3DS1$^+$ cells are, respectively, targeted as DX9$^+$ Z27$^+$ and DX9$^-$ Z27$^+$ cells. The number of individuals displaying each KIR3DL1/S1 allele combination is indicated above the density plot. (C) Box and whisker plot summarizing the frequency of Z27$^+$ DX9$^-$ NK cells in KIR3DS1$^+$ individuals as a function of the nature of the KIR3DL1 allele: null ($n = 7$), KIR3DL1*054 ($n = 7$), high ($n = 19$) and low ($n = 8$). Top and bottom whiskers represent values of the top and bottom 25% of cases respectively; boxed area, interquartile range. Statistical significance ($^*p < 0.05$; $^{**}p < 0.01$) between different groups was determined using the one-way ANOVA test. (D) Amino acid substitutions and level of Z27 binding for the KIR3DL1*004 allotype compared with the KIR3DL1*001 (reference), KIR3DL1*019 and KIR3DL1*054 (in bold), and KIR3DS1*013 allotypes are shown. Different amino acid substitutions between KIR3DL1*004 and KIR3DL1*019 on the one hand and KIR3DL1*004 and KIR3DL1*054 on the other hand are highlighted in light gray and dark gray shading respectively.

HOR) previously defined as KIR3DL1$^-$ [33]. The KIR3DL1*001, *005 genotyped and characterized DEU cell line (13th International Workshop) was used as a positive control. Sequencing of all amplified products in nine 3DL1$^-$, 3DS1$^+$ genotyped individuals showed that the KIR3DL1*054 allele was unique, with specific T^{475}, T^{550} and G^{560} nucleotides in exon 4 (data not shown). As described for KIR3DL1*004 [34, 35], no KIR3DL1 receptor was detected on the NK-cell surface for the only KIR3DL1*019, *004 individual studied (Fig. 2B). Alignment of amino acid substitutions of KIR3DL1*019 and KIR3DL1*054 compared with that of the KIR3DL1*004 allotype showed that the expected mature KIR3DL1*019 protein possesses the same amino acids involved in the intracellular retention of the null KIR3DL1*004 allotype, i.e. L^{86} and S^{182} (Fig. 2D). Only one amino acid in the D0 domain differed between KIR3DL1*019 and KIR3DL1*004. All KIR3DL1*054$^+$ individuals identified in our cohort presented the KIR3DS1 allele (Fig. 1A). In KIR3DS1$^+$ individuals, staining using the combination of Z27 and DX9 mAbs did not identify a DX9$^+$ Z27$^+$ population potentially corresponding to KIR3DL1*004, *019, or *054 allele products. Because KIR3DS1 and KIR3DL1*054 [32,36] were recognized by Z27 (a weak intensity staining pattern was observed Fig. 2B), it was difficult to selectively evaluate the expression of KIR3DL1*054 at the NK-cell surface. Interestingly, the frequency of Z27$^+$ DX9$^-$ expression was significantly higher for KIR3DS1$^+$, 3DL1*054$^+$ (28%, $n = 7$) than for KIR3DS1$^+$, 3DL1^{high} (13.26%, $n = 19$), or KIR3DS1$^+$, KIR3DL1^{low} (13.03%, $n = 8$) genotyped individuals with a p value of 0.01 and 0.03, respectively (Fig. 2C). This suggested either a cellular expression of the KIR3DL1*054 allotype, like KIR3DS1, recognized by the Z27 mAb, or a higher expression of KIR3DS1 in association with the KIR3DL1*054 allele (Fig. 2C). Different amino acid substitutions were detected between KIR3DL1*054 and KIR3DL1*004 (Fig. 2D), suggesting that KIR3DL1*054 is more similar to KIR3DS1 than to KIR3DL1. These amino acid

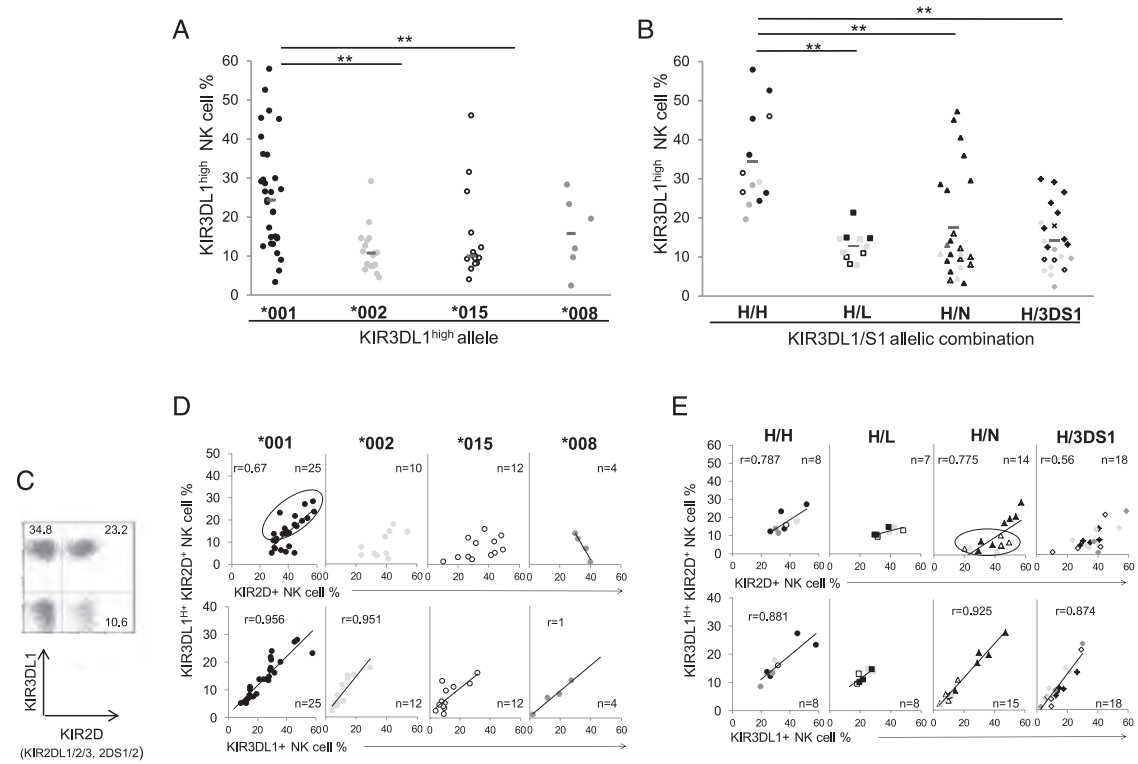

Figure 3. KIR3DL1^{high} NK-cell frequencies are determined by the number and nature of KIR3DL1^{high} alleles. (A) The KIR3DL1^{high} NK-cell frequencies out of all NK cells for 70 studied individuals as a function of the nature of the KIR3DL1^{high} allele: KIR3DL1*001 (filled black circle), KIR3DL1*002 (filled light gray circle), KIR3DL1*015 (open circle), and KIR3DL1*008 (filled dark gray circle) are shown. (B) All KIR3DL1^{high} NK-cell frequencies out of all NK cells for 70 studied individuals as a function of the second KIR3DL1 allele (high/high, high/low, and high/null) or KIR3DS1 (high/3DS1): KIR3DL1^{high} (circle), KIR3DL1^{low} (square), KIR3DL1^{null} (triangle), or KIR3DS1 (diamond). For all dots, the nature of each allele is determined by the color code used previously: KIR3DL1*001 (filled black symbols), KIR3DL1*002 (filled light gray symbol), KIR3DL1*015 (open symbol), KIR3DL1*008 (filled dark gray symbol), and KIR3DL1*017 (black cross). Statistical significance (**$p < 0.01$) between both groups was determined using the one-way ANOVA test. (C) Representative density plot of flow cytometry analysis of NK-cell ($CD3^-$ $CD56^+$) phenotype using KIR3DL1-specific Z27 and KIR2DL1/2/3- and KIR2DS1/2-specific 1A6 mAbs. (D, E) KIR3DL1^{high} $KIR2D^+$ NK-cell frequencies are compared with KIR3DL1^{high+} NK-cell frequencies and $KIR2D^+$ NK-cell frequencies as a function of (D) the nature of the KIR3DL1^{high} allele (*001, *002, *015, and *008), or of (E) the KIR3DL1/3DS1 allelic combinations (high/high, high/low, high/null, and high/3DS1). For all dots, the nature of each allele is determined by the color code used in (A, B). Spearman's rank correlation coefficients were calculated and indicated only when a significant p-value was obtained ($p < 0.05$). The number of studied individuals is indicated on each plot graph.

changes, especially in the extracellular domains, could potentially interfere with Z27/DX9 epitope recognition.

KIR3DL1^{high} NK-cell frequencies are determined by the number and nature of KIR3DL1^{high} alleles

Taking into account the nature of KIR3DL1^{high} alleles, we identified from all studied KIR3DL1^{high} individuals ($n = 70$), a higher frequency of KIR3DL1^{high} NK cells with the KIR3DL1*001 allele (24.3%, $n = 31$) than the KIR3DL1*002 allele (10.7%, $n = 17$, $p < 0.0003$), the KIR3DL1*015 allele (10%, $n = 15$, $p = 0.01$), or the KIR3DL1*008 (15.5%, $n = 6$, $p > 0.05$) (Fig. 3A). The frequency of KIR3DL1^{high} NK cells was further compared in four groups of individuals based on the nature of the second allele: KIR3DL1^{high}, KIR3DL1^{low}, KIR3DL1^{null}, and KIR3DS1. Interestingly the overall frequency of KIR3DL1^{high} NK cells in KIR3DL1 $^{high/high}$ individuals was about twice that found in heterozygous individuals (29.2%, $n = 13$ versus 12.2%, $n = 57$, $p = 3 \times 10^{-7}$) (Fig. 3B). The distribution of KIR3DL1^{high} NK-cell frequencies was heterogeneous in KIR3DL1$^{high/null}$ and KIR3DL1^{high}/3DS1 individuals, and clearly bimodal for the KIR3DL1$^{high/null}$ group. However, on average the KIR3DL1^{high} NK-cell frequencies in KIR3DL1$^{high/null}$ and KIR3DL1^{high}/3DS1 individuals were much lower than in KIR3DL1$^{high/high}$ individuals (10.8 versus 29.2%, $p = 0.0015$ and 12.5% versus 29.2%, $p = 6.2 \times 10^{-7}$, respectively). Such differences are in accordance with KIR3DL1 allelic exclusion, suggesting independent and additive generation of NK-cell subsets with the KIR3DL1 alleles being expressed in a mutually exclusive fashion. Within KIR3DL1$^{high/high}$ individuals ($n = 13$), higher KIR3DL1$^+$ NK-cell frequencies were observed for homozygous KIR3DL1*001 individuals than for those with a single or no KIR3DL1*001 allele (Fig. 3B). A similarly higher percentage of KIR3DL1^{high} NK cells was observed in individuals carrying KIR3DL1*001 versus other KIR3DL1^{high} alleles in KIR3DL1$^{high/null}$, KIR3DL1$^{high/low}$, and KIR3DL1^{high}/3DS1

individuals (Fig. 3B). Strikingly, the frequency of KIR3DL1^{high} NK cells expressing the KIR3DL1*001 allele increases to around 50% with only one copy of the KIR3DL1*001 allele, notably for KIR3DL1*001,*004 genotyped individuals (Fig. 3B). Since the KIR3DL1 NK-cell frequency can be influenced by the Bw4 environment [21], we investigated the frequency and nature of the KIR3DL1 ligand in this French population. Overall, the frequency of HLA-B Bw4$^+$ individuals was high (59%) and increased above 70% when both HLA-A and HLA-B Bw4$^+$ molecules were taken into account. Fourteen out of 18 described Bw4 ligands were observed, with a predominance of A24 (27.4%) and B44 (30.2%; Supporting Information Fig. 1A). The presence of autologous HLA-A and/or HLA-B Bw4$^+$ molecules did not affect the frequency of KIR3DL1^{high} NK cells taking into account not only all KIR3DL1^{high} individuals but also the number of KIR3DL1^{high} allele copies (2X or 1X) or KIR3DL1/3DS1 allele combinations (3DL1$^{high/low}$, 3DL1$^{high/null}$, or 3DL1^{high}/3DS1; Supporting Information Fig. 1B). Moreover, the higher frequency of KIR3DL1^{high} NK cells observed with the KIR3DL1*001 allele was independent of the Bw4 ligand number (Supporting Information Fig. 1B). Thus, the heterogeneous distribution of KIR3DL1^{high} NK-cell frequencies, especially within KIR3DL1$^{high/null}$ individuals, suggests the contribution of factors other than copy number and Bw4 environment. We observed that the KIR3DL1/S1 allele combinations were associated not only with KIR genotypes (Fig. 1A) but also with the presence of a particular KIR gene. For example, 21 out of 22 KIR3DL1^{high}/3DS1 genotyped individuals displayed the KIR2DS1 gene compared with only 2 out of 12 KIR3DL1$^{high/high}$ individuals, none out of 23 KIR3DL1$^{high/null}$ and none out of 11 KIR3DL1$^{high/low}$ individuals (data not shown). To address whether KIR2D (KIR2DL1/2/3 and KIR2DS1/2) acquisition affects the KIR3DL1 NK-cell repertoire, we determined the phenotype of NK cells by flow cytometry using KIR3DL1-specific Z27 and KIR2DL1/2/3- and KIR2DS1/2DS2-specific 1A6 mAbs (Fig. 3C). We then analyzed the proportion of KIR3DL1^{high} NK cells expressing KIR2D as a function of the nature of KIR3DL1^{high} alleles (Fig. 3D, lower panel) and as a function of the KIR3DL1/S1 allele combination (Fig. 3E, lower panel). Interestingly, a proportionate fraction of KIR3DL1^{high} KIR2D of around 50% of KIR3DL1^{high} NK cells$^+$ was observed regardless of the KIR3DL1^{high} alleles and KIR3DL1/S1 allele combinations. Indeed, a positive correlation was observed mainly for KIR3DL1*001 ($r = 0.956$), KIR3DL1*002 ($r = 0.951$), and KIR3DL1*008 (r = 1) alleles with a p-value <0.05. In contrast, the Spearman's rank test showed a low correlation between the frequency of KIR2D$^+$ KIR3DL1^{high+} NK cells and KIR2D$^+$ NK-cell frequencies, with a significant p-value (<0.05) only for the KIR3DL1*001 allele ($r = 0.67$; Fig. 3C, upper panel) and the KIR3DL1/3DS1 allele combinations (KIR3DL1$^{high/high}$, $r = 0.787$, KIR3DL1$^{high/null}$, $r = 0.775$ and KIR3DL1/3DS1, $r = 0.56$; Fig. 3D, upper panel). These data suggest a consecutive expression of KIR2D (2DL1/2/3, 2DS1/2) on KIR3DL1^{high} NK cells.

Bimodal distribution of KIR3DL1^{high} NK cells in KIR3DL1$^{high/null}$ individuals

In KIR3DL1^{high}/3DL1^{null} individuals for whom a bimodal distribution of KIR3DL1$^+$ NK cells was observed as previously mentioned, we compared the frequency and the mean fluorescence intensity of KIR3DL1^{high} NK cells (Fig. 4A). Interestingly, among the three KIR3DL1^{high} alleles identified (*001, *002, and *015), a positive correlation was noted between the frequency of KIR3DL1^{high} NK cells and membrane expression level of the KIR3DL1*001 allele ($r = 0.7033$, $p = 0.009$). KIR3DL1*004 was the unique null allele associated with KIR3DL1*001, KIR3DL1*002, and KIR3DL1*015 in our cohort. Surprisingly, the KIR3DL1*001, *004 allele combination led to both a low-frequency and mean-expression level of the KIR3DL1 receptor in 5 out of 13 individuals (Fig. 4A). Representative density plots are shown in Fig. 4B, illustrating the high and low MFI of KIR3DL1$^+$ NK cells, respectively, with high (47.3%) and low frequency (3.3%) in two representative KIR3DL1*001,*004 individuals. For comparison, a representative density plot with the expected MFI for low KIR3DL1^{high} NK-cell frequency observed for one representative KIR3DL1*002, *004 genotyped individual is also provided in Fig. 4B. The bimodal effect observed in KIR3DL1^{high,null} individuals was not influenced by the Bw4 ligand number (Supporting Information Fig. 1B). We explored KIR2DL1/S1, KIR2DS4, KIR2DL2, and KIR2DL3/2DS2 NK-cell subset frequencies as a function of KIR3DL1^{high} NK-cell frequency for these KIR3DL1^{high,null} individuals (Fig. 4C) in order to determine whether other KIR NK-cell subsets may balance the low KIR3DL1^{high} NK-cell frequency. Individuals with high KIR3DL1^{high} NK-cell frequencies presented a consistent KIR2DL1 NK-cell subset, however, the frequency of KIR2DL1 NK cells did not correlate with the frequency of KIR3DL1^{high} NK cells. Interestingly, KIR2DS4 was expressed only in individuals who displayed the KIR3DL1*015 and KIR3DL1*002 alleles (Fig. 4C). Moreover, the KIR2DS2 gene, which is in linkage disequilibrium with the KIR2DL2 gene, was present in 8 out of 23 KIR3DL1^{high,null} genotyped individuals and interestingly, KIR2DS2 was present only in KIR3DL1*001,*004 genotyped individuals who exhibited a high frequency of KIR3DL1^{high} NK cells (data not shown). The frequency of KIR2DL2$^+$ NK cells did not correlate with the frequency of KIR3DL1^{high} NK cells; however, the KIR2DL3/S2 NK-cell frequency was higher in individuals who presented a high frequency of KIR3DL1^{high} NK cells (Fig. 4C).

Because neither DX9 nor Z27 monoclonal antibodies recognize KIR3DL1 in permeabilized/fixed cells, as previously reported by Thomas et al. [32], any potential increased/decreased internalization of KIR3DL1 receptor could not have been evaluated in KIR3DL1*001, *004 genotyped individuals. However, Z27$^+$ NK cells were sorted from individuals of each group and KIR3DL1 transcripts were analyzed using primers specific for KIR3DL1*001, KIR3DL1*002, or KIR3DL1*004 (Fig. 4D). We detected not only KIR3DL1*001 or KIR3DL1*002 transcripts but also KIR3DL1*004 transcripts in all studied KIR3DL1^{high}

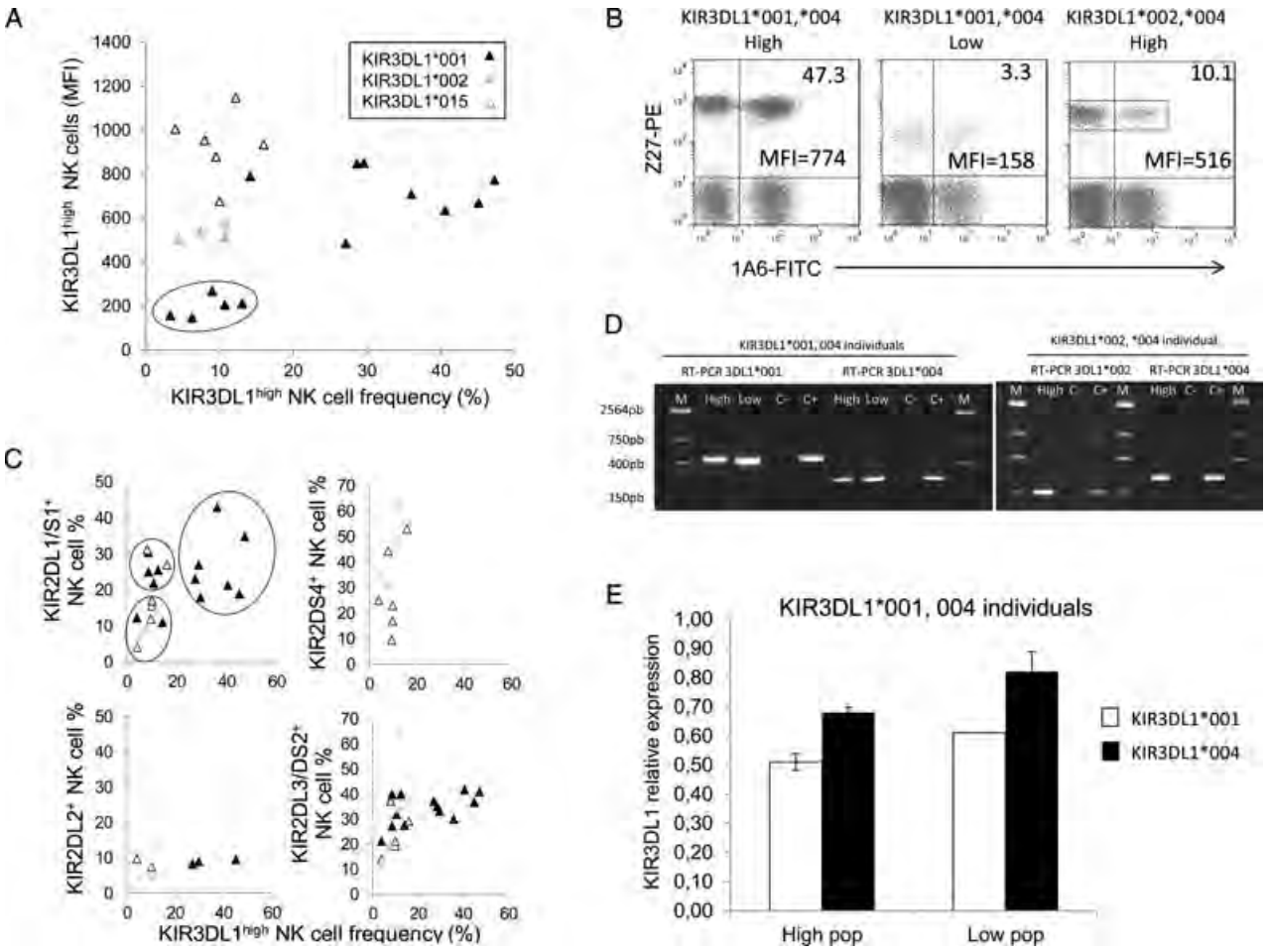

Figure 4. Detection of a similar amount of KIR3DL1^{high} and KIR3DL1^{null} transcripts in KIR3DL1^{+} NK cells regardless of the membrane expression level of KIR3DL1. (A) The KIR3DL1^{high} NK-cell frequency as a function of the KIR3DL1^{high} NK-cell MFI for KIR3DL1$^{high/null}$ individuals ($n = 23$) is shown. The nature of the KIR3DL1^{high} allele is indicated by a color code. KIR3DL1*004 is the unique null allele associated with KIR3DL1^{high} alleles in our cohort. Dots corresponding to KIR3DL1*001, *004 genotyped NK cells with a low frequency and a low mean level intensity are circled. (B) Representative flow cytometry density plots showing KIR3DL1^{+} (Z27^{+}) NK cells for two KIR3DL1*001,*004 and one KIR3DL1*002, *004 genotyped individuals. MFI and percentage of KIR3DL1^{high} NK cells is indicated in each density plot. (C) Dot representation of KIR3DL1^{high} KIR2D^{+} NK-cell frequencies as a function of KIR2DL1/S1^{+} NK-cell ($n = 22$), KIR2DS4^{+} NK-cell ($n = 10$), KIR2DL2^{+} NK-cell ($n = 8$), and KIR2DL3/2DS2^{+} NK-cell ($n = 22$) frequencies for KIR3DL1$^{high/null}$ individuals. For all dots, the nature of each allele is determined by the color code used previously. (D) Visualization of specific RT-PCR products in a 2% agarose gel in 0.5X TBE buffer with SSP-SM marker (One Lambda) for two KIR3DL1*001, 004 genotyped individuals showing different mean level intensity of the KIR3DL1^{high} NK-cell population and contrasted frequencies, and for one 3DL1*002, *004 (KIR3DL1^{high}) genotyped individual with a low KIR3DL1^{high} NK-cell frequency. RNA was extracted from sorted Z27^{+} NK cells of interest and specific RT-PCR was then performed using KIR3DL1 allele-group–specific PCR-SSP primers adapted from Gardiner et al. [19] showing a specific RT-PCR product at 456 pb for KIR3DL1*001, 277 pb for KIR3DL1*004, and 163 pb for KIR3DL1*002. Negative (C−) and positive (C+) controls were used corresponding respectively to RNA extracted from individuals with different and identical KIR3DL1 allelic typing. (E) Relative quantification of KIR3DL1*001 and KIR3DL1*004 transcripts normalized to the GAPDH reference gene in sorted NK-cell subpopulations from KIR3DL1*001, *004 genotyped individuals with KIR3DL1^{high} ($n = 4$) or KIR3DL1^{low} ($n = 2$). NK-cell populations showing a similar amount of both KIR3DL1*001 and KIR3DL1*004 transcripts whatever the membrane expression level of KIR3DL1. Data are shown as means + SD of six samples pooled from at least three independent experiments.

populations. Because the amount of KIR3DL1*001 and KIR3DL1*004 transcripts might explain the different level of expression in high and low KIR3DL1 NK-cell populations, we evaluated the expression level of KIR3DL1 transcripts by real-time RT-PCR using GAPDH transcripts as an endogenous control. We observed a similar amount of both KIR3DL1*001 and KIR3DL1*004 transcripts in high and low KIR3DL1 populations from different individuals (Fig. 4E).

The nature of the KIR3DL1^{high} allele influences KIR3DL1^{high} NK-cell responsiveness

Among the three KIR3DL1^{high} alleles identified (*001, *002, and *015) in KIR3DL1$^{high/null}$ individuals, consistent expansion of KIR3DL1^{high} NK cells was associated only with the KIR3DL1*001 allele, as mentioned earlier. Thus, we hypothesized that these three KIR3DL1^{high} receptors do not interact with the same affinity

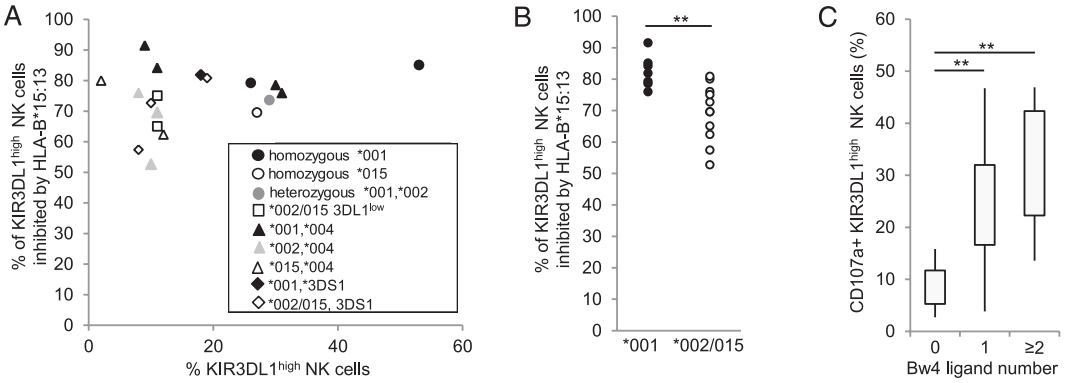

Figure 5. The nature of the KIR3DL1^{high} allele influences KIR3DL1^{high} NK-cell responsiveness. (A) A degranulation test on KIR3DL1^{high} NK cells was done only for Bw4^{+} individuals ($n = 19$) against 221 and 221-B*15:13 (Bw4 motif) cell lines. The percentage of KIR3DL1^{high} NK cells inhibited by HLA-B*15:13 (Bw4 motif) was calculated from the frequencies of KIR3DL1^{high} NK cells expressing CD107a observed following culture with 221 cells (considered as maximum) compared with those observed following culture with the 221-B*15:13 cell line. The percentage of KIR3DL1^{high} NK cells inhibited by 221-B*15:13 for low and high frequencies of KIR3DL1^{high} NK cells depending on KIR3DL1*001 (black), KIR3DL1*002 (filled light gray symbols), and KIR3DL1*015 (open symbols) alleles associated with different KIR3DL1/3DS1 alleles: KIR3DL1^{high} (circle), KIR3DL1^{low} (square), KIR3DL1^{null} (triangle), and KIR3DS1 (diamond) are shown. (B) The percentages of KIR3DL1^{high} NK cells inhibited by HLA-B*15:13 (Bw4 motif) were compared between KIR3DL1*001^{+} ($n = 7$) and KIR3DL1*002/015^{+} individuals ($n = 11$). Statistical significance (**$p < 0.001$) between both groups was determined using the one-way analysis of variance (ANOVA) test. (C) Box and whisker plot summarizing the frequency of CD107a^{+} KIR3DL1^{high} NK cells observed after 5 h of stimulation with standard HLA class I-deficient 221 cell targets as a function of Bw4 ligand number. CD107a^{+} KIR3DL1^{high} NK-cell percentage was compared between 0 and 1 Bw4 ligands and between 1 and ≥2 Bw4 ligands using the one-way ANOVA test. Top and bottom whiskers represent values of the top and bottom 25% of cases, respectively; boxed area, interquartile range, and the significant p values between groups is indicated (**$p < 0.001$, ANOVA test). PBMCs were incubated with 221 cells for 4 h with brefeldin A. To detect the frequency of KIR3DL1^{high} CD107a^{+} NK cells, NK cells were stained with anti-NKp46-allophycocyanin, Z27-PE, and CD107a-PerCP. NKG2A-FITC and 1A6-FITC were added to focus the analysis on KIR3DL1^{high} KIR2D^{-} NKG2A^{-} NK cells. The cells were analyzed by flow cytometry. Data shown are representative of 27 experiments performed.

with the Bw4 ligand and only the KIR3DL1*001 allele product constitutes a strong inhibitory receptor capable of engaging the KIR3DL1^{high} NK-cell subset toward cellular expansion. To test this hypothesis, we determined the degranulation potential (CD107a expression) of NK cells expressing one of these three KIR3DL1^{high} receptors and evaluated the capacity of these three KIR3DL1^{high} receptors to inhibit degranulation of NK cells incubated with 221-HLA-B*15:13 (Bw4 motif) target cells from 19 KIR3DL1^{high} individuals. We observed a higher frequency of KIR3DL1*001^{+} NK-cell inhibition than KIR3DL1*002^{+} or *01502^{+} NK-cell inhibition upon engagement with Bw4 ligand, regardless of the frequency of KIR3DL1^{high} NK cells or the KIR3DL1/3DS1 allele combinations (Fig. 5A). Accordingly, the percentages of KIR3DL1^{high} NK cells inhibited by HLA-B*15:13 (Bw4 motif) were higher in KIR3DL1*001^{+} than in KIR3DL1*002/015^{+} individuals (82.3%, $n = 7$ versus 69.2%, $n = 11$, $p = 0.003$, Fig. 5B). Moreover, we confirmed that the spontaneous degranulation of adult KIR3DL1^{high} NK cells against standard HLA class I-deficient 221 cells increases with the number of autologous Bw4 ligands (9.9% $n = 7$ versus 25.1%, $n = 14$, $p = 0.003$ between 0 and 1 Bw4 ligand and 25.1% $n = 14$ versus 34% $n = 6$, $p = 0.001$ between 1 and ≥2 Bw4 ligands) (Fig. 5C).

Different phenotypic profile of KIR3DL1^{high} NK cells in early life compared with that in adulthood

Because our results suggest either a selection and specific expansion of KIR3DL1^{high} NK-cell subsets or a deletion of KIR3DL1*001,*004 NK subsets, we investigated the KIR3DL1^{high} NK-cell repertoire in cord blood to gain insights into KIR3DL1 NK-cell repertoire formation during development. To this end, we evaluated the frequency of this population and the expression level of the KIR3DL1^{high} receptor taking into account the nature of the second KIR3DL1 allele (high, low, and null). Interestingly, although the KIR3DL1^{high} NK-cell frequency was significantly lower in umbilical PBMCs (9.27%, $n = 18$) than adult PBMCs (19.33%, $n = 68$, $p = 0.001$) (Fig. 6A), whatever the KIR3DL1/3DS1 allele combination analyzed (Fig. 6B), the expression level was significantly higher in cord blood NK cells (umbilical MFI = 1088, $n = 15$ versus adult MFI = 686, $n = 61$, $p = 3.5 \times 10^{-7}$) (Fig. 6C), especially for KIR3DL1$^{high/high}$ (umbilical MFI = 1258, $n = 4$ versus adult MFI = 735, $n = 11$, $p = 0.006$) and KIR3DL1^{high}/3DS1 allele combinations (umbilical MFI = 1268, $n = 6$ versus adult MFI = 689, $n = 19$, $p = 1.5 \times 10^{-5}$) reaching a MFI of 2000 (Fig. 6D). These results show that KIR3DL1^{high} is expressed at a high intensity on the NK-cell membrane in a small proportion of NK cells during early life, probably contributing to the selection or deletion of KIR3DL1 NK-cell subsets, depending on the signaling intensity received by the cells.

The Bw4 environment impacts on KIR3DL1 NK-cell frequency in cord blood samples

Considering that a functional signal via KIR3DL1 may contribute to shaping the KIR3DL1 NK-cell repertoire, we investigated the

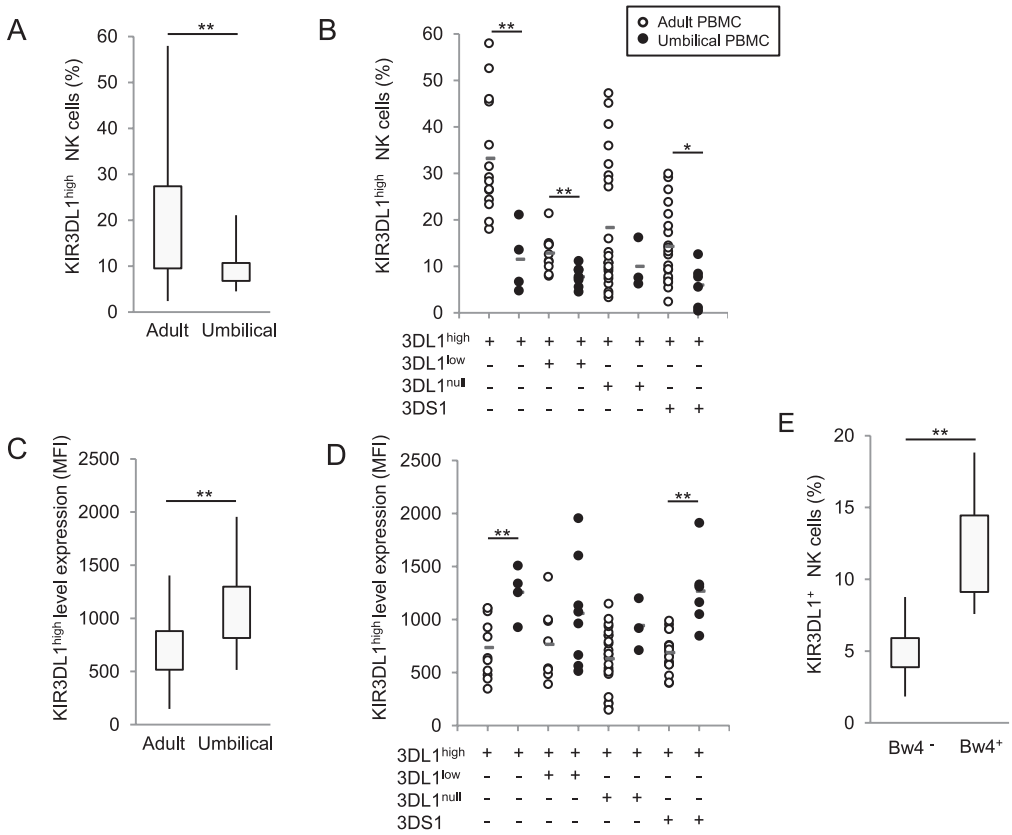

Figure 6. Phenotypic characterization of the KIR3DL1^{high} NK-cell repertoire in cord blood samples. (A) Box and whisker plot summarizing the frequency of KIR3DL1^{high} NK cells in adult ($n = 68$) and umbilical ($n = 18$) PBMCs. Top and bottom whiskers represent values of the top and bottom 25% of cases respectively; boxed area, interquartile range. (B) All KIR3DL1^{high} NK-cell frequencies as a function of the second KIR3DL1 allele or KIR3DS1: KIR3DL1$^{high/high}$ (14 adult and 4 cord blood samples), KIR3DL1$^{high/low}$ (11 adult and 8 cord blood samples), KIR3DL1$^{high/null}$ (23 adult and 3 cord blood samples), or KIR3DL1^{high}/3DS1 (20 adult and 6 cord blood samples) are shown. (C) Box and whisker plot summarizing the mean expression level of KIR3DL1 on NK cells in adult ($n = 61$) and umbilical ($n = 15$) PBMCs. (D) Dot representation of mean expression level of KIR3DL1 on NK cells as a function of the second KIR3DL1 allele or KIR3DS1: KIR3DL1$^{high/high}$ (11 adult and 4 cord blood samples), KIR3DL1$^{high/low}$ (8 adult and 8 cord blood samples), KIR3DL1$^{high/null}$ (23 adult and 3 cord blood samples), or KIR3DL1^{high}/3DS1 (19 adult and 6 cord blood samples). (E) Box and whisker plot summarizing the frequency of KIR3DL1^{+} NK cells in umbilical PBMCs as a function of Bw4^{-} ($n = 7$) or Bw4^{+} cord blood samples ($n = 10$) is shown. Statistical significance (*$p < 0.05$; **$p < 0.01$) between different groups was determined using the one-way ANOVA test. Data shown are representative of 17 experiments performed.

frequency of KIR3DL1 NK cells in early life with regards to the expression or not of autologous Bw4 ligands (Fig. 6E). Interestingly, the KIR3DL1^{+} NK-cell frequency was significantly higher in Bw4^{+} cord blood (12.33%, $n = 10$) than in the Bw4^{-} counterparts (4.97%, $n = 7$, $p = 0.0003$). These results suggest that an engagement toward one KIR3DL1 allele product expression during NK-cell development, possibly depending on functional interaction with Bw4 ligand, contributes to molding the KIR3DL1 NK-cell repertoire. Interestingly, although we confirmed that the spontaneous degranulation of adult KIR3DL1^{high} NK cells increases with the number of autologous Bw4 ligands (Fig. 5C), we did not observe a significant correlation between adult KIR3DL1^{high} NK-cell frequency and Bw4 ligand number (Supporting Information Fig. 1). This result can be partly explained by the plausible deletion of KIR3DL1 NK-cell subsets receiving excessive signals during their development, as observed for KIR3DL1$^{high/null}$ NK-cell subsets in the adult repertoire.

Discussion

In this study, we investigated the impact of allelic KIR3DL1 polymorphism on the KIR3DL1 NK-cell repertoire. In this large cohort of healthy individuals we considered possible contributing factors, including KIR3DL1/S1 allele combinations, mean expression level, Bw4 environment, and functional potential. The French population is characterized by a large KIR3DL1 allelic polymorphism with different predominant KIR3DL1 alleles such as KIR3DL1*001, *002, *004, and *054. The frequency of KIR3DL1^{high} (*001, *002) and KIR3DL1^{null} (*004, *054) alleles ranges from 20 to 50% and this contrasts with the Japanese population which is characterized by (i) only one KIR3DL1^{high} allele (*01502) overexpressed at a very high frequency (45%) compared with the next most frequent allele and (ii) the absence of the KIR3DL1*004 null allele [31]. Surprisingly, the frequency of the null KIR3DL1*004 allele was particularly high in our cohort (29%) compared with

other European or African population studies where it usually ranged from 8 to 16% [17]. Because the Bw4 ligand could differ between populations [37], we have also investigated the frequency and nature of the Bw4 ligand in the French population compared with the Japanese population [31]. The frequency of HLA-B Bw4$^+$ was higher in the French population compared with the Japanese population (59 versus 36%) and was higher than 70% if both HLA-A and HLA-B Bw4$^+$ were taken into account. Although the frequency of HLA-B51 (I^{80}) was similar in both populations (13%), HLA-B44 (T^{80}) was overrepresented in the French population (30.2 versus 7%) whereas A24 (I^{80}) was underrepresented (27.4 versus 41%). However, in both populations, the frequency of KIR3DL1^{high} NK cells was higher than that of the KIR3DL1^{low} NK-cell population and the frequency of KIR3DL1^{high} NK cells increased with gene dose. In the French population, the presence of Bw4 ligand (both HLA-A and HLA-B) or the presence of a specific HLA-Bw4 molecule such as A24, B51, B44 did not significantly increase the frequency of KIR3DL1^{high} NK cells, while in the Japanese population, the presence of HLA-Bw4$^+$ molecules significantly increased the frequency of NK cells expressing KIR3DL1 for donors with two high-binding 3DL1 allotypes, and the increase in frequency of KIR3DL1^{high} NK cells was greater in Bw4 heterozygotes than in homozygotes. The fact that the Bw4 environment did not significantly affect the KIR3DL1 NK-cell frequency in our French cohort may be due to the sample size. Overall, our study confirms that French individuals are characterized by a broad KIR3DL1 allelic polymorphism in accordance with previous studies in other European populations [17] and that the KIR3DL1 NK-cell repertoire is mainly determined by KIR3DL1 polymorphism and KIR3DL1/3DS1 allele combinations. The extensive KIR3DL1 allelic polymorphism and diversity of KIR3DL1 ligands observed in the French population suggest a coevolution of KIR3DL1 and HLA-A, HLA-B Bw4 genes as reported in other populations [37].

