UNIVERSITE DE NANTES
FACULTE DE MEDECINE

Contribution à l'analyse des mécanismes impliqués dans le maintien de la tolérance induite par transfusion spécifique de sang du donneur.

THESE DE DOCTORAT

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Sommaire

Abréviations

ADN: Acide Désoxyribonucléique

BMT: Bone Marrow Transplantation

CD: Cellules Dendritiques

CDR: Complement Determining Region

CMH: Complexe Majeur d'Histocompatibilité

CPA: Cellules présentatrices d'Antigènes

CsA: Cyclosporine A

CTLA-4:Cytotoxic T lymphocyte Associated protein 4

DIVAT: Données Informatisées et VAlidées en Transplantation

FoxP3: Forkhead box P3

GITR: Glucocorticoids induced TNF related receptor

HLA: Human leucocyte antigens

HO-1: Heme Oxygenase-1

IFN: Interféron

IL: Interleukine

ILT: Immunoglobulin Like Transcript

ITAM: Immunoreceptor Tyrosine-based Activation Motif

NK: Natural Killer

NKT: Natural Killer T cells

NO: Monoxyde d'azote

PAMPs: Pathogen Associated Molecular Patterns

PBMC: Peripheral Blood Mononuclear cells

PRRs: Pattern Recognition Receptors

TCR: T Cell Receptor

TGF: Transforming Growth Factor

TLR: Toll-Like Receptor

TNF: Tumor Necrosis Factor

TRAIL: TNF-Related-Apoptosis-Inducing Ligand

Treg: lymphocytes T régulateurs

TSD: Transfusion Spécifique du Donneur

Avant-propos

La transplantation représente à ce jour le seul moyen de remplacement d'un organe en stade terminal. Au début du XXème siècle, les techniques de greffes, notamment rénales, ont été améliorées entre autres par le chirurgien autrichien ULLMANN mais aussi par le prix Nobel Alexis CARREL. Chez l'Homme la première greffe de rein a été réalisée par le russe VORONOY en 1933. Les premières transplantations rénales réalisées avec succès furent effectuées à Paris et Boston en 1951 par KÜSS, SERVELLE, DUBOST et HUME. Malheureusement, la survie à long terme du greffon était relative et n'intervenait qu'après une greffe faite entre jumeaux (1954). Les réactions immunologiques sont alors apparues comme ayant un rôle important dans le devenir d'une transplantation. Jean Hamburger de l'hôpital Necker à Paris, montra le bénéfice d'une sélection du donneur selon certains critères de compatibilité tissulaire. C'est pourquoi, à partir de 1958, Jean Hamburger suggéra de recourir, pour effectuer cette sélection, à la comparaison des groupes d'antigènes de leucocytes faisant partie du complexe majeur d'histocompatibilité (CMH) du donneur et du receveur, le système HLA (Human Leucocyte Antigens). La greffe s'est ensuite étendue de la greffe rénale à d'autres types d'organes comme le cœur, le foie, le pancréas notamment grâce au développement des immunosuppresseurs, terme générique qui définit toute substance capable de provoquer une diminution ou une abolition des réponses immunitaires tels que la Cyclosporine A (1972). Les années 80 ont vu le nombre de greffes augmenter de façon spectaculaire: ainsi en France, le nombre d'organes transplantés est passé de 1360 en 1985 à 3500 en 1990. Depuis, par manque d'organes disponibles, le chiffre s'est stabilisé autour de 2800 greffes par an. Ce développement peut s'expliquer par un meilleur contrôle des épisodes de rejets aigus (prophylaxie et traitement), la disponibilité de médicaments immunosuppresseurs, essentiellement la Cyclosporine et le FK506 (Tacrolimus), et une sélection rigoureuse des couples donneur/receveur. C'est ainsi que depuis 1985 la survie du greffon rénal à 1 an est passée de 65 % à 85-90 %. Selon les greffes (rénales, hépatiques ou cardiaques), la survie du greffon à un an est de 90,1%, 83% et 71,7% respectivement, et à

cinq ans de 78,9%, 71,9% et 60,4% respectivement (données extraites du bilan 2004 des activités de prélèvement et de greffe en France de l'agence de la biomédecine (établissement français des greffes) sur un ensemble de patients greffés entre 1993 et 2003).Des résultats similaires sont observés aux Etats-Unis (Lechler et al. 2005).

L'évolution des connaissances dans le domaine de l'immunologie a permis le développement de nombreuses stratégies thérapeutiques et les immunosuppresseurs ont révolutionné le monde de la transplantation. Mais tous ces traitements, pour la plupart non spécifiques, ont à long terme des effets néfastes pour l'organisme (cancer, infections à répétition, etc…) et l'organe greffé lui-même (néphrotoxicité). De plus, ces immunosuppresseurs ne permettent pas de prévenir la survenue du rejet chronique, rejet qui intervient à distance de la greffe et entraîne la perte du greffon. Différents axes de recherches peuvent être identifiés. Le premier s'appuyant sur la recherche pharmacologique qui vise à diminuer la toxicité des traitements immunosuppresseurs et à augmenter leur spécificité. Un autre axe de recherche, celle des xénogreffes, vise à long terme à augmenter le nombre de greffons disponibles. Enfin, de nombreuses équipes de recherche tentent d'identifier de nouvelles stratégies thérapeutiques d'induction de tolérance via l'étude de nombreux protocoles expérimentaux qui permettent l'étude des réponses immunologiques suite à une greffe. Dans ce manuscrit, nous allons nous pencher plus précisément sur un protocole: la transfusion spécifique du donneur (TSD), qui est utilisée depuis un certain temps et qui a été testée cliniquement. Lors de ce travail de thèse, nous avons exploré la compartimentation et le caractère ambivalent de ce modèle lors du maintien de tolérance en étudiant la présence de cellules pouvant avoir une activité régulatrice et/ou cytotoxique, ainsi que le caractère «infectieux» de cette tolérance à distance de la greffe après transfusion spécifique du donneur.

Introduction

I.

La réponse immunitaire

I.1. Généralités

Le système immunitaire permet aux organismes vivants de se défendre contre les agressions extérieures (virus, bactéries) et intérieures (transformation de cellules en cellules malignes). Actuellement, on parle plutôt d'une discrimination entre le «soi» et le «non-soi» (concept qui est d'autant mieux adapté à l'immunologie de la transplantation). Au cours de l'évolution, deux types de systèmes de défense se sont mis successivement en place pour faire face au «danger».

Le premier système est un système précoce qui répond dans de très brefs délais aux agressions de façon non spécifique: l'immunité naturelle non spécifique ou innée. L'immunité naturelle non spécifique ou innée fait intervenir plusieurs mécanismes physiques, chimiques et cellulaires et repose sur une reconnaissance non spécifique de motifs caractéristiques des éléments pathogènes les PAMPs (pathogen associated molecular patterns). Ces PAMPs sont reconnus par les PRRs (pattern recognition receptors) (Janeway 1989; Medzhitov et al. 1997). Depuis quelques années, les principaux récepteurs mis en avant sont les TLR (Toll Like Receptor) (Janeway et al. 2002; Basset et al. 2003; Akira et al. 2004; Tosi 2005). La reconnaissance d'éléments par ces PRRs entraîne une cascade d'événements aboutissant à la réaction inflammatoire. Cette réaction inflammatoire fait intervenir divers facteurs solubles comme des polypeptides possédant des caractéristiques antibiotiques naturelles (Ganz 2003; Oppenheim et al. 2003; Ganz 2004 ; De Smet et al. 2005), des cytokines (pour revue (Borish et al. 2003)), des chemokines (pour revue (Esche et al. 2005)), l'activation du complément (Tosi 2005) et certains anticorps dits naturels. Elle fait également intervenir des mécanismes cellulaires: la phagocytose (Basset et al. 2003; Tosi 2005) ou la cytotoxicité (Smyth et al. 2005). Les phénomènes de lyse cellulaire ou de phagocytose (Jutras et al. 2005) induits par les récepteurs non spécifiques tels les Toll like receptors, vont permettre aux cellules présentatrices d'antigènes (CPA), comme les cellules dendritiques (CD) (Turnbull et al. 2001; Quah et al. 2005; Villadangos et al. 2005), de présenter aux cellules de la réponse adaptative, des antigènes, ce qui va permettre leur activation.

Le second système correspond à une réponse retardée dans le temps et spécifique d'une agression donnée, on parle de réponse adaptative ou acquise. La réponse immunitaire acquise se présente sous deux formes: une réponse humorale typique d'une activation des

lymphocytes B caractérisée par la sécrétion d'immunoglobulines (les anticorps) spécifiques de l'antigène et une réponse cellulaire qui correspond à l'activation des lymphocytes T.

I.2. La voie cellulaire

Les lymphocytes T forment un groupe de cellules diverses, complexes tant sur le plan de leur classification que de leurs fonctions. Il existe deux populations principales de lymphocytes T qui se distinguent par des marqueurs membranaires: le CD4 (glycoprotéine monocaténaire transmembranaire) et le CD8 (hétérodimère de glycoprotéine transmembranaire). Les lymphocytes $TCD4^+$ et les lymphocytes $TCD8^+$ vont pouvoir reconnaître des peptides présentés respectivement par des molécules du CMH de classe II et de classe I. L'activation des lymphocytes $TCD4^+$, aussi dénommés lymphocytes T auxiliaires ou T helper (Th), va permettre de démultiplier la réponse immune en stimulant soit la réponse humorale soit la réponse cellulaire. C'est par la sécrétion de cytokines diverses et variées que les $TCD4^+$ vont orienter la réponse immune (tableau 1). En se basant sur les cytokines sécrétées, on distingue deux orientations principales de la réponse immunitaire: une voie Th1 et une voie Th2. D'autres voies ont également été décrites, une voie Tr1(Th3) (Groux et al. 1997; Jiang et al. 2003) et une voie Th-17 (Harrington et al. 2005).

Th0	Th1	Th2	Th1/Th2	Tr1 (Th3)	Th-17
IL-2	IL-2	IL-4	GM-CSF	TGF-β	IL-17
	IFN-γ	IL-5	TNF-α	IL-10	
	TNF-β	IL-6	IL-3		
		IL-9	IL-10		
		IL-25	IL-13		

Tableau 1: Principales cytokines sécrétées par les lymphocytes TCD4

La réponse Th1 va stimuler la voie cellulaire et par conséquent la voie des lymphocytes T cytotoxiques, la voie Th2 quant à elle va stimuler la voie humorale via les lymphocytes B.
Les lymphocytes cytotoxiques sont les cellules effectrices capables d'agir directement sur les cellules cibles grâce à leurs propriétés cytotoxiques. Bien que traditionnellement les lymphocytes TCD4 soient associés aux lymphocytes T «helpers» et les lymphocytes TCD8 aux lymphocytes T cytotoxiques, ces deux fonctions (aide et cytotoxicité) sont retrouvées

dans ces différents sous-types cellulaires (Hahn et al. 1995). Ainsi, pendant de très nombreuses années, l'étude des lymphocytes T cytotoxiques s'est concentrée sur les cellules $TCD8^+$. On sait désormais qu'il existe des sous-populations de lymphocytes $TCD4^+$ qui peuvent, dans certaines conditions, se différencier en lymphocytes T effecteurs cytotoxiques. Il faut bien distinguer les lymphocytes T cytotoxiques $CD8^+$ et $CD4^+$ car ils diffèrent par les éléments de restriction de la reconnaissance de l'antigène, mais également par les mécanismes moléculaires qui caractérisent leur capacité cytolytique. Dans le cas des cellules $TCD8^+$, l'activité cytotoxique est due essentiellement à la libération de perforine et de granzyme dans l'environnement proche de la cellule cible et qui va conduire à son apoptose. Les lymphocytes $TCD4^+$ expriment leur potentiel cytotoxique grâce à certaines cytokines, retrouvées aussi bien sous forme soluble que membranaire, à savoir Fas ligand et le TNF α et β. Les lymphocytes $CD4^+$ cytotoxiques activés, vont induire la mort par apoptose exclusivement des cibles cellulaires qui expriment des récepteurs spécifiques comme FAS ou les récepteurs au TNF.

I.3. La reconnaissance par le TCR du complexe [CMH-peptide]

Le lymphocyte T ne reconnait pas l'antigène dans sa forme native mais seulement des fragments qui lui sont présentés à la surface des cellules par les molécules du complexe majeur d'histocompatibilité (CMH). C'est la restriction par les molécules du CMH mise en évidence par Zinkernagel et Doherty en 1974 (Zinkernagel et al. 1974). La spécificité de la réponse engendrée grâce à cette reconnaissance par le récepteur des lymphocytes T (TCR) du complexe [CMH-peptide] est un élément clé de la réponse immunitaire et mérite que l'on s'y attarde.

I.3.1. Les lymphocytes T et leur récepteur le TCR

I.3.1.1. Le récepteur des lymphocytes T: le TCR

La reconnaissance des antigènes a lieu au niveau de l'interaction du complexe [CMH-peptide] et du récepteur des lymphocytes T. Le récepteur des lymphocytes T correspond à un complexe qui comprend le complexe CD3 et le TCR (T cell receptor) (Figure 1). La fonction du CD3 est double, il doit assurer la signalisation et le transport du TCR à la membrane. Parmi les chaînes qui constituent ce complexe, trois d'entre elles (CD3γ, δ et ε) présentent un domaine extracellulaire apparenté à celui retrouvé dans les immunoglobulines, un domaine transmembranaire et un domaine intra-cytoplasmique. Les 2 autres ζ et η sont composées d'un court segment extracellulaire, d'un segment transmembranaire et d'un long segment intra-

cytoplasmique. Le complexe multimoléculaire CD3 est ainsi constitué de 3 dimères dont deux hétérodimères γε et δε et un dimère ζζ ou ζη (Malissen et al. 1996). Les parties intra-cytoplasmiques possèdent pour la plupart des motifs ITAM (Immunoreceptor Tyrosine-based Activation Motif) qui vont intervenir dans les voies de transduction du signal (activation du lymphocyte T) (Davis 1990; Horejsi et al. 2004). Le TCR est un hétérodimère formé par l'association covalente de deux chaines polypeptidiques α et β ou γ et δ. Plus de 90% des lymphocytes T de l'organisme portent des récepteurs TCRαβ (Davis 1990).

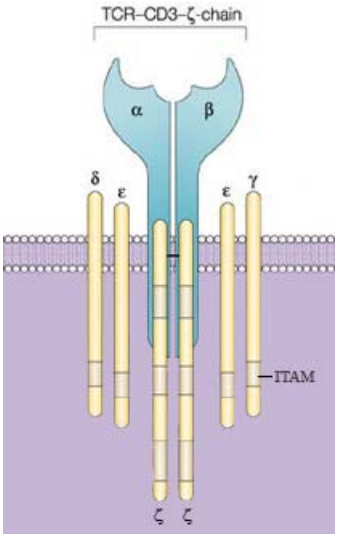

Figure 1: Le complexe TCR/CD3 (d'après Horejsi et al ; Nat Rev Immunol ; 2004)

Les chaînes α et β qui constitue le TCR sont constituées de deux régions, une constante (C) du côté C-terminal et une variable (V) du coté N-terminal (Chothia et al. 1988). La partie variable de la chaîne α est formée d'un domaine variable (V) et d'un domaine de jonction (J). La partie variable de la chaîne β est formée d'un domaine variable (V), d'un domaine de diversité (D) et d'un domaine de jonction (J). Ces domaines correspondent à des segments géniques initialement séparés sur l'ADN et réarrangés dans le thymus. La portion dite variable de ces chaines présente les trois boucles hypervariables CDR1, 2 et 3 qui sont impliquées dans l'interaction du TCR avec son ligand (Garcia et al. 1996; Reinherz et al. 1999). La région CDR3, qui est la plus hypervariable et la plus centrale, est composée de l'extrémité 3' du segment V et de l'extrémité 5' du segment J (plus le segment D pour la chaine β) et confère au TCR sa spécificité antigénique (Chothia et al. 1988; Housset et al. 2003).

I.3.1.2. La différenciation des lymphocytes T: du thymus à la périphérie

La différenciation des thymocytes en deux sous populations $CD4^+$ et $CD8^+$ a lieu dans le thymus où les précurseurs T, d'origine médullaire, subissent les processus de différenciation qui les conduisent au stade de lymphocytes T matures. Sous l'influence de médiateurs solubles, les précurseurs des lymphocytes vont successivement réarranger les gènes de leur récepteur spécifique de l'antigène. Les précurseurs des thymocytes qui pénètrent dans le thymus n'expriment à leur surface ni TCR, ni CD3, ni de molécules CD4, ni de molécules CD8. Ce sont des cellules blastiques qui vont aboutir en trois stades au lymphocyte T sanguin mature naïf.

I.3.1.2.1. Le stade I

Au stade I, ou double négatif, les thymocytes n'expriment ni le CD4, ni le CD8, ni le CD3. Ces thymocytes doubles négatifs sont concentrés dans la région sous-capsulaire du cortex thymique. Au contact du stroma thymique, ces cellules souches vont s'engager irréversiblement dans la lignée T, avec l'apparition des premiers marqueurs T (CD2, CD7). Les thymocytes les plus précoces (pro-T) n'ont pas réarrangés leurs gènes du TCR. Puis, le thymocyte devient un pré-T qui réarrange ses gènes γ, δ et β du TCR. Les réarrangements des chaînes du TCR et l'expression des molécules co-réceptrices CD4 et CD8 se font dans un ordre précis: après les gènes γ et δ, c'est le gène β du TCR qui est réarrangé. A ce stade, la cellule exprime un TCR γδ ou β pré-α associé au CD3 et il est dit double négatif $CD4^-CD8^-$ avant l'expression des molécules CD8 puis CD4 qui elle-même sera rapidement suivie par le réarrangement de la chaîne α.

I.3.1.2.2. Le stade II

Au stade II, ou double positif, les thymocytes expriment le CD4 et le CD8. C'est à ce stade que surviennent les réarrangements de la chaîne α du TCR. Cette séquence d'événements aboutit à un thymocyte double positif exprimant à la fois la molécule CD4 et la molécule CD8 ainsi qu'un TCR αβ ayant une faible affinité pour son ligand ($TCR\alpha\beta^{low}$). La contrainte de la reconnaissance spécifique par le lymphocyte T du peptide présenté par une molécule du CMH impose deux étapes de sélection lors de l'éducation thymique. Une première étape, dite de sélection positive au contact des cellules épithéliales thymiques du cortex profond, aboutit à l'élimination des lymphocytes T porteurs de TCR incapables de reconnaître les molécules du CMH de l'individu. Dans un deuxième temps, les lymphocytes T sélectionnés sont mis en présence des cellules dendritiques et des macrophages de la jonction cortico-médullaire qui

portent des peptides auto-antigéniques présents dans le thymus. Les lymphocytes T potentiellement auto-réactifs capables de reconnaître au contact ces peptides auto-antigéniques avec une forte affinité sont éliminés. Cette étape, dite de sélection négative, aboutit à un état qualifié de tolérance centrale.

I.3.1.2.3. Le stade III

Au stade III, ou simple positif $CD4^+CD8^-$ ou $CD4^-CD8^+$, les lymphocytes T sélectionnés lors de cette étape de sélection négative voient leur densité de TCR augmenter et perdent l'expression d'une des deux molécules co-réceptrices: le résultat en est l'obtention de lymphocytes T simples positifs matures $TCR\alpha\beta^{high}$ $CD4^+$ ou $TCR\alpha\beta^{high}$ $CD8^+$ prêts à être exportés en périphérie. Le stade III correspond au phénotype des cellules matures qui migrent dans la circulation périphérique (pour revue de l'ensemble du phénomène de la différenciation thymique: (Reza et al. 1998; Hunig et al. 2001; Starr et al. 2003; Liu et al. 2004; Nikolich-Zugich et al. 2004; Kappes et al. 2005).

I.3.2. Le Complexe Majeur d'Histocompatibilité (CMH)

Les molécules du CMH sont codées par un ensemble de gènes polyalléliques regroupés en deux classes I et II (Bjorkman et al. 1987; Klein et al. 2000). Ces deux types de molécules se distinguent notamment par l'origine des peptides présentés.

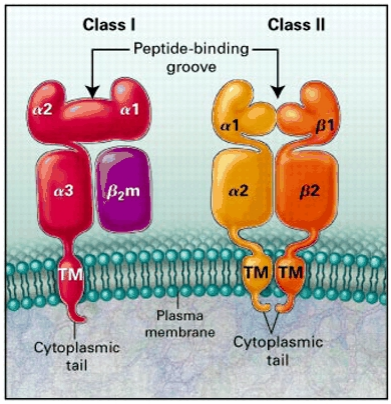

Figure 2: Les molécules du Complexe Majeur d'Histocompatibilité de type I et II (Klein et al; N Engl J Med; 2000)

I.3.2.1. Les molécules du CMH de classe I

Les molécules du CMH de classe I sont formées par l'association non covalente d'une chaîne légère non polymorphique, la β2 microglobuline et d'une chaîne lourde polymorphique α. Cette dernière comporte trois domaines extramembranaires (α1, α2 et α3), une région transmembranaire et une région intracytoplasmique (Figure 2). Les domaines α1 et α2 forment le site de liaison au peptide, avec une zone de fort polymorphisme constituant le sillon peptidique, et une zone de plus faible polymorphisme, les bordures. Le domaine α3 est impliqué dans l'association à la β2 microglobuline et constitue le site de liaison du corécepteur CD8 (Bjorkman et al. 1987; Bjorkman et al. 1990; Klein et al. 2000).

Les molécules du CMH de classe I sont fortement exprimées à la surface de la plupart des cellules nucléées de l'organisme. Chaque molécule du CMH de classe I présente un peptide de faible taille, 7 à 10 acides aminés, provenant principalement de la dégradation physiologique d'une protéine endogène (Figure 3) (Townsend et al. 1989; Rock et al. 2002; Saveanu et al. 2005; Shastri et al. 2005; Hammer et al. 2006). Ce mode de présentation, le plus courant, cohabite avec une présentation dite croisée (ou cross presentation (Ackerman et al. 2004)). Il s'agit de la présentation d'antigènes exogènes par les molécules du CMH de classe I.

Ce complexe [CMH I du soi - peptide endogène (ou exogène)] peut alors être reconnu par les lymphocytes cytotoxiques $TCD8^+$ (Ackerman et al. 2004; Heath et al. 2004; Ackerman et al. 2005; Cresswell et al. 2005; Groothuis et al. 2005; Groothuis et al. 2005; Rock et al. 2005; Shen et al. 2006).

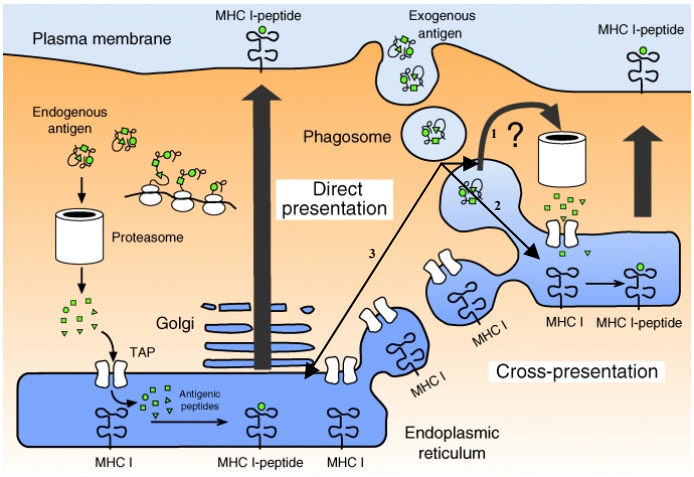

Figure 3: Représentation schématique de la présentation classique et des voies possibles de cross-présentation des antigènes par les molécules du CMH de classe I (d'après Heath et al ; Immunol Rev; 2004)

Il existe également des molécules de classe I non classiques dont le rôle reste encore peu connu (Shawar et al. 1994; Rodgers et al. 2005).

I.3.2.2. Les molécules du CMH de classe II

Les molécules du CMH de classe II sont formées par l'association d'une chaîne lourde β et d'une chaîne légère α, chacune comportant deux domaines extra-membranaires, un domaine membranaire et un domaine intracellulaire (Figure 2). La structure du sillon peptidique est proche de celle des molécules du CMH de classe I mais comporte des extrémités plus ouvertes ce qui permet la liaison de peptides plus longs (13-26 acides aminés contre 7-10) (Maffei et al. 1998).

Les molécules du CMH de classe II ont une expression généralement restreinte aux cellules présentatrices d'antigènes telles que les cellules dendritiques (Villadangos et al. 2005), les lymphocytes B, les macrophages et les cellules endothéliales. Une fois synthétisées dans le réticulum endoplasmique, les molécules du CMH de classe II sont associées avec une chaîne invariante Ii, qui empêche la fixation des antigènes endogènes (Cresswell 1996). Suite à l'action de protéases de la famille des cathepsines, les antigènes capturés sont digérés en peptides (Germain et al. 1993) ainsi que la chaîne invariante Ii. L'élimination de la chaîne invariante permet la fixation du peptide (Pierre et al. 1998) et ce complexe est présenté à la

surface de la cellule présentatrice d'antigènes. Ce complexe [CMH II du soi - peptide exogène] est reconnu par les lymphocytes auxiliaires $TCD4^{+}$.

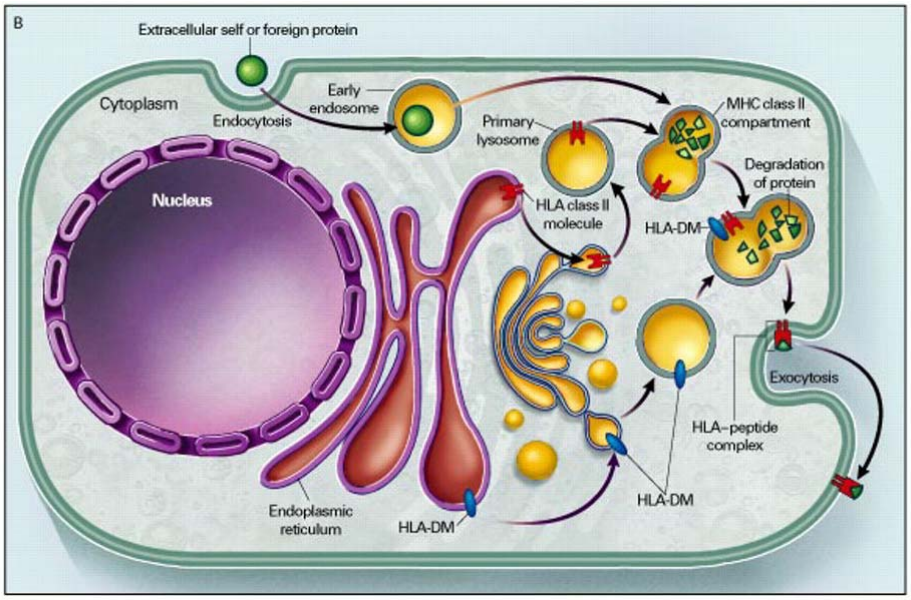

Figure 4: Représentation schématique de la voie de présentation des molécules du CMH de classe II (Klein et al; N Engl J Med; 2000)

II. L'immunologie de la transplantation

La transplantation est une situation spécifique où la greffe d'un tissu ou d'un organe allogénique, c'est-à-dire entre individus de la même espèce mais génétiquements différents, amène le système immunitaire de l'individu receveur à rencontrer les allo-antigènes du greffon. Le but de la transplantation est d'éviter les phénomènes de rejet, c'est-à-dire d'éviter que le système immunitaire reconnaisse comme étranger le greffon à court et long terme.

II.1. La greffe allogénique

Au cours de la greffe allogénique, les lymphocytes T qui reconnaissent les allo-antigènes vont jouer un rôle majeur lors du phénomène de rejet de greffe. De plus, la greffe allogénique et l'allo-reconnaissance apporte un éclairage différent sur le principe de la restriction par les molécules du CMH.

II.1.1. L'allo-reconnaissance

L'allo-reconnaissance implique des interactions [CMH-peptide]-TCR particulières. On distingue alors deux grandes voies de présentation: directe (les lymphocytes T du receveur reconnaissent les molécules du CMH du donneur présentées par les CPA du donneur) et indirecte (les lymphocytes T du receveur reconnaissent les molécules du CMH du donneur présentées par les CPA du receveur) (Figure 5).

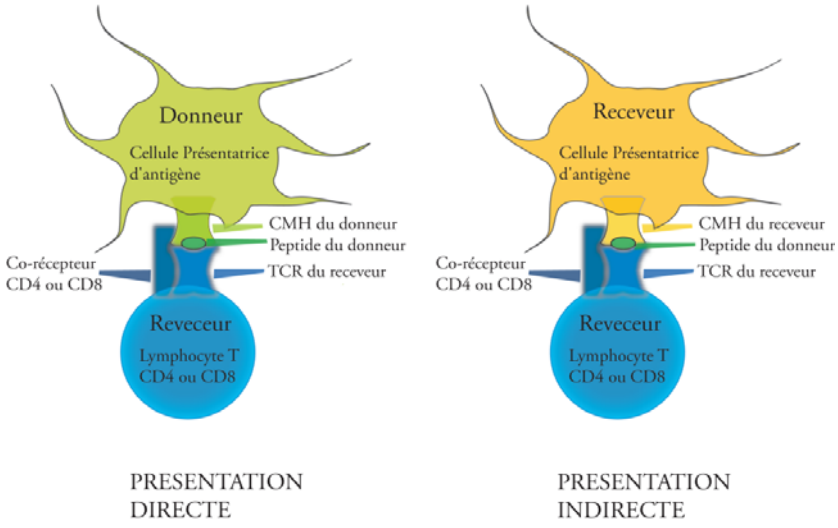

Figure 5: Schématisation de la voie d'allo-reconnaissance directe et indirecte.