The phenotypic study of KIR3DL1 allelic polymorphism in healthy individuals enabled us to show that all individuals hitherto described as negative for the KIR3DL1 gene actually possess an KIR3DL1*054 allele that is not detected with the current standard typing techniques, and a substantial proportion of individuals have at least one KIR3DL1 allele that is not, or only weakly, expressed on the NK-cell surface. The association of the KIR3DL1*054 allotype with KIR3DS1 in our cohort did not unable us to evaluate NK-cell surface expression of KIR3DL1*054 since both receptors seemed to be recognized similarly by the Z27 mAb [36]. Interestingly, in our cohort, the KIR3DL1*054 allele was mainly observed in individuals with the BB KIR genotype ($n = 9$). While the expression and function of the KIR3DL1*004 allele is well understood, [34] that of the KIR3DL1*019, KIR3DL1*054 alleles remain to be elucidated. The expected mature KIR3DL1*019 protein contains the same amino acids involved in the intracellular retention of the null KIR3DL1*004 allotype, i.e. L^{86} and S^{182}, and only one amino acid in the D0 domain differs between KIR3DL1*019 and KIR3DL1*004. Thus, we believe that the KIR3DL1*019 allele is also a null allele. However, different amino acid substitutions occur between KIR3DL1*054 and KIR3DL1*004 suggesting that KIR3DL1*054 is closer to KIR3DS1 than KIR3DL1. These amino acid changes in the extracellular domains could potentially interfere with Z27/DX9 epitope recognition. These results should shed light on the functional contribution of the frequent KIR3DL1*054 and KIR3DL1*004 receptors to different pathologies. Indeed, a high frequency of KIR3DL1-negative individuals has been reported in an autoimmune disease [38], which probably corresponded to the KIR3DL1*054 allele. Moreover, in the presence of Bw4, KIR3DL1*004 showed the most significant protection relative to all other KIR3DL1 alleles in HIV infection [29] and its functional expression at the cell surface has been recently demonstrated [35].

Our data underline the impact of KIR3DL1 allele polymorphism on its NK-cell expression pattern and on the hierarchy between all KIR3DL1 alleles, highlighting the predominance of the KIR3DL1*001 allele regardless of the presence or absence of the second allele (KIR3DL1^{low}, KIR3DL1^{null}, or KIR3DS1). In the KIR3DL1^{high,null} individuals with only one copy of the KIR3DL1*001 allele, the frequency of KIR3DL1^{high} NK cells increases up to 50% of NK cells, indicating that factors other than copy number contribute to this dominant expression. The high number of KIR3DL1*001,*004 individuals in our cohort led us to detect a bimodal distribution of KIR3DL1^{high} NK cells: A first group with a high frequency of KIR3DL1^{high} NK cells and high level of expression, and a second group with a low frequency of KIR3DL1^{high} NK cells and a low level expression. Of note, we detected the same amount of KIR3DL1*001 and KIR3DL1*004 transcripts in the low and high frequency of KIR3DL1^{high} NK cells, demonstrating that the KIR3DL1 transcript level is not linked to its protein expression at the NK-cell surface. This finding is in accordance with data demonstrating the frequent detection of KIR transcripts in T-cell clones and NK92 in the absence of the corresponding protein at the cell surface [39]. Thus, the predominance of the KIR3DL1*001 allele in our study could not be explained by its promoter properties [40]. Nonetheless, we cannot exclude the possibility of a differential internalization of KIR3DL1 receptor in both populations. Among other factors able to influence the frequency of KIR3DL1^{high} NK cells, we looked at the expression of other KIR in different groups of KIR3DL1^{high,null} individuals. Interestingly, all KIR3DL1^{high,null} individuals with KIR3DL1*002 and KIR3DL1*015 alleles showing a low frequency and high mean level expression of the KIR3DL1 receptor expressed KIR2DS4. However, all KIR3DL1*001, *004 individuals bore only nonexpressed KIR2DS4*003 as activating KIR genes (AA genotypes) and other activating KIR genes, such as KIR2DS2, were observed in four out of eight others KIR3DL1*001, *004 genotyped individuals with a high frequency and mean level expression of KIR3DL1. Thus, since the acquisition of KIR on NK cells is sequential [41], it is possible that the nature of the other KIR expressed before KIR3DL1 and probably the transduction signals received by the cells, influence the posttranscriptional regulation of the KIR3DL1*001 and KIR3DL1*004 alleles. However, the bimodal distribution cannot be entirely explained by a differential expression of activating KIR.

Another explanation of the bimodal distribution could be due to a functional selection of KIR3DL1 NK cells. Indeed, we also underlined a hierarchy between KIR3DL1^{high} alleles in their capacity to inhibit NK-cell degranulation via Bw4 engagement, and possibly

to promote in vivo maintenance or expansion of peripheral NK cells carrying the corresponding allele. As such, the frequency of KIR3DL1*001$^+$ NK-cell inhibition is higher than the frequency of KIR3DL1*002 or *015 NK-cell inhibition upon engagement with HLA-B*15:13 (Bw4 motif) expressed on transfected 221 target cells regardless of the frequency of KIR3DL1^{high} NK cells. This last point is in agreement with a previous report indicating that highly expressed KIR3DL1*002 is a better inhibitor than poorly expressed KIR3DL1*007 [27]. Based on recent data published by Taner et al. [35] and on the higher level expression of KIR3DL1 observed in cord blood, we speculate that KIR3DL1*004 can be functional, and may constitute a better signaling receptor than the KIR3DL1*002, *015, and *008 allele products, favoring its phenotypic selection during early life. Thus, for all 10 studied KIR3DL1*002/*01502, *004 genotyped individuals, we suggest that a phenotypic selection in favor of the KIR3DL1*004 receptor, which was not detected by KIR3DL1 specific Z27 and DX9 antibodies may explain the frequency of KIR3DL1^{high} observed around 10% of NK cells. For 5 KIR3DL1*001,*004 genotyped individuals, the frequency of KIR3DL1^{high} was low (around 10%) as was the mean fluorescent intensity, suggesting low expression of the KIR3DL1*001 receptor on NK cells. This low frequency seems to be due to a negative selection of this subset as previously described for T-lymphocyte selection. Selection and amplification of KIR3DL1^{high} NK cells seem to be dependent on the nature of the KIR3DL1 allele and the KIR3DL1 allele combination, which is expressed with a hierarchy favoring KIR3DL1*001, followed by KIR3DL1*004, and then the other KIR3DL1^{high} alleles (*002, *015, and *008). The engagement of two receptors such as KIR3DL1*001 and *004, thereby inducing increased signals, might lead to a negative selection of NK cells.

In this study, we did not observe any increased frequency of KIR (KIR2DL1/S1, KIR2DL2/3/S2, or KIR2DS4) NK-cell subsets balancing a low frequency of KIR3DL1^{high} NK-cell subsets in different individuals grouped by KIR3DL1 alleles or KIR3DL1/3DS1 allele combinations (data not shown). However, we observed a constant proportion of KIR3DL1^{high} NK cells expressing KIR2DL1/2/3, 2DS1/2 receptors, which suggests a subsequent acquisition of KIR2D by KIR3DL1^{high} NK cells. Previous in vitro investigations of NK-cell differentiation have been performed suggesting a sequential acquisition of KIR with the expression of KIR2DL3 before KIR2DL1 [42]. Although KIR3DL1 acquisition was observed regardless of HLA background [43], its acquisition in this sequential model has not been investigated. Even though it is now clear that KIR ligands contribute to the functional education of NK cells [4], HLA class I molecules, as KIR ligands, should partially affect the formation of the KIR NK-cell repertoire at the neonatal stage [31, 44], as observed in our study. As previously described [9], in our study we did not observe an impact of the Bw4 environment on the adult KIR3DL1 NK-cell repertoire. Other factors, and particularly individual immunological experience, seem to contribute to shaping the KIR NK-cell repertoire throughout life. The finding of amplified KIR$^+$ NKG2C$^+$ NK cells in CMV [45–47], HSV-2 [48], and HIV infections [49, 50] reinforces this hypothesis. In the case of KIR3DL1, it is conceivable that viral infections, particularly those involving viruses that negatively modulate HLA class I molecule expression such as CMV and HIV, trigger KIR3DL1 NK-cell subsets via KIR3DL1 or an undetermined coexpressed receptor. Further investigations in KIR3DL1$^{high/null}$ individuals taking into account viral status and a broader phenotype of KIR3DL1^{high} NK cells, notably including NKG2C, should help to determine the impact of viral infection on the KIR3DL1 NK-cell repertoire. Finally, this study provides new insight into the mechanisms potentially involved in shaping the KIR3DL1 NK-cell repertoire.

Materials and methods

Cells (PBMCs, cord blood samples, and cell lines)

PBMCs were isolated from citrate-phosphate-dextrose blood, collected from healthy adult volunteers by gradient centrifugation on Ficoll-Hypaque (Lymphoprep, Axis-Shield, PoC AS, Oslo, Norway). All blood donors ($n = 109$) were recruited at the Blood Transfusion Center (Etablissement Français du Sang, Nantes, France) and informed consent was obtained from all individuals. Umbilical cord blood samples ($n = 23$) were obtained at the Nantes CHU maternity unit. Informed consent was obtained from all mothers. HLA class-I–deficient 721.221 lymphoblastoid EBV-B cells (referred to as 221 cells) and Bw4 (B*15:13) transfected 221 cells (referred to as 221-Bw4$^+$) were used as controls to assess natural NK-cell cytotoxicity in functional assays. Cells were cultured in RPMI 1640 medium (Gibco, Paisley, Scotland, UK) containing glutamine (Gibco) and penicillin-streptomycin (Gibco) and supplemented with 10% fetal bovine serum (Gibco). Mycoplasma tests performed by PCR were negative for all cell lines.

HLA and KIR genotyping

Genomic DNA was extracted from PBMC and from cord blood samples using either a classical salting-out method [51] or by GenoM-6 (Qiagen, Courtaboeuf, France) using magnetic beads. Intermediate or high-resolution typing for HLA-A, HLA-B, and HLA-C was performed on all healthy donors ($n = 109$) and cord blood samples ($n = 23$) using a Sequence Based Typing kit (Abbott Molecular Park, IL, USA). All individuals were typed for the presence or absence of KIR2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 3DL1, 3DL2, 3DL3, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, and 3DS1 using the KIR genotyping SSP kit from Invitrogen (Compiègne, France), lot#003, under the conditions recommended by the manufacturer. Cord blood samples were typed using a multiplex PCR-SSP method as previously described [52]. In order to detect all KIR3DL1 alleles, especially the KIR3DL1*054 allele not amplified by the PCR-SSP Invitrogen kit lot#003, individuals negative for KIR3DL1 at the genotypic level were further typed using homemade KIR3DL1 generic primers specific for exon 3 [53], which amplified all KIR3DL1

alleles except KIR3DL1*027, *028, *030, *039, *042, *053, *057, *073 (all of which were amplified by the Invitrogen KIR PCR-SSP kit). For KIR3DL1 allelic typing, KIR3DL1 allele-group–specific PCR-SSP and sequencing of polymorphic exons were combined in order to resolve most of the remaining KIR3DL1 allele ambiguities arising as a result of the large KIR3DL1 allelic polymorphism described to date. For the subtyping of KIR3DL1 PCR-SSP coding sequences, primers designed to discriminate allele-group–specific polymorphisms were paired with KIR3DL1 locus-specific primers adapted from Gardiner et al. [19]. Depending on the allele-group–specific amplification products obtained, KIR3DL1 exons 1, 2, 3, 4, 5, or 7–9 were further sequenced to resolve remaining ambiguities as previously described [17, 24]. Sequence data files were analyzed using Assign-SBT software (Conexio Genomics, Applecross, Australia) with IPD-KIR Database version 2.3.0 (August 2010).

Identification and quantification of KIR3DL1 transcripts in the KIR3DL1$^+$ NK-cell population

For individuals genotyped as KIR3DL1*001,*004, KIR3DL1*002,*004, and KIR3DL1*002,*005, Z27$^+$ NK cells were positively selected using KIR3DL1-specific antibody Z27 (Beckman Coulter, Immunotech) and murine IgG-coupled magnetic Dynabeads according to the manufacturer's instructions (Dynal, Oslo, Norway). Beads and KIR3DL1-specific antibody were removed using goat anti-mouse IgG antiserum (EFS) as described previously [54]. Selected cells were stimulated using an in vitro model of NK-cell expansion [5]. After 2 weeks of amplification, Z27$^+$ NK-cell populations were sorted and total RNA was purified using NucleoSpin RNAXS (Macherey-Nagel). Qualitative RT-PCR was performed using a One-Step PrimeScript^{TM} RT-PCR kit (TaKaRa, Japan) with KIR3DL1 allele-group–specific PCR-SSP primers adapted from Gardiner et al. [19]. For relative quantification, KIR3DL1*001 and KIR3DL1*004 transcript levels were normalized to the GAPDH reference gene using the same primers and the One-Step SYBR® PrimeScript^{TM} RT-PCR kit (TaKaRa, Japan). For each sample, qRT-PCR was performed in triplicate on a RotorGene RG6000® RT-PCR system (Corbett Research). Amplification programs were as follows: reverse transcription at 42°C for 5 min, initial denaturation at 95°C for 5 min, 5 cycles at 97°C for 20 s, 55°C (GAPDH, KIR3DL1*004) or 62°C (KIR3DL1*001) for 30 s, 72°C for 1 min, and 30 cycles at 95°C for 15 s, 57°C (GAPDH, KIR3DL1*004) or 64°C (KIR3DL1*001) for 30 s, 72°C for 1 min. A melting curve analysis was performed after each run, with one degree increments from 72 to 95°C. The relative levels of KIR3DL1 allele transcripts were compared using unpaired *t*-tests in different sorted NK-cell subsets.

Phenotypic analysis by flow cytometry

NK-cell surface phenotype was determined by four-color flow cytometry using the following mouse antihuman mAbs: anti-KIR3DL1/S1-PE (Z27), anti-KIR3DL1-FITC (DX9) (Beckman Coulter, Immunotech), anti-KIR2DL1/2/3/2DS1/2 (1A6) [55], anti-KIR2DL3/2DS2-FITC (1F12) [55], anti-KIR2DS4-PE (FES175), anti-KIR2DL1/S1-PE (EB6), anti-KIR2DL2/3/2DS2-PE (GL183) (Beckman Coulter, Immunotech), anti-CD3-PerCP (SK7), and anti-CD56-allophycocyanin (B159) (BD Biosciences). Cells were also stained with the corresponding isotype-matched control mAb. Data were collected using a FACSCalibur (BD Biosciences) and analyzed using Flowjo 6.2 software (TreeStar).

CD107a mobilization detected by flow cytometry

KIR3DL1^{high} NK cells were tested for their cytolytic potential with the CD107a mobilization assay after stimulation with 221 or 221-Bw4$^+$ (HLA-B*15:13) transfected cell lines. Preincubated NK cells with CD107-PECy5 (H4A3, BD Biosciences) were incubated with the target cells for 5 h at an effector:target ratio of 1:1, with brefeldin A (Sigma) at 10 μg/mL for the last 4 h. The cells were surface stained with Z27-PE, NKG2A-FITC and NKp46-allophycocyanin (9E2, BD Biosciences). Data were collected using a FACSCalibur (BD Biosciences) and analyzed using Flowjo 6.2 software (TreeStar).

Statistical analyses

Comparisons of KIR3DL1$^+$ NK-cell frequencies in two different series of individuals were performed using the Student's *t*-test. Association between CD107a$^+$ KIR3DL1^{high} NK-cell frequencies and autologous Bw4 ligand number in two different series of individuals was tested using the one-way ANOVA test. *p*-values <0.05 were considered as statistically significant. Spearman's rank correlation coefficients were calculated and indicated only when a significant *p*-value was obtained ($p < 0.05$).

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Abbreviations: **KIR**: Killer Immunoglobulin-like Receptor

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Amplified NKG2C^{+} NK Cells in Cytomegalovirus (CMV) Infection Preferentially Express Killer Cell Ig-like Receptor 2DL: Functional Impact in Controlling CMV-Infected Dendritic Cells

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Amplified NKG2C$^+$ NK Cells in Cytomegalovirus (CMV) Infection Preferentially Express Killer Cell Ig-like Receptor 2DL: Functional Impact in Controlling CMV-Infected Dendritic Cells

Zakia Djaoud,* Gaëlle David,* Céline Bressollette,† Catherine Willem,* Pauline Rettman,* Katia Gagne,*,‡,§ Nolwenn Legrand,* Souad Mehlal,* Anne Cesbron,*,‡,§ Berthe-Marie Imbert-Marcille,† and Christelle Retière*

CMV infection represents a major complication in hematopoietic stem cell transplantation, which compromises graft outcome. Downregulation of HLA class I expression is one mechanism by which CMV evades T cell–mediated immune detection, rendering infected cells vulnerable to killer cell Ig-like receptor (KIR)$^+$ NK cells. In this study, we observed that the amplified NKG2C$^+$ NK cell population observed specifically in CMV seropositive individuals mainly expressed KIR2DL receptors. We have shown that HLA class I expression was downregulated on CMV-infected immature dendritic cells (iDCs), which escape to HLA-A2-pp65–specific T lymphocytes but strongly trigger the degranulation of KIR2D$^+$ NK cells. CMV infection conferred a vulnerability of C2C2$^+$ iDCs to educated KIR2DL1$^+$ and KIR2DL3$^+$ NK cell subsets. Alloreactivity of KIR2DL1$^+$ NK cell subsets against C1C1$^+$ iDCs was maintained independently of CMV infection. Unexpectedly, CMV-infected C1C1$^+$ iDCs did not activate KIR2DL3$^+$ NK cell reactivity, suggesting a potential CMV evasion to KIR2DL3 NK cell recognition. Altogether, the coexpression of KIR and NKG2C on expanded NK cell subsets could be related to a functional contribution of KIR in CMV infection and should be investigated in hematopoietic stem cell transplantation, in which the beneficial impact of CMV infection has been reported on the graft-versus-leukemia effect. ***The Journal of Immunology*****, 2013, 191: 2708–2716.**

Human CMV is a β-herpesvirus that establishes a latent infection in healthy individuals but causes a high rate of morbidity in immunocompromised patients and during fetal development. For an effective defense against CMV, both innate and adaptive immune responses are involved (1). Thanks to different inhibitory receptors, NK cells are able to detect the loss of HLA class I molecules from autologous cells, a situation that can occur when cells are perturbed by viral infection or tumor transformation (the "missing self" hypothesis) (2). Indeed, NK cells express different inhibitory receptors for self–HLA class I molecules, including CD94/NKG2A, killer cell Ig-like receptors (KIRs) and ILT2 (LILRB1). The CD94/NKG2A receptor recognizes leader peptides of most HLA-A, -B, -C, and -G molecules presented by the HLA-E molecule (3). Nevertheless, inhibitory KIRs display a more specific recognition of different HLA class I ligands. For example, HLA-C allotypes with asparagine at position 80 (C1 ligands) are recognized by KIR2DL2/3 whereas HLA-C allotypes with lysine at position 80 (C2 ligands) are recognized by KIR2DL1 (4). Additionally, ILT2 recognizes all HLA class I molecules. Moreover, the interaction between HLA class I molecules and inhibitory receptors is essential in NK cell acquisition of self-tolerance and functional abilities (5).

Soon after infection, different viral proteins downregulate HLA class I molecule expression on infected cells, leading to escape from CMV-specific T lymphocytes, but they render these infected cells vulnerable to NK cell lysis (6). Furthermore, CMV may also escape NK surveillance by maintaining the expression of nonclassical HLA class I molecules, HLA-E and HLA-G, serving as ligands for the inhibitory receptors CD94/NKG2A and ILT2 (7). Recently it has been shown that expansion of NK cells expressing the activating NKG2C counterpart is associated with CMV infection (8–12). Furthermore, CMV has developed different strategies to prevent the expression of ligands for some activating NK cell receptors (13, 14). However, no document discusses the involvement of KIR NK cells in controlling CMV infection or the potential CMV evasion to KIR NK cells. The hypothesis of a prospective role of KIR NK cells in controlling CMV is reinforced by the knowledge gained from the murine model. In fact, Ly49 receptors, analogous to KIR receptors, are mainly engaged to control murine CMV infections (15).

In allogeneic hematopoietic stem cell transplantation (HSCT), CMV infections are considered a major complication, which may compromise graft outcome. After transplantation, lymphoid T cell reconstitution is delayed, resulting in reactivation of latent viral

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Abbreviations used in this article: HSCT, hematopoietic stem cell transplantation; iDC, immature dendritic cell; KIR, killer cell Ig-like receptor; SSP, sequence-specific primer.

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infections such as CMV. However, NK cells that rapidly reconstitute hematopoiesis and play a crucial role in mediating the graft-versus-leukemia (GVL) effect constitute the first line of defense against viral infection. In HLA haploidentical HSCT, NK cell alloreactivity is due to the presence in the donor of NK cells expressing KIR that recognize HLA class I allotypes present in the donor but absent in the recipient (16). KIR genotypic studies have revealed the beneficial effect of activating KIR genes against CMV infection or reactivation in different graft contexts (16–18). During acute CMV infection, NKG2C$^+$ NK cells expand in HSCT recipients and predominately express KIR (9). However, the contribution of NKG2C in eliminating CMV-infected cells via NK cells has not been demonstrated. It is possible that NKG2C mainly constitutes a CMV infection marker. Thus, the coexpression of NKG2C with KIR on this expanded NK cell population questions the functional KIR contribution to CMV infection control. Moreover, although a recent report suggests the beneficial impact of early CMV infection on the GVL effect in HSCT (19), whether CMV modulates KIR NK cell alloreactivity in an HSCT context has not been investigated. Thus, to address these questions, we have set up a cell culture model based on a CMV-infected immature monocyte-derived dendritic cells (iDCs) as target cells, taking into account the HLA environment, to investigate not only the functional contribution of KIR NK cell subsets in controlling CMV infection but also the impact of CMV in modulating KIR NK cell alloreactivity.

Materials and Methods

PBMCs and cell lines

PBMCs were isolated from citrate-phosphate-dextrose blood from healthy adult volunteers by gradient centrifugation on Ficoll-Hypaque (Lymphoprep; Axis-Shield, Oslo, Norway). All blood donors were recruited at the Blood Transfusion Center (Etablissement Français du Sang, Nantes, France) and informed consent was obtained from all individuals. CMV serological status was determined using the chemiluminescent immunoassay LIAISON CMV IgG II (DiaSorin). EBV-B cell lines were obtained by EBV transformation of peripheral B cells using EBV supernatant harvested from the cell line B95-8 (American Type Culture Collection). B cells were isolated from PBMCs obtained from healthy individuals. The HLA class I–deficient acute myeloid leukemia K562 cell line was used to determine the degranulation potential of NK cells. K562 cells and EBV-B lymphocytes were cultured in RPMI 1640 medium (Life Technologies, Paisley, U.K.) containing glutamine (Life Technologies) and penicillin-streptomycin (Life Technologies) and supplemented with 10% FBS (Life Technologies). *Mycoplasma* tests performed by PCR were negative for all cell lines.

HLA and KIR genotyping

Genomic DNA was extracted from PBMCs using a classical salting-out method (20). HLA-A, -B, and -Cw genes were typed via molecular techniques using PCR-sequence-specific primer (SSP) kit from GenoVision (Olerup SSP HLA-A, -B, and -Cw, BioNoBis, Montfort l'Amaury, France) under the conditions recommended by the manufacturer. KIR genes were typed via molecular techniques using PCR-SSP methods under the conditions recommended by the manufacturers, as described previously (21, 22).

Isolation of monocyte and generation of iDCs

Monocytes were separated from lymphocytes by using a high-density hyperosmotic Percoll density gradient (Sigma-Aldrich) (23) and cultured for 6 d in RPMI 1640 medium (Life Technologies) containing glutamine (Life Technologies), penicillin-streptomycin (Life Technologies), and supplemented with 10% human AB serum (Life Technologies), IL-5 (200U/ml; CellGenix), and GM-CSF (1000 U/ml; CellGenix).

NK cell isolation and amplification

PBMCs were isolated as previously described (24, 25). Thereafter, NK cells were amplified after in vitro stimulation with irradiated C1$^+$ and C2$^+$ allogeneic PBMCs and EBV-B cells used as feeders following the PBMC/EBV-B cell ratio at 10:1. All amplified KIR2D NK cells were NKG2C negative.

Isolation and amplification of CMV-specific T cell lymphocytes

CMV-specific T lymphocytes were sorted from HLA-A2$^+$ CMV seropositive blood donors using anti–CD3-PerCP (SK7; BD Biosciences) and HLA-A*02:01-pp65 (NLVPMVATV) (tetramer-allophycocyanin, obtained after tetramerization of HLA-A2-pp65 monomer [platforme Structure Fédérative de Recherche François Bonamy, Nantes, France] with allophycocyanin-streptavidin [AnaSpec]). Sorted cells were stimulated with irradiated feeder cells (allogeneic PBMCs and a mix of two irradiated EBV-B cell lines) and 1 μg/ml PHA-P (L-7132; Sigma-Aldrich). After 2 wk, specific stimulation using pp65 (1 mg/ml)-loaded autologous EBV-B cells (E:T ratio of 10:1) was performed. pp65-Specific lymphocyte amplification was monitored by flow cytometry using HLA-A2-pp65-PE pentamer (Proimmune, Oxford, U.K.).

CMV preparation and infection of iDCs

Stock suspensions of CMV strain VHL/E (provided by the Prof. James Waldman, Columbus, OH) were produced as previously described (26). Of note, the full genomic sequence of VHL/E is not available and thus its genetic content has not been established. Infectivity titration of the virus was performed on iDCs. After overnight coculture of iDCs alone or with VHL/E, cells were washed and resuspended in RPMI 1640, 10% FBS.

Phenotypic analysis by flow cytometry

The NK cell surface phenotype was determined by three- or four-color flow cytometry using the following mouse anti-human mAbs: anti–KIR2DL1-FITC (143211; R&D Systems), anti–KIR2DL2/2DL3/2DS2-PE (GL183; Beckman Coulter), anti–KIR2DL1/2/3/2DS1/2-FITC (1A6) (27), anti–NKp46-allophycocyanin (9E2; Becton Dickinson), anti–NKG2C-PE (134591; R&D Systems), anti-NKG2A (Z199; Beckman Coulter), anti–CD3-PerCP (SK7; BD Biosciences), anti–CD56-allophycocyanin (B159; BD Biosciences), anti-NKG2D (ID11; BD Biosciences), anti-NKp30 (Z25; Beckman Coulter), and anti-NKp44 (Z231; Beckman Coulter). iDCs were stained with the following mouse anti-human mAbs: anti–HLA-A, -B, -C (F41-IE3H1D2; EFS), anti–HLA-C (L31; MediaPharma), anti–HLA-E-PE (3D12; Miltenyi Biotec), anti–CD40-PE (5C3; BD Biosciences), anti–CD80-PE (L307.4; BD Biosciences), anti–CD83-allophycocyanin (HB15e; BD Biosciences), anti–CD86-PE (2331; BD Biosciences), anti–HLA-A, -B, -C–AF^{647} (F41-IE3H1D2; EFS), anti–MICA/B-PE (6D4; BD Biosciences), and isotype-matched controls (IgG1 from BD Pharmingen, IgG2a from R&D Systems). Thereafter, iDCs were washed, fixed, and permeabilized for a staining with major immediate-early IE-1 protein-specific mAb (8B1.2; Millipore), which was conjugated to FITC (Sigma-Aldrich) (28).

CD107a mobilization assay detected by flow cytometry

PBMCs and NK cells were preincubated with anti-CD107a (H4A3; BD Biosciences). NK cell degranulation was assessed after incubation for 5 h alone (negative control), with K562 cells, or with uninfected or CMV-infected allogeneic iDCs (E:T ratio of 10:1). Cell surface staining was performed using the following mouse anti-human mAbs: anti–KIR2DL1/2/3/2DS1/2-FITC (27), anti–NKG2C-PE (134591; R&D Systems), anti–NKp46-allophycocyanin (9E2; Becton Dickinson), anti–KIR2DL1-FITC (143211; R&D Systems), anti–KIR2DL2/3/2DS2-PE (GL183; Beckman Coulter, Immunotech), and NKG2A-FITC or -PE (Z199; Beckman Coulter). HLA-C, HLA-E, and KIR2D neutralization were performed using anti–HLA class I (W6/32; R&D Systems), anti–HLA-E (3D12; BioLegend), and anti–KIR2DL1/2/3/2DS2 (8C11) (27) mAbs, respectively. All flow cytometry data were collected using a FACSCalibur (BD Biosciences) and analyzed using FlowJo 7.6.1 software (Tree Star).

Statistical analyses

Comparisons of NK cell frequencies between two different series of individuals were performed using ANOVA test. A p value of <0.05 was considered to be statistically significant.

Results

Preferential expression of KIR2DL on amplified NKG2C$^+$ NK cells in CMV seropositive individuals

Recently, some studies have shown that a variable expansion of NK cell subsets coexpressing NKG2C and KIR are associated with CMV infection (8–11). We therefore assessed the expression of KIR and NKG2C to determine what NK cell subsets are mainly

observed in CMV seropositive individuals ($n = 31$) compared with seronegative individuals ($n = 29$) (Fig. 1). By four-color flow cytometry, three different NK cell subsets were defined on the basis of NKG2C and KIR2D expression ($NKG2C^+KIR2D^-$, $NKG2C^+KIR2D^+$, and $NKG2C^-KIR2D^+$) using a KIR2DL1/L2/L3/2DS1/S2-specific mAb (1A6) (27) and NKG2C-specific mAb in CMV^- versus CMV^+ individuals (Fig. 1A). In agreement with previous reports, the frequencies of $NKG2C^+$ and $NKG2C^+KIR2D^+$ NK cells are significantly higher in CMV seropositive than seronegative individuals ($p = 0.004$ and $p = 0.005$, respectively) (Fig. 1B). $NKG2C^+$ NK cells did not express NKG2A and preferentially expressed CD57 (data not shown), as previously described in HSCT recipients and immunocompetent CMV seropositive individuals (9). Interestingly, expansion of the $NKG2C^+KIR2D^+$ NK cell population in CMV seropositive individuals is associated to the AA KIR genotype (Fig. 1C), although the probability is not significant ($p = 0.08$), which is confirmed by preferential expression of inhibitory KIR on the $NKG2C^+$ NK cell subset (Fig. 1D). Indeed, of the 12 individuals with an $NKG2C^+KIR2D^+$ NK cell frequency superior to the mean value (6.8%), 11 are KIR2DL3 genotyped and coexpressed KIR2DL3 and $NKG2C^+$ whatever the HLA background (2 C2C2, 4 C1C1, and 5 C1C2 individuals) (Fig. 1D). However, only one individual (C1C2) coexpressed KIR2DL1 with KIR2DL3 and NKG2C (Fig. 1D). In B^+ KIR genotyped individuals, activating KIR2DS2 was coexpressed on $NKG2C^+$ cells in one $KIR2DL3^-KIR2DL2/S2^+$ C2C2 genotyped individual (data not shown). KIR2DS1 expression was evaluated using the combination of KIR2DL1/2DL2/2DL3/2DS2-specific 8C11 and KIR2DL1/S1-specific EB6 mAbs in 2 KIR2DS1 genotyped individuals, and no KIR2DS1 expression was observed on expanded $NKG2C^+$ NK cells. Of note, all three KIR3DS1 genotyped individuals expressed KIR3DS1 on the expanded $KIR2DL3^+NKG2C^+$ NK cell subset. KIR3DL1 was coexpressed with $NKG2C^+$ and $KIR2DL3^+$ in 5 of 10 individuals expressing KIR3DL1 (data not shown).

Downregulation of HLA class I molecule expression on CMV-infected iDCs

It has been previously reported that monocyte-derived DCs are susceptible to in vitro CMV infection by the endothelial cell–adapted strain VHL/E (29). Thus, after 48 h of CMV infection, iDCs were stained with an mAb specific for the CMV major immediate-early IE-1 protein (Fig. 2A). Based on the percentage of $IE\text{-}1^+$ cells, 50% of values from 20 independent experiments are comprised between first quartile (Q1 = 39.5%) and the third quartile (Q3 = 56%) around a median of 48% (Fig. 2B). Interestingly, HLA ligands of NK receptors as HLA class I and especially HLA-C molecules (ligands of KIR2DL) were downregulated on CMV-infected iDCs. In the same line, the nonclassical HLA-E molecule (ligand of CD94/NKG2A or CD94/NKG2C), weakly expressed on iDCs, was downregulated on CMV-infected iDCs (Fig. 2C). As previously described, CMV infection does not induce maturation of iDCs (30).

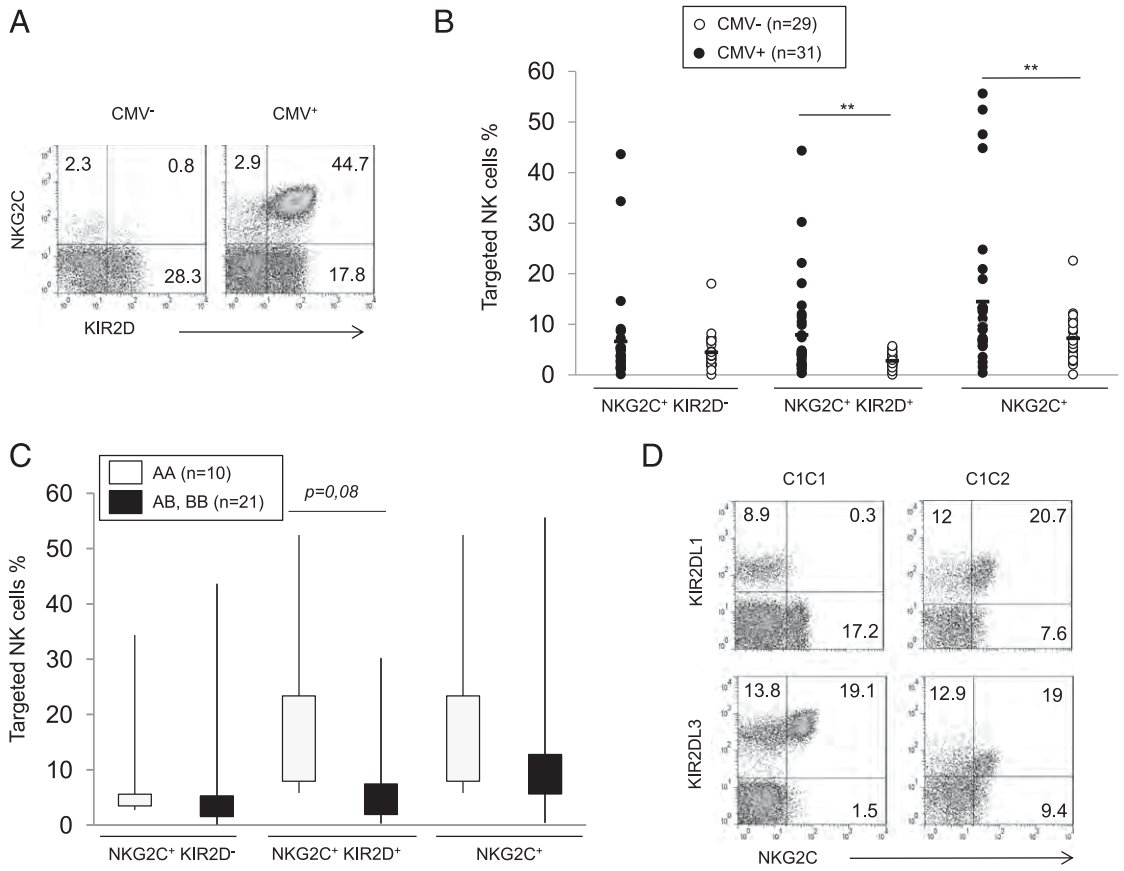

FIGURE 1. Preferential expression of KIR2DL on amplified $NKG2C^+$ NK cells in human CMV seropositive individuals. (**A**) Representative density plots illustrating NK cell phenotype from CMV^- and CMV^+ individuals. NK cells were stained with anti-CD56, anti-NKG2C, and anti-KIR2DL1/L2/L3/2DS1/S2 (1A6) mAbs. (**B**) Scatter plots displaying the $NKG2C^+KIR2D^-$, $NKG2C^+KIR2D^+$, and $NKG2C^+$ NK cell frequencies from CMV^- ($n = 29$) and CMV^+ ($n = 31$) individuals. (**C**) Summary box and whisker plot showing the frequency of $NKG2C^+KIR2D^-$, $NKG2C^+KIR2D^+$, and $NKG2C^+$ NK cells from CMV^+ AA KIR genotyped ($n = 10$) and AB and BB KIR genotyped ($n = 21$) individuals. (**D**) Representative density plots of four C1C1 and one C1C2 CMV^+ individuals. $KIR2DL3^+NKG2C^+$ and $KIR2DL1^+NKG2C^+$ NK cells are targeted with anti-NKG2C and GL183 and with anti-NKG2C and EB6, respectively. Statistical significance ($^{**}p < 0.01$) between both groups was determined using an ANOVA test.

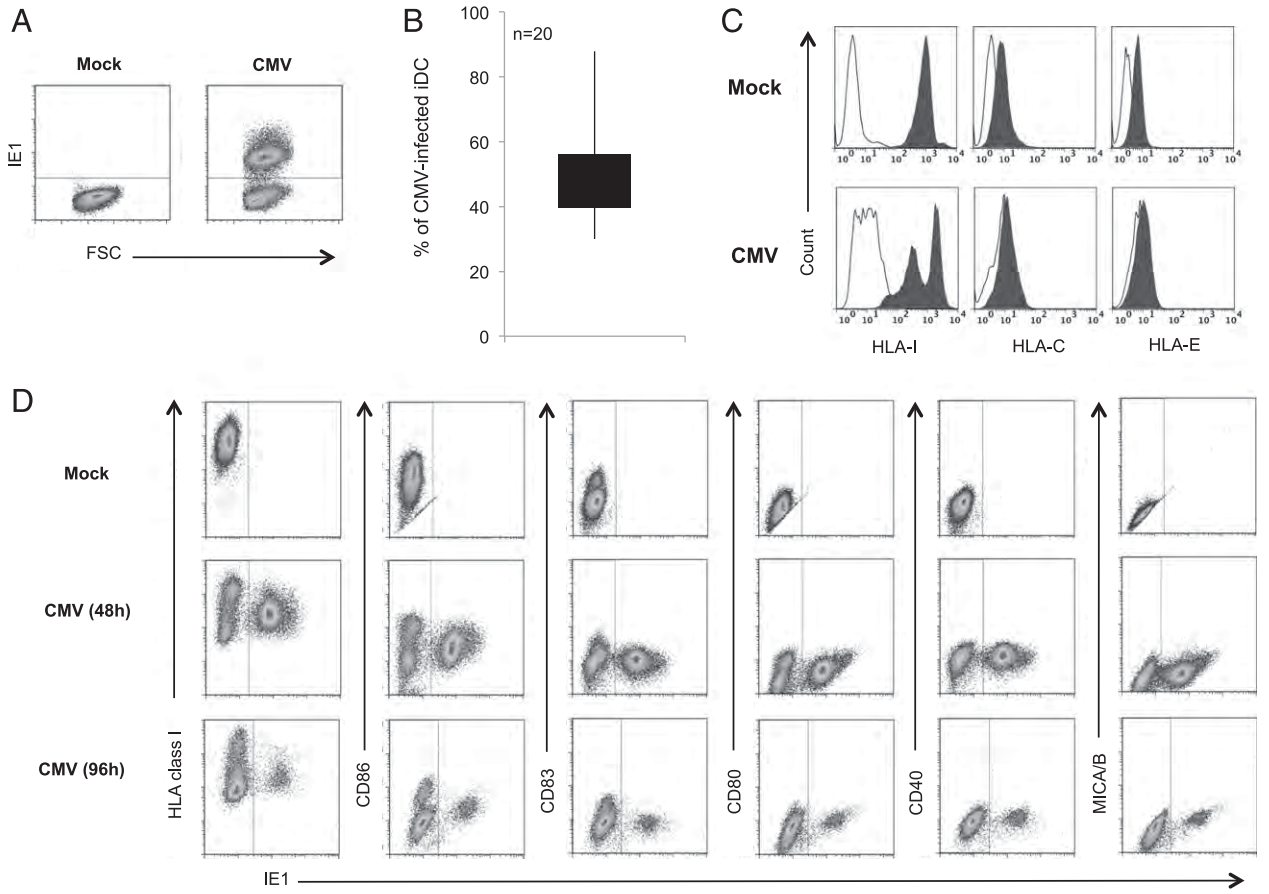

FIGURE 2. Downregulation of HLA class I molecule expression on CMV-infected iDCs. iDCs were generated from monocytes in the presence of GM-CSF and IL-4. On day 6 of the culture, they were infected with the endotheliotrope VHL/E CMV strain. Cell surface phenotype of CMV-infected iDCs was performed at 48 h postinfection by three-color flow cytometry. CMV-infected and noninfected iDCs were discriminated using FITC-conjugated IE1-specific mAb. (**A**) Representative density plots of noninfected and CMV-infected iDCs. (**B**) Summary box and whisker plot showing the frequency of $IE1^+$ iDCs for 20 experiments performed. (**C**) Forty-eight hours postinfection, iDCs were surface labeled with specific mAbs for HLA class I, HLA-C, and HLA-E (open histograms represent isotype control, and filled histograms, specific staining). Results of a representative experiment of four performed are shown (50% $IE-1^+$ cells). (**D**) Representative density plots of flow cytometry analysis of mock-infected iDCs and CMV-infected iDC phenotype using HLA class I, CD86, CD83, CD80, CD40, and MICA/B-specific mAbs.