II.1.1.1. La voie de la présentation directe

En dehors de toute sensibilisation préalable, les cellules allo-réactives préexistent chez tous les individus (environ 10% du répertoire (Benichou et al. 1996; Suchin et al. 2001)). L'allo-réactivité est conçue comme une forme particulière de «mimétisme moléculaire» (Lechler et al. 1992) où la cellule T assimile la structure tridimensionnelle du complexe [CMH allogénique-peptide] à celle d'une configuration du complexe [CMH du soi-peptide]. Une réponse immune allogénique est initiée par les leucocytes du greffon, principalement des CD, capables de migrer vers les ganglions le drainant. Rencontrant les lymphocytes T, ces CD vont initier une réponse immune dont l'intensité est décuplée (Matzinger et al. 1977). En effet le TCR ne reconnaît pas la molécule du CMH en soi mais un ensemble [CMH-peptide]. Une molécule du CMH donnée pouvant lier un très grand nombre de peptides différents, l'intensité

de l'allo-réactivité est due à la multitude de déterminants ainsi créés expliquant la fréquence (10%) des lymphocytes T précurseurs capables de répondre aux molécules du CMH allogéniques (Lindahl et al. 1977; Suchin et al. 2001).

II.1.1.2. La voie de la présentation indirecte

La présentation indirecte est un phénomène physiologique qui correspond à la voie classique pour des antigènes exogènes où les lymphocytes T du receveur reconnaissent les molécules du CMH du donneur présentées par les CPA du receveur (Figure 5). Lorsqu'il s'agit de peptides exogènes chargés par des molécules de classe I, on parle de «cross priming», mais cette occurrence ne paraît pas importante dans l'allo-réactivité (Valujskikh et al. 2002). La voie indirecte, dans les réponses allo-immunes, s'applique surtout aux lymphocytes TCD4 reconnaissant des peptides issus des molécules de classe I ou II du donneur ou d'antigènes mineurs, présentés par les molécules du CMH de classe II du receveur (Benichou et al. 1996; Game et al. 2002; Shirwan et al. 2003; Jiang et al. 2004).

II.1.1.3. Une troisième voie de présentation ?

Une troisième voie, la voie «semi-directe» qui pourrait servir de lien entre la voie directe et la voie indirecte a été décrite (Bedford et al. 1999; Tsang et al. 2003). En effet, les CD du receveur pourraient acquérir des molécules intactes du CMH du donneur à partir des tissus ou des cellules du donneur qui stimuleraient alors la voie directe d'allo-reconnaissance (Jiang et al. 2004).

II.1.2. Le rejet et ses différentes phases

Le rejet d'allogreffe est un terme général qui recouvre de nombreux mécanismes de la réponse immune dirigée contre le greffon. Il existe plusieurs types de rejet apparaissant de manière séquentielle: le rejet hyper aigu, aigu et chronique.

II.1.2.1. Le rejet hyper aigu

Le rejet hyper aigu survient dans les minutes suivant la greffe si le receveur possède déjà des anticorps préformés dirigés contre les antigènes du donneur. Seules les greffes vascularisées (cœur, poumon, rein,…) peuvent subir le rejet hyper aigu. Les anticorps préformés dirigés le plus souvent contre les protéines du système sanguin ABO ou contre les molécules du CMH étranger qui ont pu être rencontrées au cours d'une grossesse, de transfusions sanguines ou d'un rejet précédent, se lient aux cellules endothéliales et activent la voie classique du

complément conduisant à une coagulation massive. Grâce aux travaux de Dausset (Dausset 1981) et de Terasaki sur la recherche de compatibilité HLA entre le donneur et le receveur, ce rejet est désormais évité par l'analyse systématique de la compatibilité donneur/receveur avant la greffe, notamment par la vérification de la présence d'allo-anticorps préformés («cross-match»).

II.1.2.2. Le rejet aigu

En absence de rejet hyper aigu, le greffon peut subir un rejet dit aigu. Le rejet aigu est caractérisé par un fort infiltrat interstitiel de macrophages et de lymphocytes T. L'infiltrat est d'abord focalisé aux sites périvasculaires. Les lymphocytes T déclenchent une réponse dite d'hypersensibilité retardée qui a pour principaux effecteurs les lymphocytes $TCD4^+$ «helpers» (Th1). Les cytokines sécrétées (IL-2, TNF-α, IFN-γ) ont de multiples activités pro-inflammatoires qui entraînent une activation et une infiltration massive du greffon. Certaines cytokines (par exemple le TNF-α) sont directement cytotoxiques alors que d'autres comme l'IFN-γ et l'IL-1, déclenchent l'activité cytotoxique d'autres cellules infiltrantes comme les macrophages. Cette activation entraîne également une augmentation de la production de chimiokines, la synthèse d'enzymes protéolytiques, de monoxyde d'azote (NO), et d'autres facteurs solubles qui entretiennent la réponse inflammatoire (Le Moine et al. 2002).
L'accumulation d'antigènes du donneur et de lymphocytes T activés dans les ganglions drainants, permet l'activation des lymphocytes B produisant des immunoglobulines dirigées contre le greffon et qui jouent également un rôle dans le rejet aigu (Le Moine et al. 2002).
Les traitements immunosuppresseurs actuellement utilisés comme la cyclosporine A, le FK506 ou l'azathioprine préviennent le rejet aigu en ciblant les événements critiques de la réponse lymphocytaire T aux alloantigènes, tels que l'expansion clonale et la production de cytokines.

II.1.2.3. Le rejet chronique

Le problème majeur non résolu aujourd'hui est le rejet chronique, responsable d'une altération fonctionnelle progressive et la perte du greffon. Aujourd'hui, un patient recevant un greffon rénal, ayant surmonté avec succès les risques de la première année, possède un peu plus d'une chance sur deux de conserver son greffon fonctionnel 10 ans plus tard (61,9%, données extraites du bilan 2004 des activités de prélèvement et de greffe en France de l'agence de la biomédecine sur un ensemble de patients greffés entre 1993 et 2003). Le rejet chronique se

manifeste au niveau histologique par une occlusion de la lumière des artérioles et parfois des veinules à l'intérieur du greffon, résultant d'un épaississement de l'intima des vaisseaux. Cette obstruction des vaisseaux entraîne des insuffisances vasculaires, des nécroses et des fibroses des tissus greffés. Actuellement, aucun traitement ne permet de prévenir et/ou d'éliminer le rejet chronique.

II.2. La notion de tolérance

La notion de tolérance a été définie par les travaux pionniers d'Owen, Medawar et Billingham au début des années 1950 (Billingham et al. 1953). La tolérance immunologique désigne un état de non-réponse spécifique d'un antigène et est induite par un premier contact de l'organisme avec cet antigène. Plusieurs mécanismes sont impliqués dans la tolérance immunologique: la délétion clonale, l'anergie clonale et la régulation/suppression.

II.2.1. Délétion clonale

La délétion clonale des lymphocytes allo-réactifs (réagissant contre les antigènes du donneur) est envisagée comme un moyen très efficace pour obtenir un état de tolérance durable (Wells et al. 2001; Chiffoleau et al. 2003). Cette délétion peut survenir dans le thymus (délétion centrale) ou au niveau périphérique (délétion périphérique), dans les tissus lymphoïdes secondaires. La délétion thymique a ainsi été observée lors d'injections intrathymiques de peptides du CMH du donneur (Jones et al. 1998; Koksoy et al. 2005). Cette délétion des lymphocytes allo-réactifs est transitoire et est dépendante de la persistance dans le thymus des peptides du CMH.

La délétion des lymphocytes allo-réactifs en périphérie (Li et al. 1999; Wells et al. 1999) a été observée dans le cas d'injections intraveineuses ou orales d'une quantité massive d'antigènes (Kearney et al. 1994; Weiner 2001), ou après l'inhibition des fonctions accessoires ou des molécules de costimulation nécessaires à l'activation des lymphocytes T (Li et al. 1999; Wells et al. 1999; Wells et al. 2001). Lors de délétions périphériques, les cellules meurent par apoptose soit par absence de facteurs de croissance (mort passive) : ce phénomène permet le maintien de l'homéostasie lors d'infections, ne requiert pas l'interaction avec Fas ou le récepteur du TNF et résulte de l'absence de sécrétion d'IL-2 et d'expression de gènes anti-apoptotiques (Sayegh et al. 1998; Van Parijs et al. 1998); soit lors d'une stimulation répétée par les récepteurs des lymphocytes T (mort active) : ce phénomène est indépendant de la costimulation par CD28. Contrairement à la mort passive, la mort active requiert la présence d'IL-2 et est indépendante de l'expression de Bcl-2 ou Bcl-xL (gènes anti-apoptotiques) (Van

Parijs et al. 1998). L'importance du rôle de la mort cellulaire passive pour l'induction de tolérance à la transplantation a été démontrée par l'utilisation de souris transgéniques exprimant constitutivement le gène Bcl-xL dans les lymphocytes T (Wells et al. 1999). Ces souris sont résistantes à l'induction de tolérance après blocage des signaux de costimulation, efficace chez les souris sauvages. La mort cellulaire active a aussi son rôle dans la phase d'induction de tolérance. En effet, un protocole d'induction de tolérance par blocage des signaux de costimulation chez des souris déficientes en IL2 échoue (Wells et al. 1999). De plus, le rôle de l'IL-2 a aussi été démontré dans un autre modèle utilisant des inhibiteurs de la calcineurine, utilisés comme immunosuppresseurs. La Cyclosporine A et le FK506 agissent tous deux comme inhibiteurs de la calcineurine, inhibant ainsi la production d'IL-2. L'ajout d'un de ces deux immunosuppresseurs à un protocole de tolérance efficace par blocage des signaux de costimulation, abroge la survie des greffes (Li et al. 1999).

II.2.2. Anergie clonale

L'activation efficace d'un lymphocyte T naïf requiert la transduction de deux types de signaux distincts faisant intervenir le récepteur pour l'antigène et les récepteurs de costimulation. L'activation d'un lymphocyte T uniquement par la voie du récepteur en l'absence de transduction de signaux de costimulation, induit un état d'anergie qui se traduit par une inactivation de ses capacités fonctionnelles et prolifératives. Il devient alors réfractaire à une restimulation antigénique (Lechler et al. 2001; Schwartz 2003). Suite à une anergie, les lymphocytes ont une durée de vie plus courte et meurent par apoptose.

II.2.3. Régulation/suppression

La modulation de la réponse immune par des lymphocytes T régulateurs est un des mécanismes important dans le phénomène de tolérance.

II.2.3.1. Les cellules TCD4$^+$CD25$^+$

Principale population cellulaire décrite pour ses fonctions régulatrices, ces cellules ont tout d'abord été mises en évidence chez la souris (Sakaguchi et al. 1995) puis plus récemment chez l'Homme (Dieckmann et al. 2001; Jonuleit et al. 2001). Un déficit quantitatif et/ou qualitatif de ces cellules est associé à des pathologies auto-immunes chez l'animal (Sakaguchi et al. 1995; Suri-Payer et al. 1998) comme chez l'Homme (Sakaguchi 2003; Ehrenstein et al. 2004; Kriegel et al. 2004; Viglietta et al. 2004). Ces cellules régulatrices ont surtout pour fonction de maintenir la tolérance au soi mais leur rôle a été évoqué dans la tolérance en transplantation. On distingue actuellement des cellules T régulatrices (Treg) CD4$^+$CD25$^+$

«naturelles» et «acquises» (pour revue (Jonuleit et al. 2003; Cottrez et al. 2004; Sakaguchi 2004; Walsh et al. 2004)).

II.2.3.1.1. Les cellules T régulatrices «naturelles» $CD4^{\pm}CD25^{\pm}$

Elles représentent 5 à 10% des thymocytes $CD4^{+}CD8^{-}$ (Wing et al. 2002) et près de 10% des lymphocytes $TCD4^{+}$ en périphérie mais leur nombre diminue avec l'âge (Tsaknaridis et al. 2003). Elles proviennent du thymus où elles sont sélectionnées non seulement pour leur reconnaissance des molécules du CMH du soi mais aussi pour une affinité moyenne envers le complexe Antigène du soi/CMH (Jordan et al. 2001; Jonuleit et al. 2003). L'interaction CD28-B7 est indispensable dans le thymus pour la génération de ces cellules régulatrices (Tang et al. 2003) et pour leur maintien en périphérie (Tang et al. 2003).

Il n'existe pas de marqueur phénotypique discriminant permettant d'identifier précisément ces cellules (Stephens et al. 2000). Le CD25, correspondant à la chaîne α du récepteur à l'IL-2, est inductible après l'activation d'un lymphocyte $CD4^{+}$ naïf et ne permet donc pas de distinguer une cellule activée d'une cellule régulatrice. Chez l'Homme, les cellules exprimant fortement le CD25 ($CD25^{hi}$) possèderaient des propriétés suppressives (Baecher-Allan et al. 2001), alors qu'un niveau d'expression intermédiaire correspondrait aux cellules activées, mais cette distinction n'est pas absolue (Tsaknaridis et al. 2003). Elles expriment par ailleurs fréquemment les marqueurs CD45RO (marqueur «mémoire») (Baecher-Allan et al. 2001), CD122 (chaîne β du récepteur de l'IL-2) (Wing et al. 2002), CD62L (L-sélectine). Elles sont caractérisées également par une expression constitutive de CTLA-4 (CD152, ligand «régulateur» des molécules de costimulation B7) et de GITR (Glucocorticoid-Induced-TNF-R) (McHugh et al. 2002; Tsaknaridis et al. 2003). Le facteur de transcription FoxP3 (Forkhead box P3) semble être également un marqueur de ces cellules régulatrices (Sakaguchi 2003). Il jouerait un rôle dans la génération des cellules $CD4^{+}CD25^{+}$ au niveau thymique, mais interviendrait également dans les fonctions suppressives (Fontenot et al. 2003). Cependant, des travaux récents montrent que l'expression de FoxP3 n'est pas limitée aux seules cellules régulatrices chez l'Homme, il semble être également un marqueur retrouvé sur des cellules activées (Morgan et al. 2005). De plus, l'hème oxygenase 1 (HO-1) est exprimé constitutivement par les cellules régulatrices $CD4^{+}CD25^{+}$ (Pae et al. 2003). Il semblerait que ce soit FoxP3 qui induirait l'expression de HO-1. HO-1 interviendrait ensuite dans le mécanisme de suppression de ces cellules (Choi et al. 2005).

Ces cellules sont activées par leur TCR (Sanchez-Fueyo et al. 2006) et nécessitent l'IL-2 pour exercer leurs propriétés suppressives (Thornton et al. 2004). Par la suite, leur action

suppressive n'est pas dépendante de l'antigène qui les a stimulées. Cette action régulatrice s'exerce en inhibant la transcription d'IL-2 dans les autres cellules T et elle nécessite un contact cellulaire (Thornton et al. 1998; Dieckmann et al. 2001). Après leur activation, les cellules régulatrices produisent du TGF-β (Levings et al. 2002), un peu d'IL-10 et d'IL-4 (Dieckmann et al. 2001; Stephens et al. 2001). Cependant, l'action suppressive ne dépend pas directement de la sécrétion de cytokines comme l'IL-10 et le TGF-β puisque par exemple un anticorps anti-IL-10 n'annule pas l'effet suppresseur (Suri-Payer et al. 2001; Piccirillo et al. 2002; Camara et al. 2003; Trzonkowski et al. 2004). En plus de l'action sur les autres lymphocytes $CD4^+$, les lymphocytes T régulateurs $CD4^+CD25^+$ inhibent la prolifération et l'activité cytotoxique des cellules NK (Trzonkowski et al. 2004), des cellules $TCD8^+$ (Camara et al. 2003; Trzonkowski et al. 2004), des cellules NKT (Azuma et al. 2003), des monocytes-macrophages (Taams et al. 2005), les lymphocytes B (Zhao et al. 2006) et la maturation des cellules dendritiques (Misra et al. 2004).

II.2.3.1.2. Les cellules T régulatrices «acquises» $CD4^{\pm}CD25^{\pm}$

Deux types cellulaires ont été décrits: les cellules Th3 (Chen et al. 1994) et les cellules Tr1 (Groux et al. 1997). Leur mode d'action ne dépend pas du contact cellulaire, comme les cellules T régulatrices naturelles, mais est lié à l'action de cytokines, TGF-β pour les cellules Th3 (Chen et al. 1994) et IL-10 pour les cellules Tr1 (Foussat et al. 2003; Groux 2003).

Ces cellules ne sont pas caractérisées par des marqueurs phénotypiques spécifiques. FoxP3 semble induit lors de la conversion des cellules $CD4^+CD25^-$ en $CD4^+CD25^+$ aux propriétés régulatrices par le TGF-β (Fontenot et al. 2003; Hori et al. 2003; Walsh et al. 2004), mais il n'y as pas de FoxP3 dans les cellules Tr1. Les cellules dendritiques immatures peuvent également générer ces cellules régulatrices «acquises» (Jonuleit et al. 2003). Ceci a également été observé après stimulation par des cellules dendritiques plasmacytoïdes matures (Verhasselt et al. 2004) ou immatures (Wakkach et al. 2003).

Les cellules régulatrices semblent pouvoir induire elles-mêmes d'autres cellules régulatrices. Des clones T allo-réactifs pourraient être régulés par des cellules T régulatrices de même spécificité si l'allo-antigène en question était présenté par la même cellule présentatrice d'antigènes (Davies et al. 1996; Walsh et al. 2004). Ils deviennent à leur tour eux-mêmes régulateurs, via l'action de cytokines comme l'IL-10 ou le TGF-β. Les cellules régulatrices peuvent donc «transmettre» leurs propriétés régulatrices; ce phénomène est appelé la «linked immunosuppression» ou «by-stander suppression». C'est probablement ce mécanisme qui est en jeu dans la «tolérance infectieuse» et qui permet le transfert d'une tolérance d'allogreffe

d'un receveur tolérant à un deuxième receveur sans autre traitement immunosuppresseur, par simple transfert de cellules (Qin et al. 1993).

II.2.3.1.3. Implications des cellules régulatrices $CD4^{\pm}CD25^{\pm}$ dans la tolérance en allo-transplantation

Les cellules $CD4^+CD25^+$ décrites ci-dessus ne sont pas seulement impliquées dans le maintien de la tolérance au soi, même si cela reste leur fonction principale. Elles sont également retrouvées dans des modèles de tolérance en transplantation. Ceci est suggéré dès 1985 car des cellules «régulatrices» sont mises en évidence dans le pool de cellules $CD4^+$ après une greffe cardiaque chez le rat «tolérant». Ces cellules $CD4^+$ peuvent transférer la tolérance à un deuxième receveur (Hall et al. 1985). Par la suite, différents travaux ont montré que les cellules $CD4^+CD25^+$ étaient indispensables pour l'induction et/ou le maintien de la tolérance aux allo-antigènes dans certains modèles chez les rongeurs (Zhang et al. 2000; Hara et al. 2001; Taylor et al. 2001; Chiffoleau et al. 2002). Ces cellules T régulatrices sont générées au niveau des organes lymphoïdes secondaires mais elles sont également présentes dans le greffon puisque les cellules infiltrantes peuvent transférer la tolérance dans un modèle de greffe de peau (Graca et al. 2002).

Le rôle de ces cellules régulatrices a encore été souligné par la rupture de tolérance ou par l'accélération de la survenue du rejet lors de greffe de peau si les cellules $CD4^+CD25^+$ sont déplétées chez le receveur avant la greffe (Benghiat et al. 2005). Cette étude montrait, en outre, que les cellules naturelles $CD4^+CD25^+$ pouvaient exercer leur activité régulatrice sur des cellules portant des molécules de CMH allogéniques par réactivité croisée (Benghiat et al. 2005).

Chez l'Homme, les cellules $CD4^+CD25^+$ peuvent exercer une action régulatrice sur des clones allo-réactifs. Cette régulation a été mise en évidence pour des allo-antigènes présentés par voie indirecte chez des patients greffés rénaux stables. 40% d'entre eux, qui n'avaient jamais eu de rejet aigu, présentaient en effet une telle régulation des cellules $CD25^+$ contre les peptides du CMH de classe II du donneur (Salama et al. 2003). En revanche seulement 12,5% des patients qui avaient présenté un rejet aigu avaient une telle régulation. De plus, Jiang et coll. ont montré que des cellules qui exercent une activité suppressive sur des cellules spécifiques d'un allo-antigène peuvent également inhiber la prolifération d'autres cellules T interagissant avec un autre peptide allogénique présenté par la même CPA (Jiang et al. 2003). Ceci est un exemple de la «linked immunosuppression» que nous avons évoquée. En revanche, les cellules régulatrices $CD4^+CD25^+$ ne semblent pas impliquées dans l'allo-

réactivité par voie directe. Une étude menée chez 12 patients greffés rénaux a montré que la déplétion en cellules $CD4^+CD25^+$ ne modifie pas la réponse directe contre le donneur *in vitro* (Game et al. 2003).

II.2.3.1.4. Les cellules T régulatrices et le rejet chronique

Les cellules régulatrices $CD4^+CD25^+$ n'empêchent pas le développement d'un rejet chronique. Dans le modèle de transfusion spécifique du donneur, la tolérance est transférée avec l'injection de splénocytes d'un animal tolérant chez un autre receveur irradié {Koshiba, 2003 #181}. Il existe cependant histologiquement un rejet chronique à distance de la greffe {Koshiba, 2003 #181;Pirenne, 2005 #170} (Lair et al; soumis), suggérant que la présence des cellules régulatrices ne s'accompagne pas d'une tolérance vraie dans ce modèle. Plusieurs hypothèses ont été soulevées. Le rejet chronique dans la tolérance pourrait être un «effet secondaire» de la régulation. La tolérance s'est avérée être un phénomène actif lié aux cellules cytotoxiques dirigées contre les cellules du donneur {Bugeon, 1993 #164}. De plus, il a été montré que la tolérance intervient dans le contexte d'une polarisation Th2 associée au développement des lésions de rejet chronique dans les greffes {Koshiba, 2003 #181;Pirenne, 2005 #170} et que des cellules régulatrices pouvaient montrer un profil Th2 et produire de l'IL-4 {Zelenika, 2002 #331;von Herrath, 2003 #330}. Le rejet chronique apparaîtrait donc, dans ce cas, comme un effet secondaire de la tolérance. Autre hypothèse, dans certain modèle où l'on n'observe pas de polarisation Th2, le développement du rejet chronique pourrait être du à la présence d'un nombre insuffisant de cellules régulatrices {Koksoy, 2005 #87;Semiletova, 2005 #332}.

II.2.3.2. Les cellules $TCD4^+CD25^-$

Au niveau central thymique, seules les cellules $TCD4^+CD25^+$ semblent avoir un pouvoir régulateur. Le transfert de cellules $CD4^+CD45RC^-CD25^+$ provenant du thymus de rats naïfs permet de prévenir la survenue du diabète insulino-dépendant induit par une lymphopénie expérimentale (thymectomie suivie d'une série d'irradiation aux rayons γ). En revanche, le transfert de thymocytes $CD4^+CD45RC^-CD25^-$ n'a aucun effet (Stephens et al. 2000). Les cellules $TCD4^+CD45RC^-CD25^-$ et $CD25^+$ isolées de ces mêmes animaux et réinjectées chez des rats pré-diabétiques peuvent en revanche prévenir le développement du diabète (Stephens et al. 2000). Dans un modèle de transplantation entre individus différents pour leurs molécules du complexe mineur d'histocompatibilité, Graca et al ont démontré que le transfert de cellules $CD4^+CD25^-$ provenant de la rate de souris tolérant une greffe de peau permettait l'acceptation

d'une greffe de peau chez une souris non traitée. L'injection de cellules $CD4^+CD25^-$ issues de la rate d'une souris naïve étant sans effet (Graca et al. 2002). Afin d'obtenir le même effet régulateur qu'avec les cellules $CD4^+CD25^+$ de la rate, il est nécessaire d'employer 10 fois plus de cellules $CD4^+CD25^-$. Nous avons montré que des cellules $TCD25^-$ (Degauque, Lair et al; soumis) d'animaux tolérant une greffe de cœur après induction par transfusion spécifique du donneur (entre animaux incompatibles pour les molécules du CMH) étaient capables de transférer cette tolérance à des animaux naïfs. L'origine de ces cellules reste hypothétique :

1) la perte du marqueur CD25 par les cellules régulatrices $TCD25^+$. Ceci a été rapporté dans un autre modèle de souris transgéniques ou le transfert de cellules $CD4^+CD25^+$ issues de la rate et des ganglions lymphatiques de souris B6 à des souris lymphopéniques $Rag\text{-}1^{-/-}$ se traduit par une forte expansion de ces cellules qui perdent le marqueur CD25 après cinq divisions (Gavin et al. 2002).
2) «l'éducation» par les cellules $CD4^+CD25^+$ naturelles selon le phénomène de «linked immunosuppression» ou «by-stander suppression» qui a été décrit pour l'induction des cellules régulatrices acquises Tr1 et Th3.

Les mécanismes d'action de ces cellules restent encore à élucider.

II.2.3.3. Les autres cellules régulatrices

Les cellules suppressives $CD8^+CD28^-$ reconnaissent les molécules du CMH de classe I exprimées par les CPA et entraîneraient leur différenciation en CPA tolérogènes (Liu et al. 1998) en induisant notamment ILT3 et ILT4 (Cortesini et al. 2001; Chang et al. 2002). Elles inhibent également l'expression de CD40-Ligand à la surface des lymphocytes $TCD4^+$ helpers (Ciubotariu et al. 1998). Les cellules présentatrices d'antigènes ne peuvent donc plus activer complètement le lymphocyte TCD4+, les voies de costimulation étant inhibées. Elles induisent ainsi leur anergie (Jiang et al. 2004). Ces cellules suppressives expriment FoxP3 (Manavalan et al. 2004). Une augmentation du nombre de ces cellules $CD8^+CD28^-$ est trouvée chez des sujets transplantés cardiaques par rapport à des sujets normaux non greffés, et cette population cellulaire ne présente pas de cytotoxicité envers les antigènes du donneur (Colovai et al. 2003). Plus récemment, ces cellules régulatrices $CD8^+CD28^-$ ont été retrouvées après transfusions spécifiques du donneur chez le rat (Liu et al. 2004).

Certaines cellules NKT semblent avoir un rôle régulateur notamment pour la prévention des maladies auto-immunes, à médiation Th1 (Jiang et al. 2004). Elles semblent également impliquées dans des modèles de tolérance en allo-transplantation, notamment dans la

tolérance néonatale (Kawamura et al. 2002), lors du blocage de la costimulation (Seino et al. 2001) ou après un traitement non-myéloablatif et une greffe de moelle osseuse chez la souris (Higuchi et al. 2002). Chez l'Homme, les cellules NKT auraient également des propriétés régulatrices, et des déficits quantitatifs et/ou qualitatifs ont été corrélés à certaines pathologies auto-immunes, comme la sclérose en plaque (Araki et al. 2003) et le diabète (Wilson et al. 1998). Les cellules T doubles négatives $TCR\alpha\beta^{+}CD4^{-}CD8^{-}$ représentent chez l'Homme environ 1% des lymphocytes $TCD3^{+}$. Elles peuvent réguler l'action de cellules T spécifiques d'un antigène donné chez l'Homme (Fischer et al. 2005). En transplantation, elles sont impliquées dans la tolérance après transfusion spécifique du donneur dans des modèles de greffe de peau (Young et al. 2002) et de cœur (Chen et al. 2003) chez la souris en entraînant la délétion de clones $CD8^{+}$ allo-réactifs.

II.3. Les protocoles expérimentaux de tolérance

II.3.1. Les protocoles expérimentaux d'induction de tolérance périphérique

Dans le cadre de l'induction de tolérance périphérique, différentes voies sont actuellement explorées. Le blocage des co-récepteurs (utilisation d'anticorps anti-CD4 ou anti-CD8) ou du complexe CD3 permettent la survie à long terme d'allogreffes de cœur chez la souris (Chen et al. 1992; Nicolls et al. 1993; Cobbold et al. 1996). Le blocage des voies de costimulation par l'utilisation d'anticorps chez les rongeurs pour bloquer l'interaction CD28:B7 (CD80 et CD86) par le CTLA4-Ig (Lin et al. 1993; Glysing-Jensen et al. 1997; Guo et al. 2004), l'anti-CD28 (Dengler et al. 1999; Haspot et al. 2002; Haspot et al. 2005), l'anti-CD154 (Larsen et al. 1996; Wells et al. 2001; Guillot et al. 2002; Guillonneau et al. 2005), ou combiné entre eux (Jones et al. 2000; Williams et al. 2000) permettent également la survie à long terme d'allogreffes. Le blocage d'ICOS («inducible costimulator») qui permet la survie de greffes cardiaques murines (Sandner et al. 2005), celui de la molécule CD45, par l'utilisation de l'anti-CD45RB a aussi été démontré efficace pour induire la survie à long terme de greffes d'îlots de Langerhans et de greffes rénales chez la souris (Basadonna et al. 1998; Visser et al. 2004). Enfin, la dernière voie possible pour l'induction de tolérance périphérique fait intervenir la présentation des antigènes du donneur avant la greffe (transfusion spécifique du donneur). Ce protocole qui constitue la base de notre étude sera développé ultérieurement.