However, the expression of CD86 decreased on CMV-infected iDCs compared with uninfected iDCs (Fig. 2C, 2D). Moreover, the uninfected iDC subset in CMV-treated cultures present two levels of expression of HLA class I, that is, CD86 and CD83 molecules. Similar results were obtained at 96 h after infection (Fig. 2D). This could be explained by binding of viral products or soluble factors. Experiments were performed with UV-inactivated virus in the same conditions to confirm that the productive CMV infection of iDCs is necessary to modulate the biology of CMV-treated iDCs (data not shown).

CMV-infected iDCs escape to HLA-A2-pp65–specific $CD8^+$ T cell recognition but activate NK cell degranulation

Consistent with previous reports indicating that several viral proteins inhibit HLA class I expression on CMV-infected cells to escape to T lymphocyte response, we further evaluate our in vitro model in assessing the CD107a mobilization of HLA-A2-pp65–specific T lymphocytes against CMV-infected iDCs versus uninfected iDCs. HLA-A2-pp65–specific T lymphocytes, targeted using HLA-A2-pp65 pentamer (Fig. 3A), reacted against pp65-loaded $HLA\text{-}A2^+$ iDCs, but not against uninfected $HLA\text{-}A2^+$ iDCs and CMV-infected $HLA\text{-}A2^+$ iDCs (Fig. 3B). This result obtained in our cellular model is consistent with the ability of CMV to escape to T lymphocyte response by inhibiting HLA class I expression on iDCs. We therefore investigated NK cell ability to recognize decreased expression of HLA class I molecules on CMV-infected iDCs by focusing on $NKp46^+$ NK cells (Fig. 3C). Interestingly, NK cells reacted against CMV-infected iDCs almost as well as against an HLA class I–deficient K562 cell line, used as a positive control of degranulation, as illustrated for one individual (Fig. 3D) and confirmed for six individuals with a significant probability ($p = 0.01$) (Fig. 3E).

KIRs are required for a robust response against allogeneic CMV-infected iDCs

It has been reported that KIRs are required on $NKG2C^+$ NK cells for a strong production of IFN-γ (9). Nevertheless, the role of KIRs in triggering NK cell degranulation is undocumented. To investigate the functional reactivity of $NKG2C^+KIR2D^-$, $NKG2C^-KIR2D^+$, and $NKG2C^+KIR2D^+$ NK cell subsets (Fig. 4A) against allogeneic CMV-infected iDCs, degranulation assays were carried out with PBMCs from CMV^+ individuals ($n = 6$) stimulated overnight with IL-2 (Fig. 4B). As seen previously, PBMCs were incubated alone or in the presence of K562 cells, mock-infected iDCs, or CMV-infected iDCs (E:T ratio = 10:1). All of these NK cell populations reacted significantly against CMV-infected iDCs ($p = 0.02$ for $NKG2C^+KIR2D^-$ NK cells, $p = 0.0006$ for $NKG2C^-KIR2D^+$ NK cells, and $p = 3.5 \times 10^{-5}$ for $NKG2C^+KIR2D^+$ NK

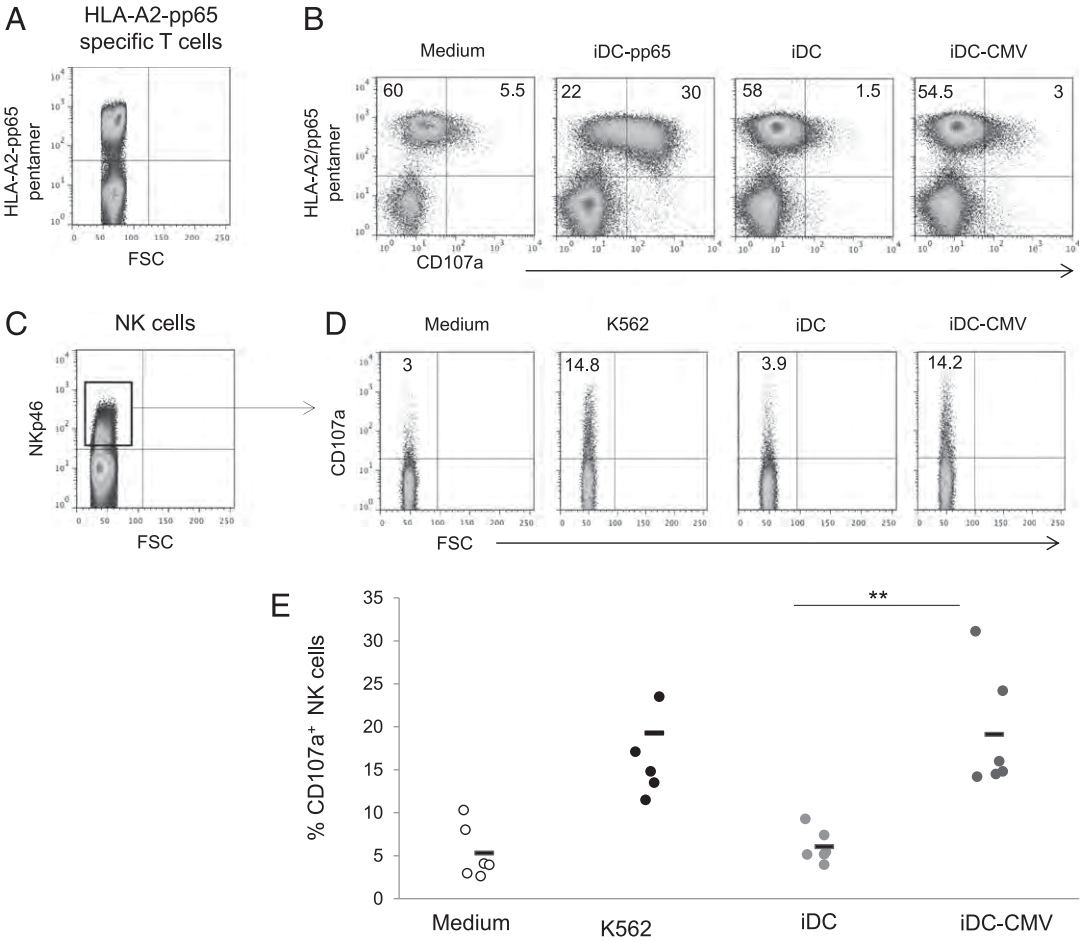

FIGURE 3. CMV-infected iDCs escape to HLA-A2-pp65–specific $CD8^+$ T cell recognition but activate NK cell degranulation. (**A**) HLA-A2-pp65–specific T cells were cell sorted and amplified in vitro from CMV seropositive individuals and targeted as $CD3^+$HLA-A2-pp65-pentamer$^+$. (**B**) After 2 wk of stimulation, these cells were phenotyped by flow cytometry and were incubated for 5 h alone or in the presence of mock-infected, pp65-loaded, or human CMV-infected allogeneic iDC HLA-$A2^+$ at an E:T ratio of 10:1. Results of CD107a mobilization obtained for a representative experiment of three performed are shown. Percentages of $CD107a^+$ HLA-A2-pp65–specific T cells observed by flow cytometry are indicated on the density plots for all conditions of stimulation. (**C**) NK cells are targeted as $NKp46^+$ cells. (**D**) PBMCs stimulated overnight with IL-2 were incubated for 5 h alone or in the presence of K562 cells as positive control or mock- or CMV-infected allogeneic iDCs at an E:T ratio of 10:1. Surface CD107a expression on $NKp46^+$ cells was analyzed by flow cytometry. Representative density plots from six experiments are shown. The percentage of $CD107a^+$ NK cells is indicated on each density plot. (**E**) Dot representation displaying the percentage of $CD107a^+$ NK cells from all six experiments. Statistical significance ($^{**}p < 0.01$) between both groups was determined using an ANOVA test.

cells). However, NK cells bearing KIR2D receptors ($NKG2C^-$ $KIR2D^+$ and $NKG2C^+KIR2D^+$ NK cell subsets) reacted more robustly than did $NKG2C^+KIR2D^-$ NK cells. Indeed, when KIR2D receptors were neutralized with anti-KIR2DL1/2/3/2DS2 mAb, $NKG2C^+KIR2D^+$ NK cell degranulation against CMV-infected iDCs was decreased by half (Fig. 4C). Otherwise, because $NKG2C^+$ NK cells are mostly $NKG2A^-$ (Fig. 4C), we neutralized HLA-E molecules with anti–HLA-E mAb to assess the impact of NKG2C on NK cell degranulation against CMV-infected iDCs. The $NKG2C^+KIR2D^+$ NK cell degranulation was slightly decreased in this blocking condition (Fig. 4C). These results suggest a predominant impact of KIR2DL in triggering NK cell response against CMV-infected iDCs. Based on our results showing a decreased expression of HLA class I molecules on CMV-infected iDCs and the expansion of $NKG2C^+KIR2D^+$ NK cell subsets in CMV infection, we hypothesized that the absence of inhibitory KIR engagement with the cognate ligand and the activating NKG2C engagement with HLA-E on human CMV–infected iDCs might both contribute to drive the expansion process.

C1C1 but not C2C2 allogeneic iDCs activate KIR2DL1$^+$ NK cell degranulation following the self-missing model

To determine the nature of KIR2DL receptors involved in the NK cell response to CMV-infected iDCs, we first focused on NK cell subsets expressing well-characterized inhibitory KIR2DL1, which recognizes HLA-C molecules of C2 group. KIR2DL1$^+$ NK cells were sorted from KIR2DS1$^-$ genotyped $C2^+$ individuals and amplified following polyclonal stimulation. The phenotype (KIR2DL, NKG2A, NKp44, NKp30, and NKG2D) of the studied NK cell lines was stable following culture alone or coculture with iDCs or CMV-infected iDCs for 5 h. All selected and amplified NK cells did not express the inhibitory receptor ILT2 (data not shown). However, most amplified NK cells expressed the NKG2A marker (Fig. 5A). Thus, NKG2A expression on KIR2D NK cells may inhibit NK cell response and override the signal mediated by KIR2D receptors. We therefore assessed the function of KIR2DL1$^+$ KIR2DL2/3/S2$^-$NKG2A$^-$ NK cells. We showed that the degranulation of $C2^+$ educated KIR2DL1$^+$ NK cells, controlled with K562, is strongly induced in contact to C1C1 allogeneic iDCs ($p = 0.002$) but not in contact to C2C2 allogeneic iDCs (Fig. 5B). These results

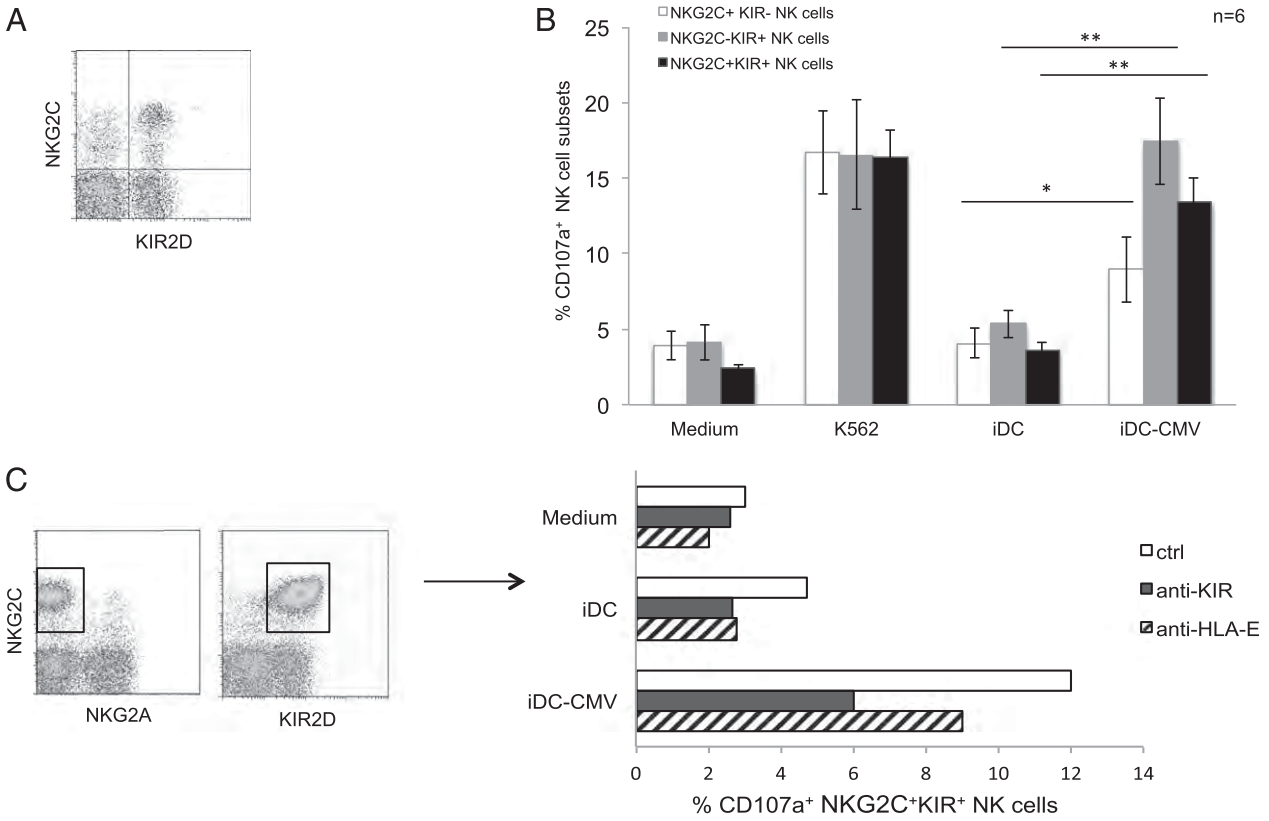

FIGURE 4. NKG2C$^+$ NK cells increased in CMV seropositive individuals require KIR2D for a robust degranulation against allogeneic CMV-infected iDCs. Degranulation assays were carried out with PBMCs from CMV$^+$ individuals ($n = 6$) stimulated overnight with IL-2. CD107a expression on NKp46$^+$ cells was analyzed by flow cytometry after incubation of PBMCs alone or in the presence of K562 cells or mock-infected or CMV-infected iDCs for 5 h at an E:T ratio of 10:1. (**A**) Representative density plot illustrating NKG2C KIR2D NK cell phenotype from CMV$^+$ individual. (**B**) Bars indicate the mean of CD107$^+$ NK cell percentages for all NKG2C$^+$KIR2D$^-$, NKG2C$^+$KIR2D$^+$, and NKG2C$^-$KIR2D$^+$ NK cell subsets observed in all culture conditions: medium, K562 cells, mock-infected, or CMV-infected allogeneic iDCs ± SD. (**C**) Degranulation assays were performed on an NKG2C$^+$KIR2D$^+$NKG2A$^-$ gated NK cell population. Results of CD107a mobilization obtained for a representative experiment of three performed were presented after culture alone (medium) or stimulation with iDCs or CMV-infected iDCs. Bars indicate CD107a$^+$NKG2C$^+$KIR2D$^+$ NK cell percentage observed by flow cytometry. KIR2D receptors were blocked with 8C11 mAb and isotype control. HLA-E molecules expressed on iDCs were blocked with 3D12 mAb and isotype control. Statistical significance (*$p < 0.05$, **$p < 0.01$) between both groups was determined using an ANOVA test.

validated our in vitro model to explore the impact of CMV infection of iDCs on KIR$^+$ NK cell degranulation.

CMV infection of C2C2 iDCs triggers alloreactivity of C2$^+$ KIR2DL1$^+$ NK cells

CMV infection of C2C2$^+$ iDCs activated significantly KIR2DL1$^+$ NK cell degranulation ($p = 0.0003$) (Fig. 5C). This result suggests that HLA class I downregulation in CMV-infected C2C2$^+$ iDCs was sufficient to trigger KIR2DL1$^+$ NK cell cytotoxicity. To confirm this point, we evaluated CMV infection of C1C1$^+$ iDCs on KIR2DL1$^+$ NK cell degranulation. Indeed, when KIR2DL1$^+$ NK cells were incubated in the presence of CMV-treated C1C1$^+$ iDCs, the frequency of CD107a$^+$KIR2DL1$^+$ NK cells was high and similar to that observed in the presence of mock-infected C1C1$^+$ iDCs (Fig. 5C). The activation of KIR2DL1$^+$ NK cell degranulation with only CMV-infected C2C2$^+$ iDCs but not CMV-infected C1C1$^+$ iDCs supports the hypothesis that the protective KIR2DL1/C2 interaction is directly targeted. Furthermore, we investigated the function of unlicensed C1C1$^+$KIR2DL1$^+$ NK cells in controlling CMV infection, taking into account published works on mice indicating that "unlicensed" NK cells dominate the response to murine CMV infection (31). However, the degranulation of C1C1$^+$KIR2DL1$^+$ NK cells, which is low against the standard HLA class I–deficient K562 cell line, was not triggered against C1C1$^+$ iDCs infected or not with CMV (data not shown). Taken together, these results support the functional contribution of C2$^+$KIR2DL1$^+$ NK cells in response to CMV-infected iDCs.

KIR2DL3$^+$ NK cells respond to CMV-infected allogeneic C2C2$^+$ iDCs but not to CMV-infected allogeneic C1C1 iDCs

Additionally, we investigated the role of KIR2DL3$^+$ NK cells in the response to CMV-infected allogeneic iDCs. KIR2DL3$^+$ NK cells were sorted and in vitro amplified from KIR2DS1$^-$/L2$^-$/S2$^-$ genotyped C1C1$^+$ individuals (Fig. 5D). Fifteen days after stimulation, we foremost assessed the degranulation of KIR2DL3$^+$KIR2DL1/L2/S2$^-$NKG2A$^-$ NK cells against allogeneic iDCs. Both C1C1$^+$ and C2C2$^+$ iDCs inhibited KIR2DL3$^+$ NK cell degranulation (Fig. 5E). Indeed, when HLA-C molecules were neutralized with anti–HLA class I mAb, C2C2$^+$ iDC-mediated KIR2DL3$^+$ NK cell degranulation was partially restored, showing the functional interaction of KIR2DL3 with C2 ligand (Fig. 5G). These results are in accordance with recently reported data showing that KIR2DL3 could recognize not only the expected group of HLA-C molecules belonging to the C1 group, but also those belonging to C2 group (32–34). To determine whether the decreased HLA class I expression on CMV-infected iDCs impacts the KIR2DL3$^+$ NK cell function, degranulation of the KIR2DL3$^+$ NK cell subset against CMV-infected iDCs has been evaluated. CMV infection of C2C2$^+$ iDCs activated significantly KIR2DL3$^+$ NK cell degranulation ($p = 0.01$) (Fig. 5F). This result may imply that HLA class I

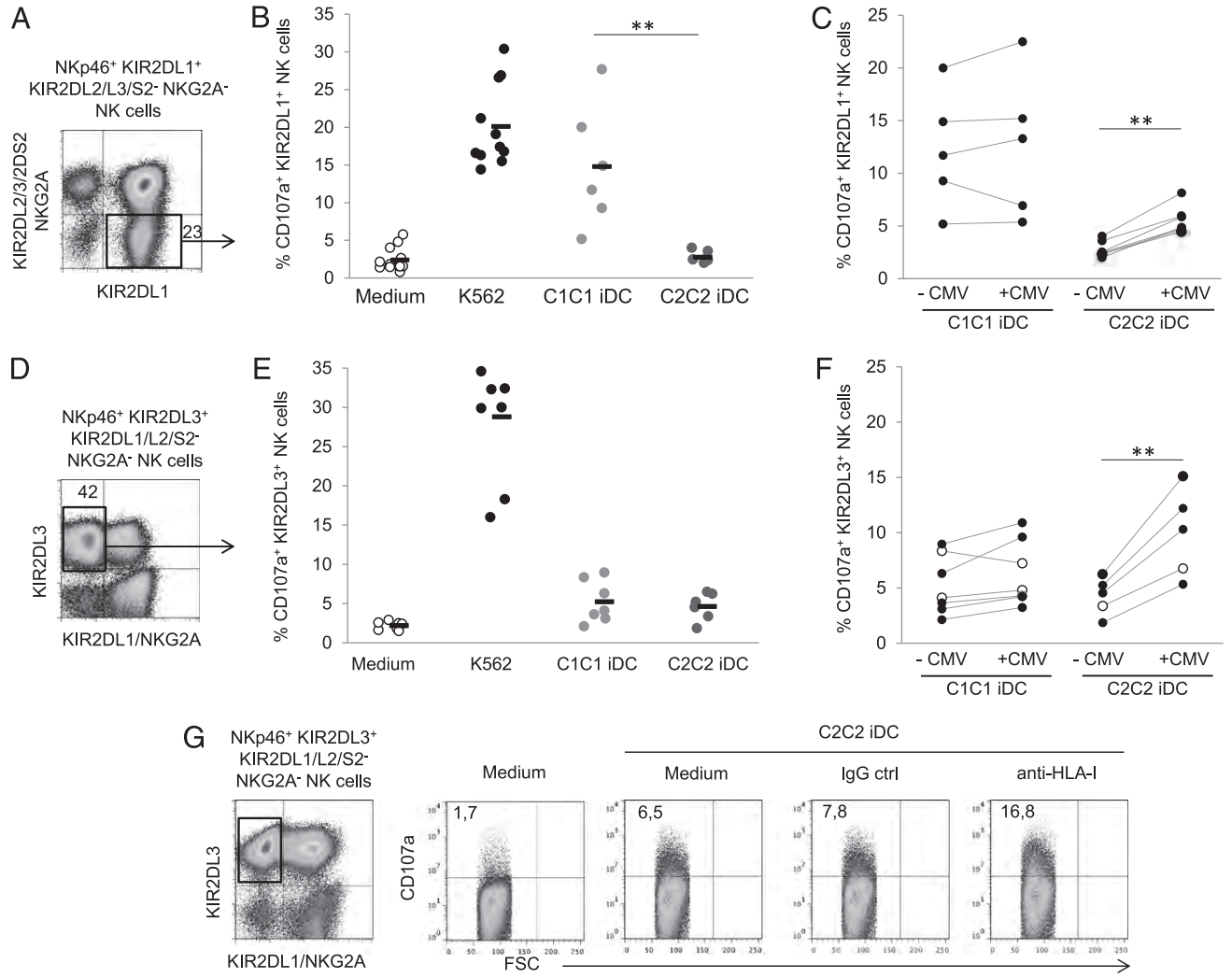

FIGURE 5. CMV-infected allogeneic C2C2$^+$ iDCs activate KIR2DL1$^+$ and KIR2DL3$^+$ NK cell subsets. Mobilization assay has been performed on sorted and in vitro–amplified KIR2DL1$^+$ NK cells from C2$^+$ individuals and KIR2DL3$^+$ NK cells from C1$^+$ individuals. (**A**) Representative density plot illustrating the phenotype of targeted KIR2DL1$^+$ NK cells in functional assay, assessed by flow cytometry using a combination of KIR2DL1-specific mAbs and a mix of KIR2DL2/3/2DS2 and NKG2A-specific mAbs. The cells were isolated from negative KIR2DS1 genotyped individuals. (**B**) Dot representation displaying the percentage of CD107a$^+$KIR2DL1$^+$ NK cells in all culture conditions: medium (n = 13), K562 cells (n = 13), C1C1$^+$ iDCs (n = 6), and C2C2$^+$ iDC (n = 7) at an E:T ratio of 10:1. (**C**) Dot representation displaying the percentage of CD107a$^+$KIR2DL1$^+$ NK cells in the presence of mock- or CMV-infected C1C1$^+$ iDCs (n = 6) or C2C2$^+$ iDCs (n = 7) at an E:T ratio of 10:1. KIR2DL1$^+$ NK cells were isolated from CMV$^-$ individuals. (**D**) Representative density plot illustrating the phenotype of targeted KIR2DL3$^+$ NK cells in functional assay, assessed by flow cytometry using a combination of KIR2DL3-specific mAbs and a mix of KIR2DL1/S1 and NKG2A-specific mAbs. The cells were isolated from negative KIR2DL2/S2 genotyped individuals. (**E**) Dot representation displaying the percentage of CD107a$^+$KIR2DL3$^+$ NK cells in all culture conditions: medium (n = 7), K562 cells (n = 7), C1C1$^+$ iDCs (n = 7), and with C2C2$^+$ iDCs (n = 6). (**F**) Dot representation displaying the percentage of CD107a$^+$KIR2DL3$^+$ NK cells in the presence of mock- or CMV-infected C1C1$^+$ iDCs (n = 7) or C2C2$^+$ iDCs (n = 5). KIR2DL3$^+$ NK cells isolated from CMV$^-$ and CMV$^+$ individuals are indicated by black and white circles, respectively. (**G**) Representative density plots of three experiments performed displaying the percentage of CD107a$^+$KIR2DL3$^+$ NK cells after coculture with C2C2$^+$ iDCs. Target cells were incubated with blocking HLA class I mAb at a concentration of 10 μg/ml for 30 min or IgG control, prior to coincubation with NK cells. Statistical significance (**$p \leq 0.01$) between both groups was determined using an ANOVA test.

downregulation on CMV-infected C2C2$^+$ iDCs is sufficient to trigger KIR2DL3$^+$ NK cell reactivity. However, CMV infection of C1C1$^+$ iDCs did not activate KIR2DL3$^+$ NK cell degranulation (Fig. 5F). These findings support the functional contribution of KIR2DL3$^+$ NK cells in response to CMV infection of C2C2$^+$ iDCs, but they suggest a potential evasion of CMV to KIR2DL3$^+$ NK cell control of C1C1$^+$ target cells.

Discussion

Recent studies have reported the expansion of NKG2C$^+$ NK cells expressing self-specific inhibitory KIR (8–10) in CMV infection. Our data provide evidence that inhibitory KIR2DL3 is preferentially coexpressed with NKG2C on amplified NK cells in CMV seropositive individuals whatever the HLA-C background, and even KIR2DL3 seems to recognize the HLA-C ligand from the C1 and C2 groups with a lower affinity to C2 ligands (32). Our results are in agreement with a previous report revealing the expansion of NKG2C$^+$KIR2DL2/2DS2/2DL3$^+$ NK cells in a T$^-$B$^+$NK$^+$ SCID patient with a CMV infection representing 80% of NK cells (35). In contrast to Charoudeh et al. (12) who reported a specific expansion of NK cells expressing the inhibitory receptors KIR2DL1 and KIR2DL3 in response to in vitro exposure to CMV, we observed preferential KIR2DL3 coexpression on NKG2C$^+$ NK cells but seldom KIR2DL1 coexpression. Similarly, we observed mainly inhibitory KIRs coexpressed on NKG2C$^+$ NK cells. Additionally, our phenotypic study showed a frequent coexpression of KIR3DL1

or KIR3DS1 on NKG2C$^+$ KIR2DL3$^+$ NK cells in CMV seropositive individuals, whatever the Bw4 environment. Thus, the predominant expression of KIR2DL3 suggests a driven clonal-like expansion of KIR2DL3$^+$NKG2C$^+$ NK cells during CMV infection that can coexpress another KIR as KIR3DL1 or KIR3DS1, as previously described for Ly49H$^+$ NK cells in mice challenged with murine CMV (36). Moreover, numerous hypotheses regarding HIV-1 infection (37) and recent data (12) suggest a potential impact of KIR3DL1/S1 in controlling CMV infection. The role of KIR3DL1/S1 engagement in CMV infection should be investigated to answer this point.

We showed that NK cells bearing KIR2D receptors react more robustly than do KIR2D$^-$NKG2C$^+$ NK cells. Indeed, KIR2D$^+$ NK cell subsets degranulated and secreted INF-γ (data not shown) more than did the KIR2D$^-$NKG2C$^+$ NK cell subset in response to CMV-infected iDCs. Our results are in accordance with previous results showing that KIRs are required for robust IFN-γ production (9). As previously described (38), degranulation of C2$^+$ educated KIR2DL1$^+$ NK cells is strongly induced in contact to allogeneic C1C1$^+$ iDCs but not in contact to C2C2$^+$ iDCs. We demonstrated that infection of C2C2$^+$ iDCs with CMV significantly enhances KIR2DL1$^+$ NK cell degranulation. Thus, HLA class I downregulation observed on CMV-infected iDCs seems sufficient to activate KIR2DL1$^+$ NK cells. Interestingly, KIR2DL1$^+$ NK cell alloreactivity against C1C1$^+$ iDCs is maintained with CMV infection. Additionally, KIR2DL1$^+$ and KIR2DL3$^+$ NK cell subsets reacted against CMV-infected C2C2$^+$ iDCs. This point could explain recent work showing that early CMV infection is associated with a reduced risk of relapse in acute myeloid leukemia patients undergoing allogeneic HCT (19). Thus, it is possible that CMV infection activates and mobilizes the NK cell subset able to recognize "missing-self" and that expanded NKG2C$^+$KIR2D$^+$ NK cells constitute boosted alloreactive NK cells that are more efficient against leukemia cells.

Recent studies performed on a large cohort has revealed the expansion of activating KIR$^+$ (KIR2DS2, KIR3DS1, KIR2DS4) NK cell subsets independently of NKG2C, suggesting a contribution of both activating KIR$^+$ and NKG2C$^+$ NK cell subsets in CMV infection (11). In a murine model, activating Ly49H recognition of the viral protein m157 drove expansion of the cells that control murine CMV infection (15). Thus, we evaluated amplified and sorted C2$^-$KIR2DS1$^+$ NK cell degranulation against C1C1$^+$ and C2C2$^+$ iDCs infected or not by CMV. In our model, we did not observe a potential activation of KIR2DS1$^+$ NK cells stimulated with CMV-infected iDCs (data not shown). However, the CMV seronegative status of the studied individuals may explain the absence of the KIR2DS1$^+$ NK cell response. Indeed, it is possible that "memory" status of NK cells expanded during CMV infection is necessary to involve KIR2DS1 engagement with viral ligands.

Interestingly, we show that KIR2DL3 recognizes not only C1 but also C2 ligands, as demonstrated in our cellular model using C1C1 or C2C2 iDCs. In contrast, KIR2DL1$^+$ NK cells recognize only C2C2$^+$ iDCs. These results are in accordance with previous works indicating that KIR2DL3 interacts with HLA-C molecules belonging to C1 and C2 groups (32). Additionally, KIR2DL3$^+$ NK cell degranulation is significantly increased in contact to CMV-infected C2C2$^+$ iDCs, probably due to the decreased expression of HLA class I molecules on CMV-infected C2C2$^+$ iDCs. Unexpectedly, KIR2DL3$^+$ NK cell degranulation is not increased in contact to CMV-infected C1C1 iDCs. This last result suggests a potential evasion of CMV to KIR2DL3$^+$ NK cell control in HLA-C1 environment as observed in our model at 48 h postinfection and confirmed at 96 h postinfection (data not shown). However, we do not exclude that KIR2DL3$^+$ NK cell degranulation is activated by CMV-infected target cells earlier or later during the viral cycle.

The existence of multiple CMV strategies to escape immune system control opens a range of hypotheses. Indeed, the high frequency of KIR2DL3$^+$ NK cells observed in CMV infection may lead to an antiviral immune pressure mediated in vivo by these KIR2DL3$^+$ NK cells. The modulation of KIR/HLA interactions by viruses has been reported for some inhibitory KIR (39–43). This suggests that viral CMV peptides may increase the affinity of C1 ligands for inhibitory KIR2DL3 and prevent the activation of specific KIR2DL3$^+$ NK cells.

Different groups have recently reported a specific NK cell phenotype marked by the acquisition of CD57, NKG2C, and KIR on NK cells in different viral infections including in nonexclusive fashion CMV (44), hantavirus (45), chikungunya virus (46), and HIV-1 (47). Interestingly, all of these viruses developed evasion strategies based on HLA class I downregulation to escape T cell recognition. Although the ligand of NKG2C is still elusive in CMV infection, it is conceivable that NKG2C$^+$KIR2DL$^+$ NK cell expansion is mutually driven by the recognition of HLA-E by CD94/NKG2C and the absence of engagement of KIR2DL with specific HLA KIR ligands on CMV-infected cells. Additionally, the large specificity of KIR2DL3 may explain its frequent expression on NKG2C$^+$ NK cells associated to CMV infection.

In conclusion, our results provide evidence that inhibitory KIR2DL3 is preferentially coexpressed with NKG2C on amplified NK cells in CMV infection. Our findings support the functional contribution of KIR2DL1$^+$ and KIR2DL3$^+$ NK cell subsets in controlling CMV infection in C2$^+$ recipients of allogeneic HSCT, suggesting a beneficial impact of CMV on GVL effect in this clinical context. In C2$^-$ recipients, the beneficial KIR2DL1$^+$ NK cell alloreactivity is maintained despite infection, whereas CMV seems to have developed a strategy to escape to KIR2DL3$^+$ NK cell control. This study opens new perspectives of investigation to determine the molecular mechanisms involved in KIR2DL3 NK cell escape employed by CMV.

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Disclosures

The authors have no financial conflicts of interest.

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Large Spectrum of HLA-C Recognition by Killer Ig–like Receptor (KIR)2DL2 and KIR2DL3 and Restricted C1 Specificity of KIR2DS2: Dominant Impact of KIR2DL2/KIR2DS2 on KIR2D NK Cell Repertoire Formation

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The interactions of killer Ig–like receptor 2D (KIR2D) with HLA-C ligands contribute to functional NK cell education and regulate NK cell functions. Although simple alloreactive rules have been established for inhibitory KIR2DL, those governing activating KIR2DS function are still undefined, and those governing the formation of the KIR2D repertoire are still debated. In this study, we investigated the specificity of KIR2DL1/2/3 and KIR2DS1/2, dissected each KIR2D function, and assessed the impact of revisited specificities on the KIR2D NK cell repertoire formation from a large cohort of 159 KIR and HLA genotyped individuals. We report that KIR2DL2$^+$ and KIR2DL3$^+$ NK cells reacted similarly against HLA-C$^+$ target cells, irrespective of C1 or C2 allele expression. In contrast, KIR2DL1$^+$ NK cells specifically reacted against C2 alleles, suggesting a larger spectrum of HLA-C recognition by KIR2DL2 and KIR2DL3 than KIR2DL1. KIR2DS2$^+$ KIR2DL2$^-$ NK cell clones were C1-reactive irrespective of their HLA-C environment. However, when KIR2DS2 and KIR2DL2 were coexpressed, NK cell inhibition via KIR2DL2 overrode NK cell activation via KIR2DS2. In contrast, KIR2DL1 and KIR2DS2 had an additive enhancing effect on NK cell responses against C1C1 target cells. KIR2DL2/3/S2 NK cells predominated within the KIR repertoire in KIR2DL2/S2$^+$ individuals. In contrast, the KIR2DL1/S1 NK cell compartment is dominant in C2C2 KIR2DL2/S2$^-$ individuals. Moreover, our results suggest that together with KIR2DL2, activating KIR2DS1 and KIR2DS2 expression limits KIR2DL1 acquisition on NK cells. Altogether, our results suggest that the NK cell repertoire is remolded by the activating and inhibitory KIR2D and their cognate ligands. ***The Journal of Immunology*****, 2013, 191: 4778–4788.**

Natural killer cells constitute the first line of defense against viral infections and tumor cells. These effectors of innate immunity discriminate self and nonself via inhibitory receptors that recognize HLA class I molecules in an allelic fashion. The absence or default of HLA class I expression on altered cells is a well-established characteristic of virally transformed and tumor cells, and leads to enhanced NK cell proliferation, cytotoxicity, and cytokine production (1). These functional abilities confer to alloreactive NK cells a preponderant contribution to cell responses in allogeneic hematopoietic stem cell transplantation (HSCT) (2, 3). Similarly, a semiallogeneic context observed in pregnancy constitutes a new HLA environment that requires a complete modulation of self HLA molecules to avoid activation of NK cells via inhibitory receptors. Among inhibitory receptors, killer Ig–like receptors (KIRs) recognize mainly HLA-C molecules. The nonclassical HLA-E molecule presents a peptide from the signal peptide of HLA class I molecules and constitutes a second line of self-presentation. This molecule is recognized by the inhibitory heterodimer CD94/NKG2A, which is acquired before KIRs on NK cells during development (4). Finally, ILT-2 recognizes all HLA class I molecules by engaging the conserved β2-microglobulin and α3 domain of HLA class I molecules. KIRs are clonally expressed (5) on NK cells, leading to large combinations of KIR expression in different proportions of each KIR NK cell subset (6). During development, NK cells acquire a functional potential that is, in part, determined by the capacities of each NK cell to engage its inhibitory receptors with self HLA class I molecules (7). In addition, the number of KIR gene copies contributes to increased NK cell responsiveness (8). Different theories have been proposed to explain the formation of the KIR NK cell repertoire. Some groups have proposed a model following a sequential acquisition of KIR that is dependent on the HLA environment (9). In parallel, based on combinatorial analysis of KIR NK cells from haplotype AA individuals, Malmberg's group (10) has proposed that the variegated

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Abbreviations used in this article: HSCT, hematopoietic stem cell transplantation; KIR, killer Ig–like receptor.

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repertoires are generated through sequential and random acquisition of KIRs in a manner that is independent of the presence or absence of cognate HLA class I molecules. Thus, the impact of self HLA class I molecules on the frequency of all KIR NK subsets is still debated. Even though NK receptor–specific genes are not located in chromosome 6 like the HLA gene complex, the HLA environment appears nevertheless an important determinant to the formation and function of the NK cell repertoire.

The KIR gene family comprises 16 genes and is located on chromosome 19. KIR2DL1, KIR2DL2, and KIR2DL3 recognize HLA-C molecules depending on the nature of amino acid at position 80 and leads to two groups of ligands: C1 (Asn^{80}) and C2 (Lys^{80}). Thus, KIR2DL1 recognizes HLA-C molecules of C2 group, and KIR2DL2 and KIR2DL3, which are allelic genes, recognize HLA-C molecules of C1group (11). KIR2DL2 presents a weaker affinity to C2 ligand (12) with the N77K80 motif (13). Activating KIR specificity is less well documented even though KIR2DS1 recognizes HLA-C of group C2 as its inhibitory counterpart (14, 15). KIR2DS3 is not expressed at the cell surface (16), and its function is questionable especially because the gene is missing from some populations such as the Yucpa group studied by Parham's group (17). If the alloreactive rules of C2 specific $KIR2DS1^+$ NK cells are well understood, those involving KIR2DS2 are less documented. This is largely explained by the absence of Abs distinguishing the activating KIR2DS2 and inhibitory KIR2DL2 forms. Studies to date have mainly relied on the use of Abs specific for KIR2DL2/3 and KIR2DS2 as the GL183, which does not allow differentiation between inhibitory and activating forms. This may induce a possible bias in the interpretation of results involving two functional receptors with opposite signals but sharing potentially the same ligands, most likely because of the high homology of the extracellular portion of KIR2DL2 and KIR2DS2. Thus, we dissected the main inhibitory KIR2DL1/2/3 and activating KIR2DS1/2 reactivity from primary NK cells from HLA and KIR genotyped individuals. Our experimental approach has been validated in studying alloreactive KIR2DL1 and KIR2DS1 NK cell subsets against a wide range of target cell lines expressing different HLA-C ligands from C1 and C2 groups. Taking into account all revisited specificities of the main inhibitory KIR2DL1/2/3, activating KIR2DS1/2 and the alloreactive rules, we analyzed phenotypically the KIR NK cell repertoire from a large cohort of 159 KIR and HLA genotyped individuals to evaluate the influence of KIR ligand expression on NK cell repertoire formation.

Materials and Methods

Cells (PBMCs and cell lines)

PBMCs were isolated as previously described (15, 18). All blood donors were recruited at the Blood Transfusion Center (Etablissement Français du Sang, Nantes, France), and informed consent was given by all donors. HLA class I–deficient 721.221 lymphoblastoid cells, referred to as 221 cells, were used as positive control to assess NK cell degranulation. HLA-C*03:04 (C1), HLA-C*08:02 (C1), HLA-C*04:01 (C2), HLA-C*02:02 (C2), HLA-C*06:02 (C2), HLA-C*07:01 (C1), and HLA-C*15:01 (C2) transfected 221 cells were used to evaluate NK cell degranulation of different KIR2D NK cell subsets. HLA-C*02:02, HLA-C*03:04, HLA-C*04:03, and HLA-C*07:01 transfected 221 cells were provided by Prof. P. Parham. HLA-C*08:02, HLA-C*02:02, HLA-C*06:02, and HLA-C*15:01 transfected 221 cells were obtained by stable transfection of 221 cell line by electroporation (BioRad) using, respectively, pcINeo–HLA-C*08:02 (provided by Dr. Agnès Moreau, INSERM Unite Mixte de Recherche 892, Nantes, France), pcINeo–HLA-C*06:02 (provided by Dr. Agnès Moreau, INSERM Unite Mixte de Recherche 892), and HLA-C*15:01 (provided by Dr. Frédérique Triebel, Immutep S.A., Chatenay-Malabry, France). All HLA-C cDNA encoded sequences were verified by DNA sequencing as described: HLA-C polymorphic exons were amplified using the HLA-C.exon 1.68G (5′-GGCCCTGACCGAGACCTG-3′) forward primer and the HLA-C.exon 4.661C (5′-CCTCAGGGTGGCCTCATG-3′) reverse primer. Amplification of exons 2 and 3 of the HLA-C locus was performed in 50 μl using 80 ng DNA, 0.2 mM dNTP (Invitrogen), 1 U Taq Gold polymerase (Applied Biosystems), 1× Taq Gold buffer (Applied), 2.5 mM $MgCl_2$ Gold (Applied), and 1 μM of each primer. Amplification presequencing was performed in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystem). The PCR amplification program included one denaturation step of 95°C for 10 min, 36 cycles of 96°C for 20 s, 60°C for 30 s, and 72°C for 3 min, followed by a final elongation step of 72°C for 10 min. Products were electrophoresed on 2% agarose gels. HLA-C cDNA sequencing was performed by the Sequencing Core Facility (SFR François Bonamy, Nantes, France) using an ABI 3730 automatic system (Applied). HLA-C cDNA sequence data files were analyzed using Assign software (Conexio Genomics, Applecross, Australia) with IPD-HLA database version 3.9.0 (July 2012). The 221 cell line and HLA-C–transfected 221 cell lines were cultured in RPMI 1640 medium (Life Technologies, Paisley, U.K.) containing glutamine (Life Technologies) and penicillin-streptomycin (Life Technologies), and supplemented with 10% FBS (Life Technologies). *Mycoplasma* tests performed by PCR were negative for all cell lines.