II.3.2. Les protocoles expérimentaux d'induction de tolérance centrale

Historiquement, ce mode d'induction date du début des années 1950 (Billingham et al. 1953). Le but est d'induire le développement d'un chimérisme, c'est à dire la co-existence de cellules hématopoïétiques du donneur et du receveur. Le taux de présence de cellules du donneur définit le macro et le microchimérisme (Wekerle et al. 2001; Wekerle et al. 2003).

- Le macrochimérisme

Dans le cadre du macrochimérisme, les cellules du donneur représentent plus de 1% des cellules hématopoïétiques. Le chimérisme peut être complet (100% cellules du donneur) ou mixte (>1% et <100% de cellules du donneur).

- Le microchimérisme

Dans le cadre du microchimérisme, les cellules du donneur représentent moins de 1% des cellules hématopoïétiques. Le microchimérisme apparaît spontanément après la transplantation d'organes, le greffon contenant des cellules hématopoïétiques (Starzl et al. 1993).

Les problèmes majeurs de ce type de protocole sont la nécessité d'une myéloablation (destruction complète ou d'une partie des cellules de la moelle osseuse de l'individu receveur). Cette destruction peut être réalisée par irradiation létale pan-corporelle, par l'utilisation d'agents cytotoxiques ou d'anticorps monoclonaux (Sharabi et al. 1989; Tomita et al. 1989; Pelot et al. 1999). Plusieurs protocoles ont été développés chez les rongeurs, mais la plupart combinent agents cytoréducteurs et utilisation d'anticorps monoclonaux déplétants ou bloquants des signaux de costimulation (Seung et al. 2003; van Pel et al. 2003; Kurtz et al. 2004). Cette combinaison permet, dans la plupart des cas, de diminuer les doses des drogues cytoréductrices utilisées, réduisant ainsi leur toxicité. Le problème de l'utilisation d'anticorps déplétants les lymphocytes T en périphérie réside dans une longue période d'immunoincompétence sévère (Wekerle et al. 2001). L'utilisation d'anticorps bloquant les signaux de costimulation des lymphocytes T, inclut dans un protocole de transfusion de moelle osseuse (BMT), permettrait d'éviter la déplétion non spécifique et massive lymphocytaire, et ainsi conserver une meilleure immunocompétence (Wekerle et al. 2001). La transfusion spécifique du donneur peut également améliorer cette situation. Ainsi une transfusion spécifique du donneur au même moment qu'une injection de cyclophosphamide (agent alkylants), suivit d'une transfusion de moelle osseuse du donneur et d'un traitement avec le busulfan (agent alkylants) permet d'inhiber la prolifération des clones allo-réactifs, et

permet l'implantation des cellules de moelle osseuse. Ce protocole permet l'induction d'un chimérisme partielle et la tolérance à des greffes de peau dans un modèle murin (Tomita et al. 2000).

Pour ma part, je me suis plus particulièrement intéressé, dans ce travail de thèse, à un protocole d'induction de tolérance: la transfusion spécifique du donneur avant la greffe.

III. La transfusion spécifique du donneur

La transfusion de sang du donneur avant la greffe (TSD) a été réalisée chez le rongeur (Soulillou et al. 1984) et le primate (van Es et al. 1978). Il a été montré qu'elle pouvait avoir un rôle bénéfique dans les greffes entre donneurs vivants apparentés (Opelz et al. 1980). Dans cette partie, nous utiliserons le terme « tolérance » dans le modèle de transplantation après transfusion spécifique du donneur. Les receveurs présentent en effet une survie prolongée de leur greffe, et ils acceptent une greffe de peau du donneur et rejettent celle d'un donneur tiers (Soulillou et al. 1984 ; Bugeon et al. 1992). Cependant certaines études récentes ont montré que les receveurs présentaient, à distance de la greffe, des signes de rejet chronique (koshiba et al. 2003), montrant de ce fait que ces animaux ne sont pas tolérants au sens immunologique. Nous discuterons de cette ambiguïté au cours de cette thèse.

III.1. L'expérience de la clinique

Dans les expériences de Billingham et al, l'injection d'allo-antigènes au cours de la période néonatale permettait d'induire une tolérance spécifique à une allogreffe de peau chez la souris (Billingham et al. 1953). Un tel état de tolérance peut être également induit chez l'animal adulte. Ainsi, dans certaines souches de souris (Peugh et al. 1988) et de rats (Fabre et al. 1972; Soulillou et al. 1984), mais également chez certains gros animaux comme le singe (van Es et al. 1978), une ou plusieurs transfusions de sang spécifique du donneur (TSD) avant la greffe peuvent induire une tolérance à une allogreffe cardiaque (Soulillou et al. 1984), rénale (Cavinato et al. 2005) ou de peau (Quezada et al. 2003).

La transfusion sanguine (spécifique et non spécifique du donneur) avant la transplantation, est également capable d'améliorer la survie d'une allogreffe chez l'Homme. Pourtant, lorsque dans les années 60 la transplantation rénale devient une alternative possible à la dialyse pour le traitement des patients souffrant d'insuffisance rénale chronique, la communauté scientifique découvrit le rôle possible des anticorps cytotoxiques spécifiques du donneur dans le phénomène de rejet hyper-aigu (Patel et al. 1969). Ces anticorps pouvant apparaître après

transfusion sanguine, il semblait évident que certains patients recevant des transfusions répétées en raison d'une anémie due à l'insuffisance rénale et à la dialyse, puissent être sensibilisés à certains allo-antigènes, et souffrir d'un taux plus élevé d'échecs de greffe. Cependant, il a été montré que les patients ayant reçu des transfusions sanguines au préalable ne présentaient pas plus de rejets que ceux ayant été peu ou pas transfusés (Morris et al. 1968). L'exposition des patients aux allo-antigènes avant la greffe peut même avoir un effet bénéfique sur la survie du greffon (Morris et al. 1968).

Dans une étude menée par Opelz et al, le taux de survie de greffes rénales à un an était de 29 % chez les patients qui n'avaient jamais été transfusés contre 66 % pour ceux qui avaient reçu plus de 10 unités de sang avant la transplantation (Opelz et al. 1973). De la même manière, des transfusions d'un individu tiers avant la transplantation améliorent de manière significative la survie de greffe à un an (Pfaff et al. 1984). Ces résultats de survie de la greffe ont été expliqués par le fait qu'il existait 10 antigènes HLA-DR communs. Ainsi un receveur transfusé avec deux unités de sang de façon aléatoire aura 40 % de chance d'être exposé au HLA-DR du rein qui lui sera transplanté (Jackson et al. 1997). L'effet bénéfique de la transfusion avec des protocoles de transfusions aléatoires peut être dû aux mêmes mécanismes que ceux impliqués dans la transfusion sanguine spécifique du donneur dans laquelle l'exposition avant la greffe au HLA-DR du rein transplanté représente un élément clé de la réussite de la transplantation (Jackson et al. 1997).

A la suite de ces résultats, la préconisation de la transfusion de sang spécifique du donneur chez les patients greffés avec un greffon donneur vivant apparenté a augmenté. Dans les années 70, de nombreuses équipes de transplantation ont engagé des protocoles de transfusions consistant à effectuer une ou plusieurs transfusions sanguines systématiques en préparation d'une transplantation rénale. Au début des années 80, il a été généralement accepté que les transfusions sanguines avant la transplantation étaient salutaires et qu'elles avaient amélioré de manière significative la survie du greffon grâce aux transfusions donneur-spécifiques et aléatoires. En 1982, il a été décidé de traiter préalablement tous les couples non HLA-identiques de donneur-receveur par transfusions de sang spécifique du donneur. En effet, le traitement de patients incompatibles traités par transfusions de sang spécifique du donneur présentaient une réponse plus faible contre les antigènes du donneur en cultures mixtes lymphocytaires que les patients non traités par transfusions de sang spécifique du donneur (Opelz et al. 1980; Salvatierra et al. 1980; Salvatierra et al. 1983). Bien que certains auteurs continuent de rappeler le risque de sensibilisation après transfusions sanguines,

notamment chez les femmes multipares, leurs bénéfices sur la survie du greffon l'emportent dans la communauté scientifique médicale. Elle sera rassurée par l'étude rétrospective d'Opelz portant sur 2580 transplantations qui montrait clairement que le taux de survie du greffon chez les patients ayant reçu entre 6 et 10 transfusions sanguines avant la greffe, était identique à celui des patients en ayant reçu plus de 20 (Opelz et al. 1980). Ainsi même un nombre faible de transfusions avant la greffe peut induire une amélioration de la survie tout en réduisant le risque de sensibilisation du receveur.

Après l'arrivée de la cyclosporine A (CsA) en 1978 (Halloran et al. 1982), l'introduction de nouveaux immunosuppresseurs, une meilleure correspondance des HLA-DR et une meilleure gestion du rejet ont réduit les avantages de la transfusion, qui est remise en question et abandonnée dans de nombreux centres. Le risque (sensibilisation, transmission d'agents infectieux) apparaît alors plus important que le bénéfice. De plus, la majorité des études sur le rôle des transfusions avant la greffe utilise comme paramètre la survie à court terme de greffons (1 an ou 2 ans). Chez les patients, transfusés ou non, ayant reçu de la Cyclosporine A, cette survie est désormais d'environ 90 % à un an, rendant difficile la détection d'un effet bénéfique des transfusions. Cependant, un effet à long terme pourrait persister comme le montre l'étude de Baatard et al (Baatard et al. 1991). Un nouvel intérêt pour les transfusions sanguines en transplantation fut suscité par deux études hollandaises montrant, *in-vitro* et *in-vivo*, que l'effet transfusionnel dépend en grande partie de l'histocompatibilité, entre le donneur et le receveur. Les patients qui partagent une molécule de HLA-DR avec la transfusion sanguine présentent une survie de leur greffon rénal ou cardiaque meilleure que celle de patients ayant reçu une transfusion différente pour les deux molécules de HLA-DR (Lagaaij et al. 1989; van Twuyver et al. 1991). Outre l'effet sur la survenue du rejet aigu, le partage d'au moins une molécule de HLA-DR se traduit par l'absence d'anticorps anti-HLA (Bayle et al. 1995) et une réduction importante de l'activité des lymphocytes T cytotoxiques dirigés contre les cellules du donneur (Lagaaij et al. 1991). L'effet bénéfique de la transfusion compatible pour au moins une molécule HLA-DR a été retrouvé chez des patients transplantés cardiaques, présentant une meilleure survie à long terme de leur greffon et moins d'épisodes de rejet (van der Mast et al. 1997; Vaessen et al. 2000). Ainsi, les différents arguments successivement mis en évidence semblent indiquer que la transfusion de sang spécifique du donneur reste, par son efficacité, sa logistique simple et l'absence de régime toxique de pré-conditionnement du patient, une stratégie tolérogènique attractive, et certains groupes continuent à l'employer dans des protocoles cliniques (Amada et al. 2005; Marti et al. 2006).

Les mécanismes immunologiques responsables de l'effet transfusionnel chez l'Homme restent, malgré les nombreuses hypothèses proposées (délétion clonale, suppression, anergie...) énigmatiques. C'est pourquoi l'étude de modèles animaux peut ainsi permettre d'éclairer ces mécanismes.

III.2. Les différents modèles expérimentaux

Dans les modèles expérimentaux, la transfusion spécifique du donneur recouvre non seulement la transfusion de sang mais aussi le transfert de différents types cellulaires. De nombreuses études ont montré que les cellules spléniques, thymiques ou de la moelle osseuse pouvaient induire une suppression/régulation des cellules allo-réactives chez l'animal adulte. Les différentes populations cellulaires constituant le sang ont également été testées pour leur capacité à entrainer une survie du greffon dans différents modèles de greffes. Ainsi, chez les rongeurs (souris ou rats), il a été montré que tous les composants cellulaires, c'est-à-dire les leucocytes (Jenkins et al. 1971; Faustman et al. 1982), les hématies (Jenkins et al. 1971; Wood et al. 1985) ou les plaquettes (Hibberd et al. 1983) étaient capables d'induire une tolérance *in-vivo*.

L'efficacité de la transfusion spécifique du donneur peut varier selon les espèces considérées. Ainsi, chez la souris, dans un modèle de transplantation d'îlots pancréatiques entre souris incompatibles pour les molécules du CMH de classe I et II, l'administration d'une transfusion spécifique du donneur quatre semaines avant la transplantation entraîne une pré-sensibilisation néfaste et un rejet accéléré du greffon (Roy-Chaudhury et al. 1997). Chez le rat, l'administration de deux transfusions quatorze et sept jours avant une greffe de cœur entre rats incompatibles pour les molécules du CMH de classe I et II permet la survie indéfinie du greffon (Soulillou et al. 1984). Ces modèles animaux sont l'exemple même de la dualité de l'effet des transfusions (pré-sensibilisation/induction de tolérance). L'efficacité de la transfusion spécifique du donneur peut aussi être différente selon les souches utilisées au sein d'une même espèce (Hamano et al. 1989; Wasowska et al. 1992; Yang et al. 1998) ou bien encore de l'organe transplanté (Okada et al. 1998; Akst et al. 2006).

Le plus souvent, les protocoles de présentation des antigènes du donneur sont associés à un blocage sélectif d'une voie de costimulation ou d'un corécepteur ce qui met en œuvre des mécanismes de régulation différents de ceux induits par la présentation seule des antigènes du donneur. Par exemple, dans le modèle murin préalablement cité d'allogreffes d'îlots pancréatiques (Roy-Chaudhury et al. 1997), l'administration conjointe d'un anticorps anti-CD40L permet la survie à long terme des îlots (Zheng et al. 1999). De nombreuses études

sont ainsi basées sur la combinaison entre la présentation des antigènes du donneur associée à une ou plusieurs autres molécules. Le tableau 2 (page suivante) montre l'hétérogénéité des modèles rencontrés dans la littérature et ce, au cours des trois dernières années.

Equipe de recherche	publication	Espèce/souche	induction	greffe
Pirenne J	Koshiba et al 2003 Kitade et al 2005	Rat Male : RA ($RT1^p$-$RT1A^u$:B/D1) > PVG (($RT1^c$-$RT1A^c$:B/Dc)	TSD (sang) à J-12	cœur
Wood KJ	Bushell et al 2003; 2005 Spriewald et al 2003	Souris: C3H/He ($H2^k$) > C57BL/10 ($H2^b$) Souris : CBA/Ca ($H2^k$) > C57BL/10 ($H2^b$) et vice versa	TSD (sang) J-27 et anti CD4 à J-28 et -27 TSD 1 où plusieurs et +/- anti CD4	peau et cœur
Flye MW	Margenthaler et al 2003 Kataoka et al 2003	Rat Male : BUF ($RT1^b$)>LEWIS ($RT1^l$) Rat Male : LEWIS ($RT1^l$) > DA ($RT1^a$)	TSD (cellules spléniques) + anti CD4 TSD (cellules spléniques) à J-7 ou J-21	cœur, rein et peau
Miyazawa M	Tahara et al 2004	Souris B10.QBR ($H2^{bq4}$)>C57BL/6 ($H2^b$)	TSD (cellules spléniques) à J-7 et DSG	peau
Noelle RJ	Quezada et al 2003; 2005	Souris $CB6F_1$ (BALB/c X C57BL/6) > C57BL/6	TSD (cellules spléniques) + anti CD154	peau
Li XK	Guo et al 2003	Rat Male : DA ($RT1^a$) > LEWIS ($RT1^l$)	TSD (cellules spléniques et de la moelle osseuse) à J0 et anti ICOS et/ou anti CTLA4Ig	cœur
Klempnauer J	Bektas et al 2005	Rat Male : LEW.1W ($RT1^u$) > LEW.1A ($RT1^a$)	TSD (sang) à J-14 et J-7	cœur
Kirk AD	Xu et al 2003 preston et al 2005	Singe Rhésus > Singe Rhésus	TSD (sang) à J-1 et anti CD154 (+ rapa ou sirolimus)	peau et rein
Noris M	Cavinato et al 2005	Rat Male : BN ($RT1^n$) > LEWIS ($RT1^l$)	TSD (cellules sanguines) à J0 + CsA	rein
Hancock WW	Lee et al 2005	Souris BALB/c ($H2^d$) > C57BL/6 ($H2^b$)	TSD (cellules) et anti CD154 (CD40L) à J0	cœur
Suciu-Foca N	Liu et al 2004	Rat Male: LEWIS ($RT1^l$) > ACI ($RT1^a$)	TSD (sang irradié: $2x10^6$ cellules)	cœur
Sayegh MH	Sandner et al 2005 Kishimoto et al 2004	Souris: C57BL/6 ($H2^b$) > B6.$CH2^{bm12}$ Souris: C57BL/6 ($H2^b$) > BALB/c ($H2^d$)	TSD (cellules spléniques) à J0 + anti B7h TSD (cellules spléniques) à J0 + anti CD154	peau et cœur
Madsen JC	Hoerbelt et al 2005	Porc miniature: $SLA^{dd}(I^d,II^d)$ > $SLA^{hh}(I^a,II^d)$	TSD (cellules sanguines) à J-14 et J-7 + CsA	cœur
Strome M	Akst et al 2006	Rat Male : BN ($RT1^n$) > LEWIS ($RT1^l$)	TSD (cellules spléniques) à J0 + CsA	appareil laryngé

Tableau 2: Liste non exhaustive des principales publications et protocoles de ces 3 dernières années

Au cours des dernières décennies, grâce à ces nombreux modèles expérimentaux, les mécanismes qui régissent l'induction de tolérance par une transfusion spécifique du donneur préalable ont été bien décrits. Nous allons essayer de dresser le bilan des mécanismes qui interviennent lors de la phase précoce de l'induction et dans le maintien à distance de la greffe de la tolérance.

III.3. La «tolérance» induite par la transfusion spécifique du donneur

III.3.1. Les acquis de notre laboratoire

La phase d'induction de « tolérance » par transfusion de sang spécifique du donneur avant la greffe (Soulillou et al. 1984) a été particulièrement étudiée dans notre laboratoire et plusieurs points importants ont été mis à jour. Ainsi, il a été montré que la différenciation et l'expansion dans le sang puis dans le greffon d'un clone $TCD8^+$ Vβ18-Dβ1-Jβ2.7 potentiellement régulateur (Douillard et al. 1996) était nécessaire. L'implication de ce clone dans l'induction de tolérance a été montrée par différents points. La déplétion des cellules $TCD8^+$ avant la greffe empêche la mise en place de la tolérance. La taille du clone Vβ18-Dβ1-Jβ2.7 augmente précocement pour devenir le clone dominant de la famille Vβ18 dans le sang et le greffon des animaux tolérants (ce clone représente 5% du répertoire des greffons tolérés) (Douillard et al. 1996). Ce clone n'est pas présent dans le greffon rejeté des animaux non traités (le clone Vβ18-Dβ1-Jβ2.7 ne représentant pas plus de 0.6% du répertoire lymphocytaire T). Enfin, la vaccination avec un ADN complémentaire codant pour le Vβ18 empêche la mise en place de la tolérance (Vignes et al. 2000). Il a été également montré que la cytokine TGF-β était nécessaire pour l'induction de la tolérance. Les greffons cardiaques d'animaux tolérants présentent une expression de TGF-β plus importante et plus précoce (transcrits et protéine) que ceux d'animaux non traités rejetant leur greffon. Le rôle de cette cytokine a été confirmée par le rejet du greffon cardiaque par des animaux ayant reçu une transfusion spécifique du donneur et traité avec un anticorps anti-TGF-β (Josien et al. 1998). Il a également été montré au laboratoire que la mise en place des phénomènes actifs de suppression nécessitait la présence des cellules dendritiques du greffon. Le traitement des donneurs par du cyclophosphamide avant la greffe permet d'éliminer les leucocytes et les cellules résidentes exprimant les molécules du CMH de classe II (cellules dendritiques interstitielles). Dans ces conditions (greffes de cœurs «déplétées» en cellules dendritiques interstitielles), la transfusion avant la greffe n'induit pas de survie à long terme (Josien et al. 1998). De plus, les greffons cardiaques tolérés présentent des niveaux plus faibles d'IL-2 et d'IFN-γ que les greffons

rejetés (Bugeon et al. 1992). L'IL-2 et l'IFN-γ sont importants pour le devenir de la greffe; ainsi, l'administration d'IFN-γ (Josien et al. 1999) ou d'IL-2 (Dallman et al. 1991) à des animaux transfusés abroge la tolérance. Notre laboratoire a montré que malgré cet infiltrat abondant, les cellules infiltrant le greffon toléré ne produisent que de faibles quantités de lymphokines (IL-2, IL-4, IL-10, IL-13 et IFN-γ), dont celles produites par les macrophages, alors que ces cytokines sont fortement accumulées dans les greffons rejetés (Bugeon et al. 1992; Josien et al. 1995; Josien et al. 1998). Par contre, il n'a pas été montré de variation pour l'expression de la perforine et du granzyme dans les greffons des deux groupes (Bugeon et al. 1993).

L'incapacité des cellules T à produire des cytokines de type Th1/Th2 et à développer une réponse immune suggère un phénomène actif de suppression. Cette suppression n'est pas induite par la transfusion de sang seule. Les animaux transfusés mais n'ayant pas été greffés, possèdent des splénocytes proliférant vigoureusement contre les cellules stimulatrices du donneur (Josien et al. 1998) et des taux détectables d'immunoglobulines dirigées contre les antigènes du donneur (Bugeon et al. 1992). Enfin, de récentes études ont montré l'importance du CX3CR1 et de son ligand la fractalkine, qui est fortement exprimé par les cellules non-T infiltrant les greffons tolérés. Ces deux molécules sont fortement exprimées dans les premiers jours suivant la greffe dans les greffons tolérants (Louvet et al. 2004). Une autre molécule, TORID, a été identifiée dans ce même modèle et serait elle aussi surexprimée par les cellules non-T infiltrant le greffon tolérant (Louvet et al. 2005).

III.3.2. Transfusion spécifique du donneur et régulation

La transfusion spécifique du donneur induit la différenciation et/ou l'activation de cellules régulatrices qui ne sont pas présentes dans tous les compartiments. Comme nous venons de le voir, un clonotype de cellules $TCD8^+$ se différentie dans le greffon et le sang des animaux tolérants (Douillard et al. 1996). Les travaux de Quiegley ont montré, dans un modèle de greffe rénale chez le rat, que des cellules suppressives se différencient chez les animaux tolérants et sont trouvées dans les ganglions lymphatiques mais non dans la rate (Quigley et al. 1989). Ainsi, la compartimentalisation des cellules régulatrices ne semble pas identique dans toutes les conditions expérimentales et peut dépendre du type de greffe (rein ou cœur), des combinaisons génétiques et des espèces utilisées. D'autres travaux ont montré la sélection de $TCD8^+FoxP3^+$ suppresseurs après transfusion spécifique du donneur (Liu et al. 2004). Ces cellules régulatrices $FoxP3^+$ sont fortement exprimées dans les greffons des animaux après

transfusion spécifique du donneur. Leur recrutement est lié à la présence d'un récepteur de chemokines, le CCR4 (Lee et al. 2005).

D'autres cellules, les lymphocytes $TCD4^{+}CD25^{+}$ ont été montrées comme ayant un grand rôle dans l'induction de la tolérance. De nombreuses études font état de leur génération après transfusion spécifique du donneur et de leur capacité à réguler la réponse allo-spécifique (Roelen et al. 1998; Bushell et al. 2003; Oluwole et al. 2003; Cavinato et al. 2005; Kitade et al. 2005). Ces études montrent que ces cellules sont présentes très rapidement voir même contemporaines à la greffe (Kitade et al. 2005). Enfin, d'autres mécanismes ont été montrés comme important dans l'induction de la tolérance par transfusion spécifique du donneur. Il s'agit entre autres, de la voie indirecte de présentation (Quezada et al. 2003; Kishimoto et al. 2004), des différentes voies de costimulation (Sho et al. 2002; Kishimoto et al. 2004), du micro-chimérisme (Wood et al. 1996; Seung et al. 2003) et également de la déviation Th1 ou Th2 qui va évoluer en fonction du stade de la phase d'induction de tolérance (Koga et al. 2000; Koshiba et al. 2003; Pirenne et al. 2005).

III.3.3. Transfusion spécifique du donneur et maintien de la tolérance

Si beaucoup d'études s'intéressent à la phase précoce de l'induction de tolérance, peu se sont intéressées aux mécanismes de tolérance qui prennent place à distance de la greffe. L'étude d'animaux tolérant leur greffon à long terme (de 30 à 100 jours après la greffe selon les modèles) nous indique que des cellules régulatrices sont présentes à distance de la greffe (Kataoka et al. 2003; Koshiba et al. 2003) et sont capables de transférer l'état de tolérance à des animaux naïfs (Kataoka et al. 2003). Ce phénomène définit le concept de tolérance infectieuse. La tolérance infectieuse se définit comme le transfert d'une tolérance d'allogreffe d'un receveur tolérant à un deuxième receveur sans traitement immunosuppresseur par simple transfert de cellules et ce sur plusieurs transferts successifs (Qin et al. 1993; Kataoka et al. 2003). Kataoka et al. ont ainsi montré que les splénocytes CD4 et CD8 issus d'animaux traités par une transfusion spécifique du donneur et tolérant leur greffon depuis plus de 60 jours sont capables de transférer l'acceptation d'une greffe par des animaux naïfs (Kataoka et al. 2003).

Cependant, les cellules régulatrices préviennent le développement du rejet aigu mais ne peuvent prévenir la survenue du rejet chronique (Koshiba et al. 2003). Koshiba et coll. ont montré que les animaux tolérants présentaient aussi des signes typiques de rejet chronique (Koshiba et al. 2003) très certainement liés à la présence de TGF-β (Shittu et al. 2002;

Koshiba et al. 2003; Spriewald et al. 2003) ou à la déviation Th1/Th2 (Koga et al. 2000; Koshiba et al. 2003; Pirenne et al. 2005).

Résultats

I. Article 1:

L'effet d'une première greffe rénale sur le devenir d'une seconde greffe chez un même receveur: Une étude expérimentale et clinique.

Titre original:

The effect of a first kidney transplant on a subsequent transplant outcome: An experimental and clinical study.

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I.1. Résumé

Nous avons étudié l'incidence d'un premier greffon non fonctionnel sur une seconde greffe, grâce aux données collectées par la base de données DIVAT sur une cohorte de 240 patients ayant reçu une transplantation rénale et dans un modèle expérimental d'allogreffe cardiaque chez le rat.

Chez le rongeur, 100 jours après le rejet d'une première greffe allogénique cardiaque, les receveurs présentent plus de lymphocytes $TCD4^{+}CD25^{+}$ sanguins que des rats naïfs. Par contre, aucune différence n'est observée au niveau de la rate. Le profil transcriptionel de diverses cytokines (IL-2, IFN-γ, IL-13, TGF-β1, IL-10 et TNF-α) est le même entre le receveur d'une greffe cardiaque LEW.1W 100 jours après le rejet et des rats naïfs. Le transfert de cellules issues du compartiment splénique des receveurs 100 jours après le rejet, ne permet pas de prolonger la survie d'une greffe de cœur allogénique chez un second receveur sans traitement additionnel. Enfin, la présence d'une première greffe cardiaque rejeté ne permet pas de prolonger la survie d'une seconde greffe de cœur. Chez les patients greffés rénaux, on a observé une forte incidence de l'immunisation anti-HLA et du pourcentage plus élevé d'anticorps (PRA: Panel Reactive Antibody) chez les receveurs retransplantés après néphrectomie de leur première greffe. Cependant, nos données ne suggèrent pas de différences significatives dans les résultats de survie de greffe entre les receveurs de deuxième greffe ayant eu ou non une néphrectomie de leur première greffe. Collectivement, les données obtenues à partir de l'étude d'un modèle expérimental et de l'étude clinique d'une grande cohorte de receveurs de seconde greffe ne suggèrent pas un effet bénéfique de la présence d'une première sur une seconde greffe.

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The effect of a first kidney transplant on a subsequent transplant outcome: An experimental and clinical study

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The effect of a first kidney transplant on a subsequent transplant outcome: An experimental and clinical study.

Background. Second kidney transplantations have a roughly similar clinical outcome to first transplantations. Nevertheless, the effect of the presence of the first, nonfunctional transplant at the time of the second transplantation may also influence its outcome and has not yet been specifically studied.

Methods. We analyzed the effect of the presence of a first graft on the outcome of a second graft in a rodent allograft model and in a cohort of 240 human second kidney allograft recipients.

Results. In rodents, 100 days subsequent to the rejection of the first graft, we observed an increase in blood but not spleen $CD4^+CD25^+$ T cells, whereas no differences were observed in transcriptional patterns. Adoptive transfer of day 100 splenocytes did not prolong graft survival. Moreover, the presence of a first rejected graft does not prolong the survival of a second graft performed at a later date. In the human context, a higher incidence of patients with anti-HLA immunization and a higher % of PRA were observed in retransplant recipients with primary allograft nephrectomy. Despite a relatively low statistical power, our data do not suggest significant differences in graft outcome between recipients of second transplants with primary allograft nephrectomy and those without.

Conclusion. Collectively, the data from both an experimental model and a large cohort of human recipients of a second graft do not suggest a beneficial effect of the presence of a first rejected graft at the time of a second transplantation.

We have recently shown that second kidney transplants have a similar outcome to first transplants when four HLA antigen matching and a stringent cross match policy are observed [1]. However, the presence of a first, nonfunctional transplant may influence the outcome of a subsequent transplantation. Indeed, previous studies have shown that the presence of a first transplant is associated with a lower incidence of panel reactive antibodies (PRA) [2]. In contrast, graft removal is often followed by a rise in anti-HLA antibodies, suggesting that the presence of a nonfunctional graft may either regulate the capacity of the recipient to mount an immune response to the donor's major histocompatibility complex antigens (MHC) or absorb a low level of antibodies [3–5]. The effect of a first kidney graft on the outcome of a second graft has not, however, been analyzed in detail. In a study performed on 127 second kidney graft recipients, Douzdjian et al [6] reported a lower incidence of acute rejection following a second transplant in recipients who still had their first graft, suggesting that some protective effects on the outcome of the second graft could occur.

Such a possibility is also supported by experimental studies performed in a rat model of chronic graft rejection model showing that, in a minor histocompatibility antigen mismatch combination (Fischer 344, $RT1^{1v1}$/LEW, $RT1^1$), a state of unresponsiveness to a second transplant is induced [7, 8]. However, in another study performed in a MHC mismatch combination (Buff/LEW), only moderate prolongation of a second graft was observed (≈10 days), and this modest effect was highly dependent on the timing of the second transplantation, which had to be performed within a week following the rejection of the first [9]. Furthermore, recent observations in mice suggest that CD25-positive ($CD25^+$) regulatory T-cells may be upregulated by inflammatory processes. For instance, Caramalho et al showed that regulatory T-cells could control adverse or inappropriate immune responses to pathogens in mice [10]. Taken together, these observations suggest that, besides the obvious initial effects of a transplant on the host immune response, which results in an early acute

[1]and [2]contributed similarly as first authors and senior investigators, respectively.

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rejection, the persisting presence of alloantigens may trigger regulatory processes. Such processes may be able to influence the host response to a subsequent graft, thus leading to a lower incidence of (or delayed) rejection.

In this paper, we analyzed the effect of the presence of the first graft on the outcome of a subsequent graft in a rodent model of cardiac allotransplantation, as well as in a series of 240 consecutive second kidney allotransplants in humans. We showed that in both the experimental model and the clinical setting, the presence of a first graft did not overtly influence the outcome of a second graft. These data, which address both theoretical and clinical issues, are discussed.

METHODS

Animals and cardiac allotransplantation

Eight-week-old male LEW.1W and LEW.1A rats weighing 250 g were purchased from Janvier (Savigny/Orge, France). These rat strains are congeneic and differed in their MHC haplotype: the LEW.1W rats were RT1u and the LEW.1A rats were RT1a. All animals were maintained under standard conditions according to European and Institutional Guidelines. LEW.1A rats were used as recipients of heterotopic cardiac transplants, performed as described [11]. Graft function was evaluated daily by abdominal palpation, and rejection was defined as the day of cessation of heart beating. LEW.1A recipients that had rejected a first LEW.1W graft received a second LEW.1W heart 100 days after the initial transplantation (D100).

Adoptive cell transfer experiments

One hundred days after the initial transplantation, spleen cells were harvested from LEW.1A recipients that had rejected a LEW.1W heart. Spleen cells depleted of erythrocytes were then either injected I.V. in transfer experiments or used for T cell purification. Blood was collected on EDTA by cardiac puncture, and peripheral blood mononuclear cells (PBMC) were separated on a density gradient (ficoll, Amersham Biosciences, Uppsala, Sweden). T cells were purified using Rat T cell enrichment columns (R&D Systems, Minneapolis, MN, USA). Purity was assessed by flow cytometry (>94%). Adoptive transfers were performed on the day of the transplantation of a LEW.1W heart into a secondary LEW.1A recipient treated with 4.5 Gy whole body irradiation before transplantation.

Flow cytometric analysis

Cell suspensions were incubated for 30 minutes at room temperature with the following fluoroscein isothiocyanate (FITC)-conjugated antibodies: W3/25 (anti-CD4), OX8 (anti-CD8), 3.2.3 (anti-NK) (all from Serotec Laboratories, Oxford, UK), and the following biotinylated antibodies: R7−3 (anti-TCRαβ), Ox39 (anti-CD25 α chain) (from Bioatlantic, Nantes, France), and revealed by Streptavidine-PE (Immunotech, Beckman Coulter, Marseille, France). Cells were washed twice in phosphate-buffered saline (PBS) and double-color staining analyzed by a FACSCalibur using Cellquest Pro® software (BD Biosciences, Mountain View, CA, USA).

Table 1. Primer sequences for cytokine transcript analysis for PCR

Cytokine	Primer sequences
IFNγ sense	5′-CAGCTCTGCCTCATGGCC-3′
IFNγ antisense	5′-GATTCTGGTGACAGCTGGTG-3′
IL-13 sense	5′-AGCAACATCACACAAGACCAG-3′
IL-13 antisense	5′-CACAACTGAGGTCCACAGCT-3′
TGF-ß1 sense	5′-CTACTGCTTCAGCTCCACAG-3′
TGF-ß1 antisense	5′-TGCACTTGCAGGAGCGCAC-3′
TNFα sense	5′-CCTTACGGAACCCCCTATATT-3′
TNFα antisense	5′-GACCCGTAGGGCGATTACAG-3′
IL-10 sense	5′-TCAGCACTGCTATGTTGCC–3′
IL-10 antisense	5′-CCTTGCTTTTATTCTCAGAGG -3′
IL-2 sense	5′-CCTTGTCAACAGCGCACCC-3′
IL-2 antisense	5′-GCTTTGACAGATGGCTATCC-3′
HPRT sense	5′-TGCTGGATTACATTAAAGCGC-3′
HPRT antisense	5′-CTTGGCTTTTCCACTTCGC-3′

The results were expressed as the intrasample ratio of cytokine/HPRT mRNA copy numbers.

Cytokine transcript analysis

Cytokine transcript analysis was performed using real-time quantitative polymerase chain reaction (PCR) for IL2, IFNγ, IL13, TGFβ1, IL10 and TNFα. Briefly, a constant amount of cDNA was amplified in 25 μL of 10X SYBR® Green PCR Core Reagent (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Amplifications were performed in an ABI Prism 7900-Perkin Elmer Sequence Detection system (Perkin-Elmer Applied Biosystems). The exact number of copies of the target cDNA sequence was deduced by comparing the measured fluorescence with a standard curve and was standardized against the level of HPRT transcripts. The primer sequences are displayed in Table 1.

Patients

Among 1513 consecutive cadaver kidney transplantations performed in adults at our institution between January 1990 and December 2002, 240 (16%) were second grafts. Of these second transplant recipients, 83 (34.6%) patients had undergone nephrectomy of the primary allograft (group I) and 157 had not (group II). Indications for transplant intracapsular nephrectomy included unexplained fever, graft pain or tenderness, persistent hematuria, graft infection, or uncontrolled hypertension [12]. While the patients were on the transplant waiting list, their serum was tested for the presence of anti-HLA antibodies systematically two and three weeks after the

first transplant nephrectomy, after every blood transfusion, and every three months systematically thereafter. Anti-HLA antibodies were detected by complement-dependent cytotoxicity assay performed on B and T cells separately on a panel of 36 representative HLA-typed donors [13]. The second transplant matching policy required a minimum of four HLA compatibilities, with priority given to patients with high levels of panel reactive antibodies (PRA) [1]. Repeated HLA mismatches were only allowed when the recipient had not developed anti-HLA class I or anti-class II antibodies specific for the second donor's lymphocytes. The pretransplant crossmatch was performed by complement-dependent cytotoxicity according to NIH guidelines. The T-cell crossmatch on historic as well as current sera had to be negative. However, a positive crossmatch on B cells, without antidonor T reactivity, was not considered as a contraindication to transplantation [14]. As autoantibodies are generally of the IgM isotype, the crossmatch was also carried out in the presence of dithiothreitol to discriminate between IgM and IgG antibodies.

Immunosuppressive protocol

All patients were treated according to the same quadruple sequential immunosuppressive protocol, consisting of an induction therapy, delayed introduction of a calcineurin inhibitor, and a "standard" maintenance therapy. Induction therapy consisted of polyclonal antilymphocyte preparations (rabbit ATG) at a dose of 1 mg/kg/day (IMTIX-SangStat, Lyon, France) administered in the immediate post-transplant period for 10 to 14 days, and associated with azathioprine (Imurel®, Glaxo Smith Kline, Marly-Le-Roy, France) (2 mg/kg/day) or mycophenolate mofetil (Cellcept®, Roche, Neuilly-sur-Seine, France) (2 g/day) and corticosteroids (prednisone, Cortancyl®, Aventis, Paris, France) at a dose of 1 mg/kg/day. Cyclosporine (Sandimmun® or Neoral®, Novartis Pharma SA, Rueil-Malmaison, France) or tacrolimus (Prograf®, Fujisawa, La Celle-Saint-Cloud, France) was begun one day before the end of induction therapy at a dose of 8 mg/kg/day or 0.1 mg/kg/day, respectively. The anticalcineurin regimen was adjusted to maintain trough levels of 150 to 250 ng/mL for cyclosporine and 10 to 15 ng/mL for tacrolimus. The prednisone dose was decreased 10 mg every five days to a maintenance dose of 10 mg/day, and slowly tapered after three months. Biopsy-confirmed AR episodes were treated with intravenous boluses of methylprednisolone for five consecutive days (5 mg/kg/day for two days, tapered by 1 mg/kg/day every day), followed by decreasing doses of oral prednisone. ATG or antilymphocyte monoclonal OKT3 antibody was used in the case of corticosteroid unresponsiveness or severe rejections (Banff grade II or III).

Data bank and statistical analysis

The clinical data were obtained from a computerized and validated standard database called the "DIVAT network" (Données Informatisées et VAlidées en Transplantation) [1, 15]. In this procedure, a specialized clinical research assistant, who is independent of the medical team, prospectively collects the pre- and post-transplant data of all patients transplanted at our institution. The database is validated annually by a medical audit and has an error level below 1%. The database includes demographic characteristics (age, donor and recipient gender), immunologic parameters [anti-T and -B peak PRA, HLA-A, -B, -DR mismatch (MM)], and transplant variables [serum creatinine and daily proteinuria until 12 months, cold ischemia time, delayed graft function (DGF) and AR]. Return to a chronic dialysis program or death with a functioning graft was considered as a graft loss. The immunosuppressive treatment was stopped within the first month after dialysis onset. AR was identified by standard clinical criteria in conjunction with a renal biopsy (except if contraindicated) analyzed according to the Banff classification. DGF was defined as the need for at least one dialysis session during the first seven days after transplantation, and/or the number of days required to reach a Cockcroft-calculated creatinine clearance of at least 10 mL/min [16]. Graft and patient survival analysis was performed using the Kaplan-Meier method, followed by log-rank analysis. Baseline characteristics among groups were compared using a chi-square test and analysis of variance (ANOVA) for categorical and continuous variables. A P value < 0.05 was considered significant.

RESULTS

Animal study

Phenotype and transcriptional patterns of recipients that had rejected a first graft, analyzed 100 days after the initial transplantation. The phenotype of spleen and blood T cells was first examined 100 days after transplantation in LEW.1A recipients that had rejected a first graft ($N = 5$), and was compared to age-matched naive LEW.1A rats. In the spleen, no variation in $CD4^+$ and $CD8^+$ T cells were apparent, and $CD25^+$ T cells were also at roughly similar levels in recipients that had rejected a first graft ($N = 5$, $16 \pm 8\%$) or naive LEW.1A ($N = 5, 12 \pm 4\%$, ns). In naive rats, $11 \pm 4\%$ of the $CD4^+$ cells were $CD25^+$ compared to $17 \pm 7\%$ (ns) in recipients that had rejected a first graft. The percentage of $CD8^+CD25^+$ spleen cells was similar in the two groups ($2.8 \pm 0.6\%$, $3.5 \pm 2\%$, respectively). In the blood, $CD25^+$ T cells were, however, significantly increased in recipients that had rejected their first graft and tested on day 100 ($16.5 \pm 4\%$, $N = 5$) compared to naive LEW.1A rats ($7 \pm 3\%$, $N = 5$, $P < 0.05$). Blood

$CD4^+CD25^+$ were found to be $10.5 \pm 3.8\%$ ($N = 5$) versus $3.4 \pm 1\%$ in normal rats ($N = 4$) ($P < 0.01$), but no differences were observed for $CD8^+CD25^+$ cells.

We next analyzed IL2, IFNγ, IL13, TGFβ1, IL6, TNFα, and IL10 spleen and blood transcript accumulation by real-time PCR in day 100 LEW.1A recipients that had rejected a first LEW.1W graft and in naive LEW.1A rats. No difference was observed for the spleen or the blood (in terms of the cytokine/HPRT transcript ratios). However there was a nonsignificant trend toward a lower accumulation of IL2 transcripts ($91.10^{-3} \pm 64.10^{-3}$, $N = 5$ versus $172.10^{-3} \pm 130.10^{-3}$, $N = 5$) in recipients that had rejected a first graft.

Taken together, these data show that recipients that had rejected a first graft had an increased pool of circulating $CD4^+$ $CD25^+$ cells. The fact that we also observed a down-regulation of IL2 transcripts in blood T cells suggest that these $CD25^+$ cells could be regulatory cells, rather than memory alloreactive cells. For these reasons, we next analyzed the survival of a second LEW.1W heart and the effect of the transfer of cells from rats that had rejected a first graft 100 days previously into a naive secondary LEW.1A recipient.

Long-term untreated LEW.1A recipients that initially reject a LEW.1W heart graft do not accept a second LEW.1W heart graft, and do not harbor regulatory cells able to prolong the survival of LEW.1W hearts in naive LEW.1A rats. Unmodified LEW.1A rat recipients rejected a MHC incompatible LEW.1W heart graft within 6.5 ± 0.6 days. A second LEW.1W transplant performed 100 days after rejection of the first graft was subject to rejection within 8 ± 1.8 days ($N = 3$) (Fig. 1). Splenocytes or PBMC of naive LEW.1A rats or of day 100 LEW.1A recipients that had initially rejected a first LEW.1W graft were then administered to subirradiated LEW.1A secondary recipients of a LEW.1W heart graft on the day of transplantation. Control subirradiated LEW.1A recipients, without further treatment, rejected their heart grafts within 16 ± 3 days ($N = 11$). The adoptive transfer of 100 ± 10^6 splenocytes from either naive or day 100 LEW.1A recipients that had initially rejected a first LEW.1W graft had no effect on graft survival ($N = 3$, 15 ± 2). Furthermore, the adoptive transfer of 20×10^6 PBMC from recipients who had rejected a first graft did not increase but rather shortened graft survival ($N = 3$, 9 ± 3 days).

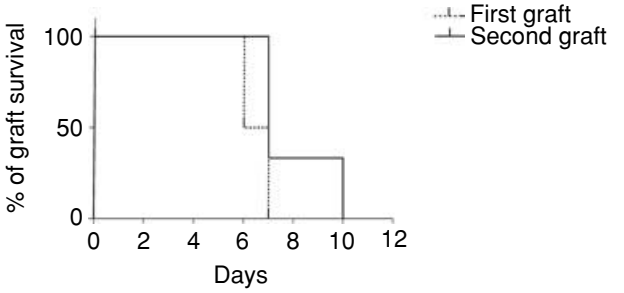

Fig. 1. Survival of second LEW.1W allografts in transplanted LEW.1A recipients that had rejected a first graft, 100 days after the first transplantation.

Table 2. Demographic characteristics and the immunosuppressive regimen of group I ($N = 83$, with first graft nephrectomy) and group II ($N = 157$, without nephrectomy)

Demographic characteristics and immunosuppressive regimen	Group I ($N = 83$)	Group II ($N = 157$)
Recipient age *years*		
Mean ± SD	43.6 ± 12	42.6 ± 13
Range	20–71	16–73
Recipient sex [*N* (%)]		
Male	50 (60%)	105 (67%)
Donor age *years*		
Mean ± SD	36.4 ± 15	39.6 ± 14
Range	9–68	6–72
Donor sex [*N* (%)]		
Male	57 (69%)	113 (72%)
Year of transplantation [*N* (%)]		
1990 to 1995	37 (44.6%)	53 (33.8%)
1996 to 2002	46 (55.4%)	104 (66.2%)
Renal disease [*N* (%)]		
Glomerulonephritis	41 (49.4%)	67 (42.7%)
Hereditary	22 (26.5%)	29 (18.5%)
Tubulointerstitial	11 (13.3%)	33 (21%)
Diabetes	2 (2.4%)	10 (6.4%)
Hypertension, vascular	4 (4.8%)	4 (2.5%)
Other, unknown	3 (3.6%)	14 (8.9%)
Hypertension [*N* (%)]		
Yes	46 (55.4%)	85 (54.1%)
No	37 (44.6%)	72 (45.9%)
Immunosuppressive regimen [*N* (%)]		
Cyclosporine, Sandimmun®	44 (53%)	59 (37.6%)
Cyclosporine, Neoral®	6 (7.2%)	22 (14%)
Tacrolimus	33 (39.8%)	76 (48.4%)
Azathioprine	41 (49.4%)	59 (37.6%)
Mycophenolate mofetil	42 (50.6%)	98 (62.4%)

Clinical study

Patient population and pre- and post-transplant immunologic characteristics. Recipients of a second renal allograft were divided into two groups: group I ($N = 83$), which had undergone primary allograft nephrectomy, and group II ($N = 157$), which had not. The demographic characteristics of the two groups were not significantly different (Table 2). The mean donor and recipient age and sex ratios, the rate of hypertension, the etiology of the original kidney disease, and the year of transplantation were similar. The characteristics of the immunosuppressive treatments did not differ between the two groups (Table 2).

No statistical difference was observed between the two groups in terms of one-year serum creatinine, daily proteinuria, cold ischemia time, and DGF (Table 3). The incidence of AR was also similar (17% for group I vs. 14% for group II). The global HLA or HLA-A/B/DR mismatch was not significantly different between the two groups: 72.3% of patients who had undergone primary allograft

Table 3. Allograft function in the patients of group I ($N = 83$, first graft nephrectomy) and II ($N = 157$, without nephrectomy) and the incidence of acute rejection episodes

Graft characteristics	Group I ($N = 83$)	Group II ($N = 157$)
Serum creatinine at 12 months *μmol/L*		
Mean ± SD	136 ± 51	144 ± 95
Range	67–282	59–979
Proteinuria at 12 months *g/24hr*		
Mean ± SD	0.30 ± 0.31	0.49 ± 1.07
Range	0–1.45	0–7.85
Cold ischemia time *hours*		
Mean ± SD	30 ± 9.4	29 ± 9.4
Range	10.8–50	9.5–50
DGF		
Mean ± SD of days with Cl. ≥10 mL/min[a]	8 ± 7.5	6.7 ± 6
First graft dialysis session[a] *Mean ± SD*	1.56 ± 2.26	1.48 ± 1.91
AR episodes [*N* (%)]	14 (17%)	22 (14%)

Cl., creatinine clearance.
[a]See **Methods** section.

Table 4. Immunologic parameters of group I ($N = 83$, with first graft nephrectomy) and group II ($N = 157$, without nephrectomy)

Immunologic parameters	Group I ($N = 83$)	Group II ($N = 157$)
Peak PRA [(% ± SD)]		
anti-T	43% ± 37[a]	33% ± 36[a]
anti-B	51% ± 32	45% ± 32
PRA [n (%)]		
anti-T ≤25%	35 (42.2%)[b]	89 (56.7%)[b]
anti-T ≥25% and <80%	27 (32.5%)	39 (25%)
anti-T ≥80%	21 (25.3%)	29 (18.3%)
Global HLA MM [*N* (%)]		
≤2	60 (72.3%)	112 (71.3%)
>2	23 (27.7%)	45 (28.7%)
HLA-A MM [*N* (%)]		
0	41 (49.4%)	72 (45.9%)
1	36 (43.4%)	72 (45.9%)
2	6 (7.2%)	13 (8.2%)
HLA-B MM [*N* (%)]		
0	25 (30.1%)	47 (30%)
1	45 (54.2%)	92 (58.6%)
2	13 (15.7%)	18 (11.4%)
HLA-DR MM [*N* (%)]		
0	47 (56.6%)	84 (53.5%)
1	34 (41%)	64 (40.8%)
2	2 (2.4%)	9 (5.7%)

[a]$P = 0.048$, ANOVA.
[b]$P < 0.05$, chi-square.

nephrectomy received a well-matched kidney (HLA MM ≤2) versus 71.3% of patients who had not undergone this procedure. Likewise, recipients were identically matched for HLA-DR. In particular, 56.6% of group I recipients versus 53.5% of group II patients had no HLA-DR mismatch (Table 4).

However, the retransplanted patients in group I were characterized by higher anti-HLA levels. In addition, the PRA peak occurred between the two transplantations for the majority of the immunized patients (anti-T PRA ≥25% for 91% of group I patients vs. 88% of group II patients). Moreover, 82% of immunized patients in the transplantectomy group exhibited a rise in their PRA levels within the first six months following surgery. Anti-T cell PRA peak was also significantly higher in group I (43%) than in group II (33%) ($P = 0.048$). No difference was observed for anti-B cell PRA. Finally, the percentage of recipients with a low level of anti-HLA immunization (anti-T PRA <25%) was significantly lower in the group of patients who had undergone nephrectomy (Table 4).

Incidence of graft survival and rejection. Recipients of a second transplant that had or had not undergone primary allograft nephrectomy showed no significant differences (see Fig. 2) in graft survival. Similar results were obtained when patient death was censored (data not shown). According to Kaplan-Meier analysis, graft survival was 92%, 81%, and 62% at 1, 5, and 10 years, respectively, for group I, and 92%, 79%, and 65% at 1, 5, and 10 years, respectively, for group II. Among the causes of secondary transplant loss, death with a functioning graft accounted for 19% versus 25%, AR for 14.3% versus 12.5%, chronic rejection for 42.8% versus 30%, vascular and recurrent renal disease for 23.8% versus 32.5%, for group I and group II, respectively. When immunologic causes of transplant failure were examined separately or together, there was no statistical difference between the two groups.

DISCUSSION

In rodents, some reports have suggested that recipients of a first heart allograft that is mismatched in terms of minor histocompatibility antigens who undergo rejection, but in whom the rejected organ is not removed, can develop mechanisms able to prolong the survival of a second graft [7]. Of course, the rat model does not entirely conform with the human situation. Indeed, in the rat model, a second graft from the same donor strain reintroduces the original donor antigens, which may lead to the induction of donor-specific regulatory cells. However, the transplantation of a third party graft, thus mimicking the human situation, is not influenced by priming with antigens of another strain [17]. In addition, a heart model was used because a life-sustaining kidney model would have required the presence of the recipient's own kidney in the long term. Moreover, in this combination, about 15% of kidney transplants are spontaneously accepted, a phenomenon which cannot be detected in the presence of a native kidney.

In an MHC-mismatched combination, the effect of retransplantation was shown to be a modest and restricted to a limited time window after transplantation [9]. Because only a few studies have explored the effects of the presence of a first rejected graft on the survival of a second graft in human recipients, and because retransplantation is now a routine procedure in humans (≈25%) and is associated with favorable survival [1], the possibility that

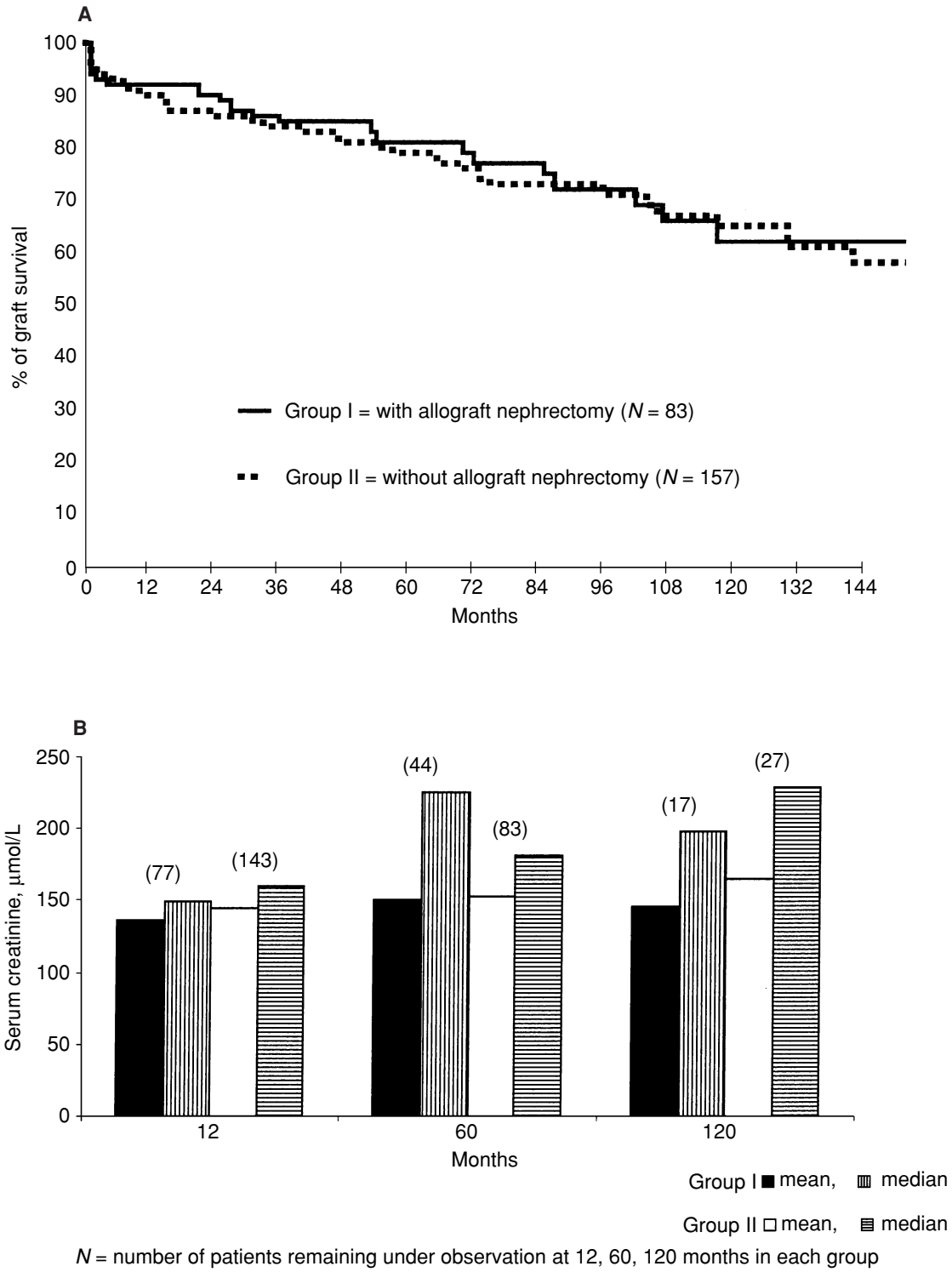

Fig. 2. Long-term graft survival for retransplantations with (group I) or without (group II) primary allograft nephrectomy (*A*). Serum creatinine (mean and median), with the number of patients remaining under observation (in brackets) at 12, 60, and 120 months in each group (*B*).

a first graft could induce a regulation of the alloimmune response to a subsequent graft, thereby contributing to this good outcome, needs to be considered. Furthermore, among the few clinical studies [2, 18, 6] that have addressed the rationale of first graft removal and its effects on the outcome of a second graft, two reported a lower incidence of rejection episodes in recipients that still had their graft in place, although this point was not detailed

[2, 6]. Another report mentioned a significantly lower graft survival when the first kidney was removed [18]. Interestingly, all these studies reported a lower level of PRA when the first transplant was not removed.

Our data show that rat recipients of a first MHC-mismatched graft exhibit some increase in circulating $CD25^+$ and $CD4^+CD25^+$ T cells in the blood, but not in the spleen. The fact that this augmentation was not associated with an increase in IL2 transcript levels could suggest the presence of regulatory T cells in these animals. However, this hypothesis was not confirmed by: (*1*) the short survival of a second graft from the original donor strain, and (*2*) the absence of graft survival prolongation following transfer of splenocytes from day 100 recipients having rejected a first graft to naive hosts. Both points also challenge the possibility of the differentiation of a subpopulation of T or non-T regulatory cells. Importantly, in this same strain combination, the transfer of 100×10^6 splenocytes from recipients treated with a donor-specific blood transfusion-induced tolerance protocol (Lair et al, in preparation), or a deoxyspergualine-induced tolerance protocol [19], consistently results in long-term survival. Therefore, despite the fact that rat recipients of a fully MHC incompatible graft may exhibit some delay in the rejection of a donor-matched subsequent graft in a limited time period following rejection of the first transplant [9], long-term recipients of such an initially rejected graft rapidly reject a second graft.

This experimental data fits with the clinical observations gathered on the largest, single-center, second kidney graft cohort analyzed. Data concerning this cohort of patients have been collected in the validated DIVAT data bank and concern grafts performed in a sufficiently recent and "clinically homogeneous" time period, in which all recipients received a treatment using a calcineurin inhibitor. Interestingly, we confirm a significant relationship between both a higher level of PRA and the percentage of immunized patients and the nephrectomy of the first transplant. However, the fact that the second graft outcome was similar in the two groups does not suggest that this low PRA and immunization rate are linked to the presence of regulatory cells. Rather, a possible absorption of antibodies onto the first rejected graft, as also suggested by the study of rejected graft eluates [20], may be involved. Furthermore, we were unable to confirm that the presence of the first graft conferred a survival advantage to the second graft. No difference in incidence of AR or in graft function and daily proteinuria was observed. Nevertheless, our data do not enable us to definitively rule out differences between the two groups due to the need for a very large sample size to accept the statistical "null hypothesis." However, despite we cannot rule out an effect of nephrectomy on the outcome of the second kidney graft, our data suggest that this effect should be necessarily small. Despite a high HLA matching policy and other factors that could affect AR incidence, both groups were similarly managed, and graft survival was identical in recipients that had or had not undergone graft nephrectomy. This higher incidence of immunization is probably not counterbalancing a possible "hidden" beneficial effect of first graft nephrectomy, since no increase in vascular rejection episodes or long-term chronic rejection (including proteinuria) was observed. Finally, the stringent HLA matching policy followed in this cohort should not minimize a potential regulatory effect. On the contrary, HLA matching (and HLA-DR matching in particular) tends to correlate with immune regulation after kidney transplantation [21].