HLA and KIR genotyping

HLA class I allele assignment and KIR gene content typing were performed as previously described (15, 18). KIR haplotypes were determined as previously described (6). The A haplotypes correspond to seven KIR genes, including KIR2DS4 as the only activating KIR gene. In contrast, B haplotypes are characterized by the presence of more than one activating KIR gene and the absence of the KIR2DS4 gene.

KIR NK cell line and clone isolation and amplification

PBMCs were stained with anti-CD3 mAb (X35, a gift from Dr. Franck Vérité, Etablissement Français du Sang, Rennes), and $CD3^+$ cells were depleted using murine IgG coupled magnetic Dynabeads according to the manufacturer's instructions (Dynal, Oslo, Norway). Then cells were labeled with appropriate mAb combinations, and the cells were sorted using a FACSCalibur equipped with a fluidic sort module (BD Biosciences). Sorted cells were amplified after in vitro stimulation with irradiated allogeneic PBMCs and HLA-C*03:04 (C1) and HLA-C*04:01 (C2) transfected 221 cell lines used as feeders following the PBMC/EBV-B cells ratio at 10:1. $KIR2DS2^+$ NK cells were cloned at 0.3 cell/well from the selected and amplified lines under limiting conditions in 96-well U-bottom microtiter plates with 50,000 irradiated autologous lymphocytes and 5000 irradiated HLA-C*03:04 (C1) and HLA-C*04:01 (C2) transfected 221 cell lines in a final volume of 100 μl. Growing colonies with a probability of monoclonality of 95% were kept for further analysis. NK cell lines and clones were cultured in RPMI 1640 medium (Life Technologies, Paisley, U.K.) containing glutamine (Life Technologies) and penicillin-streptomycin (Life Technologies), and supplemented with 10% human serum (Etablissement Français du Sang, Nantes, France) and 200 U/ml IL-2 (Chiron, Suresnes, France). Cells were maintained for 3 wk and expanded in their culture medium containing IL-2 without restimulation with feeders before functional analysis to decrease their spontaneous degranulation.

KIR2DL2 RT-PCR

Total RNA was purified from isolated $KIR2DS2^+$ NK clones using NucleoSpin RNAXS (Macherey-Nagel). Qualitative KIR2DL2 RT-PCR was performed using a One-Step PrimeScript RT-PCR kit (TaKaRa, Japan) with KIR2DL2-specific primers from Thompson et al. (19).

Phenotypic analysis by flow cytometry

The NK cell–surface phenotype was determined by three- or four-color flow cytometry using the following mouse anti-human mAbs: anti–KIR2DL1-FITC (143211; R&D Systems), anti–KIR2DL1/2DS1-PE (EB6), anti–KIR2DL2/2DS2-PE (GL183; Beckman Coulter Immunotech, Marseille, France), anti–CD3-PerCP (SK7), anti–CD56-allophycocyanin (B159; BD Biosciences), anti–KIR2DL1/2/3/2DS2-FITC (8C11), anti-KIR2DL3/2DS2 (1F12), and anti–KIR2DL1/2/3/2DS1/2-FITC (1A6), generated and characterized in our laboratory and previously described (20). The $KIR2DS2^+$ NK cell clone phenotype was determined using the following mouse anti-human mAbs: anti-KIR2DS4 (FES172; Beckman Coulter), anti-KIR3DL1/3DS1 (Z27; Beckman Coulter), anti-NKp30 (Z25; Beckman Coulter) anti-NKp44 (Z231; Beckman Coulter), anti-NKG2D (ID11; BD Biosciences), anti-2B4 (2-69; BD Biosciences), anti-ILT2 (GHI/75; BD Biosciences), anti-NKG2A (Z199; Beckman Coulter), and anti-CD16 (NKP15; BD Biosciences).

CD107a mobilization assay detected by flow cytometry

NK cells were preincubated with anti-CD107a (H4A3; BD Biosciences). NK cell degranulation was assessed after incubation for 5 h alone (negative

control), or with different target cells (E:T ratio = 10:1) with brefeldin A (Sigma) at 10 μg/ml for the last 4 h. Cell-surface staining was performed using the anti-human KIR mAbs mentioned in the previous section and NKG2A-FITC or -PE (Z199; Beckman Coulter). All flow cytometry data were collected using a FACSCalibur (BD Biosciences) and analyzed using FlowJo 7.6.1 software (TreeStar).

Statistical analyses

Comparisons of NK cell frequencies between two different series of individuals were performed using ANOVA test. The p values < 0.05 were considered to be statistically significant. Top and bottom whiskers represent values of the top and bottom 25% of cases, respectively; boxed area represents interquartile range.

Results

$C1^-$ activated $KIR2DL2^+$ and $KIR2DL3^+$ NK cells present a similar degranulation potential to their $C1^+$ counterparts

As a first step, we studied degranulation of KIR2DL NK subpopulations expressing either KIR2DL1, KIR2DL2, or KIR2DL3 using appropriate combinations of KIR-specific Abs targeting each of these receptors as previously described (20) (Fig. 1A). Thus, $KIR2DL1^+$ $KIR2DL2/3/S1/2^-$ NK cells were sorted using a combination of KIR2DL1/2/3/S2-specific 8C11 and KIR2DL2/3/S2-specific GL183 Abs from $KIR2DS1^-$ genotyped individuals (Fig. 1A). $KIR2DL2^+$ $KIR2DL1/3/S1/2^-$ NK cells were sorted using a combination of a mix of KIR2DL3/S2-specific 1F12 with KIR2DL1-specific 143211 Abs and KIR2DL2/3/S2-specific GL183 from $KIR2DS1^-$ genotyped individuals (Fig. 1A). $KIR2DL3^+$ $KIR2DL1/2/S1/2^-$ NK cells were sorted using a combination of KIR2DL2/3/S2-specific GL183 and KIR2DL1/S1-specific EB6 Abs from $KIR2DL2/S2^-$ genotyped individuals (Fig. 1A). All sorted KIR2DL NK cells were expanded after in vitro stimulation. NK cells expressing inhibitory NKG2A receptors were systematically excluded from functional studies. KIR2DL1 recognized HLA-C*04:01 (i.e., a "C2 allele"), but not HLA-C*03:04 (i.e., a "C1 allele"; Fig. 1B). However, $KIR2DL2^+$ (Fig. 1C) and $KIR2DL3^+$ NK cells (Fig. 1D) similarly recognized both HLA-C*03:04 (C1) and HLA-C*04:01 (C2) molecules as shown by the CD107a upregulation assay. The same profile has been obtained for IFN-γ production with a lower frequency of positive cells (data not shown). Moreover, $KIR2DL2^+$ and $KIR2DL3^+$ NK cells from $C1^+$ individuals showed a similar degranulation potential to NK cells from $C1^-$ individuals, as assessed against the HLA class I–deficient 221 cell line (Fig. 1C, 1D). This result was also observed for $KIR2DL2^+$ and $KIR2DL3^+$ NK cells amplified with 221 cells as feeders, eliminating a possible education of these NK cells by HLA-C molecules expressed by feeders (data not shown). In contrast, $KIR2DL1^+$ NK cells showed efficient degranulation against the 221 cell line only in $C2^+$ individuals (Fig. 1B). Although variable from one individual to another, the frequency of $CD107a^+$ $KIR2DL2^+$ or $CD107a^+$ $KIR2DL3^+$ NK cells did not correlate with the presence or absence of particular C1 or C2 alleles. Altogether, these results suggest that the stringent C2 specificity of KIR2DL1 confers an NK education only in $C2^+$ individuals; in contrast, some HLA class I molecules previously identified as C2 ligands could be recognized by KIR2DL2 and KIR2DL3 receptors and could mediate, at least to some extent, education of KIR2DL2/3 NK cells. Thus, a preactivation of these KIR2DL NK cell subsets, notably by cytokine stimulation, seems sufficient to decrease their activation threshold.

Broad HLA-C specificity of $KIR2DL2^+$ and $KIR2DL3^+$ NK cells but restricted C2 specificity of $KIR2DL1^+$ NK cells

To investigate the range of C2 specificity of KIR2DL2 and KIR2DL3, we evaluated the ability of KIR2DL NK cell subsets to recognize different C1 and C2 ligands, when compared with control $KIR2DL1^+$ cells (Fig. 1E). All HLA-C transfected 221 cell lines similarly expressed HLA class I molecules except 221-C*15:02, which is less recognized by KIR2DL2 and KIR2DL3 NK cells (Fig. 1F). The C2 specificity of KIR2DL1 was confirmed toward four different HLA ligands: HLA-C*04:01, C*02:02, C*06:02, and C*15:01 with a hierarchy of recognition. Indeed, HLA-C*04:01 inhibited more efficiently KIR2DL1 NK cell degranulation than HLA-C*02:02 and HLA-C*15:01. HLA-C*06:02 was less but variably recognized by KIR2DL1 NK cells. Although the C1 allele HLA-C*03:04 was not recognized by KIR2DL1, another C1 allele HLA-C*08:02 (C1) partially inhibited KIR2DL1 NK cell degranulation. The same hierarchy of C2 allele-induced inhibition was also observed with KIR2DL2 and KIR2DL3 NK cells. Indeed, these cells, irrespective of their C1 molecule, were strongly inhibited by HLA-C*04:01, inhibited at an intermediate level by HLA-C*15:02, and less inhibited by HLA-C*02:02 and C*06:02. In contrast with KIR2DL1 cells, HLA-C*08:02 (C1) was less recognized than HLA-C*03:04 (C1) by $KIR2DL2^+$ and $KIR2DL3^+$ NK cell subsets. These results suggest broad HLA-C specificity of KIR2DL2 and KIR2DL3 cells, and a hierarchy of recognized HLA-C ligands within each C1 and C2 group.

Stringent C2 alloreactivity of $KIR2DS1^+$ NK cells

In parallel with KIR2DL, we investigated C2 specificity of KIR2DS1 NK cells to validate our experimental approach before investigating HLA-C specificity of KIR2DS2 in this setting. We previously showed that different functional capabilities (degranulation, IFN-γ secretion, and proliferation) of $KIR2DS1^+$ NK cell lines were triggered by 221-HLA-C*04:01$^+$ (C2) cells, only among $C2^-$ individuals (15). In this study, we confirmed degranulation of sorted and in vitro expanded $KIR2DS1^+$ $KIR2DL2/3/S2^-$ $NKG2A^-$ NK cells (Fig. 2A) by a broad panel of HLA-C1 transfected 221 cells (Fig. 2B), as illustrated for one representative individual (Fig. 2C). Interestingly, HLA-C*02:02 induced stronger CD107a expression than HLA-C*04:01 and HLA-C*06:02 by $KIR2DS1^+$ NK cells. We cloned $KIR2DS1^+$ $KIR2D^-$ NK cells and confirmed the absence of $KIR2DS1^+$ NK cell alloreactivity against $C2^+$ target cells in $C2^+$ individuals (Fig. 2D). In contrast, $C2^-$ $KIR2DS1^+$ NK cell clones showed strong alloresponsiveness against HLA-C*04:01- and HLA-C*02:02- (C2) target cells (Fig. 2D). The C2 specificity of KIR2DS1 was confirmed for four clones isolated from three $C2^-$ individuals. Our results suggest that KIR2DS1 shows stringent C2 specificity, like its inhibitory counterpart, KIR2DL1.

C1 specificity of KIR2DS2

To address the specificity of KIR2DS2 NK cells and reassess the rules governing activation of $KIR2DS2^+$ NK cells, we used $KIR2DS2^+$ $KIR2DL1/3/S1^-$ NK cell lines from $KIR2DL3^-$ genotyped individuals, obtained after sorting with a combination of the KIR2DL3/S2-specific 1F12 and KIR2DL1/S1-specific EB6 Abs (20), and subsequent in vitro expansion (Fig. 3A). Because the $KIR2DS2^+$ NK cell population can coexpress the inhibitory KIR2DL2, we cloned $KIR2DS2^+$ $KIR2DL1/S1^-$ NK cells and discriminated $KIR2DS2^+$ clones from $KIR2DS2^-$ ones by KIR2DL2 RT-PCR analysis (Fig. 3B). Fig. 3C illustrates the degranulation of two $KIR2DS2^+$ ($KIR2DL2^-$ and $KIR2DL2^+$) NK cell clones isolated from the same individual against 221, 221-C1 (HLA-C*03:04), and 221-C2 (HLA-C*04:01) cell lines. In accordance with their KIR2DL2 RNA profile, the degranulation of $KIR2DS2^+$ $KIR2DL2^-$ NK cell clones was strongly activated by the 221-C1 (HLA-C*03:04) cell line, but not by 221 or 221-C2 (HLA-C*04:01) cell lines. In contrast, degranulation of $KIR2DS2^+$ $KIR2DL2^+$ NK cell clones was strongly inhibited by both 221-C1 (HLA-C*03:04) and 221-C2 (HLA-C*04:01) cell lines. This observation confirms not only the broader specificity of KIR2DL2 NK cells (Fig. 1C), but also the

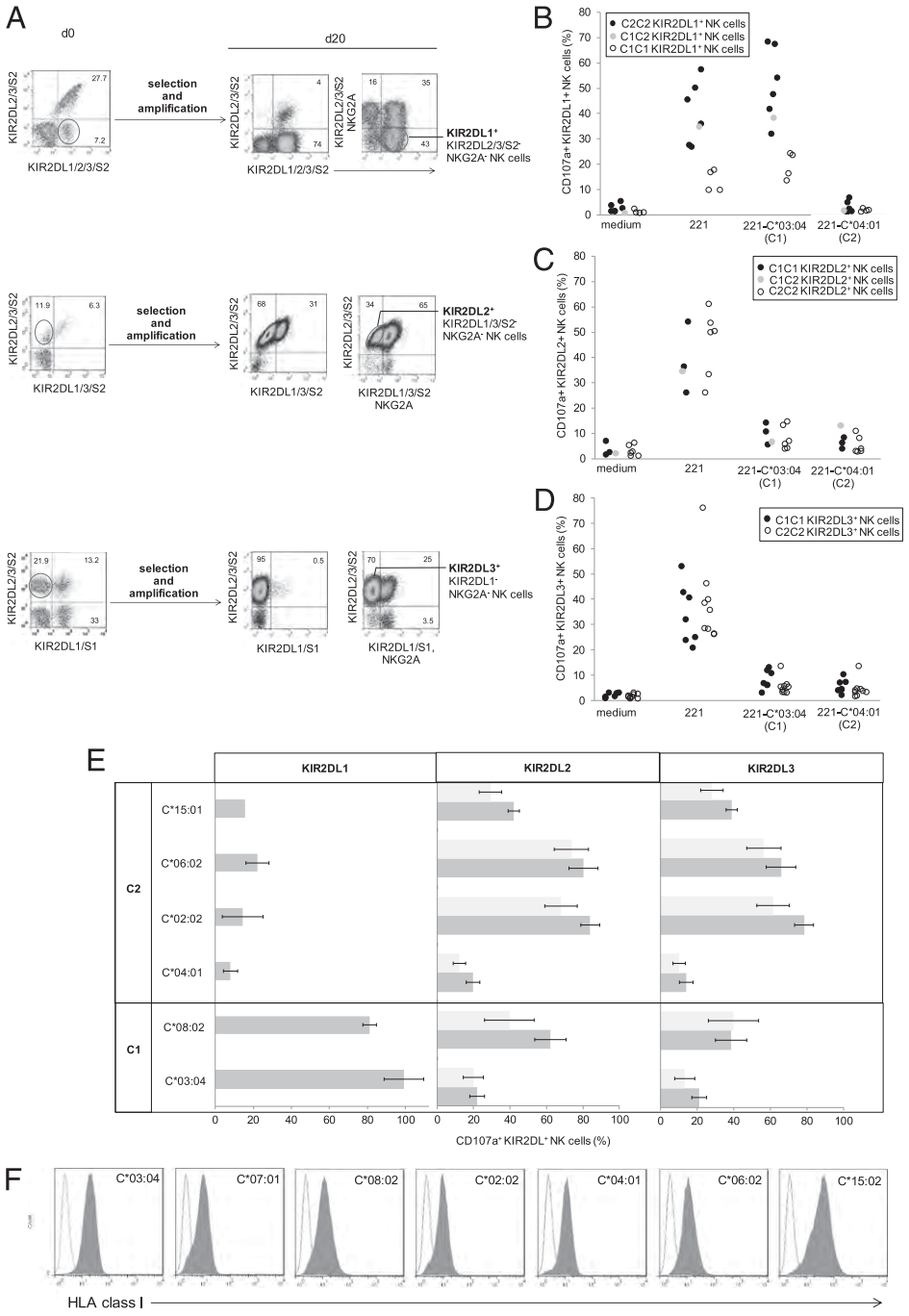

FIGURE 1. Comparable degranulation potential of $C1^-$ and $C1^+$ activated $KIR2DL2^+$ and $KIR2DL3^+$ NK cells because of a large HLA-C specificity. (**A**) After depletion of $CD3^+$ cells from PBMCs, $KIR2DL1^+$ $KIR2DL2/3/S1/2^-$ NK cells ($NKp46^+$ $8C11^+$ $GL183^-$) were cell sorted (7.2% of the population; day 0 [d0]) and amplified in vitro with irradiated allogeneic PBMCs and EBV-B cells as feeders. After 3 wk of stimulation (d20), the phenotype of these sorted and stimulated NK cells was assessed by flow cytometry and is illustrated for 1 representative $KIR2DL1^+$ genotyped individual out of 11 studied individuals. Seventy-four percent of these amplified cells are $KIR2DL1^+$ $KIR2DL2/3/S1/2^-$ NK cells ($NKp46^+$ $8C11^+$ $GL183^-$) and 43% are $NKG2A^-$. Following the same protocol, $KIR2DL2^+$ $KIR2DL1/3/S1/2^-$ NK cells ($NKp46^+$ $1F12^-$ 143211^-GL183^+) were cell sorted (11.9% of the population; d0) and amplified in vitro. After 3 wk of stimulation, the phenotype of these sorted and stimulated cells was assessed by flow cytometry and is illustrated for 1 representative $KIR2DL2^+$ $2DL3^-$ genotyped individual out of 10 studied individuals. Sixty-eight percent of these amplified cells are $KIR2DL2^+$ KIR2DL1/3/S1/2^- NK cells ($NKp46^+$ $1F12^-$ 143211^- $GL183^+$) and 34% are $NKG2A^-$. Finally, $KIR2DL3^+$ $KIR2DL1/2/S1/2^-$ NK cells ($NKp46^+$ $GL183^+$ $EB6^-$) were cell sorted (21.9% of the population; d0) and amplified in vitro. The phenotype of these sorted and stimulated cells is illustrated for 1 representative $KIR2DL3^+$ $KIR2DL2^-$ genotyped individual out of 15 studied individuals. Ninety-five percent of these amplified cells are $KIR2DL3^+$ $KIR2DL1/2/S1/2^-$ NK cells ($NKp46^+$ $GL183^+$ $EB6^-$) and 70% are $NKG2A^-$. Scatter plots displaying the $CD107a^+$ $KIR2DL^+$ NK cell frequency determined after 5-h degranulation assay at an E:T ratio of 1:1 in different stimulation conditions: medium, 221 cells, HLA-C*03:04 (C1) and HLA-C*04:01 (C2) transfected 221 cells (**B**) for selected and amplified $KIR2DL1^+$ $KIR2DL2/3/S1/2^-$ $NKG2A^-$ NK cells from $C2^+$ ($n = 7$) and $C2^-$ ($n = 4$) individuals; (**C**) for selected and amplified $KIR2DL2^+$ $KIR2DL1/3/S1/2^-$ $NKG2A^-$ NK cells from $C1^+$ ($n = 4$) and $C1^-$ ($n = 6$) individuals; and (**D**) for selected and amplified $KIR2DL3^+$ $KIR2DL1/2/S1/2^-$ $NKG2A^-$ NK cells from $C1^+$ ($n = 7$) and $C1^-$ ($n = 8$) individuals. (**E**) Bars represent the mean of $CD107a^+$ $KIR2DL^+$ NK cell percentages (± SD) evaluated with a larger panel of C1 (HLA-C*03:01 and C*08:02) and C2 (HLA-C*04:01, C*02:02, C*06:02, and C*15:01) transfected 221 cells from previously studied individuals in (B)–(D). Educated NK cells ($C1^+$ $KIR2DL2^+$, $C1^+$ $KIR2DL3^+$, and *(Figure legend continues)*

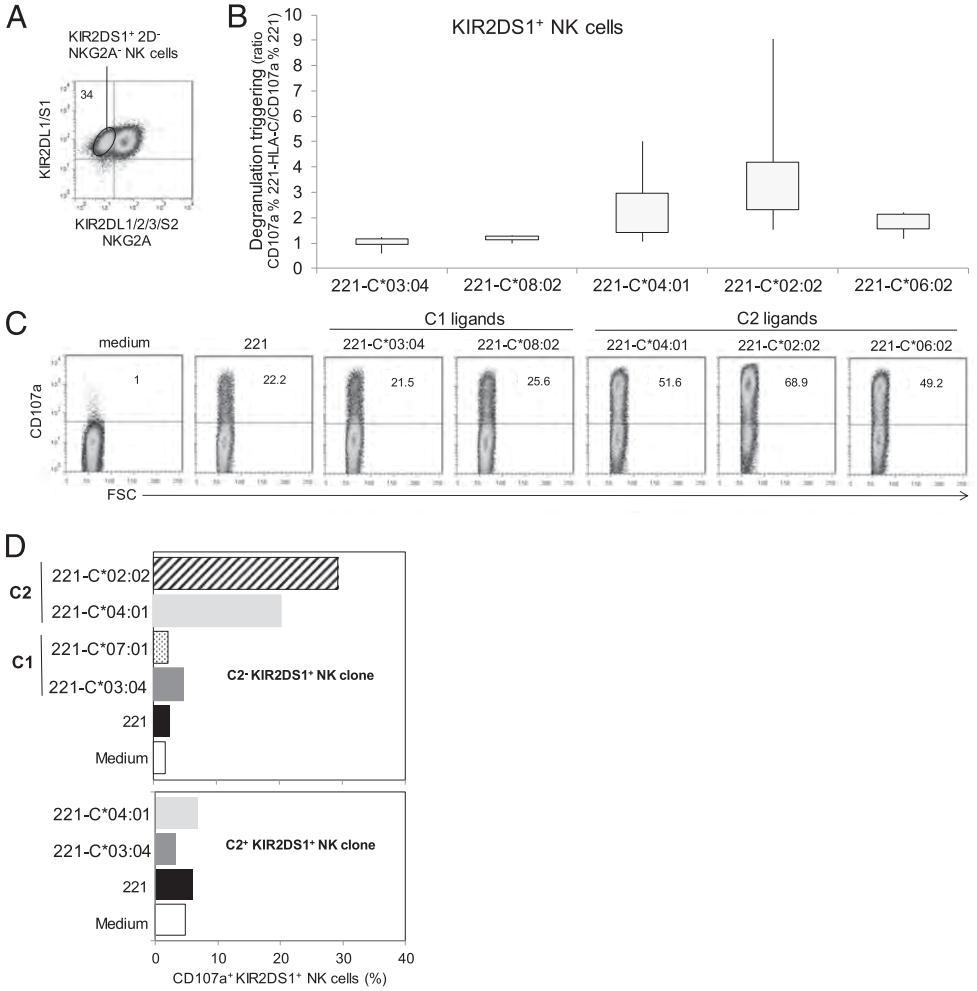

FIGURE 2. Stringent C2 alloreactivity of KIR2DS1$^+$ NK cells. (**A**) After depletion of CD3$^+$ cells from PBMCs, KIR2DS1$^+$ KIR2DL1/2/3/S2$^-$ NK cells (NKp46$^+$ EB6$^+$ 8C11$^-$) were cell sorted and amplified in vitro with irradiated allogeneic PBMCs and EBV-B cells as feeders. After 3 wk of stimulation, the phenotype of these sorted and stimulated cells was assessed by flow cytometry and is illustrated for one representative KIR2DS1$^+$ genotyped individual of eight studied individuals. Thirty-four percent of these amplified cells are KIR2DS1$^+$ KIR2DL1/2/3/S2$^-$ NKG2A$^-$ NK cells (NKp46$^+$ EB6$^+$ 8C11$^-$ NKG2A$^-$). (**B**) Summary box and whisker plot summarizing the percentages of CD107a$^+$ KIR2DS1$^+$ NK cells after 5-h incubation alone or in the presence of 221 cells as positive control, at an E:T ratio of 1:1, for eight experiments performed. To homogenize the data obtained from independent experiments, the data are presented as the ratio of the degranulation obtained with HLA-C transfected 221 cells to the degranulation obtained with untransfected 221 cells. (**C**) Representative density plots of KIR2DS1$^+$ KIR2DL1/2/3/S2$^-$ NKG2A$^-$ (NKp46$^+$ EB6$^+$ 8C11$^-$ NKG2A$^-$) NK cell degranulation observed in the different culture conditions. (**D**) Bars indicate the percentage of CD107a$^+$ KIR2DS1$^+$ NK cells from one C2$^-$ representative KIR2DS1$^+$ NK cell clone out of four clones isolated from three different C2$^-$ individuals and one C2$^+$ KIR2DS1$^+$ NK cell clone in a degranulation assay following the E:T ratio of 1:1 with different HLA-C transfected 221 target cells.

dominant effect of NKR-induced inhibitory over activating signals. To determine the impact of the HLA-C environment on C1 reactivity of KIR2DS2$^+$ NK cells, we evaluated the degranulation of 27 KIR2DS2$^+$ KIR2DL2$^-$ NK cell clones (Fig. 4A) and 14 KIR2DS2$^+$ KIR2DL2$^+$ NK cell clones (Fig. 4B) derived from 10 individuals (2 C1C1, 4 C1C2, and 4 C2C2) against 221, 221-C1 (HLA-C*03:04), and 221-C2 cells (HLA-C*04:01). Of note, we could not generate KIR2DS2$^+$ KIR2DL2$^+$ NK clones from C1C1 individuals. NK cell degranulation toward the 221 cell line varied from one KIR2DS2$^+$ NK cell clone to another. Because this variability could be linked to expression of other inhibitory or activating NKRs, we assessed expression of unengaged inhibitory receptors, like KIR3DL1 and ILT2, and activating receptors (e.g., KIR2DS4, NKp30, NKp44, NKG2D, 2B4, and CD16). For some clones, KIR3DL1 and NCR expression could explain a high reactivity toward 221 target cells of some clones, such as the 1F9 clone from D9 individual. However, this heterogeneity was most probably linked to previous in vitro stimulation of NK cell clones. A CD107a mobilization assay showed increased degranulation of KIR2DS2$^+$ NK cell clones with the 221-C1 (HLA-C*03:04) cell line in contrast with 221 and 221-C2 (HLA-C*04:01) counterparts, irrespective of the HLA-C background of individuals, even though few KIR2DS2$^+$ KIR2DL1/2/2DS1$^-$ clones were obtained from C1C1 individuals. Overall, the degranulation patterns of KIR2DS2$^+$ KIR2DL2$^-$ clones were similar in C1$^+$ (n = 11) versus C1$^-$ individuals (n = 8), because these clones were stimulated by the 221-C1 (HLA-C*03:04) cell line

C2$^+$ KIR2DL1$^+$) are indicated in light gray and uneducated NK cells (C1$^-$ KIR2DL2$^+$, C1$^-$ KIR2DL3$^+$, and C2$^-$ KIR2DL1$^+$) in dark gray. To homogenize the data obtained from independent experiments, we present the data as the degranulation obtained with HLA-C transfected 221 cells relative to the degranulation obtained with untransfected 221 cells at an E:T ratio of 1:1. (**F**) Histograms represent HLA class I expression of different HLA-C transfected 221 cell lines (filled) and 221 cell line as negative control (open).

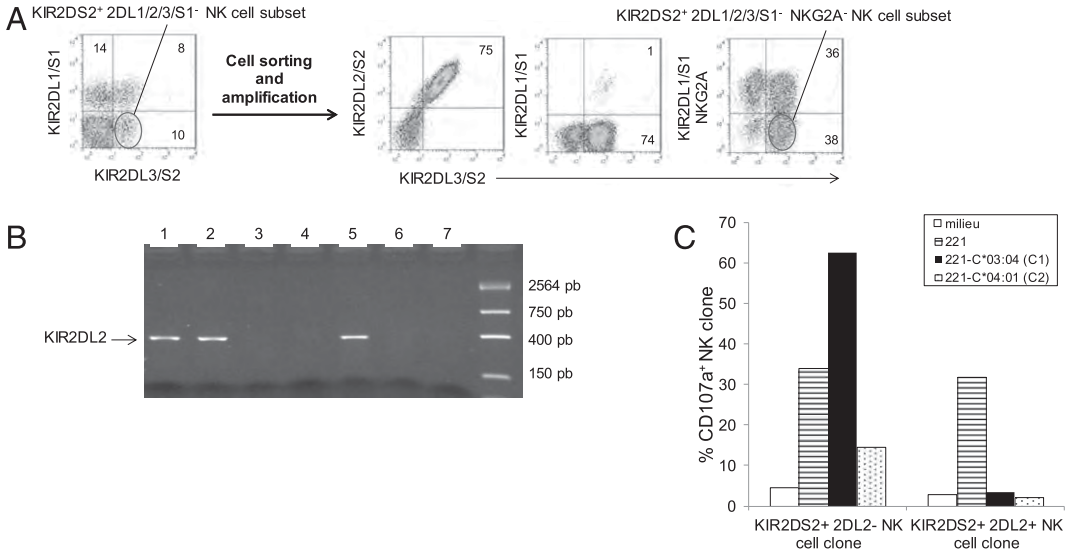

FIGURE 3. C1 specificity of KIR2DS2. (**A**) After depletion of $CD3^+$ cells from PBMCs, $KIR2DS2^+$ $KIR2DL1/3/2DS1^-$ NK cells ($NKp46^+$ $EB6^-$ $1F12^+$) were cell sorted (10% of the population; d0) from $KIR2DL3^-$ genotyped individuals and amplified in vitro with irradiated allogeneic PBMCs and EBV-B cells as feeders. After 3 wk of stimulation, the phenotype of these sorted and stimulated cells was assessed by flow cytometry and is illustrated for 1 representative $KIR2DS2^+$ genotyped individual of 10 studied individuals. Seventy-four percent of these amplified cells are $KIR2DS2^+$ $KIR2DL1/3/2DS1^-$ NK cells ($NKp46^+$ $EB6^-$ $1F12^+$) and 38% are $NKG2A^-$. (**B**) Visualization of specific KIR2DL2 RT-PCR products on a 2% agarose gel in 0.5× Tris Borate EDTA Buffer with Sequence Specific Primer Size Marker (One Lambda). RNA was extracted from $KIR2DS2^+$ $KIR2DL1/3/S1^-$ NK cell clones of interest and specific RT-PCR was then performed using KIR2DL2-specific PCR-SSP primers showing a specific RT-PCR product at 383 bp for some $KIR2DS2^+$ clones. (**C**) Histograms illustrating the degranulation of one $KIR2DS2^+$ $KIR2DL2^-$ NK cell clone and one representative $KIR2DS2^+$ $KIR2DL2^+$ NK cell clone in different culture conditions: medium, 221, HLA-C*03:04 (C1), and HLA-C*04:01 (C2) transfected 221 target cells at an ET ratio of 1:1.

(Fig. 4C), but not by 221 or 221-C2 (HLA-C*04:01) counterparts. These results suggest that, in contrast with KIR2DS1 NK cells, which are C2 alloreactive only from the $C2^-$ environment, KIR2DS2 NK cells are C1-reactive irrespective of their HLA-C haplotype. In parallel, degranulation of $KIR2DS2^+$ $KIR2DL2^+$ clones is inhibited by both 221-C1 (HLA-C*03:04) and 221-C2 (HLA-C*04:01), in line with results obtained with $KIR2DL2^+$ cell lines (Fig. 1), regardless of the HLA-C background and NK cell phenotype (Fig. 4B). The inhibitory C1-KIR2DL2 signaling bypassed the activating C1-KIR2DS2 signaling, highlighting the preponderant impact of KIR2DL2 on NK cell inhibition.

Additive effect of KIR2DL1 and KIR2DS2 against $C1^+$ target cells

Taking into account that activation of KIR2DS2 in the presence of a C1 ligand should have an additive effect on the activation mediated by the lack of engagement of KIR2DL1, we then evaluated the impact of the coexpression of KIR2DS2 and KIR2DL1 on recognition of $C1C1^+$ target cells. To this end, we sorted $KIR2DL1^+$ $KIR2DS2^+$ NK cells from KIR2DS1 and KIR2DL3 negative genotyped $C2^+$ individuals using anti-KIR2DL3/2DS2 (1F12)– and anti-KIR2DL1/S1 (EB6)–specific Abs (Fig. 5A). The sorted $KIR2DL1^+$ $KIR2DS2^+$ cells were cloned and phenotyped by KIR2DL2 RT-PCR as previously described (Fig. 3B), to discriminate $KIR2DL2^+$ NK cell clones. We observed a functionally additive effect of KIR2DL1 and KIR2DS2 on the recognition of the 221-HLA-C*03:04 (C1) cell line (Fig. 5B, 5C). C2 ligands, like HLA-C*02:02 and HLA-C*04:01, engaged KIR2DL1 receptors and inhibited $KIR2DS2^+$ $KIR2DL1^+$ NK cell degranulation.

The nature of KIR2D and HLA-C ligand affects the composition of the NK cell repertoire

To determine the functional impact of HLA-C–specific KIR2DL and KIR2DS receptor expression, we evaluated the phenotypic distribution of each KIR2D NK cell subset: $KIR2DL1/S1^+$ $KIR2DL2/3/S2^-$, $KIR2DL1/S1^-$ $KIR2DL2/3/S2^+$, and $KIR2DL1/S1^+$ $KIR2DL2/3/S2^+$ NK cell subsets within KIR2DL1/2/3/S1/2 NK cells, in a cohort of 159 KIR and HLA genotyped individuals (Supplemental Table I). Because almost all studied individuals were $KIR2DL1^+$ genotyped (96%), this analysis was performed according to KIR2DL2, KIR2DL3, and KIR2DS2 genotype and HLA-C environment (Fig. 6A). In this cohort, all $KIR2DL2^+$ individuals were $KIR2DS2^+$ genotyped; thus, we assigned the individuals in one of three groups determined by the presence of KIR2DL3, KIR2DL2/3/S2, and KIR2DL2/S2 genes. All studied populations were similarly represented, regardless of the HLA-C (C1C1, C1C2, or C2C2) environment (data not shown). However, $KIR2DL1/S1^+$ $KIR2DL2/3/S2^-$ NK cell frequency was significantly decreased in $KIR2DL2/S2^+$ individuals with a $C2^+$ haplotype (Fig. 6A, Supplemental Table II). The double-stained population frequency was not significantly different in the studied groups. The $KIR2DL2/3/S2^+$ $KIR2DL1/S1^-$ NK cell frequency was significantly higher in $KIR2DL2/S2^+$ compared with $KIR2DL3^+$ individuals (Fig. 6A, Supplemental Table II) with all HLA-C molecules, and in $KIR2DL2/3/S2^+$ compared with $KIR2DL3^+$ individuals with C1C1 and C2C2 molecules. Finally, we analyzed the distribution of each KIR2D NK cell subset, as defined by their mean frequencies in the pool of KIR2DL1/2/3/S1/2 NK cells (Fig. 6B), according to KIR2DL2, KIR2DL3, and KIR2DS2 genotypes and expression of either C1 or C2 molecules. The $KIR2DL1/S1^+$ NK cell subset predominated in $KIR2DL3^+$ individuals, and its mean frequency increased with the number of C2 alleles (mean frequencies are 36.1, 38.4, and 47.2%, respectively, in C1C1, C1C2, and C2C2 environments). In contrast, the presence of KIR2DL2 and KIR2DS2 correlated with increased mean frequencies of $KIR2DL2/3/S2^+$ NK cells, particularly in $KIR2DL2/S2^+$ individuals. Although KIR2DL2 and KIR2DL3 recognized a broad spectrum of HLA-C ligands, the higher affinity of KIR2DL2 than KIR2DL3 toward HLA-C ligands could account for decreased frequency of the $KIR2DL1^+$ NK cell pool in the former individuals. The six individuals with a $KIR2DL1^-$

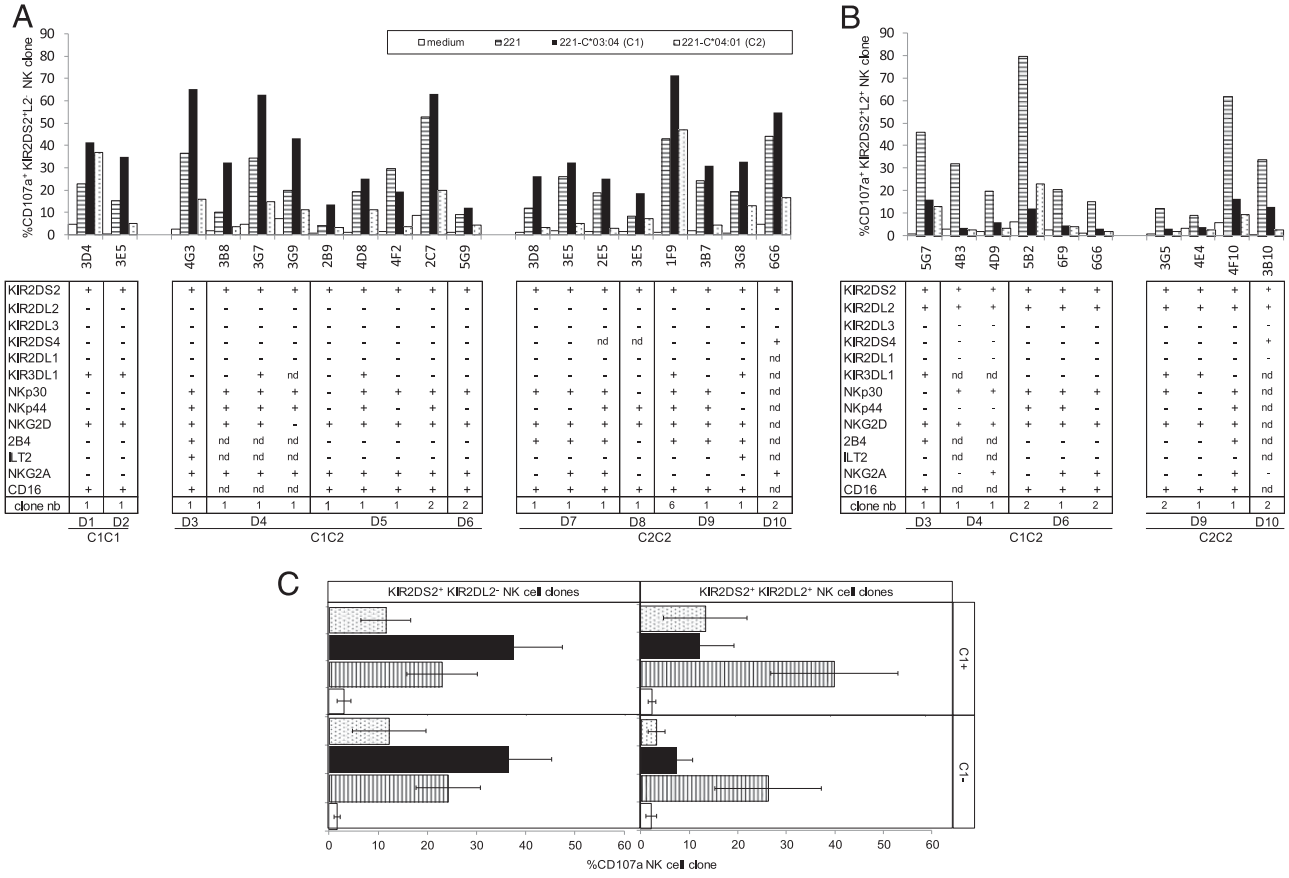

FIGURE 4. C1 reactivity of KIR2DS2$^+$ KIR2DL2$^-$ NK cell clones regardless of the HLA-C background. Histograms represent the percentage of (**A**) CD107a$^+$ KIR2DS2$^+$ KIR2DL2$^-$ NK cell clones and (**B**) KIR2DS2$^+$ KIR2DL2$^+$ NK cell clones isolated from C1C1, C1C2, and C2C2 individuals in different conditions of degranulation assay: medium, 221, HLA-C*03:04 (C1), and HLA-C*04:01 (C2) transfected 221 target cells at an ET ratio of 1:1. (**C**) Bars indicate the mean of the percentage of CD107a$^+$ KIR2DS2$^+$ KIR2DL2$^-$ NK cell clones (±SD) and CD107a$^+$ KIR2DS2$^+$ KIR2DL2$^+$ NK cell clones (±SD), grouped following C1$^+$ and C1$^-$ background, in different culture conditions: medium, 221, HLA-C*03:04 (C1), and HLA-C*04:01 (C2) transfected 221 target cells at an E:T ratio of 1:1.

genotype in our cohort were KIR2DL2/S2$^+$ KIR2DL3$^-$. We observed a significantly higher KIR2DL2/3/S2$^+$ NK cell frequency (mean = 36.1 ± 5) in these individuals when compared with KIR2DL1$^+$ KIR2DL2/S2$^+$ 2DL3$^-$ individuals (mean = 20.3 ± 5, p = 0.005; Fig. 6C), possibly as a result of compensatory KIR2DL2/S2 expression in the absence of KIR2DL1 expression. Thus, KIR2DL1 substitution by KIR2DL2 is phenotypically marked in the KIR2D repertoire and possibly allows maintenance of self C2 recognition.

Significant impact of KIR2DS1 and KIR2DS2 expression on KIR2D NK cell repertoire formation

To determine the impact of KIR2DS1 gene on the KIR2DL1/S1 NK cell compartment, we assessed the frequency of KIR2DL1$^+$ 2DS1$^-$, KIR2DL1$^+$ 2DS1$^+$, and KIR2DL1$^-$ 2DS1$^+$ NK cell subsets (Fig. 7A). Taking into account only the HLA-C environment, no difference in frequency was observed. However, a significantly decreased frequency of KIR2DL1$^+$ 2DS1$^-$ NK cells was observed particularly in B$^+$ haplotypes regardless of the HLA-C molecules, linked directly to KIR2DS1 expression (Fig. 7A, Supplemental Table II). The KIR2DL1$^-$ KIR2DS1$^+$ NK cell subset is significantly more represented in BB than AB haplotypes because of the increased number of KIR2DS1 allele copies. KIR2DS1 expression contributed to a significantly increased KIR2DL1/S1 NK cell pool, expressed with or without KIR2DL1 on NK cells (Fig.7C, 7D). Using a 1F12/GL183 Ab combination (20), we investigated the frequency of KIR2DL2$^+$ 2DL3/S2$^-$ and KIR2DL3/S2$^+$ NK cell subsets according to KIR2DL2, KIR2DL3, and KIR2DS2 genes and HLA-C environment (Fig. 7B, 7E). In accordance with the fact that KIR2DL2 and KIR2DL3 segregate as alleles (21), we observed that KIR2DL2$^+$ KIR2DL3/S2$^-$ NK cell frequency is significantly higher in KIR2DL2/S2$^+$ than KIR2DL2/3/S2$^+$ genotyped individuals with a C2C2 haplotype (Fig. 7B). However, KIR2DL2$^+$ KIR2DL3/S2$^-$ NK cell frequency was not twice in KIR2DL2/S2$^+$ genotyped individuals that was observed in KIR2DL2/3/S2$^+$ individuals with only one KIR2DL2 allele (Fig. 7E), which suggests either coexpression of KIR2DL2 with KIR2DS2, or decreased expression of KIR2DL2. Thus, the expression of the KIR2DS1 contributes to broaden significantly the pool of KIR2DL1/S1 NK cells even though it significantly limits the KIR2DL1$^+$ 2DS1$^-$ cell subset frequency. In addition, C1 reactive KIR2DS2 expression seems to function to enlarge the KIR2DL2/3/S2 NK cell pool, and it is conceivable that its additive effect with KIR2DL1 may contribute to limit KIR2DL1 expression on NK cells. In summary, our results suggest that together with KIR2DL2, activating KIR2DS1 and KIR2DS2 expression limits KIR2DL1 acquisition on NK cells.

Discussion

In this study, we revisited the HLA-C specificity of the main KIR2DL and determined C1 reactivity of KIR2DS2 from selected KIR2DL$^+$ or KIR2DS$^+$ NK cell lines and clones, and investigated these specificities on KIR2D NK cell formation. Our results are in accordance with numerous studies (9, 12, 13, 17, 22–28), and notably

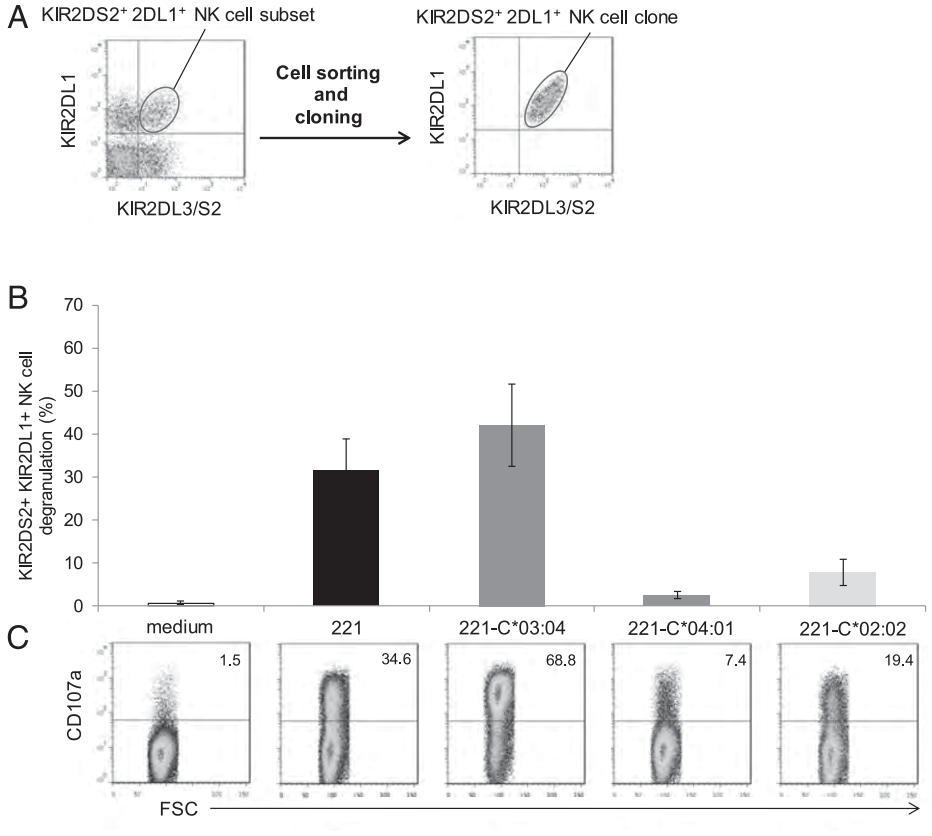

FIGURE 5. Additive effect of KIR2DL1 and KIR2DS2 against $C1^+$ target cells. (**A**) After depletion of $CD3^+$ cells from PBMCs, $KIR2DS2^+$ $KIR2DL1^+$ $KIR2DL2/3/S1^-$ NK cells ($NKp46^+$ $EB6^+$ $1F12^+$) were cell sorted from $C2^+$ $KIR2DL3/S1^-$ genotyped individuals and amplified in vitro with irradiated allogeneic PBMCs and EBV-B cells as feeders. After 3 wk of stimulation, NK cells were cloned and only $KIR2DL2^-$ NK cell clones verified by RT-PCR have been selected. The phenotype of one representative $KIR2DS2^+$ $KIR2DL1^+$ $KIR2DL2/3/S1^-$ NK cell clone is shown. (**B**) CD107a mobilization assay was performed on four $KIR2DS2^+$ $KIR2DL1^+$ $KIR2DL2/3/S1^-$ NK cell clones isolated from one $C2^+$ individual. These results were confirmed for eight $KIR2DS2^+$ $KIR2DL1^+$ $KIR2DL2/3/S1^-$ NK cell clones isolated from two different $C2^+$ individuals. Bars indicate the mean of $CD107^+$ NK cell percentages (± SD) in different culture conditions: medium, 221, HLA-C*03:04 (C1), and HLA-C*04:01 (C2) transfected 221 target cells at an E:T ratio of 10:1. (**C**) Representative density plots of $KIR2DS2^+$ $KIR2DL1^+$ $KIR2DL2/3/S1^-$ ($NKp46^+$ $EB6^+$ $1F12^+$) NK cell degranulation observed in the different culture conditions.

those obtained by Winter et al. (13) showing that KIR2DL2 and KIR2DL3 recognize C2 allotypes using NK92 infected with Vac-KIR2DL2 or -KIR2DL3. More recently, Moesta et al. (12) have shown that KIR2DL2 is a stronger receptor for HLA-C ligand than KIR2DL3. However, among C2 allotypes, we showed that HLA-C*04:01 ligand is better recognized than the other evaluated ligands (HLA-C*02:02, -C*06:02, and -C*15:01). These results are in line with a recent observation drawn from an analysis of

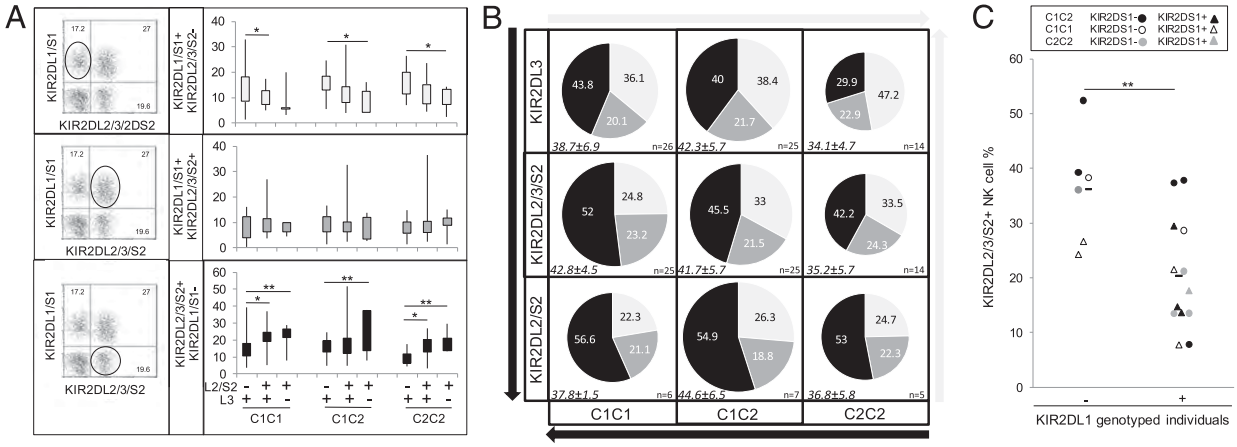

FIGURE 6. The nature of KIR2D and HLA-C ligands directs the structure of the NK cell repertoire. (**A**) Representative density plot illustrating the different KIR2D NK cell subsets in the large cohort of HLA and KIR genotyped individuals (Supplemental Table I) grouped according to the presence of KIR2DL2/3 and 2DS2 genes ($KIR2DL3^+$, $KIR2DL2/3/S2^+$, and $KIR2DL2/S2^+$) and HLA-C haplotype (C1C1, C1C2, and C2C2). $KIR2DL1/S1^+$ $KIR2DL2/3/S2^-$ ($EB6^+$ $GL183^-$) NK cell subset is indicated in white, $KIR2DL1/S1^+$ $KIR2DL2/3/S2^+$ ($EB6^+$ $GL183^+$) NK cell subset is indicated in gray, and $KIR2DL1/S1^-$ $KIR2DL2/3/S2^+$ ($EB6^-$ $GL183^+$) NK cell subset is indicated in black. Box and whisker plot summarizing the frequency of each KIR2D NK cell subset out of all NK cells. (**B**) Pie charts depict the pattern of KIR2D composition in nine groups of individuals distributed following the presence of KIR2DL2/3 and 2DS2 genes ($KIR2DL3^+$, $KIR2DL2/3/S2^+$, and $KIR2DL2/S2^+$) and HLA-C molecules (C1C1, C1C2, and C2C2). We summed $KIR2DL1/S1^+$ $KIR2DL2/3/S2^-$, $KIR2DL1/S1^-$ $KIR2DL2/3/S2^+$, and $KIR2DL1/S1^+$ $KIR2DL2/3/S2^+$ NK cell subsets, weighting them according to their frequency indicated in the pie chart. The size of the pie chart is proportional to the frequency of KIR2D NK cells (KIR2DL1/2/3/2DS1/2), and it is indicated (±SD) in the *bottom left* of each group in italics. The number of studied individuals in each group is indicated in the *bottom right*. (**C**) Scatter plots represent $KIR2DL2/3/2DS2^+$ NK cell frequency in $KIR2DL1^-$ ($n = 6$) and $KIR2DL1^+$ ($n = 13$) genotyped individuals. The mean frequency is indicated in each group by a black feature. Individuals have been identified following KIR2DS1 genotype and HLA-C molecules (C1C1, C1C2, and C2C2). Statistical significance ($^*p < 0.05$, $^{**}p \leq 0.001$) between two groups was determined using the one-way ANOVA test (Supplemental Table II).

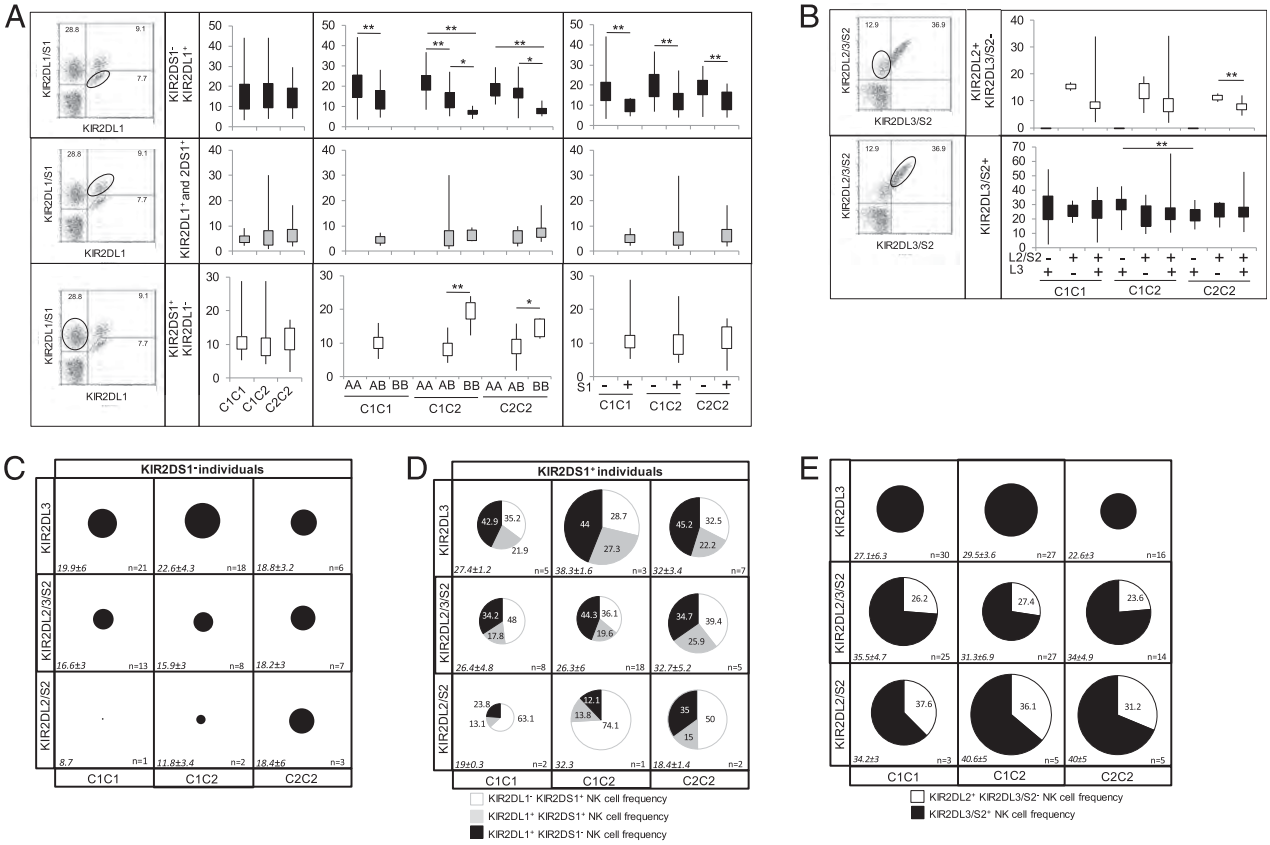

FIGURE 7. Significant impact of KIR2DS1 and KIR2DS2 expression on KIR2D NK cell repertoire formation. Representative density plot illustrating the different NK cell subsets expressing (**A**) KIR2DL1 or/and KIR2DS1, studied in the large cohort of HLA and KIR genotyped individuals (Supplemental Table I) grouped according to the HLA-C molecules (C1C1, C1C2, and C2C2), KIR haplotype (AA, AB, and BB), and presence or absence of the KIR2DS1 gene. KIR2DL1$^+$S1$^-$ (EB6^{low} 143211$^+$) NK cell subset is indicated in black, KIR2DL1$^+$S1$^+$ (EB6$^+$ 143211$^+$) NK cell subset is indicated in gray, and KIR2DL1$^-$S1$^+$ (EB6$^+$ 143211$^-$) NK cell subset is indicated in white. (**B**) Representative density plot illustrating the different NK cell subsets expressing KIR2DL2 and/or KIR2DL2/3/S2 studied in individuals grouped in function of the presence of KIR2DL2/3 and 2DS2 genes (KIR2DL3$^+$, KIR2DL2/3/S2$^+$, and KIR2DL2/S2$^+$) and HLA-C molecules (C1C1, C1C2, and C2C2). KIR2DL2$^+$ 2DL3/2DS2$^-$ (1F12$^-$ GL183$^+$) NK cell subset is indicated in white; KIR2DL2$^-$ 2DL3/2DS2$^+$ (1F12$^+$ GL183$^+$) NK cell subset is indicated in black. Box and whisker plot summarizing the frequency of each KIR2D NK cell subset out of all NK cells. Pie charts show the KIR2DL1 NK cell subset in nine groups of (**C**) KIR2DS1$^-$ genotyped individuals and (**D**) KIR2DS1$^+$ genotyped individuals distributed following the presence of KIR2DL2/3 and 2DS2 genes (KIR2DL3$^+$, KIR2DL2/3/S2$^+$, and KIR2DL2/S2$^+$) and HLA-C molecules (C1C1, C1C2, and C2C2). We summed KIR2DL1$^+$ S1$^-$, KIR2DL1$^+$ S1$^+$, and KIR2DL1$^-$ S1$^+$ NK cell subsets, weighting them according to their frequency indicated in the pie chart. (**E**) Pie charts depict the pattern of KIR2D composition in the KIR2DL2/3/2DS2 NK cell pool in nine groups of individuals distributed following the presence of KIR2DL2/3 and 2DS2 genes (KIR2DL3$^+$, KIR2DL2/3/S2$^+$, and KIR2DL2/S2$^+$) and HLA-C molecules (C1C1, C1C2, and C2C2). We summed KIR2DL2$^+$ 2DL3/S2$^-$ and KIR2DL2$^-$ 2DL3/S2$^+$ NK cell subsets, weighting them according to their frequency indicated in the pie chart. The size of the pie chart is proportional to the frequency of KIR NK cell pool and it is indicated (±SD) in the *bottom left* of each group in italics. The number of studied individuals in each group is indicated in the *bottom right*. Statistical significance (*$p < 0.05$, **$p \leq 0.001$) between two groups was determined using the one-way ANOVA test (Supplemental Table II).

a large panel of single-HLA class I molecule beads based on Luminex, which showed a broader pattern of HLA-C recognition by KIR2DL2-Fc fusion protein, even using unloaded HLA class I molecules (12). Recently, we have shown that KIR2DL3$^+$ NK cell lines equally recognized either C1C1 or C2C2 immature dendritic cells (29). The low affinity of the KIR2DL2/3 to HLA-C ligand is likely reinforced and stabilized within the immune synapse by other receptor–ligand interactions, thus allowing functional KIR-HLA interactions. Besides the wide spectrum of HLA-C recognition by KIR2DL2 and KIR2DL3, another important message of our study is the ability of activated KIR2DL2$^+$ and KIR2DL3$^+$ NK cells to recognize the "missing-self" irrespective of their HLA-C background. Our results are in agreement with those of Anfossi et al. (7), who suggested education of KIR2DL3$^+$ NK cells mainly from a C1$^+$ environment. Indeed, we observed that resting C1$^-$ KIR2DL3$^+$ NK cells are less receptive to missing-self activation (data not shown); however, KIR2DL3 engagement with C2 ligands during NK development or cytokine stimulation could enhance the response to missing-self after a preactivation, resulting in a decrease of their activation threshold. This point is essential in particular pathological contexts, such as viral infection, tumor processes, and HSCT, where activation could increase the missing-self response of poorly educated cells such as C1$^-$ KIR2DL3$^+$ NK cells or KIR2DL2$^+$ NK cells.

In contrast with other approaches based on soluble KIR-Ig proteins, KIR2D transfected cells, or a vaccinia expression system, our results were drawn from NK cells purified from different individuals with a physiological KIR2D expression. The more sensitive cellular model probably allows assessment of low-avidity interactions between of KIR2D and HLA-C, which could explain discordant results with those obtained with binding assays using KIR2D-Ig fusion proteins (13). This is typically the case for soluble KIR2DS receptors, which did not bind to any of the HLA class I molecules expressed on a large panel of transfected cells (13). In our cellular model, we showed that KIR2DS2 recognizes only C1 ligands, in contrast with its inhibitory KIR2DL2 form, in

all HLA-C environments. Failed C1/KIR2DS2 interactions using soluble KIR2DS2-Ig fusion proteins or KIR2DS2 tetramers (30) would suggest weak affinities. Thus, the few substitutions between the extracellular parts of both receptors could explain these apparent discrepancies. KIR2DS1 and KIR2DS2 show a lower affinity than their inhibitory counterparts with overlapping specificity, ensuring that inhibition signals override activation signals. However, in the absence of inhibitory KIR signals, the engagement of activating KIR2DS is sufficient to trigger NK cell responses.

The broader specificity of KIR2DL2 and KIR2DL3 toward HLA-C alleles also recognized by KIR2DL1 would explain the predominant expression of this compartment within the KIR2D NK cell repertoire. KIR2DL1 is predominantly represented in a C2C2 environment in the presence of KIR2DL3, but its representation is decreased in the presence of KIR2DL2 and KIR2DS2 genes. One possible explanation for this is that KIR2DL2 is a stronger competitor than KIR2DL3 for C2 ligands, explaining its predominant representation in the KIR2D NK cell pool in a C2C2 environment. Moreover, the high proportion of KIR2DS1$^+$ NK cells in the KIR2DL1/S1 pool could contribute to the decreased frequency of KIR2DL1$^+$ 2DS1$^-$ NK cells. Thus, all inhibitory and activating KIR2D are functional, but the strength of HLA-C affinity is different, ensuring a hierarchy between these KIR2D, and explains, in part, the formation of the NK cell repertoire depending on the HLA-C environment. In this regard, we observed in KIR2DL1$^-$ genotyped individuals that KIR2DL2/3/2DS2 expression was significantly increased compared with KIR2DL1$^+$ genotyped counterparts, suggesting an adjustment of the KIR2D repertoire formation after the distribution of each pool. This observation may not be compatible with a sequential acquisition of KIR with KIR2DL1 expression after KIR2DL2 and KIR2DL3 as proposed by Uhrberg's group (9). It is likely that the adjustment is more continuous and that the KIR2D expression can be readjusted according to other KIR2D expressed, as previously proposed (10).

The C2-expressing fetuses carried by group A KIR homozygous mothers constitute a risk factor for pre-eclampsia (31) and recurrent miscarriage (32) during pregnancy. In contrast, fetal C1 homozygosity and maternal group B KIR haplotype are protective (31, 32). Thus, it is conceivable that strong inhibitory KIR2DL2 maintains proper inhibition of NK cell responses against C1 fetal cells, whereas the weak inhibitor KIR2DL3, which is potentially efficient in C2 individuals on activated NK cells, may not properly inhibit NK cell response against allogeneic C2 ligands, resulting in pre-eclampsia or miscarriage. Moreover, although KIR2DL2 and KIR2DL3 segregate as alleles, only KIR2DL3 was associated with resistance to hepatitis C virus (33). The weaker interaction between KIR2DL3 and C1 ligand could be reinforced by particular peptides of viral or endogenous origin (34). In an HSCT context, it has been documented that KIR2DL2/3 and KIR2DS2 are predominantly expressed during hematopoietic reconstitution (35). In this activated context, KIR2DL2$^+$ and KIR2DL3$^+$ NK cells from C2C2 donors should ensure the missing-self recognition to compensate the default of functional KIR2DL1$^+$ NK cell recovery.

A previous study has defined five types of NK cell repertoire based on KIR dominant or NKG2A dominant expression, receptor-null expression, single-receptor expression, KIR-KIR coexpression, and NKG2A-KIR coexpression, and revealed a balance of KIR/NKG2A (36). This study was performed mainly from AA haplotype individuals and it would be interesting to investigate these types of repertoires taking into account the presence or absence of KIR2DL2/S2 and KIR2DS1 genes. Moreover, different affinities for the same HLA-C ligand have been shown for different KIR2DL3 allele products (12, 17), and this affinity can be modulated by the nature of loaded peptide (23). Thus, the formation of the KIR2D NK repertoire is probably finely adjusted depending on the KIR2DL polymorphism and on the other NK cell subsets. In this regard, we have recently shown the impact of KIR3DL1 allelic nature on KIR3DL1 NK repertoire formation (37). These recent investigations suggest primary expression of KIR3DL1 followed by KIR2D acquisition. The impact of allelic KIR3DL1 and a Bw4 environment on KIR2D NK cell repertoire should be studied in a larger cohort. Altogether, our results suggest that the nature of the HLA-C–specific activating and inhibitory KIR2D expressed and the ligand environment directs the structure of NK cell repertoire.

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Disclosures

The authors have no financial conflicts of interest.

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Both the nature of KIR3DL1 alleles and the KIR3DL1/S1 allele combination affect the KIR3DL1 NK-cell repertoire in the French population

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NK-cell functions are regulated by many activating and inhibitory receptors including KIR3DL1. Extensive allelic polymorphism and variability in expression can directly alter NK-cell phenotype and functions. Here we investigated the KIR3DL1$^+$ NK-cell repertoire, taking into account the allelic KIR3DL1/S1 polymorphism, KIR3DL1 phenotype, and function. All 109 studied individuals possessed at least one KIR3DL1 allele, with weak KIR3DL1*054, or null alleles being frequently present. In KIR3DL1$^{high/null}$ individuals, we observed a bimodal distribution of KIR3DL1$^+$ NK cells identified by a different KIR3DL1 expression level and cell frequency regardless of a similar amount of both KIR3DL1 transcripts, HLA background, or KIR2D expression. However, this bimodal distribution can be explained by a functional selection following a hierarchy of KIR3DL1 receptors. The higher expression of KIR3DL1 observed on cord blood NK cells suggests the expression of the functional KIR3DL1*004 receptors. Thus, the low amplification of KIR3DL1^{high}, KIR3DL1*004 NK-cell subsets during development may be due to extensive signaling via these two receptors. Albeit in a nonexclusive manner, individual immunological experience may contribute to shaping the KIR3DL1 NK-cell repertoire. Together, this study provides new insight into the mechanisms regulating the KIR3DL1 NK-cell repertoire.

Keywords: Allelic polymorphism · French population · KIR3DL1 · KIR3DL1/S1 allele combinations · NK-cell repertoire

Additional supporting information may be found in the online version of this article at the publisher's web-site

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Introduction

The effector functions of NK cells are regulated by inhibitory and activating receptors, e.g. killer cell immunoglobulin-like receptors (KIR), which are specific for allotypic determinants shared by different HLA-class I molecules [1]. In particular, HLA-Cw allotypes with Asn^{80} (C1 ligands) or Lys^{80} (C2 ligands) are, respectively, recognized by KIR2DL2/2DL3 and KIR2DL1. HLA-A and HLA-B allotypes with a Bw4 motif are recognized by KIR3DL1 whereas HLA-A3/A11 are recognized by KIR3DL2 [2,3]. Lack of inhibitory KIR engagement can trigger alloreactive KIR NK-cell cytotoxicity only within functionally competent NK cells [4]. Although the ligands and functions of inhibitory KIR receptors are well documented, this is not the case for activating KIR receptors and their ligands, except for KIR2DS1 which recognizes C2 ligands only in C2-adult individuals [5]. Moreover, the activating receptor KIR3DS1, which segregates as an allele of KIR3DL1 [6], shares more than 97% sequence homology in its extracellular domain with the KIR3DL1 receptor. However, a functional interaction with HLA-A or HLA-B allotypes sharing the Bw4 public epitope has not been demonstrated in vitro [7, 8] despite a significantly higher frequency of KIR3DS1$^+$ NK cells observed in Bw4$^+$ than in Bw4$^-$ individuals [9].

KIR genes are located on chromosome 19q13.4. To date, 14 functional KIR genes have been characterized [10]. Within the human population, genomic diversity of the KIR region is achieved on several levels. KIR gene content varies between individuals who can exhibit 7–14 inhibitory and activating KIR genes [11]. Population studies have demonstrated two major KIR haplotypes: A and B [12]. The A haplotypes correspond to 7 KIR genes, including KIR2DS4 as the only activating KIR gene. In contrast, B haplotypes are more diverse and are characterized by the presence of more than one activating KIR gene and the absence of the KIR2DS4 gene [13, 14]. KIR gene polymorphism is the largest contributor to KIR region diversity, with multiple alleles defined [15]. While polymorphism is limited in the KIR2DL1 and KIR2DL2/L3 genes, it is much broader for KIR3DL1 and can even alter NK-cell functions [16,17]. Indeed, depending on the KIR3DL1 allele present in a given individual, the level of KIR3DL1 expression differs on the NK-cell surface, i.e. null for KIR3DL1*004, low for KIR3DL1*005 and *007, and high for KIR3DL1*001, *002, *01502, and *008 alleles [18–20]. Two KIR3DL1 alleles can be transcribed in one NK-cell clone, and the proportion of clones expressing both alleles is high in comparison to clones expressing only one KIR3DL1 allele. However, based on the phenotypic pattern on all NK cells, only the nature of each allele is marked [19].

In addition to allelic polymorphism, the variability in expression levels of KIR3DL1 molecules on the NK-cell surface also varies depending on the presence or absence of autologous Bw4 molecules, promoter polymorphism, and DNA methylation [21–23]. Functional interaction between KIR3DL1 receptors and their HLA-Bw4 ligands are modulated by KIR3DL1 allotypes [24, 25] and the peptide content of HLA class I molecules [26]. Moreover, KIR3DL1$^+$ NK-cell cytotoxicity differs depending on KIR3DL1 allotypes and/or HLA-A, HLA-B Bw4$^+$ targets [3,27,28]. From a clinical standpoint, KIR3DL1 allelic polymorphism has been correlated with HIV progression and viral load [29]. We have previously shown that KIR3DL1/3DS1 gene disparities of donor/recipient pairs in hematopoietic stem cell transplantation have a significant impact on hematopoietic stem cell transplantation outcome [30].

Until now, the impact of KIR3DL1/3DS1 allele combinations on NK-cell phenotype and function has only been studied in one Japanese cohort not representative of Caucasians [31]. However, the mechanisms shaping the KIR3DL1 repertoire remain ill-defined. The link between KIR3DL1 allelic polymorphism, levels of KIR3DL1 expression at the NK-cell surface, and frequency of KIR3DL1$^+$ NK-cell subsets has yet to be clarified. This is necessary in order to better understand the mechanisms that shape the KIR3DL1$^+$ NK-cell repertoire. In this study, we hypothesize that the high KIR3DL1 allelic polymorphism, including frequent null alleles observed in Caucasians, might impact the formation of the KIR3DL1 NK-cell repertoire. We therefore analyzed the KIR3DL1 NK-cell repertoire taking into account the KIR3DL1 allelic polymorphism, KIR3DL1/KIR3DS1 allele combinations, and phenotypic patterns such as frequency, the mean level of all KIR3DL1$^+$ NK-cell subsets, the Bw4 environment, and the functional potential of these KIR3DL1$^+$ NK-cell subsets.

Results

High proportion of KIR3DL1^{high} and KIR3DL1^{null} alleles in French individuals

KIR3DL1/3DS1 allele combinations were investigated in AA ($n = 34$), AB ($n = 67$), or BB ($n = 8$) KIR genotyped individuals. Overall, 33 different KIR3DL1/3DS1 allele combinations were observed (Fig. 1A, central panel). AA and AB KIR genotyped individuals exhibited a higher KIR3DL1 allelic variability compared with BB KIR genotyped individuals. The KIR3DL1*001, *004 allele combination was the most frequent in AA KIR genotyped individuals (Fig. 1A). KIR3DL1*001, *002, *004, *005, *007, *008, *015 known alleles were the most frequent (Fig. 1A, right panel). In AA KIR genotyped individuals, the frequencies of KIR3DL1 alleles ranged from 1.61 (KIR3DL1*008, *015, *005, *009, *019) to 32.26% (KIR3DL1*004; Fig. 1A, right panel). In AB KIR genotyped individuals, the frequencies of KIR3DL1 alleles ranged from 0.71 (KIR3DL1*017, *009) to 17.73% (KIR3DL1*001; Fig. 1A, right panel). In our cohort, the highly expressed KIR3DL1*001 and null KIR3DL1*004 alleles were the most frequent, being observed at one or two doses in 39 (35%) and 32 (29%) KIR genotyped individuals, respectively (Fig. 1A, right panel). The presence of autologous HLA-A and/or HLA-B Bw4$^+$ molecules did not affect both the KIR3DL1 allele and KIR3DL1/3DS1 allele combination frequencies (data not shown).

The frequency of KIR3DL1$^+$ NK-cell populations and the KIR3DL1 expression level were further assessed using the KIR3DL1- and KIR3DS1-specific Z27 specific monoclonal antibody in 109 KIR3DL1 allele-typed individuals (Fig. 1B).

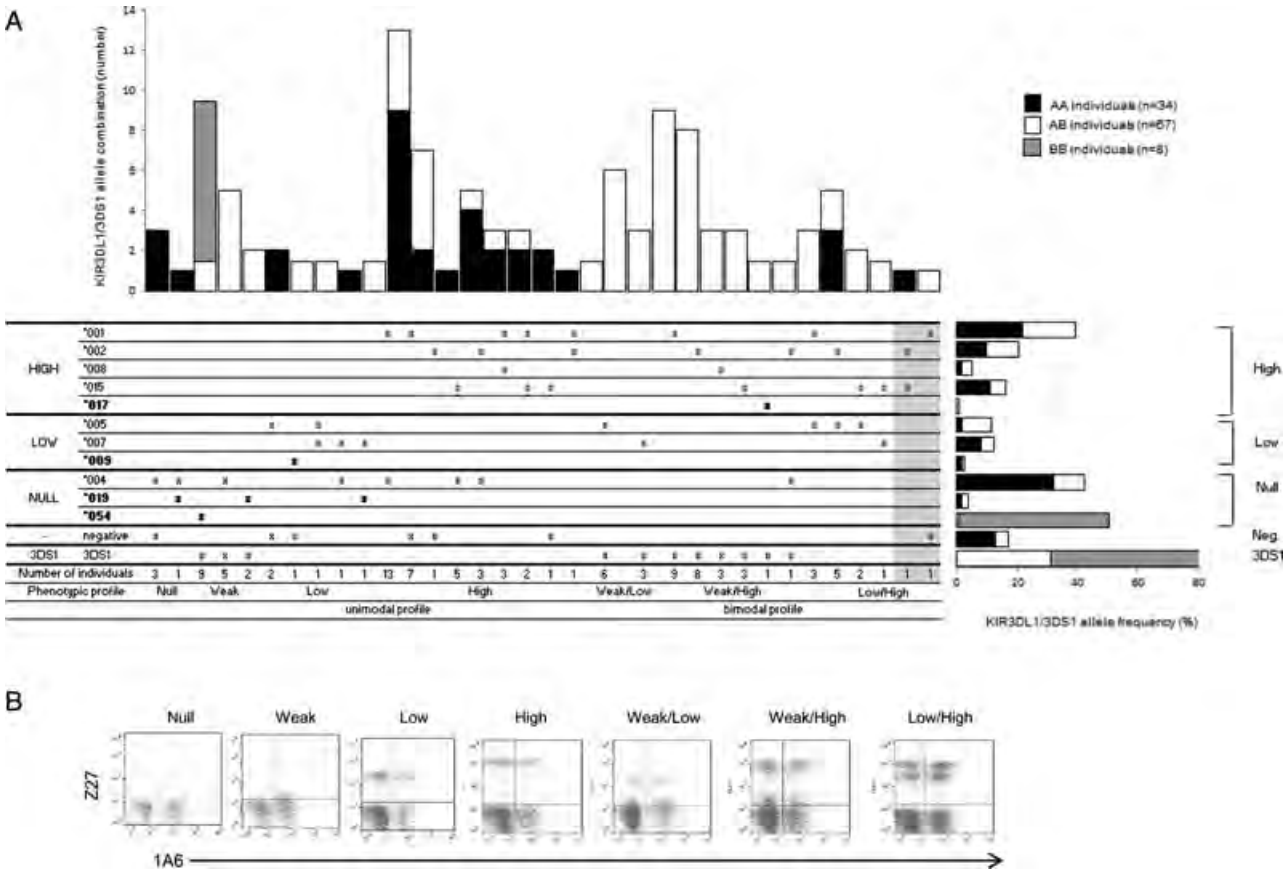

Figure 1. High proportion of KIR3DL1^{high} and KIR3DL1^{null} alleles in French individuals. (A) The number of KIR3DL1/3DS1 allele combinations and corresponding KIR3DL1/3DS1 allele frequency were established combining both allele-group–specific PCR-SSP and sequencing of polymorphic exons in AA (n = 34), AB (n = 67), and BB (n = 8) KIR genotyped individuals as described in the Materials and Methods (top). Distribution of KIR3DL1/3DS1 allele combinations and corresponding number of individuals are shown for each allele according to level of expression intensity on the NK-cell surface (i.e. null, low, high) and KIR3DS1 (weak) using the KIR3DL1/3DS1-specific Z27 mAb (bottom). New KIR3DL1 phenotypes are indicated in bold type and unexpected KIR3DL1 profiles are highlighted with gray shading. (B) Representative flow cytometry density plots of KIR3DL1$^+$ and KIR3DS1$^+$ NK cells for different KIR3DL1/S1 allele combinations identified from 109 studied individuals are shown. The staining was done using the NKp46-specific mAb to target NK cells, KIR2D-specific 1A6 mAb, and KIR3DL1/3DS1-specific Z27 mAb to evaluate KIR3DL1/3DS1$^+$ NK cells with or without KIR2D receptor expression. Data shown are representative of 109 individuals.

To examine the potential effect of KIR3DL1/3DS1 allele combinations on NK KIR3DL1$^+$ phenotype, individuals were divided into different groups based on null, low, and high KIR3DL1 expression, and weak expression of KIR3DS1. Seven different phenotypic patterns were identified: no binding pattern (KIR3DL1^{null},$^{null/null}$ genotypes), a unimodal pattern with weak intensity (KIR3DL1^{null}/KIR3DS1), a unimodal pattern with low intensity (KIR3DL1$^{low/low}$ or KIR3DL1$^{low/null}$), a unimodal pattern with high intensity (KIR3DL1$^{high/high}$ or KIR3DL1$^{high/null}$), a bimodal pattern with weak and low intensities (KIR3DS1/KIR3DL1^{low}), a bimodal pattern with weak and high intensities (KIR3DS1/KIR3DL1^{high}), and a bimodal pattern with low and high intensities (KIR3DL1$^{low/high}$). The bimodal expression patterns observed in KIR3DL1$^{low/high}$ and KIR3DS1/KIR3DL1^{high} individuals indicates the presence of NK-cell subsets expressing each of the expected KIR3DL1 alleles, and are in agreement with the reported mutually exclusive expression of KIR3DL1 and KIR3DS1 [6]. All combinations including KIR3DL1^{high}, KIR3DL1^{low}, KIR3DL1^{null}, or KIR3DS1 were observed. The in-depth analysis of KIR3DL1 alleles and NK-cell phenotypes revealed the KIR3DL1 phenotypic profile for KIR3DL1*009 and KIR3DL1*01702 alleles which are, respectively, expressed at low and high levels on the NK-cell surface and highlight unusual KIR3DL1 phenotypic profiles in a low proportion of individuals (Fig. 1A). The distribution of all KIR3DL1/3DS1 allele combinations shows that KIR3DL1^{high} alleles were abundant, and mainly associated with KIR3DL1^{null} or KIR3DS1 (Fig. 1A and B).