CONCLUSION

Taken collectively, our data obtained both in an experimental model that has been shown to be highly sensitive to regulatory T-cell modulation, and in a large cohort of human recipients of a second kidney graft, do not suggest any beneficial effect of a previous graft rejection or the presence of the first rejected graft at the time of a subsequent transplantation on the outcome of this subsequent transplant.

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Discussion

Chez les rongeurs, il a été suggéré que lorsqu'une première allogreffe cardiaque entre individus incompatibles pour les molécules du CMH est rejeté, les receveurs développent des mécanismes de régulation à l'origine de la survie d'une seconde greffe cardiaque chez le rat (Yanagiya et al. 1992). Mais, cet effet est modéré 9 jours en moyenne pour la première greffe et 19,5 jours en moyenne pour la seconde (Yanagiya et al. 1992).

La retransplantation en clinique est maintenant un procédé courant (environ 25% des transplantations) et est associée à une meilleure survie du greffon (Coupel et al. 2003). Seulement quelques études chez l'Homme ont exploré les effets de la présence d'une première greffe rejetée sur la survie d'une seconde greffe. Sumrani et Douzdjian ont rapporté une incidence limitée des épisodes de rejet de la seconde greffe chez les receveurs qui avaient toujours la première greffe en place (Sumrani et al. 1992; Douzdjian et al. 1996). Abouljoud mentionne une survie significativement réduite du second greffon quand le premier rein a été enlevé (Abouljoud et al. 1995). Il est intéressant de noter que toutes ces études ont rapporté un niveau plus faible d'immunisation (PRA: Panel Reactive Antibody) chez le receveur lorsque la première greffe est toujours présente (Sumrani et al. 1992; Abouljoud et al. 1995; Douzdjian et al. 1996). Ainsi, nous avons voulu étudier si une première greffe pouvait induire une régulation de la réponse allo-immune à une seconde greffe, contribuant ainsi à l'amélioration de la survie du greffon.

Dans notre modèle expérimental de greffe allogénique cardiaque entre rats incompatibles pour les molécules du CMH de classe I et II, nous avons étudié si la présence d'un premier greffon cardiaque rejeté permettait la différentiation d'une population de cellules régulatrices et influait sur la survie d'un second greffon. Nos résultats montrent que le nombre de cellules $TCD4^{+}CD25^{+}$, décrites comme régulatrices dans de nombreuses études, varie selon la localisation chez le receveur. Ainsi, on observe une augmentation des cellules $TCD4^{+}CD25^{+}$ circulant chez les receveurs d'une première allogreffe cardiaque rejeté comparé au sang d'un animal naïf. Tandis que dans la rate, il n'apparaît aucune différence du nombre de cellules $TCD4^{+}CD25^{+}$entre les deux groupes. Une faible diminution du taux de transcription de l'IL-2 suggère également chez les receveurs la présence de cellules régulatrices (Jonuleit et al. 2001). Cependant, cette hypothèse n'a pas été confirmée par: (1) la courte survie d'une seconde greffe cardiaque issue de la même souche que le donneur, et (2) l'absence de prolongation de survie d'une greffe après le transfert de splénocytes de receveurs ayant rejeté une première greffe à un receveur sans aucun traitement. Il est intéressant de noter que dans

cette même combinaison, le transfert de 100 millions de splénocytes d'animaux tolérants après un protocole d'induction par transfusion spécifique du donneur (Lair et al, soumis), ou un protocole d'induction de tolérance par la deoxyspergualine (Chiffoleau et al. 2002), donne une survie à long terme du greffon chez un receveur non traité. Par conséquent, nos résultats montre qu'à distance du rejet d'une première greffe, il ne semble pas y avoir de régulation vis-à-vis du second greffon allogénique et que la présence d'une première greffe rejetée n'a pas d'influence sur le devenir de la seconde greffe.

Les données expérimentales corroborent les observations cliniques. Les données concernant cette cohorte de patients ont été rassemblées dans la banque de données validée DIVAT et concernent des greffes effectuées sur une période récente et chez des receveurs ayant reçu un traitement avec inhibiteur de calcineurine. Les patients qui ont eu une néphrectomie de leur première greffe (groupe I) ont une immunisation anti-T plus élevée que les patients qui n'ont pas été néphrectomisés (groupe II). Cependant, le fait que la survie des secondes greffes soit semblable entre les deux groupes ne suggère pas que le niveau d'anticorps et le taux d'immunisation anti-T soient liés à la présence de cellules régulatrices. Une absorption des anticorps par le premier greffon rejeté, comme cela a été également suggéré précédemment dans des éluâts de greffes rejetées (Soulillou et al. 1981), est possible. Bien que nous ne puissions pas éliminer un effet de la néphrectomie sur le devenir de la seconde greffe, nos données suggèrent que malgré une forte immunisation des receveurs, l'effet de cette néphrectomie est limité, la survie de greffe étant identique et aucune augmentation des épisodes de rejet vasculaire ou de rejet chronique à long terme (protéinurie y compris) n'a été observée entre les deux groupes.

En conclusion, l'ensemble de nos données obtenues dans un modèle expérimental impliquant dans d'autres situations la présence et le rôle des cellules régulatrices, et dans une étude clinique sur une grande cohorte de patient ayant subi une seconde greffe de rein, ne suggèrent pas d'effet bénéfique de la présence d'un premier greffon rejeté sur les résultats d'une seconde transplantation.

II. Article 2:

Mise en évidence de cellules TCD25$^-$ régulatrices capable de transférer la tolérance dans un modèle de tolérance induite par transfusion spécifique du donneur.

Titre original:

Development of CD25$^-$ regulatory T cells following heart transplantation; evidence for transfer of long term survival

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Soumis

II.1.

Résumé

La survie d'une allogreffe cardiaque d'un rat LEW.1W chez un receveur LEW.1A, incompatibles pour les molécules de CMH de classe I et II, peut être induite par des transfusions de sang spécifique du donneur avant la greffe. La phase d'induction de la «tolérance» a été étudiée en détail y compris dans notre laboratoire, mais les mécanismes qui participent au maintien de la survie du greffon à distance de la greffe demeurent peu étudiés. Nous avons étudié et comparé la fonction et la capacité à transférer la «tolérance» des cellules spléniques $TCD25^+$ et $TCD25^-$ de rats «tolérants» (100 jours après transplantation). Les cellules $TCD25^+$ des animaux «tolérants», présentent une activité suppressive sur la prolifération de cellules $TCD25^-$ naïves contre des antigènes du donneur. Elles sont anergiques et ne prolifèrent pas en réponse à des antigènes du donneur. Les cellules $TCD25^-$ des animaux «tolérants» présentent des caractéristiques comparables à celles de cellules régulatrices $TCD25^+$. Elles prolifèrent peu en réponse à une stimulation par les antigènes du donneur. Elles inhibent *in vitro* la prolifération de cellules naïves $TCD25^-$, et ce de manière spécifique des antigènes du donneur. Les transcrits pour l'IFN-γ, l'IL-10 et FoxP3 sont fortement exprimés par ces cellules $TCD25^-$. De façon similaire aux cellules régulatrices naturelles $TCD4^+CD25^+$, l'activité suppressive *in vitro* des cellules $TCD25^-$ dépend de contacts cellulaires directs et n'implique pas de facteurs solubles (IL-4, IL-10, TGF-β). De plus, l'injection de cellules $TCD25^-$ de rats «tolérants» à des animaux irradiés et greffés sans traitement, permet la survie du greffon à long terme.

Ainsi, nous avons mis en évidence que les cellules régulatrices dans ce modèle ne sont pas limitées aux seules cellules $TCD4^+CD25^+$. Les cellules $TCD4^+CD25^-$ montrent également des propriétés régulatrices.

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Development of CD25(-) regulatory T cells following heart transplantation: Evidence for transfer of long-term survival.

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Donor-specific heart allograft acceptance can be induced in the MHC-mismatched LEW.1 W to LEW.1A rat by donor-specific transfusions. Whereas the induction phase of tolerance has been studied in detail, its maintenance remained poorly understood. Here, we performed a side-by-side comparison of CD25(+) and CD25(-) splenic T cells of 100-day tolerant rats. Administration of CD25(-) T cells from tolerant rats to sublethally irradiated recipients transferred long-term graft survival. These CD25(-) T cells displayed a decreased donor-specific response in the mixed lymphocyte reaction and presented suppressive activity. These CD25(-) T cells accumulated IFN-gamma, IL-10 and Foxp3 transcripts. The in vitro suppressive activity of CD25(-) T cells required both cell contact and soluble factors (IL-10 and IFN-gamma). The CD25(+) T cells from tolerant rats did not show any modification of their regulatory properties. We show that splenic CD25(-) T cells of tolerant rats contribute to the maintenance of tolerance following the transplantation. Our data show that regulatory T cells are not restricted to the CD4(+)CD25(+) T cell subset and provide new insights on the mechanisms of tolerance to allograft following donor cell priming.

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Development of CD25$^-$ regulatory T cells following heart transplantation; evidence for transfer of long term survival.[1]

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Keywords: Transplantation, Tolerance, DST, Regulatory T Cells, Rat.

Abstract

Donor-specific heart allograft acceptance can be induced in the MHC-mismatched LEW.1W to LEW.1A rat by donor-specific transfusions. Whereas the induction phase of tolerance has been studied in detail, its maintenance remained poorly understood. Here, we performed a side-by-side comparison of $CD25^+$ and $CD25^-$ splenic T cells of 100 days tolerant rats. Administration of $CD25^-$ T cells from tolerant rats to sub-lethally irradiated recipients transferred long term graft survival. These $CD25^-$ T cells displayed a decreased donor-specific response in mixed lymphocyte reaction and presented suppressive activity. These $CD25^-$ T cells accumulated IFN-γ, IL-10 and FoxP3 transcripts. Similar to natural regulatory $CD4^+CD25^+$ T cells, the *in vitro* suppressive activity of $CD25^-$ T cells required cell contact and did not involve soluble factor. The $CD25^+$ T cells from tolerant rats did not show any modification of their regulatory properties. We show that splenic $CD25^-$ T of tolerant rats contribute to the maintenance of tolerance following the transplantation. Our data show that regulatory T cells are not restricted to the $CD4^+CD25^+$ T cell subset and provide new insights on the mechanisms of tolerance to allograft following donor cell priming.

Introduction

Induction of specific tolerance for donor antigens (see [1] for review) is one of the most actively explored fields in transplantation immunology. Donor-specific blood transfusion (DST) is a clinically and experimentally proven method to induce hyporesponsiveness and does not necessarily require additional immunotherapy [2-6]. However, the use of DST clinically has become less popular with the advent of calcineurin inhibitors and because a clear understanding of the mechanisms involved had remained elusive [4, 6].

In adult rats, long term survival of MHC incompatible vascularized allografts (heart or kidney) can be obtained by priming the recipients with donor blood cells [7, 8]. We and others have shown that inhibition of early allograft rejection by DST mostly affects helper T cells functions [9-11] and requires intact resident dendritic cells at the time of transplantation [12]. Early production of TGF-β [13] and differentiation of $CD8^+$ clonal regulatory cells [14-16] are also involved. DST treatment result in a complex state where symptoms of chronic graft rejection coexist [17] with mechanisms controlling host acute anti-donor immune response. Long term recipients accept a donor derived skin graft whereas a third party graft is rejected [17]. Moreover, the spleen [18], and possibly the graft itself [19], harbor donor-specific regulatory T cells able to protect naive recipient from allogeneic heart rejection. Therefore, the immunological status of allograft recipient following DST pre-conditioning provides another example of the differentiation of regulatory cells described in mice [20-23] or in rats [18, 24] following different “tolerance” induction maneuvers (see [25] for review). Various types of cells, including $CD4^+$ and $CD8^+$ splenocytes [18, 26] harvested from long term recipients, have been described to transfer tolerance to naive syngeneic hosts when injected at the time of transplantation. Recently, Kitade and colleagues have shown that DST induces — rapidly posttransplant— the developpement of alloantigen-specific Tregs in lymphoid tissues and in the graft, mainly with a $CD4^+CD45RC^-$ phenotype [27]. However, the exact nature of

the cells, the possible cell interactions involved in the maintenance of long term tolerance (>100 days post-transplantation) and in its transfer, and their mechanisms of action remain a matter of debate.

In this paper, we focused on $CD25^-$ T cells and their potential role in tolerance maintenance and transfer. We first analyzed *in vitro* regulatory patterns of T cells harvested from spleen of LEW.1A tolerant recipients for a LEW.1W MHC incompatible heart graft 100 days after transplantation. We show that $CD25^+$ T cells from tolerant recipients inhibit proliferation of $CD25^-$ T cells with a magnitude similar to that observed with naive $CD25^+$ T cells. In contrast, we show that in tolerant recipients, normally strongly alloreactive $CD25^-$ T cells have now acquired hypo-responsiveness and the capacity to inhibit proliferation of naive $CD25^-$ T cells against donor APCs. This inhibition was cell-contact-dependant. Finally, we show that, in roughly half of the case, $CD25^-$ T cells from tolerant recipients can transfer graft acceptance to a naive host. Taken together, our data suggest that $CD25^-$ T cells are involved in the maintenance and transfer of tolerance, and likely contribute to the control of alloreactivity [28]. In addition, our data challenge some of the concepts on regulatory cells involved in the long term maintenance of tolerance to an allograft.

Results

Tolerant animals contain allogeneic hyporesponsive cells in both the $CD25^+$ and $CD25^-$ T cell compartments.

We first investigated the alloreactive response of T cells of tolerant heart allograft recipients (day 100 following surgery). Splenic T cells from tolerant recipients proliferate less than T cells from naive animals when stimulated with LEW.1W APCs (Fig.1.A). We performed a side-by-side comparison of the effector ($CD25^-$ T cells) and regulatory ($CD25^+$ T cells) compartments. $CD25^+$ T cells from both tolerant and naive animals did not proliferate when stimulated with LEW.1W APCs (Fig.1.A). However, $CD25^-$ T cells from tolerant animals proliferated 70% less than naive $CD25^-$ T cells (Fig.1.A; $p<0.001$). Proliferation against third-party BN APCs was not significantly reduced for $CD25^-$ T cells from tolerant rats compared to naive rats (Fig.1.B). Thus, $CD25^-$ T cells from tolerant rats were specifically hyporesponsive to the donor antigens.

Allograft-induced $CD25^-$ T cells inhibit naive alloreactive T cell proliferation.

We then analyzed if the $CD25^-$ T cells from tolerant animals were able to inhibit naive $CD25^-$ T cell alloreactivity, and if the inhibitory properties of the regulatory $CD25^+$ compartment were modified. Proliferation of naive $CD25^-$ T cells to LEW.1W APCs was tested in the presence of $CD25^-$ T cells from either tolerant or naive rats. $CD25^-$ T cells from tolerant rats significantly and dose dependently inhibited the alloreactive response of naive $CD25^-$ T cells in the coculture system. At a ratio 1:1 ($1x10^5$ naive $CD25^-$ T responder cell cultured with $1x10^5$ $CD25^-$ T cell from tolerant recipient), the mean percentage of proliferation was 44% ± 9% when $CD25^-$ T cells from tolerant animals were added to the coculture system (Fig.2.A; $p<0.0001$). In contrast, the mean percentage of proliferation was 166% ± 18% when $CD25^-$ T cells of naive animals were added. $CD25^+$ T cells of tolerant animals were also able to inhibit

proliferation when added to the MLR (Fig.2.B). The level of inhibition was similar to that observed with naive $CD25^+$ regulatory T cells, showing that the $CD25^-$ T subset only has acquired a new inhibitory phenotype in the tolerant recipients. The regulatory properties of $CD25^-$ T cells from tolerants recipients was donor specific since no inhibition was observed when $CD25^-$ T cells from naive or tolerant animals were added to a MLR using a third party (BN) APCs (Fig.2.C).

$CD25^-$ T cells from tolerant rats transferred tolerance to naive rats.

$CD25^-$ T cells from tolerant animals were administered into sub-lethally irradiated LEW.1A secondary recipients of a LEW.1W heart grafts on the day of transplantation. Without cell transfer, sub-lethally irradiated LEW.1A recipients rejected their heart graft in 17+/-1 days (excepted for 2 out of 17 animals). Adoptive transfer of $50x10^6$ purified $CD25^-$ T cells from tolerant animals significantly prolong graft survival ($p<0.01$ vs. sub-irradiated controls; Fig.3) and induced long term survival (>100 days) in 4 out 9 recipients. Adoptive transfer of $50x10^6$ T cells from naive LEW.1A rat (data not shown) did not prolong graft survival. Finally, $50x10^6$ purified $CD25^-$ T cells from tolerant animals did not protect BN graft from rejection (n=3; Fig.3). Thus, $CD25^-$ T cells from tolerant animals are able to prolong and eventually to transfer tolerance in a donor-antigen specific manner.

Previous studies from our laboratory have shown that $2x10^6$ $CD4^+CD25^+$ T cells from desoxyspergualine derivate-treated animals was efficient in transferring tolerance in the same strain combinaison [29]. Adoptive transfer of 1 to $4x10^6$ $CD4^+CD25^+$ T cells from tolerant animals did not prolong significantly heart survival to LEW.1A hosts (17 days; n=4; Fig.3).

***In vitro* suppressive activity of $CD25^-$ T cells from tolerant animals is cell-contact dependent.**

The mechanisms involved in the hyporesponse of $CD25^-$ T cells to allostimulation were then investigated. The inhibition of IDO, iNOS (Fig.4.A) and specific blocking antibodies against

IL-4, IL-10, or TGF-β (Fig.4.A) did not restore the alloreactivity of $CD25^-$ T cells of tolerant rats. However, addition of IL-2 significantly restored $CD25^-$ T cell responses of tolerant rats to donor APCs ($p<0.05$; Fig.4.A), suggesting an anergy-like state and indicating that $CD25^-$ T cells have recover CD25 marker in the culture (data not shown).

To analyze if suppressive $CD25^-$ T cells of tolerant animals were acting through direct cell to cell contact, transwell membrane were used. Figure 4.B. shows that the suppressive activity of $CD25^-$ T cells from tolerant animals was abrogated by the semi-permeable membrane suggesting a cell-contact dependant mechanism of the proliferation inhibition. The absence of soluble mediator was further suggested by the effect of adding to the coculture different inhibitors. Again, inhibition of IDO, iNOS (Fig.4.C) and anti-IL-4, anti-IL-10 or anti-TGF-β antibodies (Fig.4.C) did not significantly affect *in vitro* suppressive activity of $CD25^-$ T cells from tolerant animals.

$CD25^-$ T cells from tolerant rats accumulate IFN-γ, IL-10 and FoxP3 transcripts.

Next, the transcriptional profiles of purified $CD25^-$ T cells from spleens of tolerant animals were compared with those from naive LEW.1A animals by real-time PCR. IFN-γ and IL-10 cytokine transcripts were significantly increased in tolerant animals, (9 fold and 64 fold respectively, $p<0.05$; Fig.5.A). Tolerant animals exhibited similar levels of IL-2, TNF-α, IL-13, and TGF-β 1 transcripts as naive animals. In addition, because the transcriptional factor FoxP3 has been shown to program regulatory $CD25^+$ T cells development and function [30-32], we analysed its expression by regulatory $CD25^-$ T cells population. FoxP3 transcripts accumulated significantly but moderately (3-fold) in $CD25^-$ T cells of tolerant animals vs. $CD25^-$ T cells of naive animals ($p<0.001$; Fig.5.B).

Discussion

In this study, we investigated the nature of T regulatory cells and the potential regulatory role of $CD25^-$ T cells in tolerant rat recipients of MHC incompatible heart transplant following pre-graft donor cell priming. In mice and rat, regulatory $CD25^+$ T cells have been shown to control host anti-graft responses in recipients tolerant to skin allograft following multiple pre-transplant donor-specific transfusions [20, 27]. $CD25^-$ T cells have been also suggested to harbor a regulatory population able to control responses against minor MHC incompatible antigen ([33] and [28] for review). Here, we show that regulatory $CD25^-$ T cells are present in long term tolerant recipients (>100 days) of MHC incompatible grafts, and we provide some of their functional characteristics. These $CD25^-$ T cells exhibit *in vitro* and *in vivo* regulatory effects including the transfer of tolerance in a fully MHC incompatible model.

Our data show that $CD25^-$ T cells in tolerant animals share several characteristics of $CD4^+$ regulatory T cells induced by immature DCs *in vitro* [34], and exhibit more similarity to the natural regulatory $CD4^+CD25^+$ cells than to Tr1 regulatory cells. However, we also show that they share a key property with Tr1 cells [35]: the capacity to produce both high levels of IL-10 and FoxP3 transcripts, but not of IL-2. Their other characteristics are close to some of the well established properties of natural $CD25^+$ regulatory T cells, such as the need for cellular contact [36], the absence of *in vitro* IL-4, IL-10 mediated effect [36-39], the reversal of the inhibition by exogenous IL-2 [40] and the absence of CDR3 length distribution biases [41-43]. The origin of the regulatory $CD25^-$ T cells are not yet known, and further experiments are needed to better understand their similarities and differences with $CD4^+$ regulatory T cells induced by immature DCs *in vitro*.

The acquisition of antigen specific regulatory functions by $CD25^-$ T cells in tolerant animals could reveal an education [44, 45] of conventional alloreactive $CD25^-$ T cells by “natural” regulatory $CD25^+$ T cells [37, 38]. This hypothesis has to reconcile with the apparent absence

of expansion of the regulatory $CD25^+$ T cells pool in tolerant recipients. Indeed, no increase in the percentage of $CD25^+$ T cells from tolerant animals was noted nor in their inhibitory properties. Furthermore, $4x10^6$ of $CD4^+CD25^+$ T cells from tolerant animals were not able to prolong graft survival following transfer to a naive host. Recently, Pirenne and colleagues have published a detailed and careful description of Tregs induced by DST alone [27], in a closely related DST model. These authors found that both $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells had the ability to transfer tolerance. As mentioned by these authors, caution should be used when extrapolating the data to different experimental settings because the operating mechanisms may depend upon the strain combination and the species used. In our experiments, despite the fact that the amount of $CD25^-$ or $CD25^+$ T cells transferred roughly correspond to a "spleen equivalent" in terms of numbers of cells transferred, the number of $CD25^+$ T transferred may be still insufficient. However, previous studies from our laboratory have shown that a roughly similar quantity of purified $CD4^+CD25^+$ T cells from desoxyspergualine derivate-treated animals was highly efficient in transferring tolerance in the same strain combination suggesting that different mechanisms operate in different tolerance induction models, even in the same genetic background [29].

Regulatory $CD25^-$ T cells derived from induced regulatory $CD25^+$ T cells have been suggested by other studies [46-49]. These studies have shown that $CD25^-$ T cells with regulatory function originally derived from $CD25^+$ precursors [49] and that CD25 expression may not always be a stable marker for regulatory T cells in periphery [49]. $CD25^-$ T cell population also prevented the rejection of a skin graft between minor MHC mismatch mice only if derived from tolerant mice, but not from naive ones [47]. Similarly, in our model, $CD25^-$ T cells exhibited *in vitro* regulatory functions only when derived from tolerant animals and not from naive animals. $CD25^-$ T cells from tolerant rats prolong graft survival and transfer tolerance to roughly half of naive recipients whereas large numbers ($50x10^6$) of T cells from either naive or rejecting LEW.1A rats (sacrificed 95 days after the rejection of their

graft) do not [48]. Graca *et al.* have suggested that some "tolerant" $CD25^+$ regulatory cells may loose the expression of CD25 and endow the $CD25^-$ population with new regulatory properties. Finally, Gavin *et al*. demonstrated that during the homeostasis process, $CD4^+$ $CD25^+$ T cells that had divided more than five times no longer expressed the CD25 marker but remained highly potent for suppression [46].

Previous works from our laboratory showed that, after donor cell priming in the same strain combination, high levels of TGF-β1 mRNA and active protein are found in the graft in the first days following transplantation (i.e. during tolerance induction phase). Moreover, injection of a neutralizing anti TGF-β1 antibody abrogated allograft tolerance [13]. The role of TGF-β1 has also been shown in another strain combination using another protocol to induce tolerance [50]. Fantini *et al.* have demonstrated that TGF-β1 *in vitro* can induce a conversion of naive $CD4^+CD25^-$ T cells into regulatory T cells able to inhibit alloreactive T cells, with induction of FoxP3 [51]. Here, we show *in vivo* that regulatory $CD25^-$ T cells from tolerant rats also accumulate FoxP3 transcripts whereas naive $CD25^-$ T cells do not. To which extend the early up regulation of TGF-β1 observed in our model [13] may have influenced the differentiation of regulatory $CD25^-$ T cells is, however, unknown. Kinetic studies are also needed to test when these cells appear and particularly if they are present at the induction phase of tolerance or only later during maintenance phase.

$CD25^-$ T regulatory cells exhibited an increased level of IL-10 transcripts. Late (day 90) IL-10 and IL-4 expression has also been reported by Koshiba *et al.* in tolerated grafts (and not in rejected ones) in DST-treated long term recipients of heart allografts [52]. Despite the IL-10 transcript up-regulation, our data do not suggest that this cytokine is instrumental at least in their *in vitro* regulatory functions since they are unaffected by the addition of blocking antibodies against IL-10 in the coculture system. A similar pattern has been recently reported for a new T cell regulatory subset in the rat ($CD8^+$ $CD45RC^{low}$) [53]. However, our data do

not exclude that IL-10 production by these cells could have an *in vivo* role in the maintenance of tolerance. Similarly, IFN-γ production may have a potential role *in vivo*. IFN-γ KO mice have been shown to be refractory to tolerance induction [54], and in rats we previously demonstrated that a blocking anti-rat IFN-γ antibody inhibited rather than potentialized the effect of DST on a subsequent heart transplant [55].

In summary, this study provides a detailed description, *in vitro and in vivo*, of the features of regulatory $CD25^-$ T cells induced by DST. Regulatory $CD25^-$ T cells from long term allograft tolerant recipients are able to transfer graft survival to fully MHC incompatible naive recipients and extend our knowledge on their mechanisms of action.

Material & Method

Animal Model, Induction of Tolerance and *in vivo* transfer experiments.

Naive adult MHC mismatched congeneic LEW.1A ($RT1^a$), LEW.1W ($RT1^u$) and BN ($RT1^n$) rats were purchased from Janvier (Savigny/Orge, France). LEW.1W or BN heart grafts were implanted heterotypically onto LEW.1A recipient abdominal aorta and vena cava by using standard microsurgical techniques [56]. Graft was monitored by daily abdominal palpation and rejection was defined as complete cessation of heartbeat.

Long term survival of heart graft was obtained by two donor specific transfusion (DST) of 1 mL of heparinized LEW.1W blood to LEW.1A recipients on preTx days –14 and –7 [7].

Adoptive cell transfers were performed the day of the transplantation on a LEW.1A secondary naive recipient sub irradiated (4.5 Gy) 3 days before transplantation.

Reagents

All cells were grown in RPMI 1640 (Sigma, St Louis, USA) supplemented with 10% heat-inactivated autologous serum, 100 U/ml penicillin, 100 U/ml streptomycin, 2mM L-glutamine (Sigma), 0.1mM non-essential amino acids (Sigma), 1mM sodium pyruvate (Sigma) and 50µM β-ME (Sigma). Antibodies, anti-CD25 (Ox39) or anti-CD4 (W3.25), were obtained from the European Collection of Animal Cell Cultures (Salisbury, U.K). MARG 2a-7 (IgG2a), 2b-3(IgG2b), 2c-5(IgG2c) and 1-2 (IgG1) antibodies were purchased from TECHNOPHARM (Villejuif, France) and fluorescein isothiocyanate-conjugated (FITC) IgM was purchased from Jackson Immunology Research (Villepinte, France).

Purification of T cells and subsets, and mixed lymphocyte reaction (MLR)

Purified T cells were obtained from spleen of naive LEW.1A rats or from tolerant DST-treated LEW.1A rats either with rat $CD3^+$ T cell small enrichment columns (R&D Systems,

UK) or after warm nylon wool adherence and depletion of Ox6 (MHC II) 3.2.3 (CD161) positive cells using Dynal-magnetic strategy (Invitrogen SARL, Cergy Pontoise, France). T cell purity, systematically assessed using flow-cytometry, was >95% of TCR^+, with no detectable MHC class II^+ cells. Cells were labelled with biotinylated-Ox39, Ox35-PE and R7.3-FITC and $CD4^+CD25^+$ T cells were sorted with FACSARIA (purity > 99%). Negative selection of $CD25^-$ T cells was performed using Ox39 mAb followed by incubation with Dynabeads® Pan Mouse IgG according to the manufacture's recommendations (purity >95%).

Mixed lymphocyte reaction (MLR) and transwell experiments.