The KIR3DL1*054 allele is frequently present and mainly associated with KIR3DS1

All KIR3DS1$^+$ individuals for whom no KIR3DL1 allele was identified at the genotypic level had the KIR3DL1*054 allele [32], as illustrated in Figure 2A for two individuals and two well-characterized cell lines (13th International Workshop; WT51 and

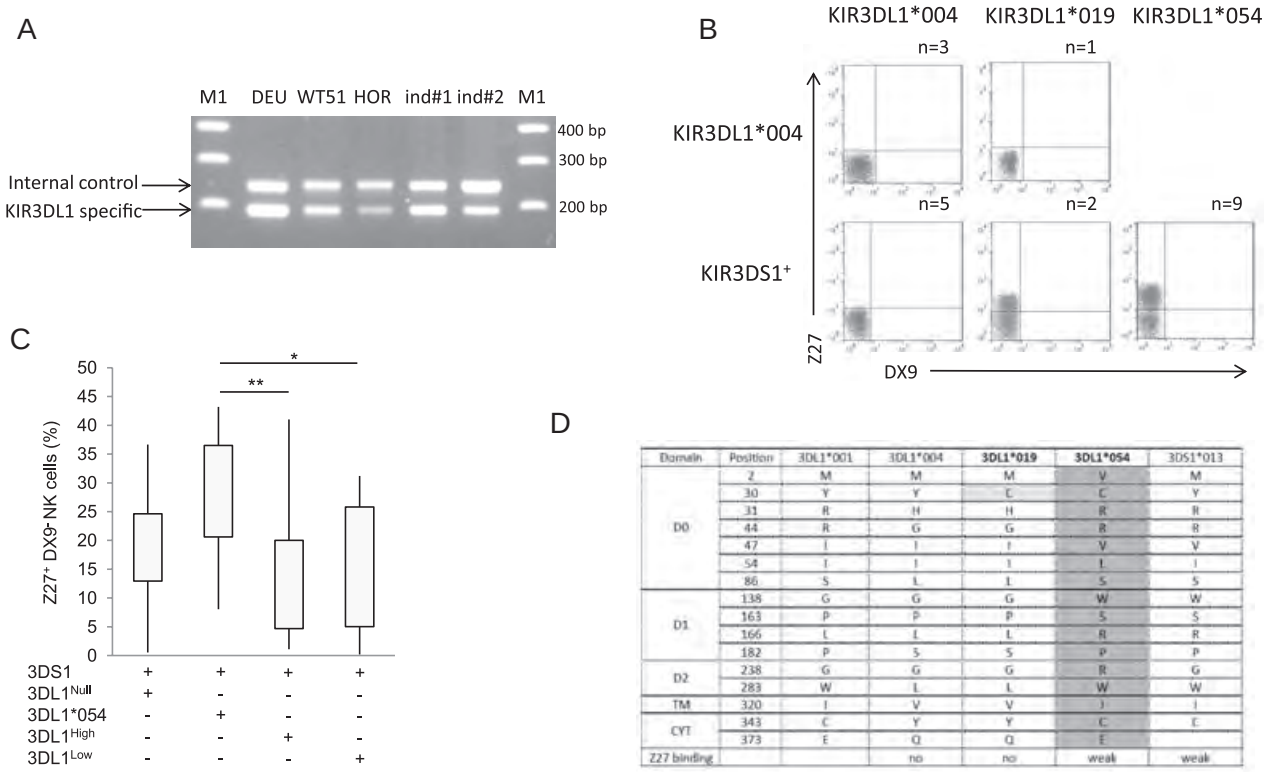

Figure 2. The KIR3DL1*054 allele is frequently present and mainly associated to KIR3DS1. (A) Visualization of KIR3DL1 PCR amplification products in a 2% Seakem® (Lonza) agarose gel in 0.5X TBE buffer with a 100 bp Ext. ladder marker (Lonza, M1). The figure shows one KIR3DL1$^+$ control Workshop DNA (DEU), two KIR3DL1$^-$ control Workshop DNAs (WT51, HOR) and two individuals previously determined as KIR3DL1$^-$ using the KIR genotyping SSP kit from Invitrogen. Both an internal control band at 256 bp and a specific PCR product amplifying exon 3 at 197 bp using homemade KIR3DL1 primers [53] can be observed. (B) Representative flow cytometry density plots of KIR3DL1$^+$ and KIR3DS1$^+$ NK cells for different KIR3DL1/S1 allele combinations integrating KIR3DL1*004, KIR3DL1*019, or KIR3DL1*054 with the KIR3DS1 or KIR3DL1*004 allele are shown. Density plots are gated on CD3$^-$ CD56$^+$ NK cells. KIR3DL1$^+$ and KIR3DS1$^+$ cells are, respectively, targeted as DX9$^+$ Z27$^+$ and DX9$^-$ Z27$^+$ cells. The number of individuals displaying each KIR3DL1/S1 allele combination is indicated above the density plot. (C) Box and whisker plot summarizing the frequency of Z27$^+$ DX9$^-$ NK cells in KIR3DS1$^+$ individuals as a function of the nature of the KIR3DL1 allele: null ($n = 7$), KIR3DL1*054 ($n = 7$), high ($n = 19$) and low ($n = 8$). Top and bottom whiskers represent values of the top and bottom 25% of cases respectively; boxed area, interquartile range. Statistical significance ($^*p < 0.05$; $^{**}p < 0.01$) between different groups was determined using the one-way ANOVA test. (D) Amino acid substitutions and level of Z27 binding for the KIR3DL1*004 allotype compared with the KIR3DL1*001 (reference), KIR3DL1*019 and KIR3DL1*054 (in bold), and KIR3DS1*013 allotypes are shown. Different amino acid substitutions between KIR3DL1*004 and KIR3DL1*019 on the one hand and KIR3DL1*004 and KIR3DL1*054 on the other hand are highlighted in light gray and dark gray shading respectively.

HOR) previously defined as KIR3DL1$^-$ [33]. The KIR3DL1*001, *005 genotyped and characterized DEU cell line (13th International Workshop) was used as a positive control. Sequencing of all amplified products in nine 3DL1$^-$, 3DS1$^+$ genotyped individuals showed that the KIR3DL1*054 allele was unique, with specific T^{475}, T^{550} and G^{560} nucleotides in exon 4 (data not shown). As described for KIR3DL1*004 [34, 35], no KIR3DL1 receptor was detected on the NK-cell surface for the only KIR3DL1*019, *004 individual studied (Fig. 2B). Alignment of amino acid substitutions of KIR3DL1*019 and KIR3DL1*054 compared with that of the KIR3DL1*004 allotype showed that the expected mature KIR3DL1*019 protein possesses the same amino acids involved in the intracellular retention of the null KIR3DL1*004 allotype, i.e. L^{86} and S^{182} (Fig. 2D). Only one amino acid in the D0 domain differed between KIR3DL1*019 and KIR3DL1*004. All KIR3DL1*054$^+$ individuals identified in our cohort presented the KIR3DS1 allele (Fig. 1A). In KIR3DS1$^+$ individuals, staining using the combination of Z27 and DX9 mAbs did not identify a DX9$^+$ Z27$^+$ population potentially corresponding to KIR3DL1*004, *019, or *054 allele products. Because KIR3DS1 and KIR3DL1*054 [32,36] were recognized by Z27 (a weak intensity staining pattern was observed Fig. 2B), it was difficult to selectively evaluate the expression of KIR3DL1*054 at the NK-cell surface. Interestingly, the frequency of Z27$^+$ DX9$^-$ expression was significantly higher for KIR3DS1$^+$, 3DL1*054$^+$ (28%, $n = 7$) than for KIR3DS1$^+$, 3DL1^{high} (13.26%, $n = 19$), or KIR3DS1$^+$, KIR3DL1^{low} (13.03%, $n = 8$) genotyped individuals with a p value of 0.01 and 0.03, respectively (Fig. 2C). This suggested either a cellular expression of the KIR3DL1*054 allotype, like KIR3DS1, recognized by the Z27 mAb, or a higher expression of KIR3DS1 in association with the KIR3DL1*054 allele (Fig. 2C). Different amino acid substitutions were detected between KIR3DL1*054 and KIR3DL1*004 (Fig. 2D), suggesting that KIR3DL1*054 is more similar to KIR3DS1 than to KIR3DL1. These amino acid

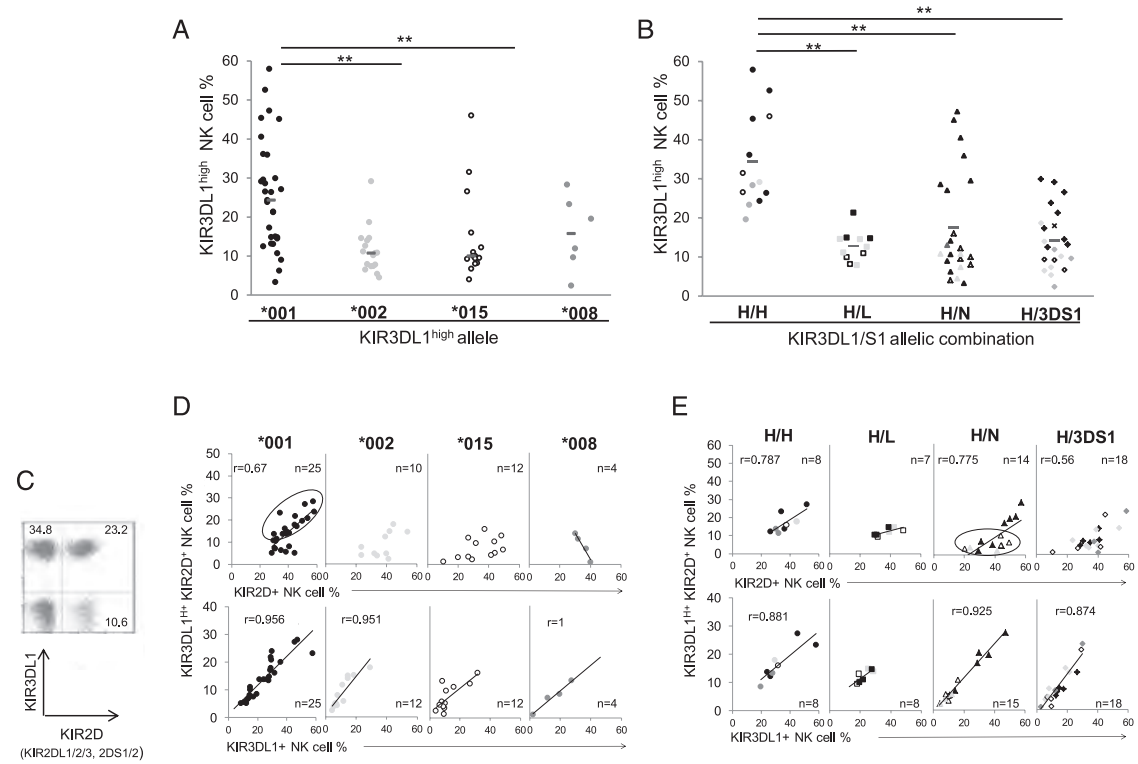

Figure 3. KIR3DL1^{high} NK-cell frequencies are determined by the number and nature of KIR3DL1^{high} alleles. (A) The KIR3DL1^{high} NK-cell frequencies out of all NK cells for 70 studied individuals as a function of the nature of the KIR3DL1^{high} allele: KIR3DL1*001 (filled black circle), KIR3DL1*002 (filled light gray circle), KIR3DL1*015 (open circle), and KIR3DL1*008 (filled dark gray circle) are shown. (B) All KIR3DL1^{high} NK-cell frequencies out of all NK cells for 70 studied individuals as a function of the second KIR3DL1 allele (high/high, high/low, and high/null) or KIR3DS1 (high/3DS1): KIR3DL1^{high} (circle), KIR3DL1^{low} (square), KIR3DL1^{null} (triangle), or KIR3DS1 (diamond). For all dots, the nature of each allele is determined by the color code used previously: KIR3DL1*001 (filled black symbols), KIR3DL1*002 (filled light gray symbol), KIR3DL1*015 (open symbol), KIR3DL1*008 (filled dark gray symbol), and KIR3DL1*017 (black cross). Statistical significance (**$p < 0.01$) between both groups was determined using the one-way ANOVA test. (C) Representative density plot of flow cytometry analysis of NK-cell ($CD3^-$ $CD56^+$) phenotype using KIR3DL1-specific Z27 and KIR2DL1/2/3- and KIR2DS1/2-specific 1A6 mAbs. (D, E) KIR3DL1^{high} $KIR2D^+$ NK-cell frequencies are compared with KIR3DL1^{high+} NK-cell frequencies and $KIR2D^+$ NK-cell frequencies as a function of (D) the nature of the KIR3DL1^{high} allele (*001, *002, *015, and *008), or of (E) the KIR3DL1/3DS1 allelic combinations (high/high, high/low, high/null, and high/3DS1). For all dots, the nature of each allele is determined by the color code used in (A, B). Spearman's rank correlation coefficients were calculated and indicated only when a significant p-value was obtained ($p < 0.05$). The number of studied individuals is indicated on each plot graph.

changes, especially in the extracellular domains, could potentially interfere with Z27/DX9 epitope recognition.

KIR3DL1^{high} NK-cell frequencies are determined by the number and nature of KIR3DL1^{high} alleles

Taking into account the nature of KIR3DL1^{high} alleles, we identified from all studied KIR3DL1^{high} individuals ($n = 70$), a higher frequency of KIR3DL1^{high} NK cells with the KIR3DL1*001 allele (24.3%, $n = 31$) than the KIR3DL1*002 allele (10.7%, $n = 17$, $p < 0.0003$), the KIR3DL1*015 allele (10%, $n = 15$, $p = 0.01$), or the KIR3DL1*008 (15.5%, $n = 6$, $p > 0.05$) (Fig. 3A). The frequency of KIR3DL1^{high} NK cells was further compared in four groups of individuals based on the nature of the second allele: KIR3DL1^{high}, KIR3DL1^{low}, KIR3DL1^{null}, and KIR3DS1. Interestingly the overall frequency of KIR3DL1^{high} NK cells in KIR3DL1 $^{high/high}$ individuals was about twice that found in heterozygous individuals (29.2%, $n = 13$ versus 12.2%, $n = 57$, $p = 3 \times 10^{-7}$) (Fig. 3B). The distribution of KIR3DL1^{high} NK-cell frequencies was heterogeneous in KIR3DL1$^{high/null}$ and KIR3DL1^{high}/3DS1 individuals, and clearly bimodal for the KIR3DL1$^{high/null}$ group. However, on average the KIR3DL1^{high} NK-cell frequencies in KIR3DL1$^{high/null}$ and KIR3DL1^{high}/3DS1 individuals were much lower than in KIR3DL1$^{high/high}$ individuals (10.8 versus 29.2%, $p = 0.0015$ and 12.5% versus 29.2%, $p = 6.2 \times 10^{-7}$, respectively). Such differences are in accordance with KIR3DL1 allelic exclusion, suggesting independent and additive generation of NK-cell subsets with the KIR3DL1 alleles being expressed in a mutually exclusive fashion. Within KIR3DL1$^{high/high}$ individuals ($n = 13$), higher KIR3DL1$^+$ NK-cell frequencies were observed for homozygous KIR3DL1*001 individuals than for those with a single or no KIR3DL1*001 allele (Fig. 3B). A similarly higher percentage of KIR3DL1^{high} NK cells was observed in individuals carrying KIR3DL1*001 versus other KIR3DL1^{high} alleles in KIR3DL1$^{high/null}$, KIR3DL1$^{high/low}$, and KIR3DL1^{high}/3DS1

individuals (Fig. 3B). Strikingly, the frequency of KIR3DL1^{high} NK cells expressing the KIR3DL1*001 allele increases to around 50% with only one copy of the KIR3DL1*001 allele, notably for KIR3DL1*001,*004 genotyped individuals (Fig. 3B). Since the KIR3DL1 NK-cell frequency can be influenced by the Bw4 environment [21], we investigated the frequency and nature of the KIR3DL1 ligand in this French population. Overall, the frequency of HLA-B Bw4$^+$ individuals was high (59%) and increased above 70% when both HLA-A and HLA-B Bw4$^+$ molecules were taken into account. Fourteen out of 18 described Bw4 ligands were observed, with a predominance of A24 (27.4%) and B44 (30.2%; Supporting Information Fig. 1A). The presence of autologous HLA-A and/or HLA-B Bw4$^+$ molecules did not affect the frequency of KIR3DL1^{high} NK cells taking into account not only all KIR3DL1^{high} individuals but also the number of KIR3DL1^{high} allele copies (2X or 1X) or KIR3DL1/3DS1 allele combinations (3DL1$^{high/low}$, 3DL1$^{high/null}$, or 3DL1^{high}/3DS1; Supporting Information Fig. 1B). Moreover, the higher frequency of KIR3DL1^{high} NK cells observed with the KIR3DL1*001 allele was independent of the Bw4 ligand number (Supporting Information Fig. 1B). Thus, the heterogeneous distribution of KIR3DL1^{high} NK-cell frequencies, especially within KIR3DL1$^{high/null}$ individuals, suggests the contribution of factors other than copy number and Bw4 environment. We observed that the KIR3DL1/S1 allele combinations were associated not only with KIR genotypes (Fig. 1A) but also with the presence of a particular KIR gene. For example, 21 out of 22 KIR3DL1^{high}/3DS1 genotyped individuals displayed the KIR2DS1 gene compared with only 2 out of 12 KIR3DL1$^{high/high}$ individuals, none out of 23 KIR3DL1$^{high/null}$ and none out of 11 KIR3DL1$^{high/low}$ individuals (data not shown). To address whether KIR2D (KIR2DL1/2/3 and KIR2DS1/2) acquisition affects the KIR3DL1 NK-cell repertoire, we determined the phenotype of NK cells by flow cytometry using KIR3DL1-specific Z27 and KIR2DL1/2/3- and KIR2DS1/2DS2-specific 1A6 mAbs (Fig. 3C). We then analyzed the proportion of KIR3DL1^{high} NK cells expressing KIR2D as a function of the nature of KIR3DL1^{high} alleles (Fig. 3D, lower panel) and as a function of the KIR3DL1/S1 allele combination (Fig. 3E, lower panel). Interestingly, a proportionate fraction of KIR3DL1^{high} KIR2D of around 50% of KIR3DL1^{high} NK cells$^+$ was observed regardless of the KIR3DL1^{high} alleles and KIR3DL1/S1 allele combinations. Indeed, a positive correlation was observed mainly for KIR3DL1*001 ($r = 0.956$), KIR3DL1*002 ($r = 0.951$), and KIR3DL1*008 (r = 1) alleles with a p-value <0.05. In contrast, the Spearman's rank test showed a low correlation between the frequency of KIR2D$^+$ KIR3DL1^{high+} NK cells and KIR2D$^+$ NK-cell frequencies, with a significant p-value (<0.05) only for the KIR3DL1*001 allele ($r = 0.67$; Fig. 3C, upper panel) and the KIR3DL1/3DS1 allele combinations (KIR3DL1$^{high/high}$, $r = 0.787$, KIR3DL1$^{high/null}$, $r = 0.775$ and KIR3DL1/3DS1, $r = 0.56$; Fig. 3D, upper panel). These data suggest a consecutive expression of KIR2D (2DL1/2/3, 2DS1/2) on KIR3DL1^{high} NK cells.

Bimodal distribution of KIR3DL1^{high} NK cells in KIR3DL1$^{high/null}$ individuals

In KIR3DL1^{high}/3DL1^{null} individuals for whom a bimodal distribution of KIR3DL1$^+$ NK cells was observed as previously mentioned, we compared the frequency and the mean fluorescence intensity of KIR3DL1^{high} NK cells (Fig. 4A). Interestingly, among the three KIR3DL1^{high} alleles identified (*001, *002, and *015), a positive correlation was noted between the frequency of KIR3DL1^{high} NK cells and membrane expression level of the KIR3DL1*001 allele ($r = 0.7033$, $p = 0.009$). KIR3DL1*004 was the unique null allele associated with KIR3DL1*001, KIR3DL1*002, and KIR3DL1*015 in our cohort. Surprisingly, the KIR3DL1*001, *004 allele combination led to both a low-frequency and mean-expression level of the KIR3DL1 receptor in 5 out of 13 individuals (Fig. 4A). Representative density plots are shown in Fig. 4B, illustrating the high and low MFI of KIR3DL1$^+$ NK cells, respectively, with high (47.3%) and low frequency (3.3%) in two representative KIR3DL1*001,*004 individuals. For comparison, a representative density plot with the expected MFI for low KIR3DL1^{high} NK-cell frequency observed for one representative KIR3DL1*002, *004 genotyped individual is also provided in Fig. 4B. The bimodal effect observed in KIR3DL1^{high,null} individuals was not influenced by the Bw4 ligand number (Supporting Information Fig. 1B). We explored KIR2DL1/S1, KIR2DS4, KIR2DL2, and KIR2DL3/2DS2 NK-cell subset frequencies as a function of KIR3DL1^{high} NK-cell frequency for these KIR3DL1^{high,null} individuals (Fig. 4C) in order to determine whether other KIR NK-cell subsets may balance the low KIR3DL1^{high} NK-cell frequency. Individuals with high KIR3DL1^{high} NK-cell frequencies presented a consistent KIR2DL1 NK-cell subset, however, the frequency of KIR2DL1 NK cells did not correlate with the frequency of KIR3DL1^{high} NK cells. Interestingly, KIR2DS4 was expressed only in individuals who displayed the KIR3DL1*015 and KIR3DL1*002 alleles (Fig. 4C). Moreover, the KIR2DS2 gene, which is in linkage disequilibrium with the KIR2DL2 gene, was present in 8 out of 23 KIR3DL1^{high,null} genotyped individuals and interestingly, KIR2DS2 was present only in KIR3DL1*001,*004 genotyped individuals who exhibited a high frequency of KIR3DL1^{high} NK cells (data not shown). The frequency of KIR2DL2$^+$ NK cells did not correlate with the frequency of KIR3DL1^{high} NK cells; however, the KIR2DL3/S2 NK-cell frequency was higher in individuals who presented a high frequency of KIR3DL1^{high} NK cells (Fig. 4C).

Because neither DX9 nor Z27 monoclonal antibodies recognize KIR3DL1 in permeabilized/fixed cells, as previously reported by Thomas et al. [32], any potential increased/decreased internalization of KIR3DL1 receptor could not have been evaluated in KIR3DL1*001, *004 genotyped individuals. However, Z27$^+$ NK cells were sorted from individuals of each group and KIR3DL1 transcripts were analyzed using primers specific for KIR3DL1*001, KIR3DL1*002, or KIR3DL1*004 (Fig. 4D). We detected not only KIR3DL1*001 or KIR3DL1*002 transcripts but also KIR3DL1*004 transcripts in all studied KIR3DL1^{high}

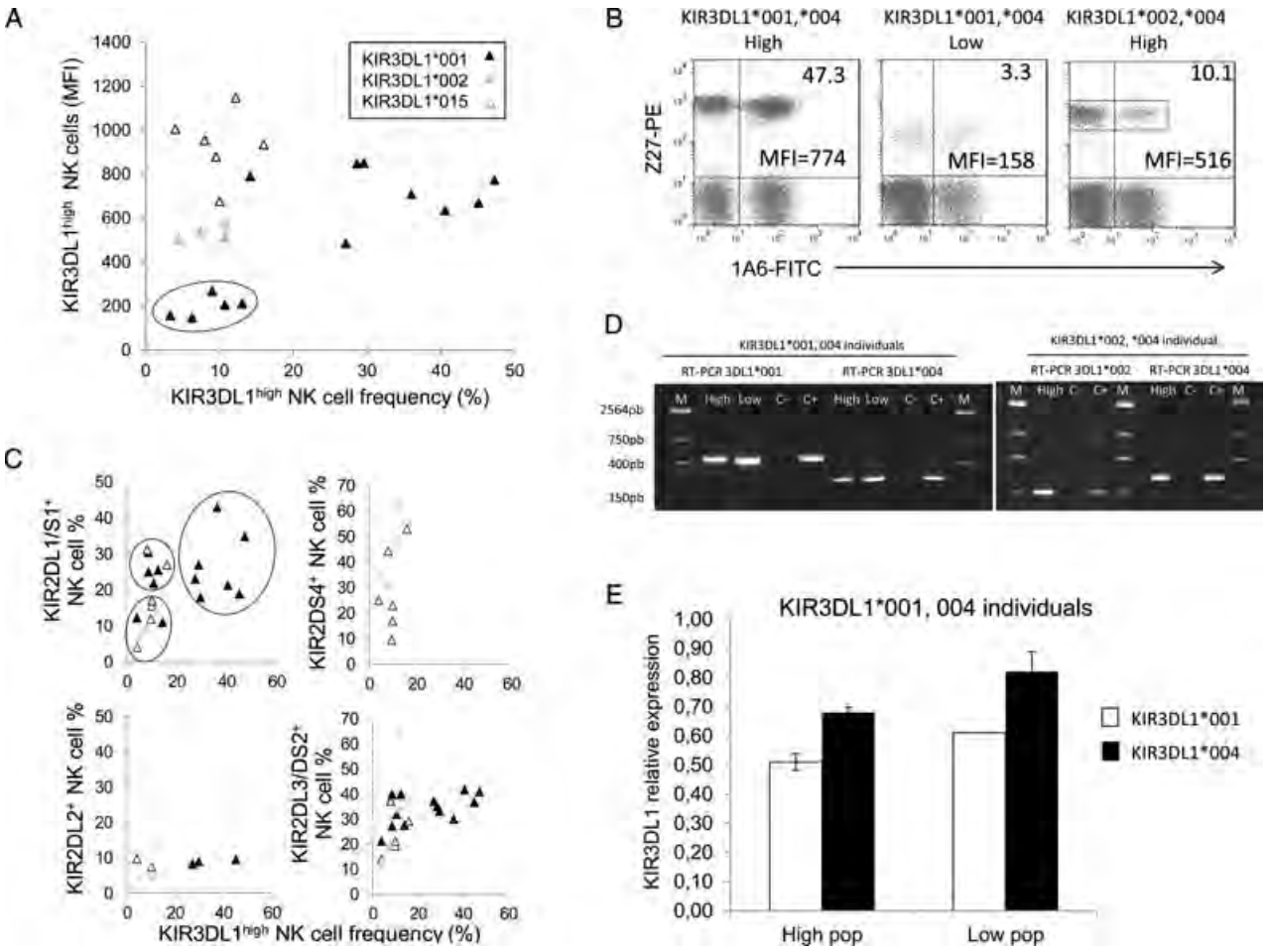

Figure 4. Detection of a similar amount of KIR3DL1^{high} and KIR3DL1^{null} transcripts in KIR3DL1^{+} NK cells regardless of the membrane expression level of KIR3DL1. (A) The KIR3DL1^{high} NK-cell frequency as a function of the KIR3DL1^{high} NK-cell MFI for KIR3DL1$^{high/null}$ individuals ($n = 23$) is shown. The nature of the KIR3DL1^{high} allele is indicated by a color code. KIR3DL1*004 is the unique null allele associated with KIR3DL1^{high} alleles in our cohort. Dots corresponding to KIR3DL1*001, *004 genotyped NK cells with a low frequency and a low mean level intensity are circled. (B) Representative flow cytometry density plots showing KIR3DL1^{+} (Z27^{+}) NK cells for two KIR3DL1*001,*004 and one KIR3DL1*002, *004 genotyped individuals. MFI and percentage of KIR3DL1^{high} NK cells is indicated in each density plot. (C) Dot representation of KIR3DL1^{high} KIR2D^{+} NK-cell frequencies as a function of KIR2DL1/S1^{+} NK-cell ($n = 22$), KIR2DS4^{+} NK-cell ($n = 10$), KIR2DL2^{+} NK-cell ($n = 8$), and KIR2DL3/2DS2^{+} NK-cell ($n = 22$) frequencies for KIR3DL1$^{high/null}$ individuals. For all dots, the nature of each allele is determined by the color code used previously. (D) Visualization of specific RT-PCR products in a 2% agarose gel in 0.5X TBE buffer with SSP-SM marker (One Lambda) for two KIR3DL1*001, 004 genotyped individuals showing different mean level intensity of the KIR3DL1^{high} NK-cell population and contrasted frequencies, and for one 3DL1*002, *004 (KIR3DL1^{high}) genotyped individual with a low KIR3DL1^{high} NK-cell frequency. RNA was extracted from sorted Z27^{+} NK cells of interest and specific RT-PCR was then performed using KIR3DL1 allele-group–specific PCR-SSP primers adapted from Gardiner et al. [19] showing a specific RT-PCR product at 456 pb for KIR3DL1*001, 277 pb for KIR3DL1*004, and 163 pb for KIR3DL1*002. Negative (C−) and positive (C+) controls were used corresponding respectively to RNA extracted from individuals with different and identical KIR3DL1 allelic typing. (E) Relative quantification of KIR3DL1*001 and KIR3DL1*004 transcripts normalized to the GAPDH reference gene in sorted NK-cell subpopulations from KIR3DL1*001, *004 genotyped individuals with KIR3DL1^{high} ($n = 4$) or KIR3DL1^{low} ($n = 2$). NK-cell populations showing a similar amount of both KIR3DL1*001 and KIR3DL1*004 transcripts whatever the membrane expression level of KIR3DL1. Data are shown as means + SD of six samples pooled from at least three independent experiments.

populations. Because the amount of KIR3DL1*001 and KIR3DL1*004 transcripts might explain the different level of expression in high and low KIR3DL1 NK-cell populations, we evaluated the expression level of KIR3DL1 transcripts by real-time RT-PCR using GAPDH transcripts as an endogenous control. We observed a similar amount of both KIR3DL1*001 and KIR3DL1*004 transcripts in high and low KIR3DL1 populations from different individuals (Fig. 4E).

The nature of the KIR3DL1^{high} allele influences KIR3DL1^{high} NK-cell responsiveness

Among the three KIR3DL1^{high} alleles identified (*001, *002, and *015) in KIR3DL1$^{high/null}$ individuals, consistent expansion of KIR3DL1^{high} NK cells was associated only with the KIR3DL1*001 allele, as mentioned earlier. Thus, we hypothesized that these three KIR3DL1^{high} receptors do not interact with the same affinity

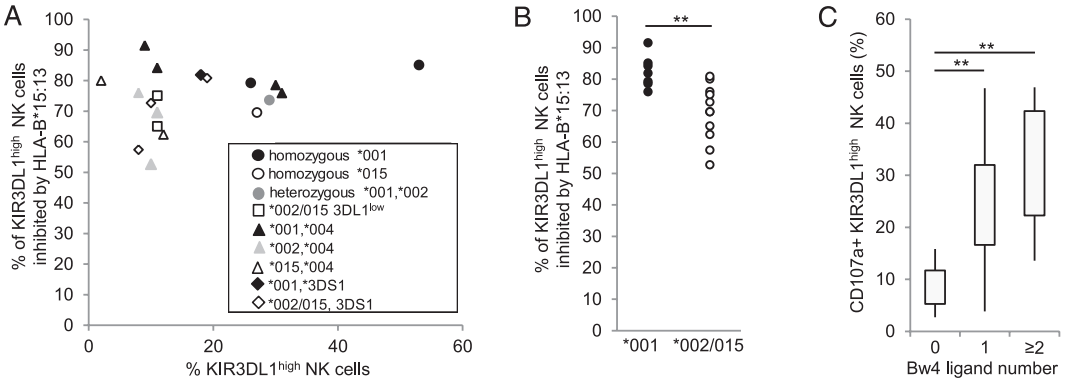

Figure 5. The nature of the KIR3DL1^{high} allele influences KIR3DL1^{high} NK-cell responsiveness. (A) A degranulation test on KIR3DL1^{high} NK cells was done only for Bw4^{+} individuals ($n = 19$) against 221 and 221-B*15:13 (Bw4 motif) cell lines. The percentage of KIR3DL1^{high} NK cells inhibited by HLA-B*15:13 (Bw4 motif) was calculated from the frequencies of KIR3DL1^{high} NK cells expressing CD107a observed following culture with 221 cells (considered as maximum) compared with those observed following culture with the 221-B*15:13 cell line. The percentage of KIR3DL1^{high} NK cells inhibited by 221-B*15:13 for low and high frequencies of KIR3DL1^{high} NK cells depending on KIR3DL1*001 (black), KIR3DL1*002 (filled light gray symbols), and KIR3DL1*015 (open symbols) alleles associated with different KIR3DL1/3DS1 alleles: KIR3DL1^{high} (circle), KIR3DL1^{low} (square), KIR3DL1^{null} (triangle), and KIR3DS1 (diamond) are shown. (B) The percentages of KIR3DL1^{high} NK cells inhibited by HLA-B*15:13 (Bw4 motif) were compared between KIR3DL1*001^{+} ($n = 7$) and KIR3DL1*002/015^{+} individuals ($n = 11$). Statistical significance (**$p < 0.001$) between both groups was determined using the one-way analysis of variance (ANOVA) test. (C) Box and whisker plot summarizing the frequency of CD107a^{+} KIR3DL1^{high} NK cells observed after 5 h of stimulation with standard HLA class I-deficient 221 cell targets as a function of Bw4 ligand number. CD107a^{+} KIR3DL1^{high} NK-cell percentage was compared between 0 and 1 Bw4 ligands and between 1 and ≥2 Bw4 ligands using the one-way ANOVA test. Top and bottom whiskers represent values of the top and bottom 25% of cases, respectively; boxed area, interquartile range, and the significant p values between groups is indicated (**$p < 0.001$, ANOVA test). PBMCs were incubated with 221 cells for 4 h with brefeldin A. To detect the frequency of KIR3DL1^{high} CD107a^{+} NK cells, NK cells were stained with anti-NKp46-allophycocyanin, Z27-PE, and CD107a-PerCP. NKG2A-FITC and 1A6-FITC were added to focus the analysis on KIR3DL1^{high} KIR2D^{-} NKG2A^{-} NK cells. The cells were analyzed by flow cytometry. Data shown are representative of 27 experiments performed.

with the Bw4 ligand and only the KIR3DL1*001 allele product constitutes a strong inhibitory receptor capable of engaging the KIR3DL1^{high} NK-cell subset toward cellular expansion. To test this hypothesis, we determined the degranulation potential (CD107a expression) of NK cells expressing one of these three KIR3DL1^{high} receptors and evaluated the capacity of these three KIR3DL1^{high} receptors to inhibit degranulation of NK cells incubated with 221-HLA-B*15:13 (Bw4 motif) target cells from 19 KIR3DL1^{high} individuals. We observed a higher frequency of KIR3DL1*001^{+} NK-cell inhibition than KIR3DL1*002^{+} or *01502^{+} NK-cell inhibition upon engagement with Bw4 ligand, regardless of the frequency of KIR3DL1^{high} NK cells or the KIR3DL1/3DS1 allele combinations (Fig. 5A). Accordingly, the percentages of KIR3DL1^{high} NK cells inhibited by HLA-B*15:13 (Bw4 motif) were higher in KIR3DL1*001^{+} than in KIR3DL1*002/015^{+} individuals (82.3%, $n = 7$ versus 69.2%, $n = 11$, $p = 0.003$, Fig. 5B). Moreover, we confirmed that the spontaneous degranulation of adult KIR3DL1^{high} NK cells against standard HLA class I-deficient 221 cells increases with the number of autologous Bw4 ligands (9.9% $n = 7$ versus 25.1%, $n = 14$, $p = 0.003$ between 0 and 1 Bw4 ligand and 25.1% $n = 14$ versus 34% $n = 6$, $p = 0.001$ between 1 and ≥2 Bw4 ligands) (Fig. 5C).

Different phenotypic profile of KIR3DL1^{high} NK cells in early life compared with that in adulthood

Because our results suggest either a selection and specific expansion of KIR3DL1^{high} NK-cell subsets or a deletion of KIR3DL1*001,*004 NK subsets, we investigated the KIR3DL1^{high} NK-cell repertoire in cord blood to gain insights into KIR3DL1 NK-cell repertoire formation during development. To this end, we evaluated the frequency of this population and the expression level of the KIR3DL1^{high} receptor taking into account the nature of the second KIR3DL1 allele (high, low, and null). Interestingly, although the KIR3DL1^{high} NK-cell frequency was significantly lower in umbilical PBMCs (9.27%, $n = 18$) than adult PBMCs (19.33%, $n = 68$, $p = 0.001$) (Fig. 6A), whatever the KIR3DL1/3DS1 allele combination analyzed (Fig. 6B), the expression level was significantly higher in cord blood NK cells (umbilical MFI = 1088, $n = 15$ versus adult MFI = 686, $n = 61$, $p = 3.5 \times 10^{-7}$) (Fig. 6C), especially for KIR3DL1$^{high/high}$ (umbilical MFI = 1258, $n = 4$ versus adult MFI = 735, $n = 11$, $p = 0.006$) and KIR3DL1^{high}/3DS1 allele combinations (umbilical MFI = 1268, $n = 6$ versus adult MFI = 689, $n = 19$, $p = 1.5 \times 10^{-5}$) reaching a MFI of 2000 (Fig. 6D). These results show that KIR3DL1^{high} is expressed at a high intensity on the NK-cell membrane in a small proportion of NK cells during early life, probably contributing to the selection or deletion of KIR3DL1 NK-cell subsets, depending on the signaling intensity received by the cells.

The Bw4 environment impacts on KIR3DL1 NK-cell frequency in cord blood samples

Considering that a functional signal via KIR3DL1 may contribute to shaping the KIR3DL1 NK-cell repertoire, we investigated the

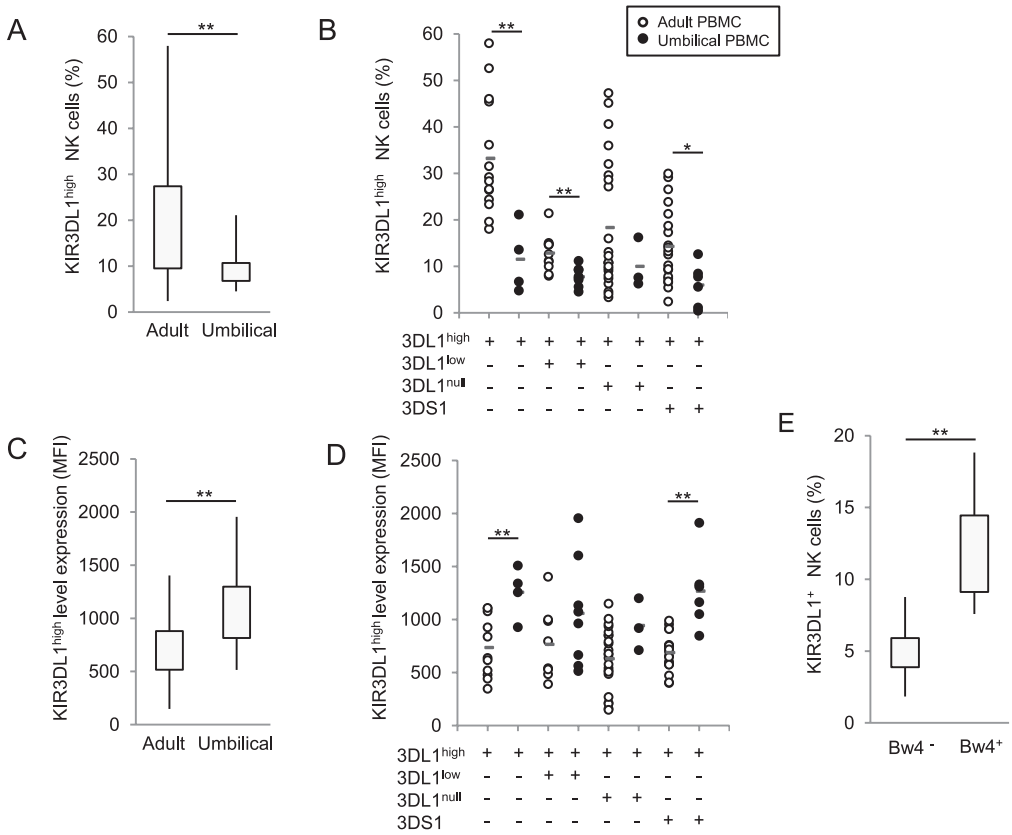

Figure 6. Phenotypic characterization of the KIR3DL1^{high} NK-cell repertoire in cord blood samples. (A) Box and whisker plot summarizing the frequency of KIR3DL1^{high} NK cells in adult ($n = 68$) and umbilical ($n = 18$) PBMCs. Top and bottom whiskers represent values of the top and bottom 25% of cases respectively; boxed area, interquartile range. (B) All KIR3DL1^{high} NK-cell frequencies as a function of the second KIR3DL1 allele or KIR3DS1: KIR3DL1$^{high/high}$ (14 adult and 4 cord blood samples), KIR3DL1$^{high/low}$ (11 adult and 8 cord blood samples), KIR3DL1$^{high/null}$ (23 adult and 3 cord blood samples), or KIR3DL1^{high}/3DS1 (20 adult and 6 cord blood samples) are shown. (C) Box and whisker plot summarizing the mean expression level of KIR3DL1 on NK cells in adult ($n = 61$) and umbilical ($n = 15$) PBMCs. (D) Dot representation of mean expression level of KIR3DL1 on NK cells as a function of the second KIR3DL1 allele or KIR3DS1: KIR3DL1$^{high/high}$ (11 adult and 4 cord blood samples), KIR3DL1$^{high/low}$ (8 adult and 8 cord blood samples), KIR3DL1$^{high/null}$ (23 adult and 3 cord blood samples), or KIR3DL1^{high}/3DS1 (19 adult and 6 cord blood samples). (E) Box and whisker plot summarizing the frequency of KIR3DL1^{+} NK cells in umbilical PBMCs as a function of Bw4^{-} ($n = 7$) or Bw4^{+} cord blood samples ($n = 10$) is shown. Statistical significance (*$p < 0.05$; **$p < 0.01$) between different groups was determined using the one-way ANOVA test. Data shown are representative of 17 experiments performed.

frequency of KIR3DL1 NK cells in early life with regards to the expression or not of autologous Bw4 ligands (Fig. 6E). Interestingly, the KIR3DL1^{+} NK-cell frequency was significantly higher in Bw4^{+} cord blood (12.33%, $n = 10$) than in the Bw4^{-} counterparts (4.97%, $n = 7$, $p = 0.0003$). These results suggest that an engagement toward one KIR3DL1 allele product expression during NK-cell development, possibly depending on functional interaction with Bw4 ligand, contributes to molding the KIR3DL1 NK-cell repertoire. Interestingly, although we confirmed that the spontaneous degranulation of adult KIR3DL1^{high} NK cells increases with the number of autologous Bw4 ligands (Fig. 5C), we did not observe a significant correlation between adult KIR3DL1^{high} NK-cell frequency and Bw4 ligand number (Supporting Information Fig. 1). This result can be partly explained by the plausible deletion of KIR3DL1 NK-cell subsets receiving excessive signals during their development, as observed for KIR3DL1$^{high/null}$ NK-cell subsets in the adult repertoire.

Discussion

In this study, we investigated the impact of allelic KIR3DL1 polymorphism on the KIR3DL1 NK-cell repertoire. In this large cohort of healthy individuals we considered possible contributing factors, including KIR3DL1/S1 allele combinations, mean expression level, Bw4 environment, and functional potential. The French population is characterized by a large KIR3DL1 allelic polymorphism with different predominant KIR3DL1 alleles such as KIR3DL1*001, *002, *004, and *054. The frequency of KIR3DL1^{high} (*001, *002) and KIR3DL1^{null} (*004, *054) alleles ranges from 20 to 50% and this contrasts with the Japanese population which is characterized by (i) only one KIR3DL1^{high} allele (*01502) overexpressed at a very high frequency (45%) compared with the next most frequent allele and (ii) the absence of the KIR3DL1*004 null allele [31]. Surprisingly, the frequency of the null KIR3DL1*004 allele was particularly high in our cohort (29%) compared with

other European or African population studies where it usually ranged from 8 to 16% [17]. Because the Bw4 ligand could differ between populations [37], we have also investigated the frequency and nature of the Bw4 ligand in the French population compared with the Japanese population [31]. The frequency of HLA-B Bw4$^+$ was higher in the French population compared with the Japanese population (59 versus 36%) and was higher than 70% if both HLA-A and HLA-B Bw4$^+$ were taken into account. Although the frequency of HLA-B51 (I^{80}) was similar in both populations (13%), HLA-B44 (T^{80}) was overrepresented in the French population (30.2 versus 7%) whereas A24 (I^{80}) was underrepresented (27.4 versus 41%). However, in both populations, the frequency of KIR3DL1^{high} NK cells was higher than that of the KIR3DL1^{low} NK-cell population and the frequency of KIR3DL1^{high} NK cells increased with gene dose. In the French population, the presence of Bw4 ligand (both HLA-A and HLA-B) or the presence of a specific HLA-Bw4 molecule such as A24, B51, B44 did not significantly increase the frequency of KIR3DL1^{high} NK cells, while in the Japanese population, the presence of HLA-Bw4$^+$ molecules significantly increased the frequency of NK cells expressing KIR3DL1 for donors with two high-binding 3DL1 allotypes, and the increase in frequency of KIR3DL1^{high} NK cells was greater in Bw4 heterozygotes than in homozygotes. The fact that the Bw4 environment did not significantly affect the KIR3DL1 NK-cell frequency in our French cohort may be due to the sample size. Overall, our study confirms that French individuals are characterized by a broad KIR3DL1 allelic polymorphism in accordance with previous studies in other European populations [17] and that the KIR3DL1 NK-cell repertoire is mainly determined by KIR3DL1 polymorphism and KIR3DL1/3DS1 allele combinations. The extensive KIR3DL1 allelic polymorphism and diversity of KIR3DL1 ligands observed in the French population suggest a coevolution of KIR3DL1 and HLA-A, HLA-B Bw4 genes as reported in other populations [37].

The phenotypic study of KIR3DL1 allelic polymorphism in healthy individuals enabled us to show that all individuals hitherto described as negative for the KIR3DL1 gene actually possess an KIR3DL1*054 allele that is not detected with the current standard typing techniques, and a substantial proportion of individuals have at least one KIR3DL1 allele that is not, or only weakly, expressed on the NK-cell surface. The association of the KIR3DL1*054 allotype with KIR3DS1 in our cohort did not unable us to evaluate NK-cell surface expression of KIR3DL1*054 since both receptors seemed to be recognized similarly by the Z27 mAb [36]. Interestingly, in our cohort, the KIR3DL1*054 allele was mainly observed in individuals with the BB KIR genotype ($n = 9$). While the expression and function of the KIR3DL1*004 allele is well understood, [34] that of the KIR3DL1*019, KIR3DL1*054 alleles remain to be elucidated. The expected mature KIR3DL1*019 protein contains the same amino acids involved in the intracellular retention of the null KIR3DL1*004 allotype, i.e. L^{86} and S^{182}, and only one amino acid in the D0 domain differs between KIR3DL1*019 and KIR3DL1*004. Thus, we believe that the KIR3DL1*019 allele is also a null allele. However, different amino acid substitutions occur between KIR3DL1*054 and KIR3DL1*004 suggesting that KIR3DL1*054 is closer to KIR3DS1 than KIR3DL1. These amino acid changes in the extracellular domains could potentially interfere with Z27/DX9 epitope recognition. These results should shed light on the functional contribution of the frequent KIR3DL1*054 and KIR3DL1*004 receptors to different pathologies. Indeed, a high frequency of KIR3DL1-negative individuals has been reported in an autoimmune disease [38], which probably corresponded to the KIR3DL1*054 allele. Moreover, in the presence of Bw4, KIR3DL1*004 showed the most significant protection relative to all other KIR3DL1 alleles in HIV infection [29] and its functional expression at the cell surface has been recently demonstrated [35].

Our data underline the impact of KIR3DL1 allele polymorphism on its NK-cell expression pattern and on the hierarchy between all KIR3DL1 alleles, highlighting the predominance of the KIR3DL1*001 allele regardless of the presence or absence of the second allele (KIR3DL1^{low}, KIR3DL1^{null}, or KIR3DS1). In the KIR3DL1^{high,null} individuals with only one copy of the KIR3DL1*001 allele, the frequency of KIR3DL1^{high} NK cells increases up to 50% of NK cells, indicating that factors other than copy number contribute to this dominant expression. The high number of KIR3DL1*001,*004 individuals in our cohort led us to detect a bimodal distribution of KIR3DL1^{high} NK cells: A first group with a high frequency of KIR3DL1^{high} NK cells and high level of expression, and a second group with a low frequency of KIR3DL1^{high} NK cells and a low level expression. Of note, we detected the same amount of KIR3DL1*001 and KIR3DL1*004 transcripts in the low and high frequency of KIR3DL1^{high} NK cells, demonstrating that the KIR3DL1 transcript level is not linked to its protein expression at the NK-cell surface. This finding is in accordance with data demonstrating the frequent detection of KIR transcripts in T-cell clones and NK92 in the absence of the corresponding protein at the cell surface [39]. Thus, the predominance of the KIR3DL1*001 allele in our study could not be explained by its promoter properties [40]. Nonetheless, we cannot exclude the possibility of a differential internalization of KIR3DL1 receptor in both populations. Among other factors able to influence the frequency of KIR3DL1^{high} NK cells, we looked at the expression of other KIR in different groups of KIR3DL1^{high,null} individuals. Interestingly, all KIR3DL1^{high,null} individuals with KIR3DL1*002 and KIR3DL1*015 alleles showing a low frequency and high mean level expression of the KIR3DL1 receptor expressed KIR2DS4. However, all KIR3DL1*001, *004 individuals bore only nonexpressed KIR2DS4*003 as activating KIR genes (AA genotypes) and other activating KIR genes, such as KIR2DS2, were observed in four out of eight others KIR3DL1*001, *004 genotyped individuals with a high frequency and mean level expression of KIR3DL1. Thus, since the acquisition of KIR on NK cells is sequential [41], it is possible that the nature of the other KIR expressed before KIR3DL1 and probably the transduction signals received by the cells, influence the posttranscriptional regulation of the KIR3DL1*001 and KIR3DL1*004 alleles. However, the bimodal distribution cannot be entirely explained by a differential expression of activating KIR.

Another explanation of the bimodal distribution could be due to a functional selection of KIR3DL1 NK cells. Indeed, we also underlined a hierarchy between KIR3DL1^{high} alleles in their capacity to inhibit NK-cell degranulation via Bw4 engagement, and possibly

to promote in vivo maintenance or expansion of peripheral NK cells carrying the corresponding allele. As such, the frequency of KIR3DL1*001$^+$ NK-cell inhibition is higher than the frequency of KIR3DL1*002 or *015 NK-cell inhibition upon engagement with HLA-B*15:13 (Bw4 motif) expressed on transfected 221 target cells regardless of the frequency of KIR3DL1^{high} NK cells. This last point is in agreement with a previous report indicating that highly expressed KIR3DL1*002 is a better inhibitor than poorly expressed KIR3DL1*007 [27]. Based on recent data published by Taner et al. [35] and on the higher level expression of KIR3DL1 observed in cord blood, we speculate that KIR3DL1*004 can be functional, and may constitute a better signaling receptor than the KIR3DL1*002, *015, and *008 allele products, favoring its phenotypic selection during early life. Thus, for all 10 studied KIR3DL1*002/*01502, *004 genotyped individuals, we suggest that a phenotypic selection in favor of the KIR3DL1*004 receptor, which was not detected by KIR3DL1 specific Z27 and DX9 antibodies may explain the frequency of KIR3DL1^{high} observed around 10% of NK cells. For 5 KIR3DL1*001,*004 genotyped individuals, the frequency of KIR3DL1^{high} was low (around 10%) as was the mean fluorescent intensity, suggesting low expression of the KIR3DL1*001 receptor on NK cells. This low frequency seems to be due to a negative selection of this subset as previously described for T-lymphocyte selection. Selection and amplification of KIR3DL1^{high} NK cells seem to be dependent on the nature of the KIR3DL1 allele and the KIR3DL1 allele combination, which is expressed with a hierarchy favoring KIR3DL1*001, followed by KIR3DL1*004, and then the other KIR3DL1^{high} alleles (*002, *015, and *008). The engagement of two receptors such as KIR3DL1*001 and *004, thereby inducing increased signals, might lead to a negative selection of NK cells.

In this study, we did not observe any increased frequency of KIR (KIR2DL1/S1, KIR2DL2/3/S2, or KIR2DS4) NK-cell subsets balancing a low frequency of KIR3DL1^{high} NK-cell subsets in different individuals grouped by KIR3DL1 alleles or KIR3DL1/3DS1 allele combinations (data not shown). However, we observed a constant proportion of KIR3DL1^{high} NK cells expressing KIR2DL1/2/3, 2DS1/2 receptors, which suggests a subsequent acquisition of KIR2D by KIR3DL1^{high} NK cells. Previous in vitro investigations of NK-cell differentiation have been performed suggesting a sequential acquisition of KIR with the expression of KIR2DL3 before KIR2DL1 [42]. Although KIR3DL1 acquisition was observed regardless of HLA background [43], its acquisition in this sequential model has not been investigated. Even though it is now clear that KIR ligands contribute to the functional education of NK cells [4], HLA class I molecules, as KIR ligands, should partially affect the formation of the KIR NK-cell repertoire at the neonatal stage [31, 44], as observed in our study. As previously described [9], in our study we did not observe an impact of the Bw4 environment on the adult KIR3DL1 NK-cell repertoire. Other factors, and particularly individual immunological experience, seem to contribute to shaping the KIR NK-cell repertoire throughout life. The finding of amplified KIR$^+$ NKG2C$^+$ NK cells in CMV [45–47], HSV-2 [48], and HIV infections [49, 50] reinforces this hypothesis. In the case of KIR3DL1, it is conceivable that viral infections, particularly those involving viruses that negatively modulate HLA class I molecule expression such as CMV and HIV, trigger KIR3DL1 NK-cell subsets via KIR3DL1 or an undetermined coexpressed receptor. Further investigations in KIR3DL1$^{high/null}$ individuals taking into account viral status and a broader phenotype of KIR3DL1^{high} NK cells, notably including NKG2C, should help to determine the impact of viral infection on the KIR3DL1 NK-cell repertoire. Finally, this study provides new insight into the mechanisms potentially involved in shaping the KIR3DL1 NK-cell repertoire.

Materials and methods

Cells (PBMCs, cord blood samples, and cell lines)

PBMCs were isolated from citrate-phosphate-dextrose blood, collected from healthy adult volunteers by gradient centrifugation on Ficoll-Hypaque (Lymphoprep, Axis-Shield, PoC AS, Oslo, Norway). All blood donors ($n = 109$) were recruited at the Blood Transfusion Center (Etablissement Français du Sang, Nantes, France) and informed consent was obtained from all individuals. Umbilical cord blood samples ($n = 23$) were obtained at the Nantes CHU maternity unit. Informed consent was obtained from all mothers. HLA class-I–deficient 721.221 lymphoblastoid EBV-B cells (referred to as 221 cells) and Bw4 (B*15:13) transfected 221 cells (referred to as 221-Bw4$^+$) were used as controls to assess natural NK-cell cytotoxicity in functional assays. Cells were cultured in RPMI 1640 medium (Gibco, Paisley, Scotland, UK) containing glutamine (Gibco) and penicillin-streptomycin (Gibco) and supplemented with 10% fetal bovine serum (Gibco). Mycoplasma tests performed by PCR were negative for all cell lines.

HLA and KIR genotyping

Genomic DNA was extracted from PBMC and from cord blood samples using either a classical salting-out method [51] or by GenoM-6 (Qiagen, Courtaboeuf, France) using magnetic beads. Intermediate or high-resolution typing for HLA-A, HLA-B, and HLA-C was performed on all healthy donors ($n = 109$) and cord blood samples ($n = 23$) using a Sequence Based Typing kit (Abbott Molecular Park, IL, USA). All individuals were typed for the presence or absence of KIR2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 3DL1, 3DL2, 3DL3, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, and 3DS1 using the KIR genotyping SSP kit from Invitrogen (Compiègne, France), lot#003, under the conditions recommended by the manufacturer. Cord blood samples were typed using a multiplex PCR-SSP method as previously described [52]. In order to detect all KIR3DL1 alleles, especially the KIR3DL1*054 allele not amplified by the PCR-SSP Invitrogen kit lot#003, individuals negative for KIR3DL1 at the genotypic level were further typed using homemade KIR3DL1 generic primers specific for exon 3 [53], which amplified all KIR3DL1

alleles except KIR3DL1*027, *028, *030, *039, *042, *053, *057, *073 (all of which were amplified by the Invitrogen KIR PCR-SSP kit). For KIR3DL1 allelic typing, KIR3DL1 allele-group–specific PCR-SSP and sequencing of polymorphic exons were combined in order to resolve most of the remaining KIR3DL1 allele ambiguities arising as a result of the large KIR3DL1 allelic polymorphism described to date. For the subtyping of KIR3DL1 PCR-SSP coding sequences, primers designed to discriminate allele-group–specific polymorphisms were paired with KIR3DL1 locus-specific primers adapted from Gardiner et al. [19]. Depending on the allele-group–specific amplification products obtained, KIR3DL1 exons 1, 2, 3, 4, 5, or 7–9 were further sequenced to resolve remaining ambiguities as previously described [17, 24]. Sequence data files were analyzed using Assign-SBT software (Conexio Genomics, Applecross, Australia) with IPD-KIR Database version 2.3.0 (August 2010).

Identification and quantification of KIR3DL1 transcripts in the KIR3DL1$^+$ NK-cell population

For individuals genotyped as KIR3DL1*001,*004, KIR3DL1*002,*004, and KIR3DL1*002,*005, Z27$^+$ NK cells were positively selected using KIR3DL1-specific antibody Z27 (Beckman Coulter, Immunotech) and murine IgG-coupled magnetic Dynabeads according to the manufacturer's instructions (Dynal, Oslo, Norway). Beads and KIR3DL1-specific antibody were removed using goat anti-mouse IgG antiserum (EFS) as described previously [54]. Selected cells were stimulated using an in vitro model of NK-cell expansion [5]. After 2 weeks of amplification, Z27$^+$ NK-cell populations were sorted and total RNA was purified using NucleoSpin RNAXS (Macherey-Nagel). Qualitative RT-PCR was performed using a One-Step PrimeScript^{TM} RT-PCR kit (TaKaRa, Japan) with KIR3DL1 allele-group–specific PCR-SSP primers adapted from Gardiner et al. [19]. For relative quantification, KIR3DL1*001 and KIR3DL1*004 transcript levels were normalized to the GAPDH reference gene using the same primers and the One-Step SYBR® PrimeScript^{TM} RT-PCR kit (TaKaRa, Japan). For each sample, qRT-PCR was performed in triplicate on a RotorGene RG6000® RT-PCR system (Corbett Research). Amplification programs were as follows: reverse transcription at 42°C for 5 min, initial denaturation at 95°C for 5 min, 5 cycles at 97°C for 20 s, 55°C (GAPDH, KIR3DL1*004) or 62°C (KIR3DL1*001) for 30 s, 72°C for 1 min, and 30 cycles at 95°C for 15 s, 57°C (GAPDH, KIR3DL1*004) or 64°C (KIR3DL1*001) for 30 s, 72°C for 1 min. A melting curve analysis was performed after each run, with one degree increments from 72 to 95°C. The relative levels of KIR3DL1 allele transcripts were compared using unpaired *t*-tests in different sorted NK-cell subsets.

Phenotypic analysis by flow cytometry

NK-cell surface phenotype was determined by four-color flow cytometry using the following mouse antihuman mAbs: anti-KIR3DL1/S1-PE (Z27), anti-KIR3DL1-FITC (DX9) (Beckman Coulter, Immunotech), anti-KIR2DL1/2/3/2DS1/2 (1A6) [55], anti-KIR2DL3/2DS2-FITC (1F12) [55], anti-KIR2DS4-PE (FES175), anti-KIR2DL1/S1-PE (EB6), anti-KIR2DL2/3/2DS2-PE (GL183) (Beckman Coulter, Immunotech), anti-CD3-PerCP (SK7), and anti-CD56-allophycocyanin (B159) (BD Biosciences). Cells were also stained with the corresponding isotype-matched control mAb. Data were collected using a FACSCalibur (BD Biosciences) and analyzed using Flowjo 6.2 software (TreeStar).

CD107a mobilization detected by flow cytometry

KIR3DL1^{high} NK cells were tested for their cytolytic potential with the CD107a mobilization assay after stimulation with 221 or 221-Bw4$^+$ (HLA-B*15:13) transfected cell lines. Preincubated NK cells with CD107-PECy5 (H4A3, BD Biosciences) were incubated with the target cells for 5 h at an effector:target ratio of 1:1, with brefeldin A (Sigma) at 10 μg/mL for the last 4 h. The cells were surface stained with Z27-PE, NKG2A-FITC and NKp46-allophycocyanin (9E2, BD Biosciences). Data were collected using a FACSCalibur (BD Biosciences) and analyzed using Flowjo 6.2 software (TreeStar).

Statistical analyses

Comparisons of KIR3DL1$^+$ NK-cell frequencies in two different series of individuals were performed using the Student's *t*-test. Association between CD107a$^+$ KIR3DL1^{high} NK-cell frequencies and autologous Bw4 ligand number in two different series of individuals was tested using the one-way ANOVA test. *p*-values <0.05 were considered as statistically significant. Spearman's rank correlation coefficients were calculated and indicated only when a significant *p*-value was obtained ($p < 0.05$).

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Abbreviations: **KIR**: Killer Immunoglobulin-like Receptor

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Amplified NKG2C^{+} NK Cells in Cytomegalovirus (CMV) Infection Preferentially Express Killer Cell Ig-like Receptor 2DL: Functional Impact in Controlling CMV-Infected Dendritic Cells

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Amplified NKG2C$^+$ NK Cells in Cytomegalovirus (CMV) Infection Preferentially Express Killer Cell Ig-like Receptor 2DL: Functional Impact in Controlling CMV-Infected Dendritic Cells

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CMV infection represents a major complication in hematopoietic stem cell transplantation, which compromises graft outcome. Downregulation of HLA class I expression is one mechanism by which CMV evades T cell–mediated immune detection, rendering infected cells vulnerable to killer cell Ig-like receptor (KIR)$^+$ NK cells. In this study, we observed that the amplified NKG2C$^+$ NK cell population observed specifically in CMV seropositive individuals mainly expressed KIR2DL receptors. We have shown that HLA class I expression was downregulated on CMV-infected immature dendritic cells (iDCs), which escape to HLA-A2-pp65–specific T lymphocytes but strongly trigger the degranulation of KIR2D$^+$ NK cells. CMV infection conferred a vulnerability of C2C2$^+$ iDCs to educated KIR2DL1$^+$ and KIR2DL3$^+$ NK cell subsets. Alloreactivity of KIR2DL1$^+$ NK cell subsets against C1C1$^+$ iDCs was maintained independently of CMV infection. Unexpectedly, CMV-infected C1C1$^+$ iDCs did not activate KIR2DL3$^+$ NK cell reactivity, suggesting a potential CMV evasion to KIR2DL3 NK cell recognition. Altogether, the coexpression of KIR and NKG2C on expanded NK cell subsets could be related to a functional contribution of KIR in CMV infection and should be investigated in hematopoietic stem cell transplantation, in which the beneficial impact of CMV infection has been reported on the graft-versus-leukemia effect. ***The Journal of Immunology*****, 2013, 191: 2708–2716.**

Human CMV is a β-herpesvirus that establishes a latent infection in healthy individuals but causes a high rate of morbidity in immunocompromised patients and during fetal development. For an effective defense against CMV, both innate and adaptive immune responses are involved (1). Thanks to different inhibitory receptors, NK cells are able to detect the loss of HLA class I molecules from autologous cells, a situation that can occur when cells are perturbed by viral infection or tumor transformation (the "missing self" hypothesis) (2). Indeed, NK cells express different inhibitory receptors for self–HLA class I molecules, including CD94/NKG2A, killer cell Ig-like receptors (KIRs) and ILT2 (LILRB1). The CD94/NKG2A receptor recognizes leader peptides of most HLA-A, -B, -C, and -G molecules presented by the HLA-E molecule (3). Nevertheless, inhibitory KIRs display a more specific recognition of different HLA class I ligands. For example, HLA-C allotypes with asparagine at position 80 (C1 ligands) are recognized by KIR2DL2/3 whereas HLA-C allotypes with lysine at position 80 (C2 ligands) are recognized by KIR2DL1 (4). Additionally, ILT2 recognizes all HLA class I molecules. Moreover, the interaction between HLA class I molecules and inhibitory receptors is essential in NK cell acquisition of self-tolerance and functional abilities (5).

Soon after infection, different viral proteins downregulate HLA class I molecule expression on infected cells, leading to escape from CMV-specific T lymphocytes, but they render these infected cells vulnerable to NK cell lysis (6). Furthermore, CMV may also escape NK surveillance by maintaining the expression of nonclassical HLA class I molecules, HLA-E and HLA-G, serving as ligands for the inhibitory receptors CD94/NKG2A and ILT2 (7). Recently it has been shown that expansion of NK cells expressing the activating NKG2C counterpart is associated with CMV infection (8–12). Furthermore, CMV has developed different strategies to prevent the expression of ligands for some activating NK cell receptors (13, 14). However, no document discusses the involvement of KIR NK cells in controlling CMV infection or the potential CMV evasion to KIR NK cells. The hypothesis of a prospective role of KIR NK cells in controlling CMV is reinforced by the knowledge gained from the murine model. In fact, Ly49 receptors, analogous to KIR receptors, are mainly engaged to control murine CMV infections (15).

In allogeneic hematopoietic stem cell transplantation (HSCT), CMV infections are considered a major complication, which may compromise graft outcome. After transplantation, lymphoid T cell reconstitution is delayed, resulting in reactivation of latent viral

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Abbreviations used in this article: HSCT, hematopoietic stem cell transplantation; iDC, immature dendritic cell; KIR, killer cell Ig-like receptor; SSP, sequence-specific primer.

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infections such as CMV. However, NK cells that rapidly reconstitute hematopoiesis and play a crucial role in mediating the graft-versus-leukemia (GVL) effect constitute the first line of defense against viral infection. In HLA haploidentical HSCT, NK cell alloreactivity is due to the presence in the donor of NK cells expressing KIR that recognize HLA class I allotypes present in the donor but absent in the recipient (16). KIR genotypic studies have revealed the beneficial effect of activating KIR genes against CMV infection or reactivation in different graft contexts (16–18). During acute CMV infection, NKG2C$^+$ NK cells expand in HSCT recipients and predominately express KIR (9). However, the contribution of NKG2C in eliminating CMV-infected cells via NK cells has not been demonstrated. It is possible that NKG2C mainly constitutes a CMV infection marker. Thus, the coexpression of NKG2C with KIR on this expanded NK cell population questions the functional KIR contribution to CMV infection control. Moreover, although a recent report suggests the beneficial impact of early CMV infection on the GVL effect in HSCT (19), whether CMV modulates KIR NK cell alloreactivity in an HSCT context has not been investigated. Thus, to address these questions, we have set up a cell culture model based on a CMV-infected immature monocyte-derived dendritic cells (iDCs) as target cells, taking into account the HLA environment, to investigate not only the functional contribution of KIR NK cell subsets in controlling CMV infection but also the impact of CMV in modulating KIR NK cell alloreactivity.

Materials and Methods

PBMCs and cell lines

PBMCs were isolated from citrate-phosphate-dextrose blood from healthy adult volunteers by gradient centrifugation on Ficoll-Hypaque (Lymphoprep; Axis-Shield, Oslo, Norway). All blood donors were recruited at the Blood Transfusion Center (Etablissement Français du Sang, Nantes, France) and informed consent was obtained from all individuals. CMV serological status was determined using the chemiluminescent immunoassay LIAISON CMV IgG II (DiaSorin). EBV-B cell lines were obtained by EBV transformation of peripheral B cells using EBV supernatant harvested from the cell line B95-8 (American Type Culture Collection). B cells were isolated from PBMCs obtained from healthy individuals. The HLA class I–deficient acute myeloid leukemia K562 cell line was used to determine the degranulation potential of NK cells. K562 cells and EBV-B lymphocytes were cultured in RPMI 1640 medium (Life Technologies, Paisley, U.K.) containing glutamine (Life Technologies) and penicillin-streptomycin (Life Technologies) and supplemented with 10% FBS (Life Technologies). *Mycoplasma* tests performed by PCR were negative for all cell lines.

HLA and KIR genotyping

Genomic DNA was extracted from PBMCs using a classical salting-out method (20). HLA-A, -B, and -Cw genes were typed via molecular techniques using PCR-sequence-specific primer (SSP) kit from GenoVision (Olerup SSP HLA-A, -B, and -Cw, BioNoBis, Montfort l'Amaury, France) under the conditions recommended by the manufacturer. KIR genes were typed via molecular techniques using PCR-SSP methods under the conditions recommended by the manufacturers, as described previously (21, 22).

Isolation of monocyte and generation of iDCs

Monocytes were separated from lymphocytes by using a high-density hyperosmotic Percoll density gradient (Sigma-Aldrich) (23) and cultured for 6 d in RPMI 1640 medium (Life Technologies) containing glutamine (Life Technologies), penicillin-streptomycin (Life Technologies), and supplemented with 10% human AB serum (Life Technologies), IL-5 (200U/ml; CellGenix), and GM-CSF (1000 U/ml; CellGenix).

NK cell isolation and amplification

PBMCs were isolated as previously described (24, 25). Thereafter, NK cells were amplified after in vitro stimulation with irradiated C1$^+$ and C2$^+$ allogeneic PBMCs and EBV-B cells used as feeders following the PBMC/EBV-B cell ratio at 10:1. All amplified KIR2D NK cells were NKG2C negative.

Isolation and amplification of CMV-specific T cell lymphocytes

CMV-specific T lymphocytes were sorted from HLA-A2$^+$ CMV seropositive blood donors using anti–CD3-PerCP (SK7; BD Biosciences) and HLA-A*02:01-pp65 (NLVPMVATV) (tetramer-allophycocyanin, obtained after tetramerization of HLA-A2-pp65 monomer [platforme Structure Fédérative de Recherche François Bonamy, Nantes, France] with allophycocyanin-streptavidin [AnaSpec]). Sorted cells were stimulated with irradiated feeder cells (allogeneic PBMCs and a mix of two irradiated EBV-B cell lines) and 1 μg/ml PHA-P (L-7132; Sigma-Aldrich). After 2 wk, specific stimulation using pp65 (1 mg/ml)-loaded autologous EBV-B cells (E:T ratio of 10:1) was performed. pp65-Specific lymphocyte amplification was monitored by flow cytometry using HLA-A2-pp65-PE pentamer (Proimmune, Oxford, U.K.).

CMV preparation and infection of iDCs

Stock suspensions of CMV strain VHL/E (provided by the Prof. James Waldman, Columbus, OH) were produced as previously described (26). Of note, the full genomic sequence of VHL/E is not available and thus its genetic content has not been established. Infectivity titration of the virus was performed on iDCs. After overnight coculture of iDCs alone or with VHL/E, cells were washed and resuspended in RPMI 1640, 10% FBS.

Phenotypic analysis by flow cytometry

The NK cell surface phenotype was determined by three- or four-color flow cytometry using the following mouse anti-human mAbs: anti–KIR2DL1-FITC (143211; R&D Systems), anti–KIR2DL2/2DL3/2DS2-PE (GL183; Beckman Coulter), anti–KIR2DL1/2/3/2DS1/2-FITC (1A6) (27), anti–NKp46-allophycocyanin (9E2; Becton Dickinson), anti–NKG2C-PE (134591; R&D Systems), anti-NKG2A (Z199; Beckman Coulter), anti–CD3-PerCP (SK7; BD Biosciences), anti–CD56-allophycocyanin (B159; BD Biosciences), anti-NKG2D (ID11; BD Biosciences), anti-NKp30 (Z25; Beckman Coulter), and anti-NKp44 (Z231; Beckman Coulter). iDCs were stained with the following mouse anti-human mAbs: anti–HLA-A, -B, -C (F41-IE3H1D2; EFS), anti–HLA-C (L31; MediaPharma), anti–HLA-E-PE (3D12; Miltenyi Biotec), anti–CD40-PE (5C3; BD Biosciences), anti–CD80-PE (L307.4; BD Biosciences), anti–CD83-allophycocyanin (HB15e; BD Biosciences), anti–CD86-PE (2331; BD Biosciences), anti–HLA-A, -B, -C–AF^{647} (F41-IE3H1D2; EFS), anti–MICA/B-PE (6D4; BD Biosciences), and isotype-matched controls (IgG1 from BD Pharmingen, IgG2a from R&D Systems). Thereafter, iDCs were washed, fixed, and permeabilized for a staining with major immediate-early IE-1 protein-specific mAb (8B1.2; Millipore), which was conjugated to FITC (Sigma-Aldrich) (28).

CD107a mobilization assay detected by flow cytometry

PBMCs and NK cells were preincubated with anti-CD107a (H4A3; BD Biosciences). NK cell degranulation was assessed after incubation for 5 h alone (negative control), with K562 cells, or with uninfected or CMV-infected allogeneic iDCs (E:T ratio of 10:1). Cell surface staining was performed using the following mouse anti-human mAbs: anti–KIR2DL1/2/3/2DS1/2-FITC (27), anti–NKG2C-PE (134591; R&D Systems), anti–NKp46-allophycocyanin (9E2; Becton Dickinson), anti–KIR2DL1-FITC (143211; R&D Systems), anti–KIR2DL2/3/2DS2-PE (GL183; Beckman Coulter, Immunotech), and NKG2A-FITC or -PE (Z199; Beckman Coulter). HLA-C, HLA-E, and KIR2D neutralization were performed using anti–HLA class I (W6/32; R&D Systems), anti–HLA-E (3D12; BioLegend), and anti–KIR2DL1/2/3/2DS2 (8C11) (27) mAbs, respectively. All flow cytometry data were collected using a FACSCalibur (BD Biosciences) and analyzed using FlowJo 7.6.1 software (Tree Star).

Statistical analyses

Comparisons of NK cell frequencies between two different series of individuals were performed using ANOVA test. A p value of <0.05 was considered to be statistically significant.

Results

Preferential expression of KIR2DL on amplified NKG2C$^+$ NK cells in CMV seropositive individuals

Recently, some studies have shown that a variable expansion of NK cell subsets coexpressing NKG2C and KIR are associated with CMV infection (8–11). We therefore assessed the expression of KIR and NKG2C to determine what NK cell subsets are mainly

observed in CMV seropositive individuals ($n = 31$) compared with seronegative individuals ($n = 29$) (Fig. 1). By four-color flow cytometry, three different NK cell subsets were defined on the basis of NKG2C and KIR2D expression ($NKG2C^+KIR2D^-$, $NKG2C^+KIR2D^+$, and $NKG2C^-KIR2D^+$) using a KIR2DL1/L2/L3/2DS1/S2-specific mAb (1A6) (27) and NKG2C-specific mAb in CMV^- versus CMV^+ individuals (Fig. 1A). In agreement with previous reports, the frequencies of $NKG2C^+$ and $NKG2C^+KIR2D^+$ NK cells are significantly higher in CMV seropositive than seronegative individuals ($p = 0.004$ and $p = 0.005$, respectively) (Fig. 1B). $NKG2C^+$ NK cells did not express NKG2A and preferentially expressed CD57 (data not shown), as previously described in HSCT recipients and immunocompetent CMV seropositive individuals (9). Interestingly, expansion of the $NKG2C^+KIR2D^+$ NK cell population in CMV seropositive individuals is associated to the AA KIR genotype (Fig. 1C), although the probability is not significant ($p = 0.08$), which is confirmed by preferential expression of inhibitory KIR on the $NKG2C^+$ NK cell subset (Fig. 1D). Indeed, of the 12 individuals with an $NKG2C^+KIR2D^+$ NK cell frequency superior to the mean value (6.8%), 11 are KIR2DL3 genotyped and coexpressed KIR2DL3 and $NKG2C^+$ whatever the HLA background (2 C2C2, 4 C1C1, and 5 C1C2 individuals) (Fig. 1D). However, only one individual (C1C2) coexpressed KIR2DL1 with KIR2DL3 and NKG2C (Fig. 1D). In B^+ KIR genotyped individuals, activating KIR2DS2 was coexpressed on $NKG2C^+$ cells in one $KIR2DL3^-KIR2DL2/S2^+$ C2C2 genotyped individual (data not shown). KIR2DS1 expression was evaluated using the combination of KIR2DL1/2DL2/2DL3/2DS2-specific 8C11 and KIR2DL1/S1-specific EB6 mAbs in 2 KIR2DS1 genotyped individuals, and no KIR2DS1 expression was observed on expanded $NKG2C^+$ NK cells. Of note, all three KIR3DS1 genotyped individuals expressed KIR3DS1 on the expanded $KIR2DL3^+NKG2C^+$ NK cell subset. KIR3DL1 was coexpressed with $NKG2C^+$ and $KIR2DL3^+$ in 5 of 10 individuals expressing KIR3DL1 (data not shown).

Downregulation of HLA class I molecule expression on CMV-infected iDCs

It has been previously reported that monocyte-derived DCs are susceptible to in vitro CMV infection by the endothelial cell–adapted strain VHL/E (29). Thus, after 48 h of CMV infection, iDCs were stained with an mAb specific for the CMV major immediate-early IE-1 protein (Fig. 2A). Based on the percentage of $IE\text{-}1^+$ cells, 50% of values from 20 independent experiments are comprised between first quartile (Q1 = 39.5%) and the third quartile (Q3 = 56%) around a median of 48% (Fig. 2B). Interestingly, HLA ligands of NK receptors as HLA class I and especially HLA-C molecules (ligands of KIR2DL) were downregulated on CMV-infected iDCs. In the same line, the nonclassical HLA-E molecule (ligand of CD94/NKG2A or CD94/NKG2C), weakly expressed on iDCs, was downregulated on CMV-infected iDCs (Fig. 2C). As previously described, CMV infection does not induce maturation of iDCs (30).

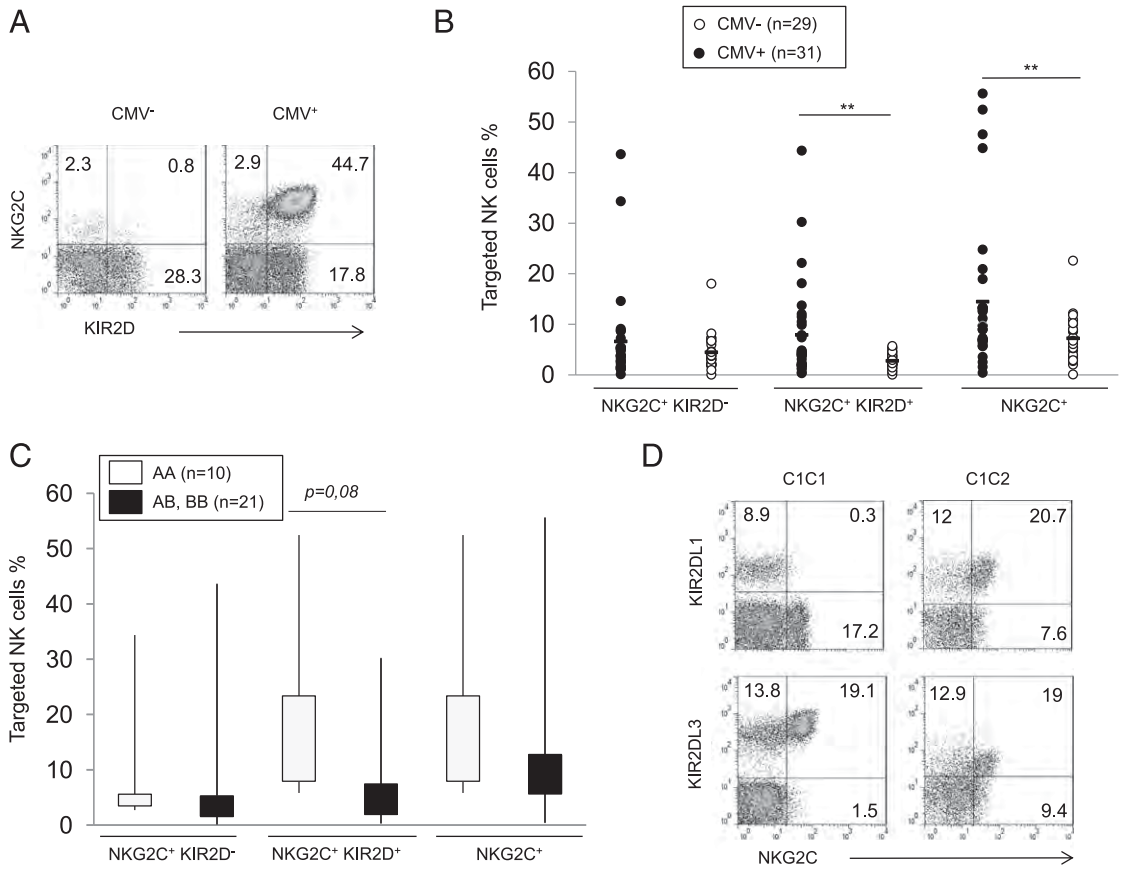

FIGURE 1. Preferential expression of KIR2DL on amplified $NKG2C^+$ NK cells in human CMV seropositive individuals. (**A**) Representative density plots illustrating NK cell phenotype from CMV^- and CMV^+ individuals. NK cells were stained with anti-CD56, anti-NKG2C, and anti-KIR2DL1/L2/L3/2DS1/S2 (1A6) mAbs. (**B**) Scatter plots displaying the $NKG2C^+KIR2D^-$, $NKG2C^+KIR2D^+$, and $NKG2C^+$ NK cell frequencies from CMV^- ($n = 29$) and CMV^+ ($n = 31$) individuals. (**C**) Summary box and whisker plot showing the frequency of $NKG2C^+KIR2D^-$, $NKG2C^+KIR2D^+$, and $NKG2C^+$ NK cells from CMV^+ AA KIR genotyped ($n = 10$) and AB and BB KIR genotyped ($n = 21$) individuals. (**D**) Representative density plots of four C1C1 and one C1C2 CMV^+ individuals. $KIR2DL3^+NKG2C^+$ and $KIR2DL1^+NKG2C^+$ NK cells are targeted with anti-NKG2C and GL183 and with anti-NKG2C and EB6, respectively. Statistical significance ($^{**}p < 0.01$) between both groups was determined using an ANOVA test.