According to experiments, $1x10^5$ $CD25^-$ or $CD25^+$ or whole T cells purified from LEW.1A rats were cultured in triplicate in U-bottom 96-well plates (0.2mL) with $2x10^4$ of APC, obtained as described elsewhere [57], from either LEW.1W or BN rats for 5 days at 37°C/7% CO_2. For inhibition assessment, increase numbers of $CD25^+$ or $CD25^-$ T lymphocytes purified from naive or tolerant rats were added to a MLR using naive $CD25^-$ T cells as responder's cells. Transwell experiments were done in 24-well plates. $6x10^5$ $CD25^-$ T cells of naive LEW.1A rats were stimulated with $3x10^5$ of APC from LEW.1W rats. Additionally, $6x10^5$ $CD25^-$ T cells of either naive or tolerant LEW.1A plus 10^5 of APC from LEW.1W rats were placed in Transwell chambers (Millicell, 0.4mm; Millipore) in the same well.

When indicated, MLRs or cocultures were cultured in the presence of the following mouse blocking mAbs: anti-rat IL-4 (Ox81; ECACC, Wiltshire, United Kingdom), anti-TGF-β1 (clones 2G7) [58], anti-rat IL-10 and an irrelevant control (3G8, anti-human CD16; American Type Culture Collection, Bethesda, MD). Rabbit neutralizing anti-rat IL-10 and rabbit IgG were both kindly provided by J. Khalife (Institut Pasteur, Lille France). Anti IL-10 and anti-rat IL-4 were used at 10 µg/mL, and anti-rat TGF-β 1 at 100 µg/mL. 5 µL of L-N Methyl

Arginine (Sigma) or 1mg/mL of 1-methyltriptophane (Sigma) were added to the culture. Human recombinant IL-2 was used as indicated at 100 U/mL.

All cultures were pulsed with [^{3}H] T for the last 12 hours of culture and the results were expressed as specific c.p.m (c.p.m MLR-(c.p.m APC alone + c.p.m cells alone)).

Quantitative PCR of cytokine transcripts.

Transcript analysis was performed using real-time quantitative PCR for HPRT, IL-2, TNF-α, IFN-γ, IL-10, IL-13, TGF-β 1 and FoxP3. Total RNA was isolated using RNeasy Mini Kit (Qiagen®, Courtaboeuf, France). 2 µg RNA was reverse-transcribed as previously described [59]. A constant amount of cDNA was amplified in 25 µl SYBR® Green PCR Core Reagent (Applied Biosystems, Foster city, CA) with 1mg/mL of BSA, 3 mM $MgCl_2$, 200 µM of each dNTP, 0.6 U AmpliTaq Gold® polymerase, and 300 nM of each primer in 1X SYBR® Green PCR buffer. Amplifications were performed using an ABI Prism 7700 machine (Applied Biosystems). HPRT was used as an endogenous control to normalize RNA amounts. Transcript levels were calculated according to the 2^{-ddCt} method as described by the manufacturer (ABI PRISM 7700 user bulletin PE Applied Biosystems Foster City 2:11-24, 1997) and expressed in Arbitrary Unit (AU). Profiles for transcript levels were further confirmed by using beta-actine gene as another endogenous control to normalize RNA amounts. Primer sequences are available upon request.

Statistical analysis

Mann-Whitney test or Kruskal-Wallis test followed by a Dunn's post-hoc test were performed. Survival curves were analyzed with Kaplan-Meier test. P values less than .05 were considered as significant.

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Legends

Figure 1. $CD25^-$ T cells from long term tolerant recipients are hyporesponsive in a donor specific antigen manner. Splenic T cells or T cells subsets ($CD25^+$ or $CD25^-$) were obtained from tolerant recipients (black bar) or naive (white bar) LEW.1A rats and were stimulated with LEW.1W rat APCs (A) or third-part APCs (B). Results are representatives of four independent experiments and bars represent mean +/- SEM. Statistical significance was tested using the Mann-Whitney test.

Figure 2. $CD25^-$ T cells of long term tolerant animals inhibit alloreactive naive T cells. Various ratios of $CD25^-$ T cells (A) or $CD25^+$ T cells (B), obtained from tolerant recipients (black bar) or naive (white bar) LEW.1A rats, were added to $1x10^5$ naive $CD25^-$ T cells in LEW.1W MLRs. Bars represent mean +/- SEM and are representatives of one out of at least four independent experiments (n=24 for $CD25^-$ T cells exp. and n=4 for $CD25^+$ T cells exp.). Efficiency of inhibition is also displayed as percentage of proliferation of naive $CD25^-$ T cells in MLRs. Statistical significance was tested using the Mann-Whitney test. C. Antigen specificity of inhibition observed with $CD25^-$ T cells from tolerant animals was assessed by adding various ratios of $CD25^-$ T cells to $1x10^5$ naive $CD25^-$ T cells in MLRs using BN APCs as stimulators (n=6).

Figure 3. $CD25^-$ T cells of tolerant animals transfer tolerance to naive LEW.1A recipients. Survival of LEW.1W heart graft: ◇, untreated sub-lethally irradiated LEW.1A recipients (MST = 17 days, n=17); ■, sub irradiated LEW.1A recipients transferred with $50x10^6$ $CD25^-$ T cells from tolerant animals (MST > 100 days, n=9; $p<0.05$); ●, sub-lethally irradiated LEW.1A recipients transferred with 1 to $4x10^6$ $CD4^+CD25^+$ T from tolerant animals (MST = 17 days, n=4). ▲, sub irradiated LEW.1A recipients transferred with $50x10^6$ $CD25^-$ T cells from tolerant animals and grafted with BN heart graft (MST =10 days, n=3). Statistical significance was tested using the Kaplan-Meier test.

Figure 4. Suppression mediated by alloantigen-induced CD25$^-$ T cells is cell contact dependent. A. Anti IL-4, anti IL-10 or anti TGF- β antibodies (n=5), or inhibitors of IDO (L-NMMA) or iNOS (1-MT) activity (n=3), or IL-2 (n=11) were added to a tolerant CD25$^-$ T cells in MLR. Bars represent mean +/- SEM. Statistical significance was tested using the Kruskal-Wallis test. B. Transwell chambers were used to prevent direct cell-contact between suppressive CD25$^-$ T cells (tolerant animals; n=3) and alloreactive CD25$^-$ T cells (naive animals; n=3). Bars represent cpm +/- SEM obtained in the lower chamber. C. Anti IL-4, anti IL-10 or anti TGF- β antibodies (n=6), or inhibitors of IDO (L-NMMA) or iNOS (1-MT) activity (n=7) were added to $1x10^5$ CD25$^-$ T cells from tolerant recipients cultured with $1x10^5$ CD25$^-$ T cells from naive rats in MLRs using LEW.1W APCs as stimulators. Bars represent mean +/- SEM.

Figure 5. CD25$^-$ T cells from long term tolerant rats accumulate IFN- gamma, IL-10 and FoxP3 transcripts. Real-time RT-PCR analysis of cytokines (IL-2, TNF-α, IFN-γ, IL-10, IL-13, TGF-β1) (A) and of FoxP3 (B) expression in CD25$^-$ T cells from either tolerant (n=6) or naive (n=6) LEW.1A rats. Bars represent mean +/- SEM and statistical significance was tested using the Mann-Whitney test.

Figure 1.

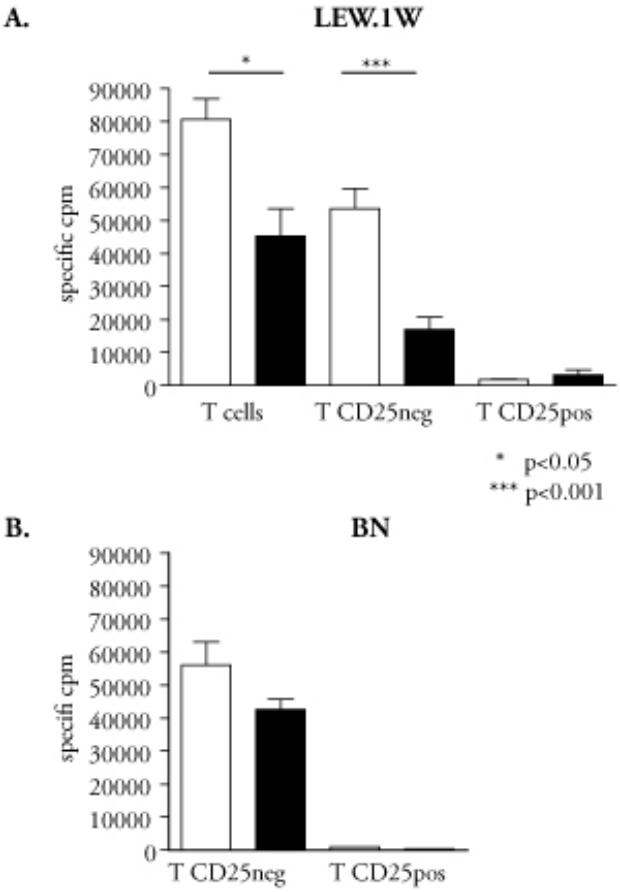

A.
LEW.1W
*
***
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
specific cpm
T cells
T CD25neg
T CD25pos
* p<0.05
*** p<0.001
B.
BN
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
specifi cpm
T CD25neg
T CD25pos

Figure 2.

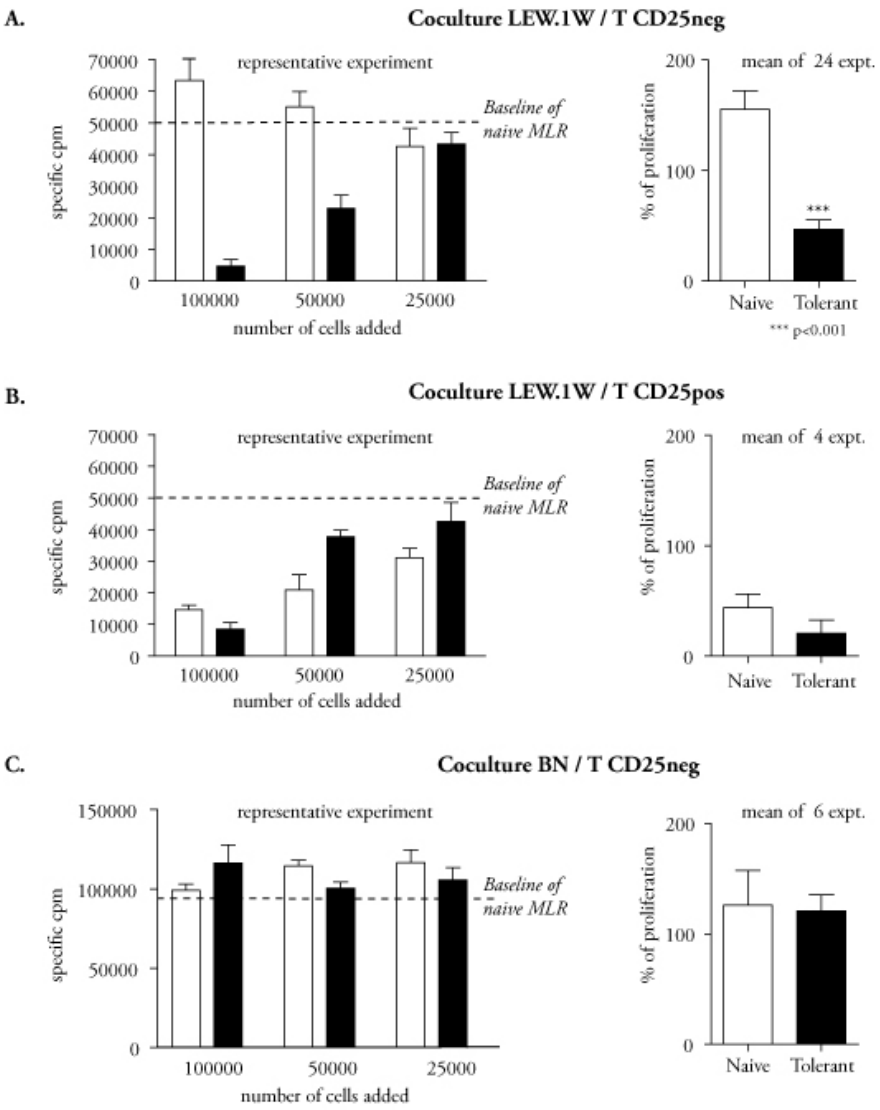

A.
Coculture LEW.1W / T CD25neg
representative experiment
specific cpm
70000
60000
50000
40000
30000
20000
10000
0
Baseline of naive MLR
100000
50000
25000
number of cells added
mean of 24 expt.
% of proliferation
200
100
0
***
Naive
Tolerant
*** p<0.001
B.
Coculture LEW.1W / T CD25pos
representative experiment
specific cpm
70000
60000
50000
40000
30000
20000
10000
0
Baseline of naive MLR
100000
50000
25000
number of cells added
mean of 4 expt.
% of proliferation
200
100
0
Naive
Tolerant
C.
Coculture BN / T CD25neg
representative experiment
specific cpm
150000
100000
50000
0
Baseline of naive MLR
100000
50000
25000
number of cells added
mean of 6 expt.
% of proliferation
200
100
0
Naive
Tolerant

Figure 3.

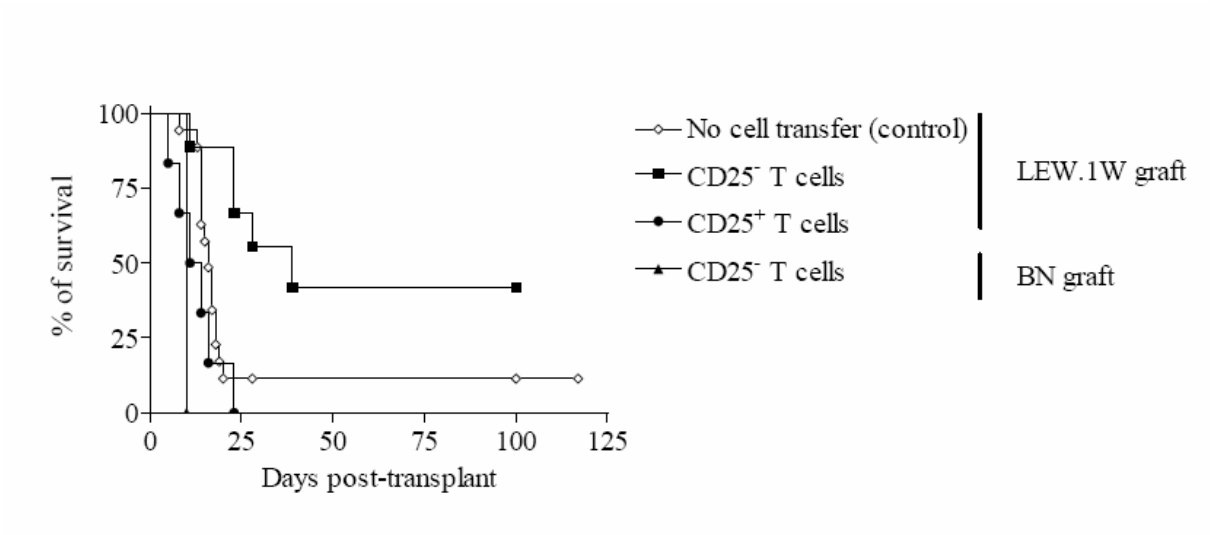

100
75
50
25
0
% of survival
0
25
50
75
100
125
Days post-transplant
No cell transfer (control)
CD25- T cells
CD25+ T cells
CD25- T cells
LEW.1W graft
BN graft

Figure 4.

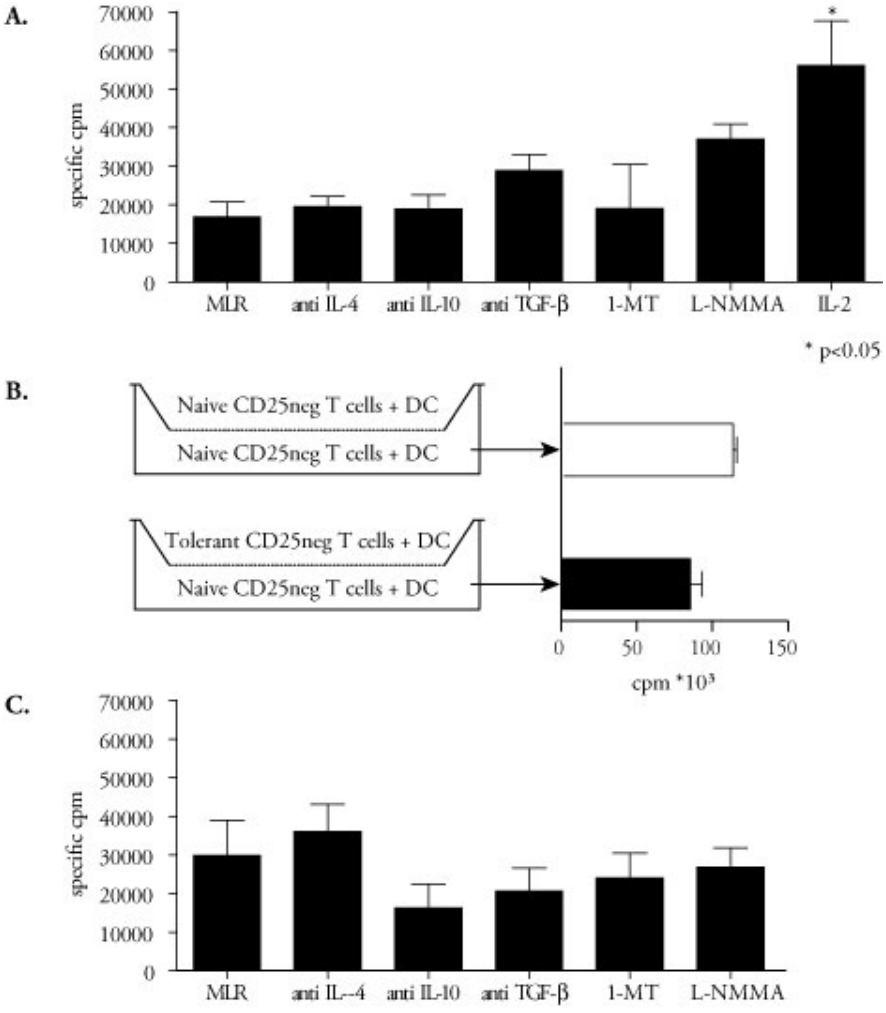
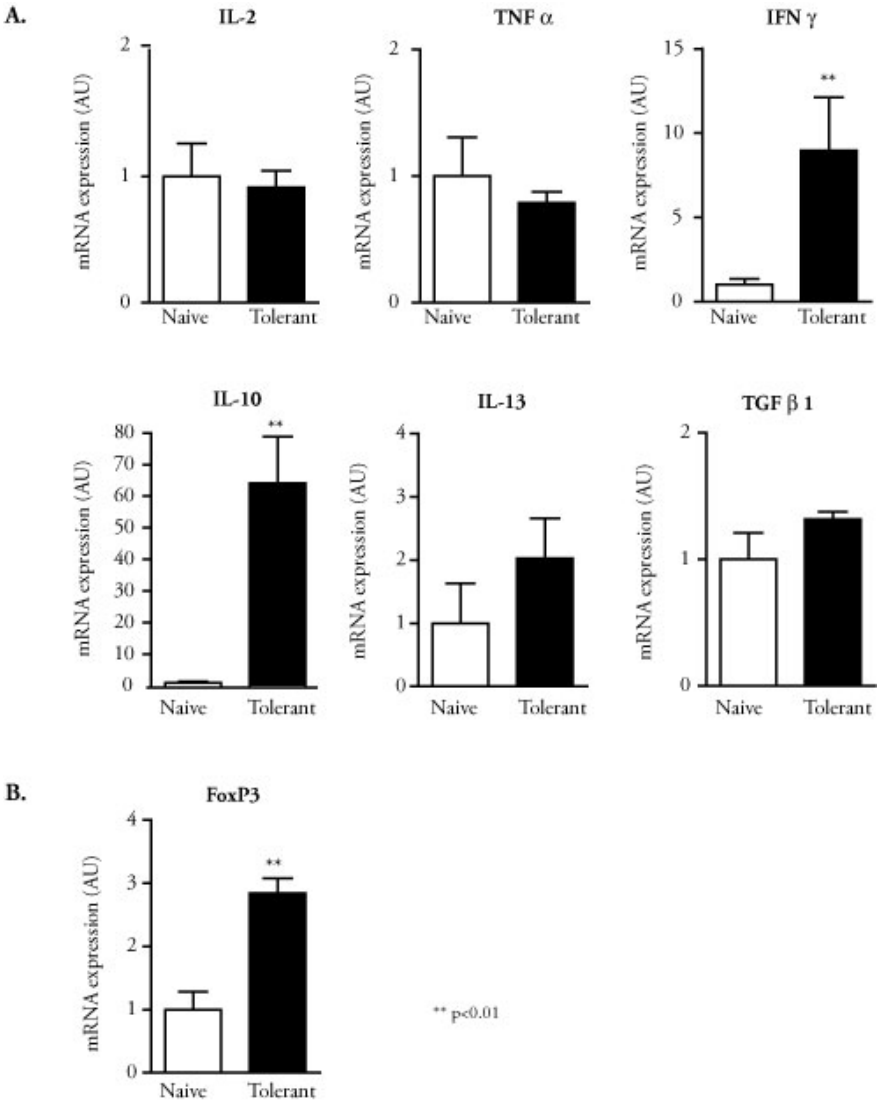

A.
specific cpm
70000
60000
50000
40000
30000
20000
10000
0
*
MLR
anti IL-4
anti IL-10
anti TGF-β
1-MT
L-NMMA
IL-2
* p<0.05
B.
Naive CD25neg T cells + DC
Naive CD25neg T cells + DC
Tolerant CD25neg T cells + DC
Naive CD25neg T cells + DC
0
50
100
150
cpm *10³
C.
specific cpm
70000
60000
50000
40000
30000
20000
10000
0
MLR
anti IL--4
anti IL-10
anti TGF-β
1-MT
L-NMMA

Figure 5.


A.
IL-2
TNF α
IFN γ
IL-10
IL-13
TGF β 1
mRNA expression (AU)
Naive
Tolerant
**
B.
FoxP3
** p<0.01

II.2.

Discussion

Dans cette étude, nous avons cherché à caractériser les cellules régulatrices T présentes dans la rate de rats «tolérants» une greffe cardiaque allogénique après un protocole de transfusion spécifique du donneur (TSD). Cette étude montre que les cellules $TCD25^-$ chez les animaux «tolérants» présentent des propriétés régulatrices *in vitro* et *in vivo*. Ces cellules $TCD25^-$ sont comparables de par leurs fonctions aux cellules régulatrices $CD4^+CD25^+$ plus classiquement décrites, à savoir une activité suppressive de la prolifération de cellules $TCD25^-$ naïves contre des antigènes du donneur qui dépend de contacts cellulaires directs, elles sont anergiques et ne prolifèrent pas en réponse à des antigènes du donneur. Cependant, ces cellules $TCD25^-$ partagent avec les cellules régulatrices Tr1 une de leur principale propriété: la production à un niveaux élevés de transcrits codant pour l'IL-10 mais pas de transcrits pour l'IL-2 (Groux et al. 1997). Ces cellules $TCD25^-$ sont caractérisées également par la présence du marqueur FoxP3. Leurs fonctions se rapprochent de celles des cellules régulatrices classiques $TCD25^+$. Elles nécessitent un contact cellulaire directe pour agir (Thornton et al. 1998), il est également possible de rétablir la réponse proliférative vis-à-vis du donneur en MLR par l'ajout d'IL-2 exogène (Sakaguchi et al. 1995). Enfin, ces cellules ne montrent pas de variation dans la distribution de la longueur du CDR3 (Pacholczyk et al. 2002; Romagnoli et al. 2002; Kasow et al. 2004). L'origine des cellules T CD25- régulatrices demeure inconnue. De nombreuses publications évoquent la possibilité d'une conversion des cellules T effectrices en cellules T régulatrices. Certaines études (Stephens et al. 2000; Gavin et al. 2002; Graca et al. 2002) ont également suggéré que les cellules régulatrices $TCD25^-$ proviendraient des cellules régulatrices $TCD25^+$. Graca et al ont montré que les cellules régulatrices $CD25^+$ peuvent perdre l'expression du marqueur CD25 (Graca et al. 2002). De plus, Gavin et al ont démontré que pendant le processus d'homéostasie, les cellules $TCD4^+CD25^+$ qui s'étaient divisées plus de cinq fois n'exprimaient plus le marqueur CD25 mais conservaient leurs propriétés suppressives (Gavin et al. 2002). Ces études suggèrent que l'expression du marqueur CD25 n'est pas toujours un marqueur fiable pour les cellules T régulatrices en périphérie (Stephens et al. 2000).

Récemment, Pirenne et al (Kitade et al. 2005), ont montré, dans un modèle équivalent de transfusion spécifique du donneur, que les cellules $TCD4^+CD25^+$ et les cellules $TCD4^+CD25^-$ avaient la capacité de transférer la tolérance à un animal greffé non traité. Cependant, comme le précise les auteurs, il faut être prudent car les mécanismes mis en jeu peuvent dépendre du modèle utilisé. Dans nos expériences, malgré le fait que la quantité de cellules $TCD25^-$ ou

TCD25$^+$ transférées correspond, en terme de nombres de cellules transférées, à la proportion des cellules présentes naturellement dans une rate d'un animal contrôle non traité, le nombre de TCD25$^+$ transférées peut être encore insuffisant. Cependant, des études précédentes de notre laboratoire ont montré qu'une quantité semblable de cellules TCD4$^+$CD25$^+$ d'animaux traités par la deoxyspergualine étaient capables de transférer la tolérance dans la même combinaison LEW.1W sur LEW.1A suggérant que différents mécanismes interviennent selon les modèles d'induction de tolérance (Chiffoleau et al. 2002).

Dans notre modèle, les cellules TCD25$^-$ d'animaux «tolérants» ont des fonctions régulatrices *in vitro*, propriétés que n'ont pas les cellules TCD25$^-$ mais les TCD25$^+$ d'animaux naïfs. Les cellules TCD25$^-$ issues de la rate des rats «tolérants» prolongent la survie d'une greffe ou induisent une «tolérance» à des animaux irradiés et greffés sans traitement lorsqu'elles sont transférées, alors qu'on ne peut pas transférer cette «tolérance» avec des TCD25$^-$ d'animaux naïfs.

Les travaux de notre laboratoire ont montré l'importance du TGF-β1 lors de la phase d'induction de «tolérance» suite à une transfusion spécifique du donneur (Josien et al. 1998). De plus, Fantini et al ont démontré que le TGF-β1 *in vitro* pouvait induire une conversion des cellules naïves TCD4$^+$CD25$^-$ en cellules régulatrices avec l'induction du marqueur FoxP3 (Fantini et al. 2004). Dans cette étude, nous montrons *in vivo* une augmentation des transcrits FoxP3 portés par les cellules régulatrices TCD25$^-$ des animaux «tolérants» contrairement aux cellules TCD25$^-$ des animaux non traités. Cependant, nous ne savons pas dans notre modèle dans quelle mesure la présence précoce du TGF-β1 peut influencer la génération des cellules régulatrices TCD25$^-$.

Nous avons montré également que ces cellules régulatrices TCD25$^-$ avaient un niveau élevé de transcrits IL-10. Or, Malgré une sur-régulation de l'IL-10, nos résultats ne suggèrent pas que cette cytokine soit impliquée dans la fonction régulatrice de ces cellules *in vitro* puisque cette fonction n'est pas modifiée par l'addition d'anticorps bloquant l'IL-10 dans un système ou les TCD25$^-$ des animaux «tolérants» inhibent une MLR entre des TCD25$^-$ naïfs de rat LEW.1A et des cellules présentatrices d'antigènes LEW.1W. Cependant, nos résultats n'excluent pas que la production d'IL-10 par ces cellules pourrait avoir un rôle *in vivo* dans le maintien de la «tolérance» comme l'ont montré Koshiba et al, qui observent une forte expression d'IL-10 et d'IL-4 dans les greffes tolérées des receveurs d'allogreffe cardiaque après transfusion spécifique du donneur à distance de la greffe (Koshiba et al. 2003). Et récemment, le même type de profil a été publié pour une autre population de cellules T régulatrices CD8$^+$CD45RC^{low} chez le rat (Xystrakis et al. 2004).

De même, la production d'IFN-γ a un rôle *in vivo.* En effet, chez le rat, il a été montré dans notre laboratoire qu'un anticorps bloquant anti-IFN-γ de rat bloque l'effet de la transfusion spécifique du donneur (Paineau et al. 1991).

III. Article 3:

Maintien de tolérance et compartimentation des phénomènes de régulation, chez les rats présentant une prolongation de survie après transfusion spécifique du donneur.

Titre original:

Distinct lymphoid compartments are playing different critical roles in DST-treated long term recipients.

David Lair*, Nicolas Degauque*, Patrick Miqueu, Vojislav Jovanovic, Marina Guillet, Emmanuel Mériau, Anne Moreau, Jean-Paul Soulillou* and Sophie Brouard*.

Soumis

III.1.

Résumé de l'article

Deux transfusions spécifiques du donneur (TSD) 14 et 7 jours avant la greffe entraînent la survie d'une greffe de cœur LEW.1W chez un receveur LEW.1A incompatible pour les molécules du CMH de classe I et II. Dans ce modèle, nous avons caractérisé les différents compartiments immuns impliqués dans la survie à long terme du greffon, leur capacité à transférer la tolérance et à protéger du rejet chronique. Les splénocytes et les lymphocytes T de la rate des receveurs traités par TSD transfèrent la survie à long terme d'une greffe chez 100% des animaux greffés irradiés non traités. Nous avons pu mettre en évidence que les lymphocytes T de la rate se caractérisent par un répertoire du TCR non altéré, une augmentation du nombre de cellules $TCD4^{+}CD25^{+}$, des propriétés suppressives, une diminution de la réponse anti-donneur et un taux normal de transcription des molécules perforine et granzyme par rapport à des animaux naïfs. En revanche, la situation rencontrée dans le sang est très différente. Les cellules monocytaires obtenues après gradient de densité à partir du sang périphérique transfèrent la survie à long terme d'une greffe chez 50% des receveurs secondaires irradiés non traités, alors que les lymphocytes T purifiés, eux, ne transfèrent pas cette «tolérance». On a pu noter pour cette population, une forte altération du répertoire du TCR, principalement portée par la population $CD8^{+}$, un nombre normal de cellules $TCD4^{+}CD25^{+}$, une réponse anti-donneur normale, un phénotype activé des lymphocytes T et un taux accru de transcription des molécules perforine et granzyme. Cependant, tous les animaux, que ce soit les receveurs traités par TSD ou bien les receveurs secondaires après transfert de cellules du sang ou de la rate, montrent des signes de rejet chronique. Ces résultats montrent que les cellules T régulatrices capables de transférer la survie à long terme ne s'accumulent pas au niveau sanguin, qui apparaît principalement comme un réservoir de cellules cytotoxiques. Par contre, les cellules de la rate, qui montrent un profil régulateur et qui sont capables de transférer la tolérance ne peuvent pas empêcher la survenue du rejet chronique.

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Functional Compartmentalization Following Induction of Long-Term Graft Survival with Pregraft Donor-Specific Transfusion.

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Long-term survival is achieved in rat recipients by pre-graft donor-specific blood transfusion. We characterized the immune compartments in long-term survivors and analyzed them for capacity to transfer tolerance and protect against chronic rejection. Splenocytes and spleen T cells from treated recipients transferred long-term graft survival to 100% of secondary recipients. In contrast, blood transferred graft survival to only 50% of recipients whereas blood T cells had no effect. An unaltered TCR repertoire, an increase in suppressive CD4(+)CD25(+) T cells, a decrease in antidonor T-cell proliferative response and normal perforin-granzyme levels were the hallmarks of the spleen T cells. Blood T cells were characterized by a strongly altered CD8(+) repertoire, normal CD4(+)CD25(+) T cell number with unchanged antidonor T-cell proliferative response, an activated T-cell phenotype and an increase in perforin-granzyme levels. However, following the transfer of blood or spleen cells into secondary recipients, all grafts displayed chronic rejection. These findings provide evidence that distinct compartments play critical roles in DST recipients. Regulatory cells do not accumulate in blood, which appears to be a reservoir for cytotoxic T cells. Spleen T cells, which display a regulatory-like profile and transfer graft survival, are not able to prevent chronic rejection.

PMID: 17217443 [PubMed - as supplied by publisher]

FUNCTIONAL COMPARTMENTALIZATION FOLLOWING INDUCTION OF LONG-TERM GRAFT SURVIVAL WITH PRE-GRAFT DONOR-SPECIFIC TRANSFUSION.

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Abbreviations:

LEW.1A: Lewis 1A

LEW.1W: Lewis 1W

BN: Brown Norway

TCR: T Cell Receptor

PCR: Polymerase Chain Reaction

PBS: Phosphate Buffered Saline

DST: Donor Specific Transfusion

FCS: Fetal Calf Serum

I.V: intravenously

PBMC: Peripheral Blood Mononuclear Cells

CDR3-LD: Complementary Determining Region 3 Length Distribution

MLR: Mixed Leukocyte Reaction

Gy: Gray

APC: Antigen Presenting Cells

Abstract

Long term survival of vascularised organs is achieved in rat recipients of MHC incompatible heart transplants by pre-graft donor specific transfusion (DST). We used this model to characterize the different immune compartments in long-term survivors and to analyze the capacity of cells from each compartment to transfer tolerance and to protect against chronic rejection. Splenocytes and purified spleen T cells from DST-treated recipients transferred long-term graft survival to 100% of naive secondary sub-irradiated untreated recipients. In contrast, peripheral blood lymphocytes transferred long-term graft survival to only 50% of naive secondary sub-irradiated untreated recipients, whereas purified blood T cells from DST-treated recipients had no effect. An unaltered TCR repertoire usage, an increase in $CD4^+CD25^+$ T cells with suppressive properties, a decrease in the anti-donor T cell proliferative response and a normal level of perforin-granzyme transcript expression were the hallmarks of the spleen T cells of DST-treated recipients. In contrast, the blood T cells of DST-treated recipients was characterized by strongly altered $CD8^+$ TCR repertoire usage, normal $CD4^+CD25^+$ T cell levels with unchanged anti-donor T cell proliferative responses, an activated T cell phenotype and an increased perforin-granzyme transcript expression . However, following transfer of blood or spleen cells into secondary naive recipients, the grafts from the animals that reached 100 days survival all displayed signs of chronic rejection. These findings provide compelling evidence that distinct lymphoid compartments play critical and different roles in DST-treated long-term graft recipients. Regulatory T cells capable of transferring long-term survival do not accumulate in the blood, which appears to be mainly a reservoir for cytotoxic T cells accumulating perforin/granzyme transcripts. Nevertheless, the spleen T cells, which display a regulatory-like profile and transfer long-term graft survival in secondary recipients, are not able to prevent the onset of chronic rejection.

Introduction

A major goal in transplantation is to achieve an immunological state of allo-antigen donor-specific tolerance in the absence of chronic immunosuppression (1). Although this has been achieved in a number of animal models (2-4), it remains difficult to reproduce in large animals (5, 6) and in the clinic (7, 8). However, understanding the precise cellular and molecular mechanisms of induction and maintenance of immune tolerance to allografts in adult organisms is of paramount importance for the conception of clinical protocols. It is now known that, in addition to clonal anergy, active suppression may largely contribute to graft survival (9). Thus, $CD4^+CD25^+$ T cells with regulatory activities, the most extensively studied population to date, have been shown to play an important regulatory role in several animal models of autoimmune diseases (10) and transplantation (11). $CD4^+CD25^+$ T cells with regulatory activities *in vitro* have also been found to be present in decreased numbers in human peripheral blood of patients suffering from chronic rejection (12-14). Nevertheless, although several studies have defined the cellular mechanisms by which regulation may develop (9, 11, 12, 15-17), few studies have analyzed their anatomic compartmental distribution and where the "tolerization" phenomenon takes place (18) (19).

Because donor-specific transfusion does not necessarily require other immunotherapies to induce long-term allograft survival (20-22) and the beneficial effect of this method of tolerance induction in allograft models is well recognized (8, 21, 23). Several studies have also shown the large implication of $CD4^+CD25^+$ in this model (2, 4, 19, 24). We used this model to characterize the different immune compartments of long-term allograft survivors and to analyze these compartments for their capacity to transfer tolerance and/or to protect against chronic rejection.

Materials and methods

Animals: Eight-week-old male, 250g congenic LEW.1W ($RT1^u$) and LEW.1A ($RT1^a$) rats were purchased from Janvier (Savigny/Orge, France). All animals were maintained under standard conditions according to European and Institutional Guidelines.

Model of DST-induced long-term survival of heart allografts: LEW.1A rat recipients were transfused I.V. with 1 ml of blood from a LEW.1W donor 14 and 7 days before transplantation. Heterotopic LEW.1W cardiac transplantation was performed as previously described (25). Graft function was evaluated daily by abdominal palpation and rejection was defined as the day of cessation of heart beating and confirmed by laparotomy.

Purification of T cells and subsets: Spleen cells were harvested from DST-treated recipients and rejecting recipients one hundred days after transplantation. Cells were isolated by passing the spleen through a stainless steel mesh. Erythrocytes were depleted by osmotic shock. Mononuclear cells were washed twice in PBS and resuspended in sterile RPMI 1640 (Life Technologies, Grand Island, NY). T cells were purified from spleen cells using Rat T cell enrichment columns (R&D Systems, Lille, France). Purity was checked by flow-cytometry and was typically ≥ 94%. $CD4^+$ or $CD8^+$ cells were enriched by negative selection with purified anti-CD8 mAbs or anti-CD4 mAbs respectively, follow by incubation with Pan Mouse IgG magnetic Dynabeads® (Dynal, Invitrogen, Cergy Pontoise, France). Negative selection of $CD25^-$ T cells was performed using the Ox39 mAb followed by incubation with Pan Mouse IgG Dynabeads® (purity >95%). Blood from DST-treated recipients was collected in EDTA tubes by cardiac puncture. PBMC were separated on a Ficoll® layer (Amersham biosciences, Uppsala, Sweden) and T cells were purified using the same protocol as described above. Non-T cells were purified from PBMC: mononuclear cells were isolated as described above and T cells depleted by negative selection with a purified anti-TCRαβ mAb (R7.3) and superparamagnetic beads (Dynal, Oslo, Norway).

***Adoptive Cell transfer*:** Spleen cells, spleen T cells, PBMC or blood T cells from DST-treated recipients, naive LEW.1A rats and rejecting recipients were injected I.V on the day of transplantation into a sub-irradiated (4.5 Gy whole body irradiation) LEW.1A secondary naive recipient 3 days before transplantation.

Flow cytometry analysis: cells from DST-treated recipients, naive rats and rejecting recipients were incubated for 30 min at room temperature with FITC-conjugated W3/25 mAb (anti-CD4) (Serotec Laboratories, Oxford, U.K.) and the biotinylated antibodies R7-3 (anti-TCRαβ) and OX39 (anti-CD25α chain) (Bioatlantic, Nantes, France), and were subsequently revealed by Streptavidine-PE (Immunotech, Beckman coulter, Marseille, France). Cells were washed twice in phosphate-buffered saline (PBS) and two-color staining analyzed by a FACSCalibur using Cellquest Pro® software (BD Biosciences, Mountain View, CA). The same protocol was used for complete phenotyping of cells from DST-treated recipients, naive rats and rejecting recipients. The FITC-conjugated antibody R7-3 (anti-TCRαβ) (Bioatlantic, Nantes, France) and the following biotinylated antibodies were used: OX34 (anti-CD2), W3/25 (anti-CD4), OX19 (anti-CD5), OX8 (anti-CD8), JJ319 (anti-CD28), OX33 (anti-CD45RA), OX22 (anti-CD45RC), OX85 (anti-CD62L), OX26 (anti-CD71), OX7 (anti-CD90), OX40 (anti-CD134), OX2 (anti-CD200), OX6 (anti-RT1B class II), OX17 (anti-RTID classII) and OX62 (anti-integrin αE) (Bioatlantic, Nantes, France), and revealed by Streptavidine-PE.

Mixed leukocyte reactions (MLR) and inhibition assays: Irradiated (35 Gy) antigen-presenting cell-enriched cell populations from donor-type LEW.1W or third-party BN (RT1.^{n}) rats served as stimulator cells. 1.10^5 purified responder T cells from the spleen and blood of DST-treated recipients, naive rats or rejecting recipients and 2.10^4 irradiated stimulatory cells were plated in 96-well round-bottom plates in triplicate in 200 µl of RPMI 1640 supplemented with 2 mM L-glutamine, 5.10^{-5} M 2-ME, 1 mM sodium pyruvate (Life

Technologies), 1% nonessential amino acids, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10% heat-inactivated FCS (Life Technologies). Cultures were incubated at 37°C, in 5% CO2, for 5 days and pulsed for the final 8 hours with 0.5 μCi of [^{3}H]TdR (Amersham, Les Ulis, France). The cells were then harvested on glass fiber filters and [^{3}H]TdR incorporation was measured using standard scintillation procedures (Packard Institute, Meriden, CT). For inhibition assessment, increasing numbers of $CD25^+$ T lymphocytes purified from naive LEW.1A rats or DST-treated recipients were added to the MLR using naive $CD25^-$ T cells as responder cells. Cultures were pulsed with [^{3}H]TdR for the final 12 hours of culture. For the inhibition assay, the results were expressed as specific c.p.m (c.p.m MLR-(c.p.m DC alone + c.p.m added cells alone + c.p.m $CD25^-$ alone)).

Histology analysis: Tissue sections (heart grafts) were embedded in paraffin and colored with HPS (hematoxylin phloxin safran). The following quantitative criteria were analyzed: mononuclear cell interstitial inflammation (0: no interstitial parenchyma inflammation, 1: 1-5%, 2: 5-25%, 3: 25-50%, 4: >50%), interstitial fibrosis (0: interstitial fibrosis in up to 5% of the graft area, 1: mild 6 to 25%, 2: moderate 26 to 50%, 3: severe >50%), vascular narrowing (0: no vascular changes, 1: vascular narrowing of up to 25% of the luminal area, 2: 26-50%, 3: severe vascular changes above 50%), vasculitis (0: no vascular lesions, 1: mild endothelitis, 2: inflammatory endarteritis, 3: fibrous endarteritis) and percentage of pathological arterial sections per heart.

RNA extraction and cDNA synthesis: Total RNA was isolated by the Trizol reagent procedure (Invitrogen). Two μg RNA were reverse transcribed using a cDNA synthesis kit (Roche, Indianapolis, IN) and diluted to a final volume of 100 μl.

TCR repertoire analysis: Complementary Determining Region 3 length distribution (CDR3-LD) alteration was analyzed using Immunoscope® software (26). Global CDR3-LD alterations were measured according to Gorochov et al. (27). The profiles obtained from 11

naive rats were used as controls. Vβ/HPRT transcript ratios were measured by real-time PCR (28). Data were displayed as a bidimentional TopView or as a tridimentional TcLandscape® (29, 30) where the x-axis displays the 21 Vβ families, the z-axis shows the Vβ transcripts/HPRT transcripts ratios and the y-axis gives the 13 possible CDR3 lengths per Vβ family. Percentages of alterations were represented as a color code, ranging from deep blue (values -30%) to dark red (30%) in the integrated landscapes (29, 30). MatLab® 5.3 software (The MathWorks Inc, Natick; Maryland;) was used to compute and display the data.

Cytokine transcript analysis: Transcript analysis was performed using real-time quantitative PCR for HPRT, Cβ, IL-2, IFN-γ, IL-13, TGF-β1, IL-10 and TNF-α. A constant amount of cDNA was amplified in 25 µl of 10X SYBR® Green PCR Core Reagent (Applied Biosystems, Foster City, CA). Amplifications were performed in an ABI Prism 7900-Perkin Elmer Sequence Detection system (Perkin-Elmer Applied Biosystems, Foster City, CA) as describe elsewhere (16). The exact cDNA copy number was deduced from the comparison of measured fluorescence with the standard curve and standardized against the level of HPRT transcripts. Primer sequences are available upon request.

Results were expressed as the intra-sample ratio of cytokine to HPRT mRNA copy number. Perforin-1 and granzyme quantification was performed using labeled TaqMan® Gene Expression Assays (granzyme B: assay ID Rn00821752_g1 and perforin 1; Rn00569095_m1, Applied Biosystems).

Statistical analyses: Different statistical approaches were used to compare the CDR3-LD alterations in the blood and spleen of DST-treated recipients, naive rats and rejecting recipients. A Box-and-Whisker Plot representation was used to more easily visualize the global differences between the groups. The Kruskal-Wallis test followed by a Dunn's post-hoc test was performed to compare perturbations between two or three groups respectively. Statistical significance was considered for $p<0.05$. Graft survival was analyzed using the

Kaplan-Meier method, p values were considered as significant when <0.05. The Mann-Whitney test was performed to compare transcript accumulation, p values were considered significant when <0.05. Analyses were performed using the STATGRAPHICS PLUS 5.1 software (Rockville, Maryland, Manugistics Inc).

Results

Numerous reports have shown that regulatory T cells differentiate rapidly in the spleen, graft (19) and lymph node (19, 31) after allotransplantation and in long-term recipients (>100 days) (32) following various maneuvers aimed at inducing tolerance. The regulatory mechanisms described have also been reported to be dependant on the strain combinations used (21, 33, 34). In this study, we investigated the ability to identify the regulatory T cell population in the spleen and blood of DST-treated recipients through a combined investigation of the T cell TCR Vβ repertoire, phenotype and function.

TCR usage compartmentalization in the blood and spleen of DST-treated recipients.

At 100 days post transplantation the spleen and blood TCR Vβ repertoires of DST-treated recipients were compared to those of naive and untransfused LEW.1A control rats that had previously rejected a LEW.1W heart graft (6.4 ± 1.7 days after transplantation ; referred to as rejecting recipients in the manuscript).

The spleens of DST-treated recipients displayed a roughly gaussian TCR repertoire usage (14.6 ± 3.4 % alteration) with low Vβ/HPRT ratios (13.5 ± 10.6) (Figure 1.a). These patterns were not statistically different form those of naive rats (7.3 ± 1.5 % alteration with low Vβ/HPRT ratios) (*data not shown*). We have recently described that both $CD25^+$ and $CD25^-$ regulatory T cells can be found 100 days after transplantation in this model, and that $CD25^-$ T cells displaying *in vitro* suppressive activity are able to prolong graft survival in a donor-antigen specific manner when transferred to a secondary, untreated host (*Degauque et al., Submitted*). We thus studied the T cell repertoire of purified $CD25^+$ and CD25- T cells. Both spleen $CD25^+$ and $CD25^-$ T cells from DST-treated recipients and naive rats exhibited a gaussian CDR3-LD usage (Figure 1.b).

In contrast, blood T cells from DST-treated recipients displayed a highly altered CDR3-LD profile (27.8 ± 8 % alteration; Figure 2.a) compared to naive rats (*data not shown*) and to rejecting recipients (15.2 ± 3 %) (Figure 2.b), with high Vβ/HPRT ratios (35.9 ± 23.7) . Such a profile depicted a highly restricted repertoire and clonal selection. To better characterize the subpopulation that had an oligoclonal repertoire, blood $CD4^+$ and $CD8^+$ T cells were isolated from DST-treated recipients and analyzed separately. Blood $CD4^+$ T cells displayed a more or less resting repertoire (Figure 2.c; 14.6 ± 3.2 % alteration) with most clonal selections concerning blood $CD8^+$ T cells (Figure 2.d; 22.9 ± 2 % alteration) (Figure 2.e; $p<0.05$).

We have previously shown that in a similar strain combination, the induction of tolerance following DST was associated with a public Vβ18, Jβ2.7 monoclonal expansion of graft infiltrating $CD8^+$ T cells (35). However, this clone was not observed in the blood. CDR3 lengths of identified altered Vβ families (Vβ5, 6, 8.2, 11 and 14) in the blood were next compared between DST-treated recipients. As shown in Figure 3, although recurrent blood Vβ and CDR3 length alterations were found in the DST-treated recipients , no public alteration was reproducibly found in all the animals tested.

Finally, the Box-and-Whisker Plot method was used to globally compare the TCR Vβ repertoire of the blood T cells purified from DST-treated recipients, naive animals and rejecting recipients. This method is helpful for the handling of large data sets. The data in figure 4 confirm that the TCR Vβ repertoires in the blood T cells of DST-treated recipients were globally more altered than those of naive or rejecting recipients ($p<0.01$).

Altogether, these data showed that DST-treated recipients displayed a highly altered and heterogenous blood $CD8^+$ TCR repertoire usage and a gaussian spleen TCR repertoire usage, suggesting compartmentalization of TCR repertoire usage. This also shows that transplantation performed in inbred rat strains only differing for the MHC involves private TCR repertoire usage.

Differential capacity of spleen and blood T and non-T cells to transfer long-term survival.

Without any treatment, 80% of sub-irradiated untreated LEW.1A recipients rejected their grafts (Figure 5). Adoptive transfer of $100x10^6$ splenocytes from DST-treated recipients indefinitely prolonged graft survival in all sub-irradiated untreated LEW.1A recipients (Figure 5). Identical results were obtained when purified spleen T cells ($30x10^6$, n=6) from DST-treated recipients were transferred to secondary recipients (Figure 5). In contrast, 20 - $40x10^6$ PBMC from DST-treated recipients induced long-term graft survival in only 50 % of secondary recipients (Figure 5). Purified blood T cells ($20x10^6$, n=4) had no effect (Figure 5). In contrast, the adoptive transfer of $20x10^6$ blood non-T cells (n=10) from DST-treated recipients induced an indefinite graft survival in 40% of secondary sub-irradiated LEW 1A recipients.

As a control, transfer of $100x10^6$ splenocytes, $40x10^6$ purified spleen T cells and $20x10^6$ blood non-T cells from rejecting recipients (n=3) or naive LEW.1A rats (n=3) had no significant effect (12 ± 1 days and 13 ± 2 days respectively) on graft survival prolongation. Altogether, these data showed that long-term graft survival may be induced in 100% of sub-irradiated secondary graft recipients by splenocytes and spleen T cells and in 50% of animals by PBMC from DST-treated recipients only. Blood T cells had no effect, suggesting that the blood T cells with an oligoclonal repertoire were not selected regulatory T cells.

The long-term surviving grafts of secondary recipients display signs of chronic rejection.

DST is known to result in a complex state where histological lesions of chronic graft rejection coexist with mechanisms controlling acute host anti-donor immune responses (36). Thus, the possible occurrence of chronic graft rejection lesions was also analyzed in the secondary recipients that survived 100 days after splenocyte or PBMC cell transfer from DST-treated recipients (Figure 6). All long-term surviving grafts from secondary recipients, that had received blood or spleen cells displayed histological lesions of chronic rejection (Figure 6.a,.b) with inflammatory endarteritis and interstitial fibrosis (Figure 6.c).

Spleen T cells from DST-treated recipients proliferate poorly against donor-antigen cells whereas blood T cells proliferate similarly to naive T cells.

In the DST-treated recipients, regulatory T cells differentiated in different compartments, as assessed by their various capacities to transfer long-term survival to secondary recipients. To investigate the regulatory mechanisms taking place, we compared the alloreactive response of T cells from DST-treated recipients taking into account their origin (blood or spleen). When cultured against donor APC, purified spleen T cells from DST-treated recipients proliferated significantly less ($p<0.05$) than those from naive rats (Figure 7.a). In contrast, no difference was observed when purified blood T cells from DST-treated recipients or naive LEW.1A rats were cultured with naive donor APC (Figure 7.a).

DST-treated recipients displayed increased numbers of spleen $CD4^+CD25^+$ T cells and normal numbers of blood $CD4^+CD25^+$ T cells.

An increase in the percentage of regulatory $CD4^+CD25^+$ T cells has been described in various tolerance-related models (2). We performed an exhaustive cell phenotype analysis in the spleen and blood of DST-treated recipients compared to naive and rejecting recipients (Figure

7.b). A significant increase in $CD4^+CD25^+$ T cells was observed in the spleen of DST-treated recipients (10.8 ± 2.8%) as compared to naive rats (5.3 ± 0.8%, *p<0.05*) and rejecting recipients (6.5 ± 3.6%, *data not shown*). In contrast blood from DST-treated recipients showed normal $CD4^+CD25^+$ T cell numbers as compared to naive rats (6.3 ± 1.2 % vs 3.9 ± 1%). No difference was observed for the other markers tested (see Materials and Methods section) in the spleen and blood from the different groups (*data not shown*).

$CD4^+CD25^+$ spleen T cells from DST-treated recipients exhibit inhibitory properties similar to $CD4^+CD25^+$ spleen T cells from naive animals.

Because DST-treated recipients exhibit an increased percentage of $CD4^+CD25^+$ spleen T cells, we assessed whether their ability to inhibit proliferation of naive $CD25^-$ T cells was also increased. Proliferation of naive $CD25^-$ T cells against LEW.1W APCs was tested with or without $CD4^+CD25^+$ spleen T cells from either DST-treated recipients or naive rats. $CD4^+CD25^+$ spleen T cells from both DST-treated recipients and naive rats significantly and dose-dependently inhibited the alloreactive response of naive $CD25^-$ T cells in the coculture system (Figure 7.c). Thus, DST-treated recipients exhibited an increased number of $CD4^+CD25^+$ spleen regulatory T cells with intact suppressive function on a cell per cell basis.

The blood T cells of DST-treated recipients display an activated phenotype.

The Cβ, IL-2, IFN-γ, IL-10, IL-13, TGF-β1 and TNF-α transcript levels were compared in the spleen (n=3) and blood (n=5) of DST-treated recipients (Figure 8.a) using quantitative RT-PCR. Blood from DST-treated recipients showed consistently higher levels of Cβ, IL-2, IFN-γ, IL-10, IL-13, TGF-β1 and TNF-α cytokines than splenocytes although statistical significance was reached only for IFN-γ (*p<0.05*) (Figure 8.a), suggesting an active

phenotype in DST-treated recipients. Finally, perforin and granzyme transcript levels were measured by RT-PCR in the spleen and blood of DST-treated recipients and compared with those of naive rats (Figure 8.b,.c). Low transcript levels of perforin and granzyme were observed in the spleen and blood of naive rats (Figure 8.b,.c, *baseline level*); similar low levels were detected in splenocytes from DST-treated recipients 100 days after transplantation (Figure 8.b,.c). In contrast, blood from DST-treated recipients 100 days after transplantation accumulated significantly more cytotoxic transcripts as compared to the level detected in the spleen ($p<0.05$; Figure 8.b,.c).

Altogether, these data showed that spleen T cells characterized by a gaussian TCR Vβ repertoire were hyporesponsive to donor APC, displayed suppressive activity *in vitro* and were able to transfer long-term survival to secondary recipients. In contrast, blood T cells, which displayed a highly oligoclonal T cell repertoire, retained their alloreactivity both *in vitro* and *in vivo*, had an activated-cytotoxic phenotype and were unable to transfer long-term survival.

Discussion

Tolerance in transplantation is associated with inactivation of the recipient's immune response to donor antigens, while retaining normal responsiveness to other unrelated antigens (37). In humans, the beneficial effect of donor specific transfusion (DST) on allograft survival was recognized several decades ago (8, 21, 23) and DST has become a classical model to induce tolerance or long-term graft acceptance in both experimental rodent models and humans (20, 22, 38). Nevertheless, the immunological mechanisms of the DST effect are only poorly understood, although it is clear that leukocyte depleted blood transfusions are ineffective (39).

The mechanisms underlying the beneficial effects of DST may include clonal deletion (40), anergy (15), generation of suppressive/regulatory cells (2, 9, 38), regulation of cytokine production (36, 41, 42), microchimerism (43, 44) or a combination thereof (34). The presence of donor-committed cytotoxic T lymphocyte infiltration in allografts early after DST does not favor a major role for clonal deletion of alloreactive cells (4, 41, 45-47). Moreover, once established, DST induced-tolerance can be perpetuated by adoptive transfer of regulatory cells into secondary naive recipients (2, 4). This phenomenon, which is linked to the "education" of the naive recipient immune cells and may be transferred over multiple generations to secondary recipients (2, 4), is referred to as "infectious tolerance" (48-50) and implicates the presence and role of regulatory/suppressive cells.

There is now a large body of evidence showing that, whereas $CD8^+$ T cells may play a role (17, 51-53), $CD4^+$ T cells are the major regulatory population involved in the DST effect (2, 4, 24). Nevertheless, their mode of action, their compartmentalization and the extent to which they efficiently inhibit effectors or cytotoxic cells thereby preventing chronic rejection, remains obscure (36, 54).

The presence of regulatory cells in the different immune compartments of DST-treated recipients was reported by several studies showing that infectious tolerance can be transferred

with splenocytes, lymph nodes, graft infiltrating cells or even blood (4, 24, 55-58). In this study, we analyzed in detail two major compartments in DST recipients and we showed that both spleen cells and peripheral blood mononuclear cells from DST-treated recipients were able to transfer long-term graft survival to 100% and 50% of naive host recipients respectively. We also showed that the nature of the regulatory cells differs between the two compartments. Whereas T cells purified from the spleen were able to transfer long-term survival, purified blood T cells failed to do so and long-term graft survival was reached only after transfer of blood non T cells. Moreover, T cells varied in their phenotypic and functional characteristics between these two compartments: blood T cells had an activated phenotype with increased perforin/granzyme transcript levels and a preserved proliferative response to donor antigens. They also exhibited a highly altered TCR repertoire. T cells from splenocytes, on the other hand, displayed an increased proportion of $CD4^+CD25^+$ T cells with suppressive properties *in vitro*, a decreased proliferative response to donor antigen and an unaltered TCR repertoire.

Although the contribution of regulatory T cells in the transfer and maintenance of long-term graft acceptance in this model is well established (4, 59), the manner in which regulatory T cells control effector mechanisms and the degree to which they protect against chronic rejection is unclear. We showed that spleen T cells, which displayed a regulatory-like profile and transferred long-term graft survival to secondary recipients, were unable to prevent the onset of chronic rejection. Several other studies have shown that chronic rejection develops in apparently tolerant animals, in models of both heart (36, 42) and kidney (60) transplantation, whereas signs of chronic rejection were absent in syngeneic grafts (36). Chronic rejection, which represents the leading cause of progressive failure of organ transplants over time, is thought to be influenced by alloantigen-dependent and independent factors. In the present study, both DST-induced tolerant recipients (*data not shown*) and secondary recipients

displayed severe signs of chronic rejection. This observation suggest that the potentially regulatory T cells are not sufficient to protect the graft from chronic rejection. However, we cannot exclude the possibility that regulatory T cells able to inhibit acute rejection (as shown by the effect of DST itself or by the transfer of cells from DST-treated recipients) can also contribute to chronic rejection. Indeed, tolerance has been shown to be an "active" phenomenon associated with cytotoxic cells directed against donor cells (61) and has also been shown to develop in the context of a Th2 polarization, which has itself been associated with the development of chronic rejection lesions in functioning grafts (36, 42). In our model, no Th1/Th2 deviation was observed in DST-treated recipients thus suggesting that the potentially regulatory T cells are not likely sufficient to protect the graft from chronic rejection following DST induction.