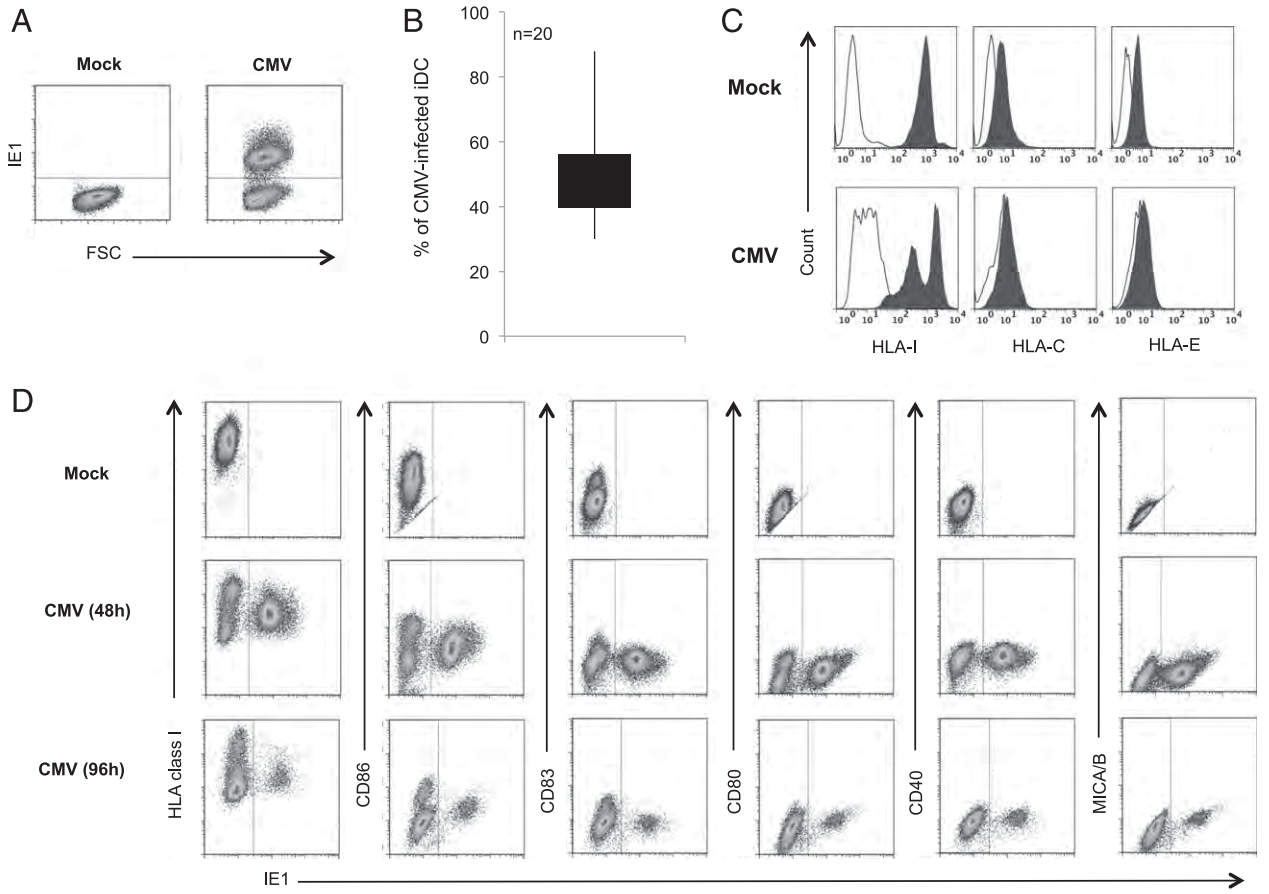

FIGURE 2. Downregulation of HLA class I molecule expression on CMV-infected iDCs. iDCs were generated from monocytes in the presence of GM-CSF and IL-4. On day 6 of the culture, they were infected with the endotheliotrope VHL/E CMV strain. Cell surface phenotype of CMV-infected iDCs was performed at 48 h postinfection by three-color flow cytometry. CMV-infected and noninfected iDCs were discriminated using FITC-conjugated IE1-specific mAb. (**A**) Representative density plots of noninfected and CMV-infected iDCs. (**B**) Summary box and whisker plot showing the frequency of $IE1^+$ iDCs for 20 experiments performed. (**C**) Forty-eight hours postinfection, iDCs were surface labeled with specific mAbs for HLA class I, HLA-C, and HLA-E (open histograms represent isotype control, and filled histograms, specific staining). Results of a representative experiment of four performed are shown (50% $IE-1^+$ cells). (**D**) Representative density plots of flow cytometry analysis of mock-infected iDCs and CMV-infected iDC phenotype using HLA class I, CD86, CD83, CD80, CD40, and MICA/B-specific mAbs.

However, the expression of CD86 decreased on CMV-infected iDCs compared with uninfected iDCs (Fig. 2C, 2D). Moreover, the uninfected iDC subset in CMV-treated cultures present two levels of expression of HLA class I, that is, CD86 and CD83 molecules. Similar results were obtained at 96 h after infection (Fig. 2D). This could be explained by binding of viral products or soluble factors. Experiments were performed with UV-inactivated virus in the same conditions to confirm that the productive CMV infection of iDCs is necessary to modulate the biology of CMV-treated iDCs (data not shown).

CMV-infected iDCs escape to HLA-A2-pp65–specific $CD8^+$ T cell recognition but activate NK cell degranulation

Consistent with previous reports indicating that several viral proteins inhibit HLA class I expression on CMV-infected cells to escape to T lymphocyte response, we further evaluate our in vitro model in assessing the CD107a mobilization of HLA-A2-pp65–specific T lymphocytes against CMV-infected iDCs versus uninfected iDCs. HLA-A2-pp65–specific T lymphocytes, targeted using HLA-A2-pp65 pentamer (Fig. 3A), reacted against pp65-loaded $HLA\text{-}A2^+$ iDCs, but not against uninfected $HLA\text{-}A2^+$ iDCs and CMV-infected $HLA\text{-}A2^+$ iDCs (Fig. 3B). This result obtained in our cellular model is consistent with the ability of CMV to escape to T lymphocyte response by inhibiting HLA class I expression on iDCs. We therefore investigated NK cell ability to recognize decreased expression of HLA class I molecules on CMV-infected iDCs by focusing on $NKp46^+$ NK cells (Fig. 3C). Interestingly, NK cells reacted against CMV-infected iDCs almost as well as against an HLA class I–deficient K562 cell line, used as a positive control of degranulation, as illustrated for one individual (Fig. 3D) and confirmed for six individuals with a significant probability ($p = 0.01$) (Fig. 3E).

KIRs are required for a robust response against allogeneic CMV-infected iDCs

It has been reported that KIRs are required on $NKG2C^+$ NK cells for a strong production of IFN-γ (9). Nevertheless, the role of KIRs in triggering NK cell degranulation is undocumented. To investigate the functional reactivity of $NKG2C^+KIR2D^-$, $NKG2C^-KIR2D^+$, and $NKG2C^+KIR2D^+$ NK cell subsets (Fig. 4A) against allogeneic CMV-infected iDCs, degranulation assays were carried out with PBMCs from CMV^+ individuals ($n = 6$) stimulated overnight with IL-2 (Fig. 4B). As seen previously, PBMCs were incubated alone or in the presence of K562 cells, mock-infected iDCs, or CMV-infected iDCs (E:T ratio = 10:1). All of these NK cell populations reacted significantly against CMV-infected iDCs ($p = 0.02$ for $NKG2C^+KIR2D^-$ NK cells, $p = 0.0006$ for $NKG2C^-KIR2D^+$ NK cells, and $p = 3.5 \times 10^{-5}$ for $NKG2C^+KIR2D^+$ NK

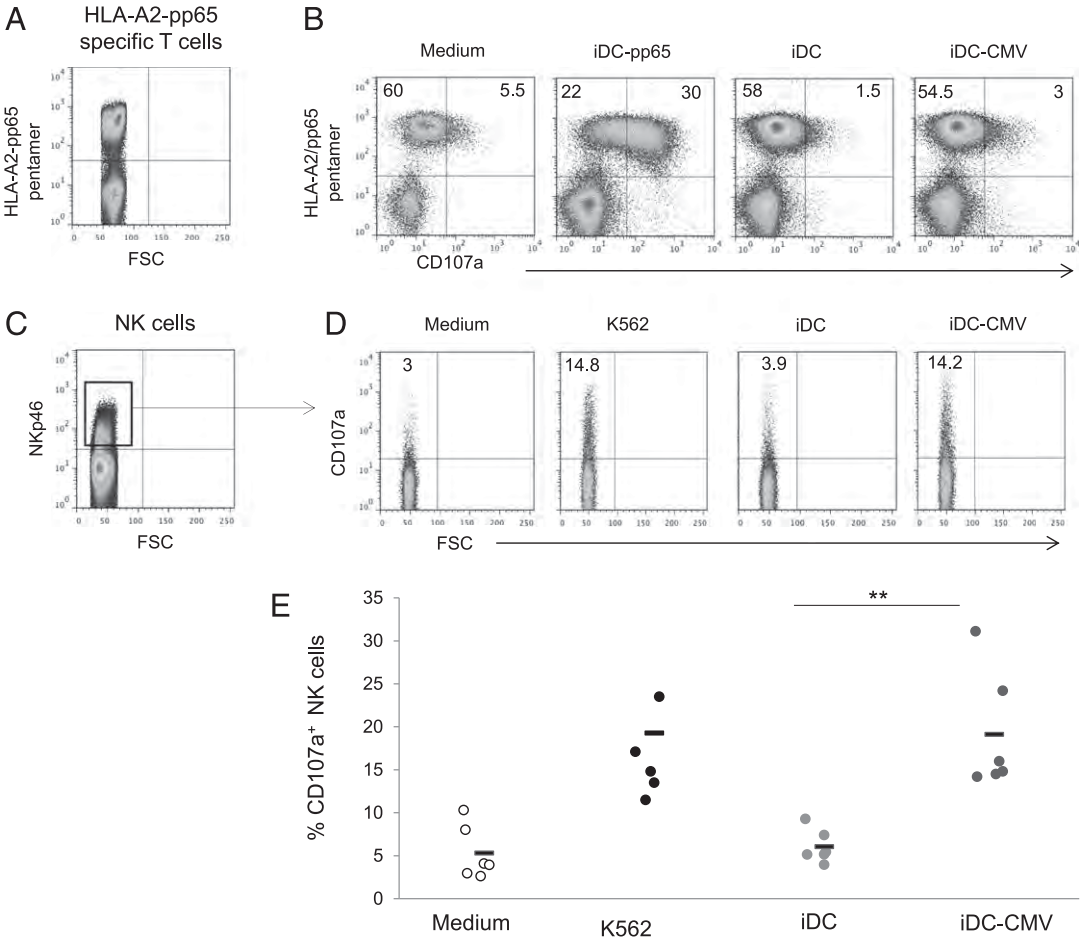

FIGURE 3. CMV-infected iDCs escape to HLA-A2-pp65–specific $CD8^+$ T cell recognition but activate NK cell degranulation. (**A**) HLA-A2-pp65–specific T cells were cell sorted and amplified in vitro from CMV seropositive individuals and targeted as $CD3^+$HLA-A2-pp65-pentamer$^+$. (**B**) After 2 wk of stimulation, these cells were phenotyped by flow cytometry and were incubated for 5 h alone or in the presence of mock-infected, pp65-loaded, or human CMV-infected allogeneic iDC HLA-$A2^+$ at an E:T ratio of 10:1. Results of CD107a mobilization obtained for a representative experiment of three performed are shown. Percentages of $CD107a^+$ HLA-A2-pp65–specific T cells observed by flow cytometry are indicated on the density plots for all conditions of stimulation. (**C**) NK cells are targeted as $NKp46^+$ cells. (**D**) PBMCs stimulated overnight with IL-2 were incubated for 5 h alone or in the presence of K562 cells as positive control or mock- or CMV-infected allogeneic iDCs at an E:T ratio of 10:1. Surface CD107a expression on $NKp46^+$ cells was analyzed by flow cytometry. Representative density plots from six experiments are shown. The percentage of $CD107a^+$ NK cells is indicated on each density plot. (**E**) Dot representation displaying the percentage of $CD107a^+$ NK cells from all six experiments. Statistical significance ($^{**}p < 0.01$) between both groups was determined using an ANOVA test.

cells). However, NK cells bearing KIR2D receptors ($NKG2C^-$ $KIR2D^+$ and $NKG2C^+KIR2D^+$ NK cell subsets) reacted more robustly than did $NKG2C^+KIR2D^-$ NK cells. Indeed, when KIR2D receptors were neutralized with anti-KIR2DL1/2/3/2DS2 mAb, $NKG2C^+KIR2D^+$ NK cell degranulation against CMV-infected iDCs was decreased by half (Fig. 4C). Otherwise, because $NKG2C^+$ NK cells are mostly $NKG2A^-$ (Fig. 4C), we neutralized HLA-E molecules with anti–HLA-E mAb to assess the impact of NKG2C on NK cell degranulation against CMV-infected iDCs. The $NKG2C^+KIR2D^+$ NK cell degranulation was slightly decreased in this blocking condition (Fig. 4C). These results suggest a predominant impact of KIR2DL in triggering NK cell response against CMV-infected iDCs. Based on our results showing a decreased expression of HLA class I molecules on CMV-infected iDCs and the expansion of $NKG2C^+KIR2D^+$ NK cell subsets in CMV infection, we hypothesized that the absence of inhibitory KIR engagement with the cognate ligand and the activating NKG2C engagement with HLA-E on human CMV–infected iDCs might both contribute to drive the expansion process.

C1C1 but not C2C2 allogeneic iDCs activate KIR2DL1$^+$ NK cell degranulation following the self-missing model

To determine the nature of KIR2DL receptors involved in the NK cell response to CMV-infected iDCs, we first focused on NK cell subsets expressing well-characterized inhibitory KIR2DL1, which recognizes HLA-C molecules of C2 group. KIR2DL1$^+$ NK cells were sorted from KIR2DS1$^-$ genotyped $C2^+$ individuals and amplified following polyclonal stimulation. The phenotype (KIR2DL, NKG2A, NKp44, NKp30, and NKG2D) of the studied NK cell lines was stable following culture alone or coculture with iDCs or CMV-infected iDCs for 5 h. All selected and amplified NK cells did not express the inhibitory receptor ILT2 (data not shown). However, most amplified NK cells expressed the NKG2A marker (Fig. 5A). Thus, NKG2A expression on KIR2D NK cells may inhibit NK cell response and override the signal mediated by KIR2D receptors. We therefore assessed the function of KIR2DL1$^+$ KIR2DL2/3/S2$^-$NKG2A$^-$ NK cells. We showed that the degranulation of $C2^+$ educated KIR2DL1$^+$ NK cells, controlled with K562, is strongly induced in contact to C1C1 allogeneic iDCs ($p = 0.002$) but not in contact to C2C2 allogeneic iDCs (Fig. 5B). These results

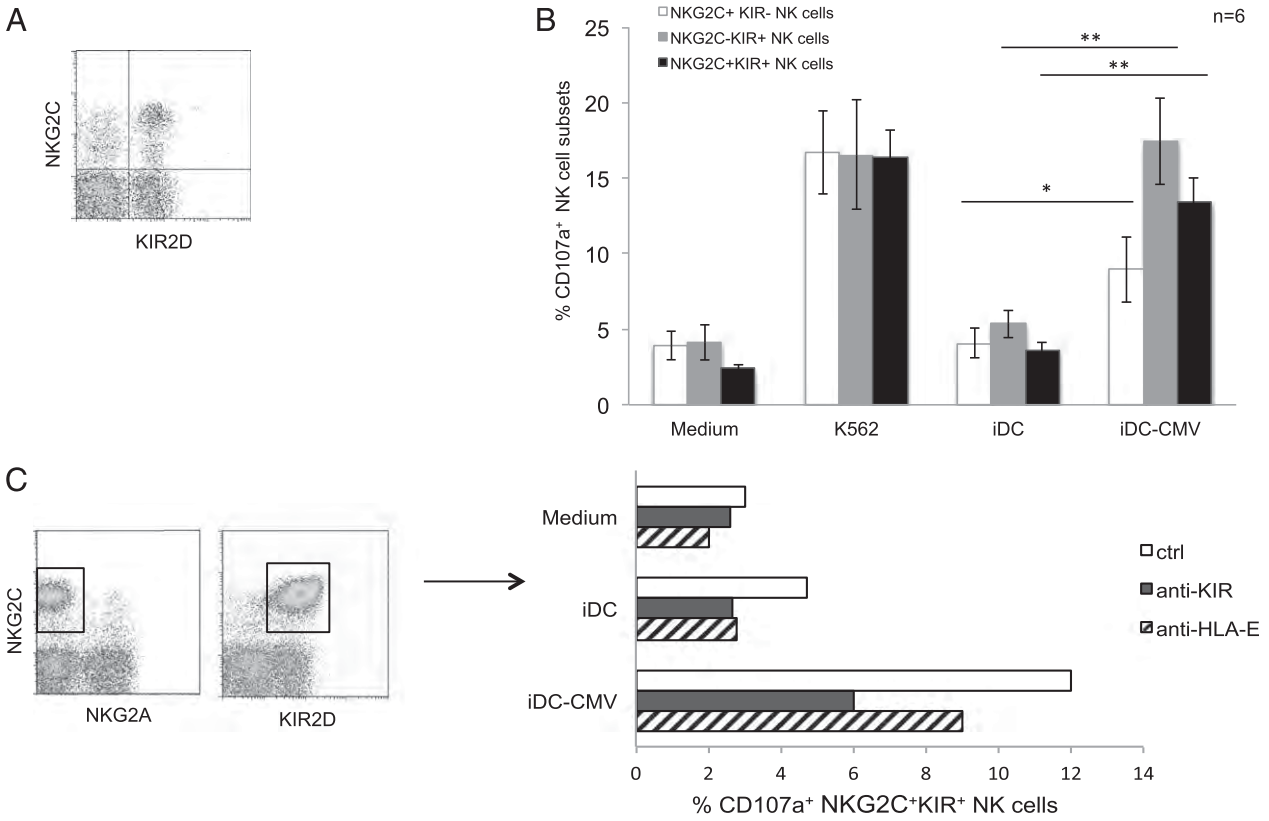

FIGURE 4. NKG2C$^+$ NK cells increased in CMV seropositive individuals require KIR2D for a robust degranulation against allogeneic CMV-infected iDCs. Degranulation assays were carried out with PBMCs from CMV$^+$ individuals ($n = 6$) stimulated overnight with IL-2. CD107a expression on NKp46$^+$ cells was analyzed by flow cytometry after incubation of PBMCs alone or in the presence of K562 cells or mock-infected or CMV-infected iDCs for 5 h at an E:T ratio of 10:1. (**A**) Representative density plot illustrating NKG2C KIR2D NK cell phenotype from CMV$^+$ individual. (**B**) Bars indicate the mean of CD107$^+$ NK cell percentages for all NKG2C$^+$KIR2D$^-$, NKG2C$^+$KIR2D$^+$, and NKG2C$^-$KIR2D$^+$ NK cell subsets observed in all culture conditions: medium, K562 cells, mock-infected, or CMV-infected allogeneic iDCs ± SD. (**C**) Degranulation assays were performed on an NKG2C$^+$KIR2D$^+$NKG2A$^-$ gated NK cell population. Results of CD107a mobilization obtained for a representative experiment of three performed were presented after culture alone (medium) or stimulation with iDCs or CMV-infected iDCs. Bars indicate CD107a$^+$NKG2C$^+$KIR2D$^+$ NK cell percentage observed by flow cytometry. KIR2D receptors were blocked with 8C11 mAb and isotype control. HLA-E molecules expressed on iDCs were blocked with 3D12 mAb and isotype control. Statistical significance (*$p < 0.05$, **$p < 0.01$) between both groups was determined using an ANOVA test.

validated our in vitro model to explore the impact of CMV infection of iDCs on KIR$^+$ NK cell degranulation.

CMV infection of C2C2 iDCs triggers alloreactivity of C2$^+$ KIR2DL1$^+$ NK cells

CMV infection of C2C2$^+$ iDCs activated significantly KIR2DL1$^+$ NK cell degranulation ($p = 0.0003$) (Fig. 5C). This result suggests that HLA class I downregulation in CMV-infected C2C2$^+$ iDCs was sufficient to trigger KIR2DL1$^+$ NK cell cytotoxicity. To confirm this point, we evaluated CMV infection of C1C1$^+$ iDCs on KIR2DL1$^+$ NK cell degranulation. Indeed, when KIR2DL1$^+$ NK cells were incubated in the presence of CMV-treated C1C1$^+$ iDCs, the frequency of CD107a$^+$KIR2DL1$^+$ NK cells was high and similar to that observed in the presence of mock-infected C1C1$^+$ iDCs (Fig. 5C). The activation of KIR2DL1$^+$ NK cell degranulation with only CMV-infected C2C2$^+$ iDCs but not CMV-infected C1C1$^+$ iDCs supports the hypothesis that the protective KIR2DL1/C2 interaction is directly targeted. Furthermore, we investigated the function of unlicensed C1C1$^+$KIR2DL1$^+$ NK cells in controlling CMV infection, taking into account published works on mice indicating that "unlicensed" NK cells dominate the response to murine CMV infection (31). However, the degranulation of C1C1$^+$KIR2DL1$^+$ NK cells, which is low against the standard HLA class I–deficient K562 cell line, was not triggered against C1C1$^+$ iDCs infected or not with CMV (data not shown). Taken together, these results support the functional contribution of C2$^+$KIR2DL1$^+$ NK cells in response to CMV-infected iDCs.

KIR2DL3$^+$ NK cells respond to CMV-infected allogeneic C2C2$^+$ iDCs but not to CMV-infected allogeneic C1C1 iDCs

Additionally, we investigated the role of KIR2DL3$^+$ NK cells in the response to CMV-infected allogeneic iDCs. KIR2DL3$^+$ NK cells were sorted and in vitro amplified from KIR2DS1$^-$/L2$^-$/S2$^-$ genotyped C1C1$^+$ individuals (Fig. 5D). Fifteen days after stimulation, we foremost assessed the degranulation of KIR2DL3$^+$KIR2DL1/L2/S2$^-$NKG2A$^-$ NK cells against allogeneic iDCs. Both C1C1$^+$ and C2C2$^+$ iDCs inhibited KIR2DL3$^+$ NK cell degranulation (Fig. 5E). Indeed, when HLA-C molecules were neutralized with anti–HLA class I mAb, C2C2$^+$ iDC-mediated KIR2DL3$^+$ NK cell degranulation was partially restored, showing the functional interaction of KIR2DL3 with C2 ligand (Fig. 5G). These results are in accordance with recently reported data showing that KIR2DL3 could recognize not only the expected group of HLA-C molecules belonging to the C1 group, but also those belonging to C2 group (32–34). To determine whether the decreased HLA class I expression on CMV-infected iDCs impacts the KIR2DL3$^+$ NK cell function, degranulation of the KIR2DL3$^+$ NK cell subset against CMV-infected iDCs has been evaluated. CMV infection of C2C2$^+$ iDCs activated significantly KIR2DL3$^+$ NK cell degranulation ($p = 0.01$) (Fig. 5F). This result may imply that HLA class I

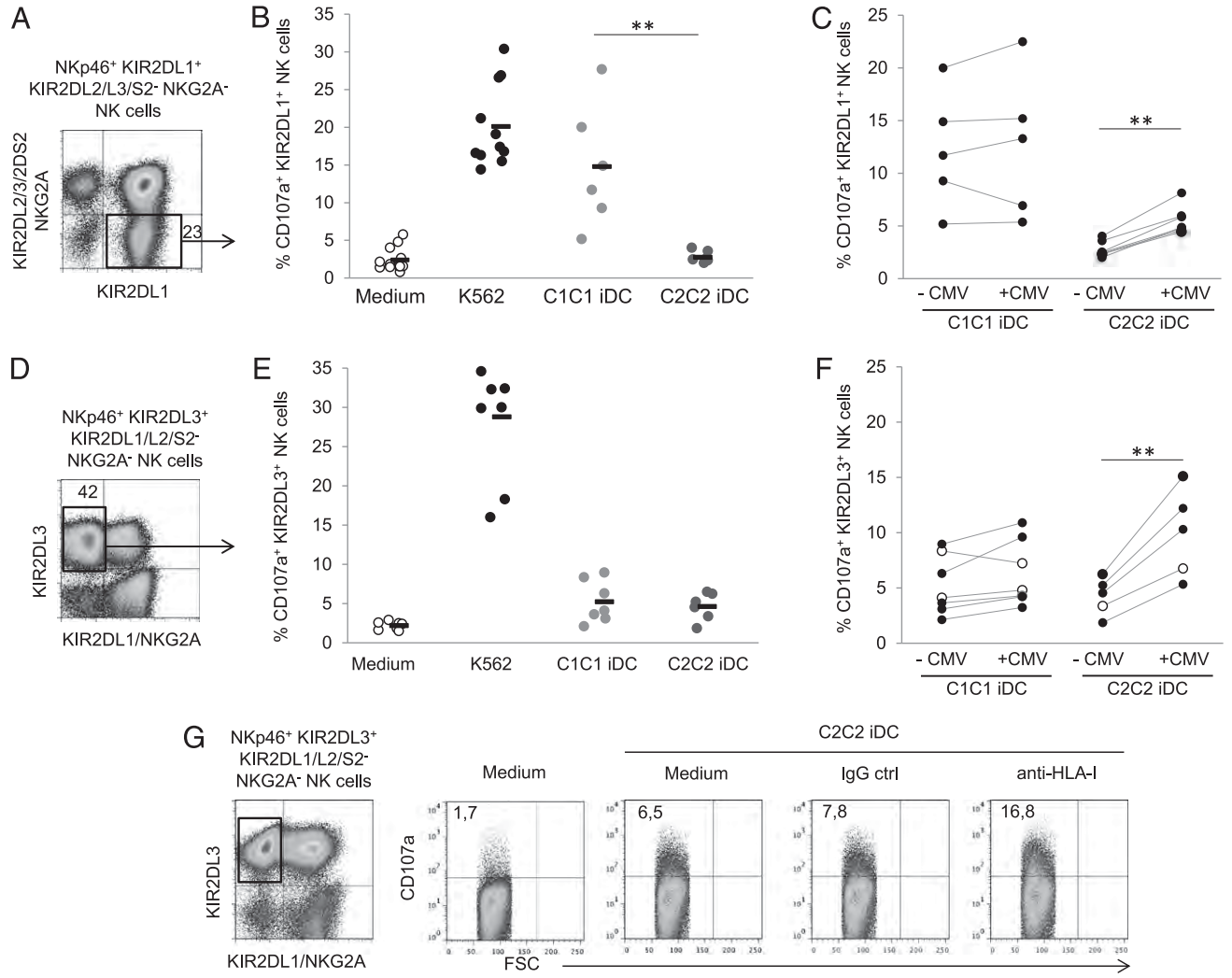

FIGURE 5. CMV-infected allogeneic C2C2$^+$ iDCs activate KIR2DL1$^+$ and KIR2DL3$^+$ NK cell subsets. Mobilization assay has been performed on sorted and in vitro–amplified KIR2DL1$^+$ NK cells from C2$^+$ individuals and KIR2DL3$^+$ NK cells from C1$^+$ individuals. (**A**) Representative density plot illustrating the phenotype of targeted KIR2DL1$^+$ NK cells in functional assay, assessed by flow cytometry using a combination of KIR2DL1-specific mAbs and a mix of KIR2DL2/3/2DS2 and NKG2A-specific mAbs. The cells were isolated from negative KIR2DS1 genotyped individuals. (**B**) Dot representation displaying the percentage of CD107a$^+$KIR2DL1$^+$ NK cells in all culture conditions: medium (n = 13), K562 cells (n = 13), C1C1$^+$ iDCs (n = 6), and C2C2$^+$ iDC (n = 7) at an E:T ratio of 10:1. (**C**) Dot representation displaying the percentage of CD107a$^+$KIR2DL1$^+$ NK cells in the presence of mock- or CMV-infected C1C1$^+$ iDCs (n = 6) or C2C2$^+$ iDCs (n = 7) at an E:T ratio of 10:1. KIR2DL1$^+$ NK cells were isolated from CMV$^-$ individuals. (**D**) Representative density plot illustrating the phenotype of targeted KIR2DL3$^+$ NK cells in functional assay, assessed by flow cytometry using a combination of KIR2DL3-specific mAbs and a mix of KIR2DL1/S1 and NKG2A-specific mAbs. The cells were isolated from negative KIR2DL2/S2 genotyped individuals. (**E**) Dot representation displaying the percentage of CD107a$^+$KIR2DL3$^+$ NK cells in all culture conditions: medium (n = 7), K562 cells (n = 7), C1C1$^+$ iDCs (n = 7), and with C2C2$^+$ iDCs (n = 6). (**F**) Dot representation displaying the percentage of CD107a$^+$KIR2DL3$^+$ NK cells in the presence of mock- or CMV-infected C1C1$^+$ iDCs (n = 7) or C2C2$^+$ iDCs (n = 5). KIR2DL3$^+$ NK cells isolated from CMV$^-$ and CMV$^+$ individuals are indicated by black and white circles, respectively. (**G**) Representative density plots of three experiments performed displaying the percentage of CD107a$^+$KIR2DL3$^+$ NK cells after coculture with C2C2$^+$ iDCs. Target cells were incubated with blocking HLA class I mAb at a concentration of 10 μg/ml for 30 min or IgG control, prior to coincubation with NK cells. Statistical significance (**$p \leq 0.01$) between both groups was determined using an ANOVA test.

downregulation on CMV-infected C2C2$^+$ iDCs is sufficient to trigger KIR2DL3$^+$ NK cell reactivity. However, CMV infection of C1C1$^+$ iDCs did not activate KIR2DL3$^+$ NK cell degranulation (Fig. 5F). These findings support the functional contribution of KIR2DL3$^+$ NK cells in response to CMV infection of C2C2$^+$ iDCs, but they suggest a potential evasion of CMV to KIR2DL3$^+$ NK cell control of C1C1$^+$ target cells.

Discussion

Recent studies have reported the expansion of NKG2C$^+$ NK cells expressing self-specific inhibitory KIR (8–10) in CMV infection. Our data provide evidence that inhibitory KIR2DL3 is preferentially coexpressed with NKG2C on amplified NK cells in CMV seropositive individuals whatever the HLA-C background, and even KIR2DL3 seems to recognize the HLA-C ligand from the C1 and C2 groups with a lower affinity to C2 ligands (32). Our results are in agreement with a previous report revealing the expansion of NKG2C$^+$KIR2DL2/2DS2/2DL3$^+$ NK cells in a T$^-$B$^+$NK$^+$ SCID patient with a CMV infection representing 80% of NK cells (35). In contrast to Charoudeh et al. (12) who reported a specific expansion of NK cells expressing the inhibitory receptors KIR2DL1 and KIR2DL3 in response to in vitro exposure to CMV, we observed preferential KIR2DL3 coexpression on NKG2C$^+$ NK cells but seldom KIR2DL1 coexpression. Similarly, we observed mainly inhibitory KIRs coexpressed on NKG2C$^+$ NK cells. Additionally, our phenotypic study showed a frequent coexpression of KIR3DL1

or KIR3DS1 on NKG2C$^+$ KIR2DL3$^+$ NK cells in CMV seropositive individuals, whatever the Bw4 environment. Thus, the predominant expression of KIR2DL3 suggests a driven clonal-like expansion of KIR2DL3$^+$NKG2C$^+$ NK cells during CMV infection that can coexpress another KIR as KIR3DL1 or KIR3DS1, as previously described for Ly49H$^+$ NK cells in mice challenged with murine CMV (36). Moreover, numerous hypotheses regarding HIV-1 infection (37) and recent data (12) suggest a potential impact of KIR3DL1/S1 in controlling CMV infection. The role of KIR3DL1/S1 engagement in CMV infection should be investigated to answer this point.

We showed that NK cells bearing KIR2D receptors react more robustly than do KIR2D$^-$NKG2C$^+$ NK cells. Indeed, KIR2D$^+$ NK cell subsets degranulated and secreted INF-γ (data not shown) more than did the KIR2D$^-$NKG2C$^+$ NK cell subset in response to CMV-infected iDCs. Our results are in accordance with previous results showing that KIRs are required for robust IFN-γ production (9). As previously described (38), degranulation of C2$^+$ educated KIR2DL1$^+$ NK cells is strongly induced in contact to allogeneic C1C1$^+$ iDCs but not in contact to C2C2$^+$ iDCs. We demonstrated that infection of C2C2$^+$ iDCs with CMV significantly enhances KIR2DL1$^+$ NK cell degranulation. Thus, HLA class I downregulation observed on CMV-infected iDCs seems sufficient to activate KIR2DL1$^+$ NK cells. Interestingly, KIR2DL1$^+$ NK cell alloreactivity against C1C1$^+$ iDCs is maintained with CMV infection. Additionally, KIR2DL1$^+$ and KIR2DL3$^+$ NK cell subsets reacted against CMV-infected C2C2$^+$ iDCs. This point could explain recent work showing that early CMV infection is associated with a reduced risk of relapse in acute myeloid leukemia patients undergoing allogeneic HCT (19). Thus, it is possible that CMV infection activates and mobilizes the NK cell subset able to recognize "missing-self" and that expanded NKG2C$^+$KIR2D$^+$ NK cells constitute boosted alloreactive NK cells that are more efficient against leukemia cells.

Recent studies performed on a large cohort has revealed the expansion of activating KIR$^+$ (KIR2DS2, KIR3DS1, KIR2DS4) NK cell subsets independently of NKG2C, suggesting a contribution of both activating KIR$^+$ and NKG2C$^+$ NK cell subsets in CMV infection (11). In a murine model, activating Ly49H recognition of the viral protein m157 drove expansion of the cells that control murine CMV infection (15). Thus, we evaluated amplified and sorted C2$^-$KIR2DS1$^+$ NK cell degranulation against C1C1$^+$ and C2C2$^+$ iDCs infected or not by CMV. In our model, we did not observe a potential activation of KIR2DS1$^+$ NK cells stimulated with CMV-infected iDCs (data not shown). However, the CMV seronegative status of the studied individuals may explain the absence of the KIR2DS1$^+$ NK cell response. Indeed, it is possible that "memory" status of NK cells expanded during CMV infection is necessary to involve KIR2DS1 engagement with viral ligands.

Interestingly, we show that KIR2DL3 recognizes not only C1 but also C2 ligands, as demonstrated in our cellular model using C1C1 or C2C2 iDCs. In contrast, KIR2DL1$^+$ NK cells recognize only C2C2$^+$ iDCs. These results are in accordance with previous works indicating that KIR2DL3 interacts with HLA-C molecules belonging to C1 and C2 groups (32). Additionally, KIR2DL3$^+$ NK cell degranulation is significantly increased in contact to CMV-infected C2C2$^+$ iDCs, probably due to the decreased expression of HLA class I molecules on CMV-infected C2C2$^+$ iDCs. Unexpectedly, KIR2DL3$^+$ NK cell degranulation is not increased in contact to CMV-infected C1C1 iDCs. This last result suggests a potential evasion of CMV to KIR2DL3$^+$ NK cell control in HLA-C1 environment as observed in our model at 48 h postinfection and confirmed at 96 h postinfection (data not shown). However, we do not exclude that KIR2DL3$^+$ NK cell degranulation is activated by CMV-infected target cells earlier or later during the viral cycle.

The existence of multiple CMV strategies to escape immune system control opens a range of hypotheses. Indeed, the high frequency of KIR2DL3$^+$ NK cells observed in CMV infection may lead to an antiviral immune pressure mediated in vivo by these KIR2DL3$^+$ NK cells. The modulation of KIR/HLA interactions by viruses has been reported for some inhibitory KIR (39–43). This suggests that viral CMV peptides may increase the affinity of C1 ligands for inhibitory KIR2DL3 and prevent the activation of specific KIR2DL3$^+$ NK cells.

Different groups have recently reported a specific NK cell phenotype marked by the acquisition of CD57, NKG2C, and KIR on NK cells in different viral infections including in nonexclusive fashion CMV (44), hantavirus (45), chikungunya virus (46), and HIV-1 (47). Interestingly, all of these viruses developed evasion strategies based on HLA class I downregulation to escape T cell recognition. Although the ligand of NKG2C is still elusive in CMV infection, it is conceivable that NKG2C$^+$KIR2DL$^+$ NK cell expansion is mutually driven by the recognition of HLA-E by CD94/NKG2C and the absence of engagement of KIR2DL with specific HLA KIR ligands on CMV-infected cells. Additionally, the large specificity of KIR2DL3 may explain its frequent expression on NKG2C$^+$ NK cells associated to CMV infection.

In conclusion, our results provide evidence that inhibitory KIR2DL3 is preferentially coexpressed with NKG2C on amplified NK cells in CMV infection. Our findings support the functional contribution of KIR2DL1$^+$ and KIR2DL3$^+$ NK cell subsets in controlling CMV infection in C2$^+$ recipients of allogeneic HSCT, suggesting a beneficial impact of CMV on GVL effect in this clinical context. In C2$^-$ recipients, the beneficial KIR2DL1$^+$ NK cell alloreactivity is maintained despite infection, whereas CMV seems to have developed a strategy to escape to KIR2DL3$^+$ NK cell control. This study opens new perspectives of investigation to determine the molecular mechanisms involved in KIR2DL3 NK cell escape employed by CMV.

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Disclosures

The authors have no financial conflicts of interest.

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Thèse de Doctorat

Pauline RETTMAN

Contribution à l'étude des cellules Natural Killer dans le contexte de la double-greffe de sang de cordon

Contribution to the study of Natural Killer cells in the context of double-umbilical cord blood transplantation

Résumé

La dUCBT est réalisée chez l'adulte en l'absence d'un donneur de CSH HLA compatible et seul l'un des 2 SC injectés reconstitue l'hématopoïèse du patient dans 95% des cas. Cependant, les mécanismes immunologiques impliqués dans la dominance d'un seul SC n'ont pas été identifiés. Les cellules NK étant les premières à réapparaitre après dUCBT, les incompatibilités HLA pourraient être propices aux réponses NK KIR alloréactives. Nous avons étudié les incompatibilités KIR et KIR ligand sur une cohorte locale (n=50 dUCBT) et montré l'association de la combinaison $KIR3DL1^{+}/Bw4^{-}$ dans le sens GvG à une meilleure prise mais à une incidence plus élevée de rechute. L'impact de cette combinaison n'a pas été confirmé sur un effectif national plus large (n=293), dû à l'hétérogénéité des pratiques cliniques des centres greffeurs et la complexité immunologique de la dUCBT. L'étude locale a révélé l'intérêt du génotypage KIR dès J+14 après dUCBT pour définir le cordon dominant. Nos résultats confirment que le répertoire NK KIR est constitué à partir d'un seul UCB dès J+14 après dUCBT. Pour mieux comprendre la biologie des NK de SC en dUCBT, nous avons étudié le phénotype et la fonction de SC (n=73) en parallèle à l'adulte (n=178). La formation du répertoire NK de SC est ainsi modulée par la nature des gènes KIR exprimés et semble peu dépendant de l'environnement HLA. La faible lyse spontanée des cellules NK de SC n'est pas corrélée à la fréquence de cellules NK KIR^{+} suggérant un répertoire en cours d'éducation fonctionnelle.
Ce projet contribue à une meilleure connaissance de la biologie des cellules NK de SC permettant d'appréhender leur implication dans le contexte clinique de la dUCBT.

Mots clés
Cellules NK - KIR - HLA - Greffe de CSH
Greffe de sang de cordon

Abstract

dUCBT is performed in adult recipients in absence of HLA matched HSC donor and only one of the two injected cord blood unit (UCB) reconstitutes the hematopoiesis in 95% of cases. However, immunologic mechanisms involved in one full UCB dominance are not yet identified. As NK cells are the first to reappear in high frequency after dUCBT, HLA incompatibilities could drive KIR+NK cell alloreactivities. We then studied KIR and KIR ligand incompatibilities on a local cohort of 50 dUCBT and showed that the KIR3DL1+/Bw4- combination in GvG direction is associated with better neutrophil reconstitution and a higher incidence of relapse. However, the impact of this combination has not been confirmed on a larger national cohort (n=293), mainly due to the heterogeneity of centers' clinical and biological protocols, and the immunologic complexity of dUCBT. The local study highlighted the interest of KIR genotyping early after dUCBT (d+14) to define the dominant UCB. Our results confirmed that NK KIR repertoire is often constituted from only one UCB as early as d+14 after dUCBT. To better understand cord blood NK cell biology in dUCBT, we studied phenotype and function of cord blood samples (n=73) in comparison to adult PBMC (n=178). We show that UCB NK cell repertoire formation is modulated by the nature of expressed KIR genes and does not seem to be HLA-dependent. The weak spontaneous lysis of cord blood NK cell is not correlated with KIR+ NK cell, suggesting that cord blood NK repertoire is being functionally educated.
This thesis project contributes to the understanding of cord blood NK cell biology allowing to better apprehend their implication in the clinical context of dUCBT.

Key Words
NK cells - KIR - HLA - HSCT - UCBT