Finally, DST treatment results in the differentiation of regulatory cells in spleen T cells and in the non-T cell compartment in the blood, since blood T cells did not transfer graft survival prolongation to secondary recipients. These data suggest that blood is not a reservoir for regulatory T cells. They also suggest that selected clonal expansion TCR Vβ repertoire (particularly CD8 T cells) rather represent potentially cytopathic T cells than regulatory T cells. The differentiation of potentially cytopathic T cells together with long-term graft survival do not fulfill the paradigm of tolerance but constitutes another example of the complex state that results from tolerance induction by DST

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Legends

Figure 1: TCR repertoire in different spleen cell subsets of DST-treated recipients: (a). TCR repertoire analysis in spleens (n=4) of DST-treated recipients (left-hand panel: Tclandscapes ; right-hand panel: TopViews). The percentages indicate the global variation of CDR3-LD compared to naive animals (average of 11 CDR3-LD naive profiles). Circles indicate recurrent Vβ families. (b). TCR repertoire analysis of T cells, $CD25^{+}$ T cells and $CD25^{-}$ T cells from the spleens of DST-treated recipients and naive rats (n=3).

Figure 2: **TCR repertoire in the PBMC of DST-treated recipients and rejecting recipients and in $CD4^{+}$ and $CD8^{+}$ T cells of DST-treated recipients:** (a). TCR repertoire analysis in the blood of DST-treated recipients (n=5; left-hand panel: Tclandscapes; right-hand panel: TopViews). The percentages indicate the global variation of CDR3-LD compared to naive animals (average of 11 CDR3-LD naive profiles). Circles indicate recurrent Vβ families. (b). TCR repertoire analysis in the blood (TopViews) of rejecting recipients (n=4). (c). $CD4^{+}$ (n=4) and (d). $CD8^{+}$ (n=5) T cell TCR repertoire analysis TopViews in the blood of DST-treated recipients. The percentages indicate the global variation of CDR3-LD compared to naive animals (average of 11 CDR3-LD naive profiles). (e). Percentage of alteration in $CD8^{+}$ and $CD4^{+}$ T cells in the blood of DST-treated recipients.

Figure 3: Recurrent DST-treated recipient blood Vβ and CDR3 length alterations.

Figure 4: Synthetic representation of the percentages of Vβ family alterations in the blood T cells of DST-treated recipients, naive recipients and rejecting recipients represented per individual by the Box-and-Whisker Plot method. The median of the data

is represented by a vertical line. The 2 whiskers represent the lower and the upper quartiles. Points beyond the whiskers are considered as outliers (squares). The mean is indicated by a cross.

Figure 5: Graft survival after different cell transfers into naive secondary LEW.1W recipients. As a control, transfer of $100x10^6$ splenocytes or $40x10^6$ purified spleen T cells from rejecting recipients (n=3) or naive LEW.1A rats (n=3) had no significant effect (12 ± 1 days, 13 ± 2 days respectively) on graft survival prolongation.

Figure 6: Histological analysis of grafts from secondary rat recipients after spleen cell transfer (a). and PBMC transfer (b). from DST-treated recipients. (c). The table shows Banff scores for mononuclear cell interstitial inflammation, interstitial fibrosis, vasculitis, vascular narrowing and percentage of pathological arterial sections per heart.

Figure 7: (a). Proliferative response of spleen (n= 8) and blood (n=5) T cells from DST-treated recipients and naive LEW.1A rats against LEW.1W APC. **(**b). Percentage of $CD4^+CD25^+$ T cells in the spleen and blood of naive LEW.1A rats and DST-treated recipients. Results are representatives of four independent experiments and bars represent mean ± SEM. Statistical significance was tested using the Mann-Whitney test. (c). No increase in the suppressive functions of the spleen $CD4^+CD25^+$ T cells in DST-treated recipients: Various ratios of spleen $CD4^+CD25^+$ T cells from DST-treated recipients (black bar) or naive (white bar) LEW.1A rats, were added to naive $CD25^-$ T cells from LEW.1W in MLRs. Bars represent mean ± SEM and are representatives of one of four independent experiments.

Figure 8: (a).Cytokine transcript accumulation in the spleen (n=3)) and blood T cells (n=5) of DST-treated recipients. Bars represent mean ± SEM. Statistical significance was tested using the Mann-Whitney test. (b). Perforin and (c). Granzyme transcript levels in the blood and spleen of DST-treated recipients. Bars represent mean ± SEM. Statistical significance was tested using the Mann-Whitney test.

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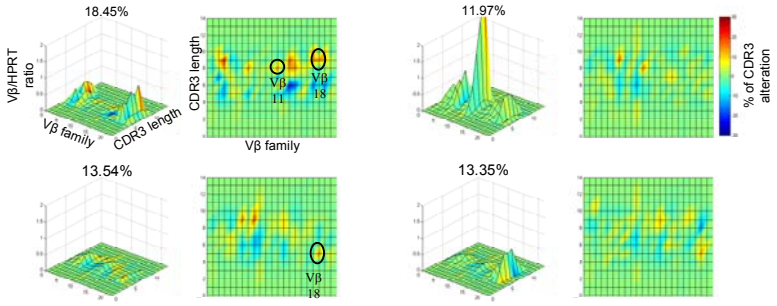
Figure 1

a.


18.45%
Vβ/HPRT ratio
Vβ family
CDR3 length
CDR3 length
Vβ 11
Vβ 18
Vβ family
11.97%
% of CDR3 alteration
13.54%
Vβ 18
13.35%

b.

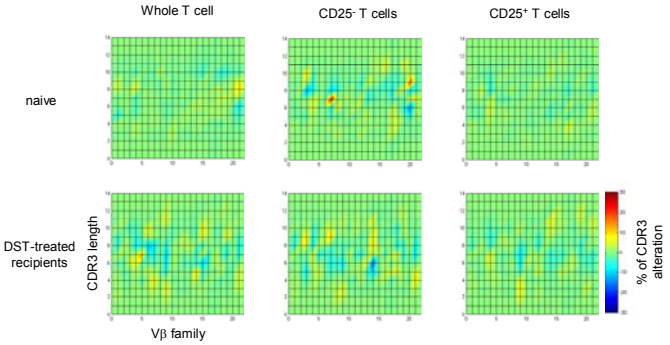

Whole T cell
CD25⁻ T cells
CD25⁺ T cells
naive
DST-treated recipients
CDR3 length
Vβ family
% of CDR3 alteration

Figure 2

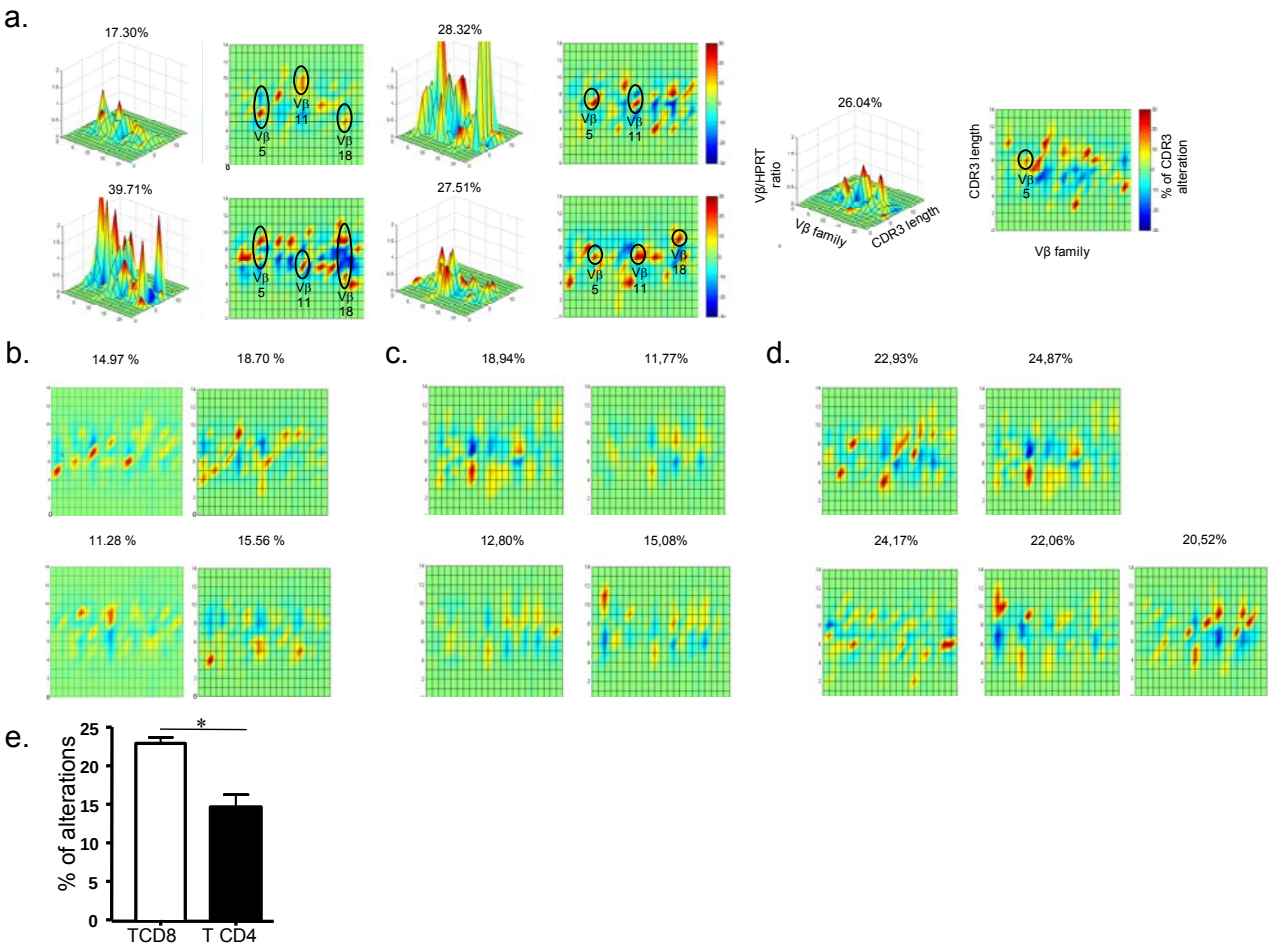

a.
17.30%
28.32%
39.71%
27.51%
26.04%
Vβ/HPRT ratio
Vβ family
CDR3 length
% of CDR3 alteration
b.
14.97 %
18.70 %
11.28 %
15.56 %
c.
18,94%
11,77%
12,80%
15,08%
d.
22,93%
24,87%
24,17%
22,06%
20,52%
e.
% of alterations
TCD8
T CD4
*

Figure 3

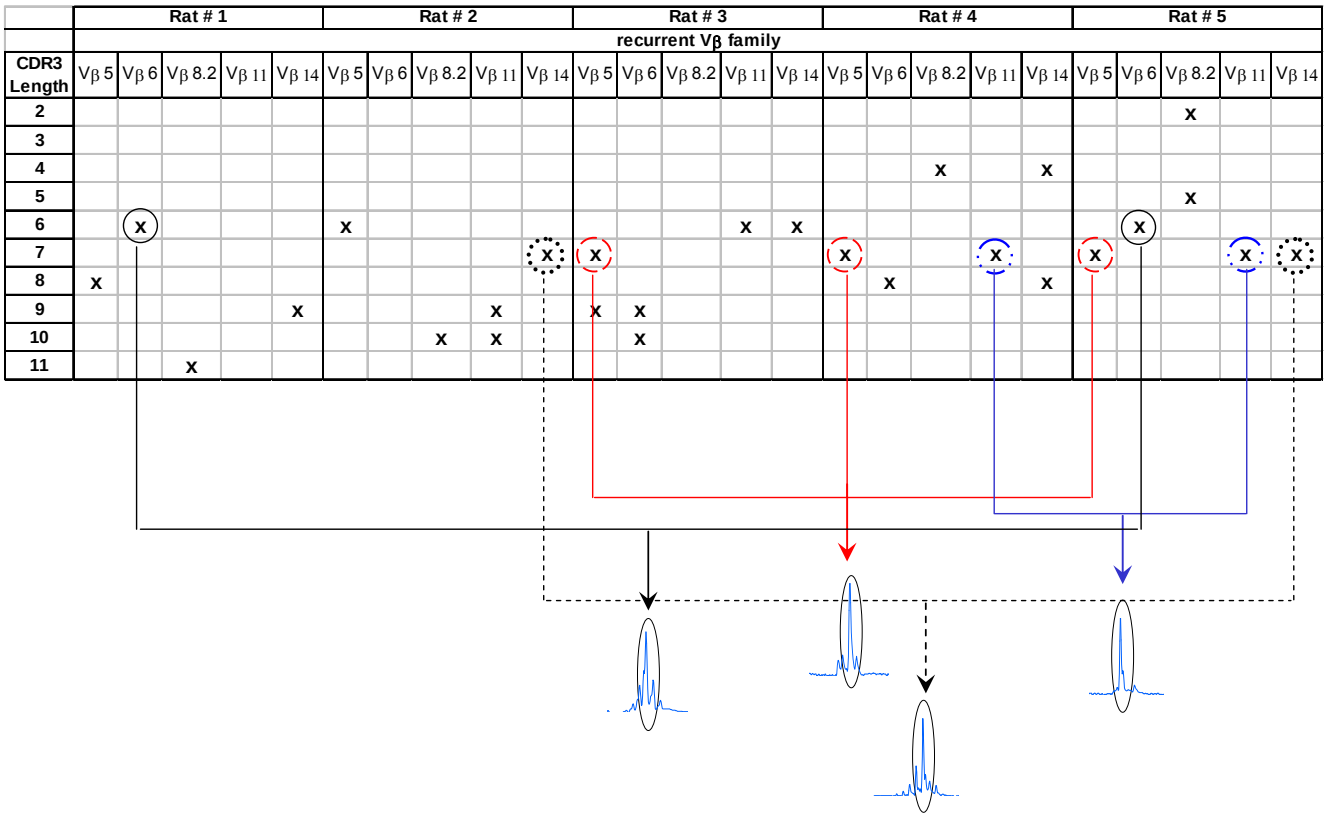

Rat # 1
Rat # 2
Rat # 3
Rat # 4
Rat # 5
recurrent Vβ family
CDR3 Length
Vβ 5
Vβ 6
Vβ 8.2
Vβ 11
Vβ 14
2
3
4
5
6
7
8
9
10
11

Figure 4

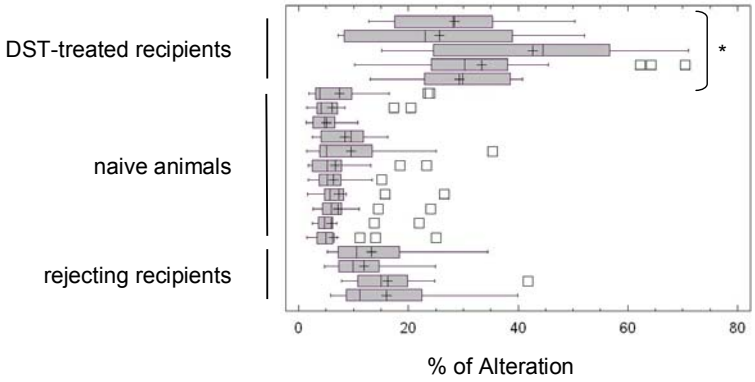

DST-treated recipients
naive animals
rejecting recipients
*
0
20
40
60
80
% of Alteration

Figure 5

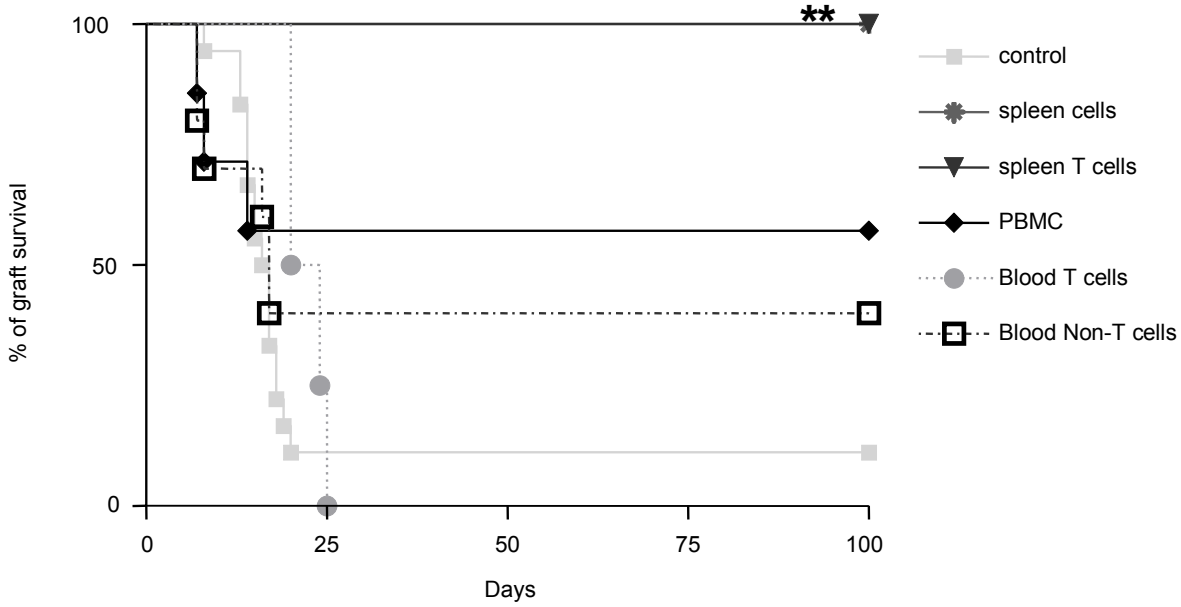
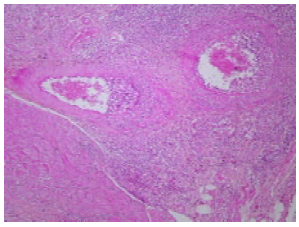
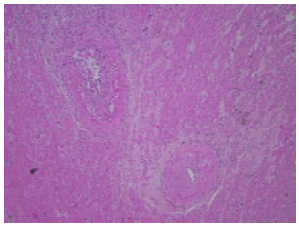

**
100
50
0
% of graft survival
0
25
50
75
100
Days
control
spleen cells
spleen T cells
PBMC
Blood T cells
Blood Non-T cells

Figure 6

a. 

b. 

c.

		mononuclear cell interstitial inflammation	interstitial fibrosis	vasculitis	vascular narrowing	% of pathological arterial sections per heart
Transfer of splenocytes	#1	1	1	2	1	10
	#2	1	1	2	1	8
Transfer of PBMC	#1	2	1	2	3	50
	#2	2	1	2 and 3	3	60
	#3	2	1	1	1	50
	#4	4	3	2	1	50

Figure 7

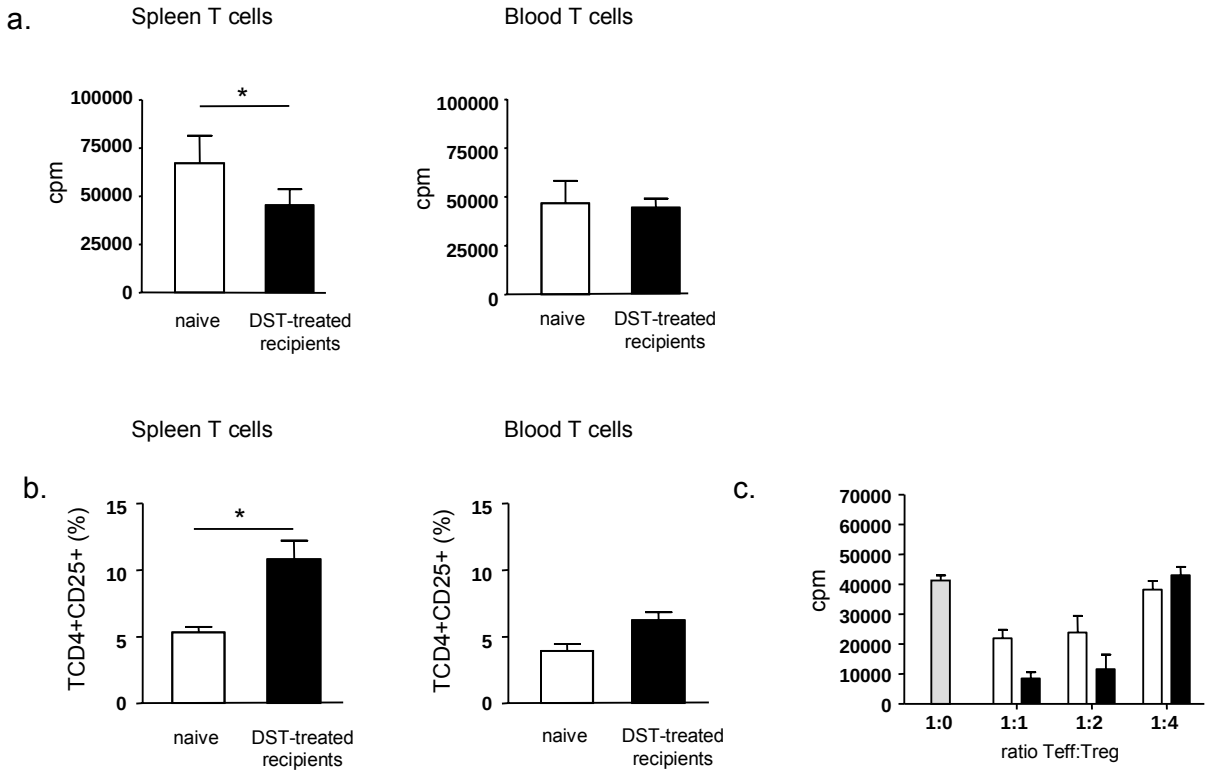
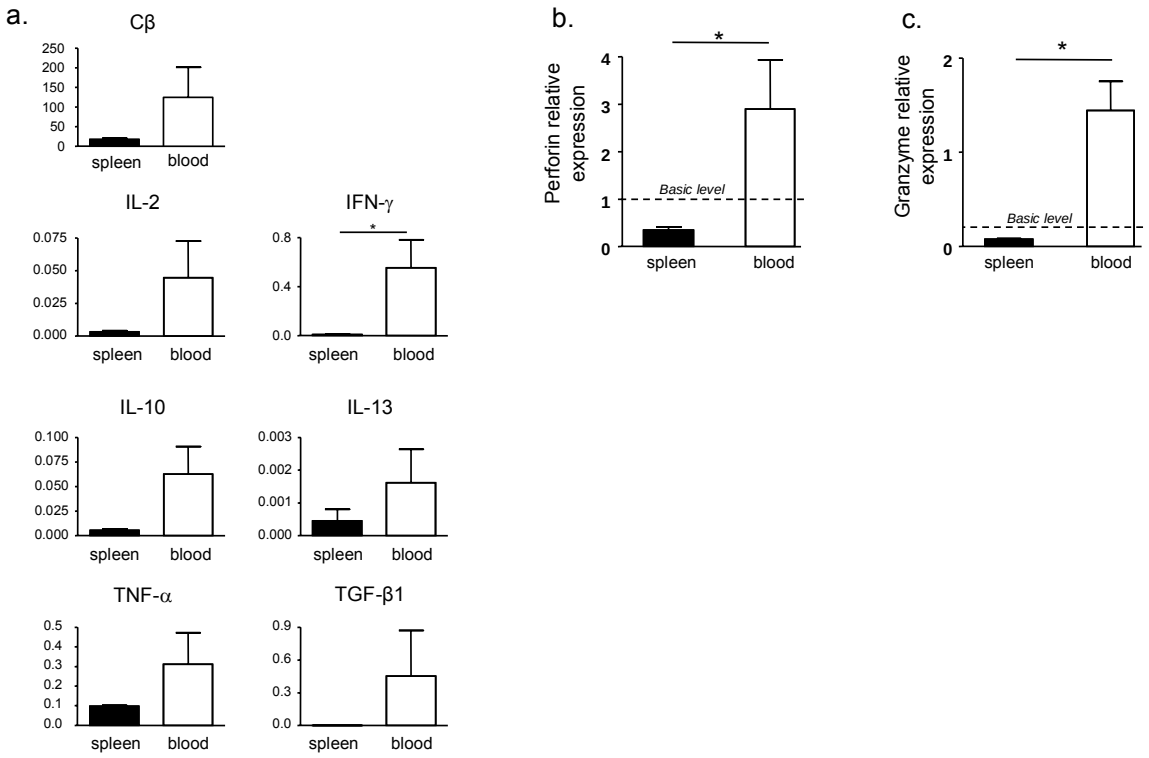

a.
Spleen T cells
Blood T cells
cpm
100000
75000
50000
25000
0
*
naive
DST-treated recipients
b.
Spleen T cells
Blood T cells
TCD4+CD25+ (%)
15
10
5
0
*
naive
DST-treated recipients
c.
cpm
70000
60000
50000
40000
30000
20000
10000
0
1:0
1:1
1:2
1:4
ratio Teff:Treg

Figure 8



III.2.

Discussion

La tolérance en transplantation est associée à une absence de la réponse immune vis-à-vis des antigènes du donneur, tout en maintenant une réponse normale aux autres antigènes (Margenthaler et al. 2003). Chez l'Homme, l'effet bénéfique de la transfusion spécifique du donneur (TSD) sur la survie de l'allogreffe a été montré il y a plusieurs décennies (Soulillou et al. 1984; van Rood et al. 1990; Flye et al. 1995). La TSD est devenue un modèle classique d'induction de «tolérance» ou d'acceptation à long terme du greffon dans des modèles expérimentaux (Johnson et al. 1984; Quigley et al. 1989; Soulillou 1998). Néanmoins, les mécanismes immunologiques de la TSD sont mal compris, seule certitude: le sang déplété en leucocytes n'entraîne pas de tolérance (Persijn et al. 1979). Les mécanismes fondamentaux impliqués dans la TSD peuvent inclure la suppression clonale (Terasaki 1984), l'anergie (Dallman et al. 1991), la génération de cellules suppressives / régulatrices (Quigley et al. 1989; Bushell et al. 2003; Suciu-Foca et al. 2003), la régulation de la production de cytokine (Yang et al. 1998; Koshiba et al. 2003; Pirenne et al. 2005), le microchimérisme (Seung et al. 2003; Wood 2003) ou encore une combinaison de ces différents mécanismes (Yang et al. 1998). La présence de lymphocytes T cytotoxiques spécifiques du donneur dans le greffon peu après la TSD ne suggère pas la suppression clonale des cellules allo-réactives (Chevalier et al. 1987; Dallman et al. 1987; Bugeon et al. 1992; Yang et al. 1998; Kataoka et al. 2003). D'ailleurs, une fois établi, l'état de «tolérance» induit par la TSD peut être perpétué par le transfert de cellules régulatrices à des receveurs sans traitement (Bushell et al. 2003; Kataoka et al. 2003). Ce phénomène, lié à l'«éducation» des cellules naïves des receveurs et qui peut être transféré sur de multiples générations chez des receveurs secondaires (Bushell et al. 2003; Kataoka et al. 2003) désigné sous le terme de «tolérance infectieuse» (Qin et al. 1993; Cobbold et al. 1998; Kupiec-Weglinski et al. 1998), plaide en grande partie pour la présence et le rôle de cellules régulatrices/suppressives. Bien que de nombreuses preuves montrent que les cellules $TCD8^+$ peuvent jouer un rôle dans ce modèle (Douillard et al. 1999; Kosiewicz et al. 2004; Stock et al. 2004; Xystrakis et al. 2004), les cellules $TCD4^+$ restent les principales cellules régulatrices impliquées dans les effets de la TSD (Onodera et al. 1998; Bushell et al. 2003; Kataoka et al. 2003). Néanmoins, leur mode d'action, leur compartimentation et leur capacité à empêcher le rejet chronique demeurent obscures (Graca et al. 2002; Koshiba et al. 2003). La présence de cellules régulatrices dans les différents compartiments immuns des receveurs traités par TSD a été rapportée par plusieurs études qui ont montré que la tolérance

infectieuse peut être transférée avec les splénocytes (Onodera et al. 1998; Graca et al. 2000; Kataoka et al. 2003), les ganglions lymphatiques (Zhai et al. 2001), les cellules infiltrant le greffon (Kataoka et al. 2003) ou même le sang (Bektas et al. 2005). Dans cette étude, nous avons analysé en détails deux compartiments principaux des receveurs et nous avons montré que les cellules de la rate et les cellules mononucléées du sang périphérique des animaux traités par TSD pouvaient transférer la survie à long terme d'une greffe chez des receveurs greffés sans traitement dans 100 et 50% des cas respectivement. Nous avons également montré que la nature des cellules régulatrices est différente entre ces deux compartiments. En effet, les lymphocytes T de la rate peuvent transférer la survie à long terme, alors que des lymphocytes T du sang n'y parviennent pas. Seul le transfert de cellules non-T issues du sang des animaux traités par TSD a permis une survie à long terme de la greffe. De plus, les lymphocytes T ont des caractéristiques phénotypiques et fonctionnelles différentes entre ces deux compartiments. Les lymphocytes T du sang ont un phénotype activé avec des niveaux élevés de transcripts des molécules perforine/granzyme, une réponse prolifération préservée contre les antigènes de donneur et un répertoire du TCR fortement modifié. En revanche, les lymphocytes T de la rate montrent une augmentation du nombre de cellules $TCD4^{+}CD25^{+}$, des propriétés suppressives *in vitro*, une diminution de la réponse prolifération contre les antigènes du donneur et un répertoire du TCR comparable à celui d'un animal naïf.

Si la contribution des cellules régulatrices dans le transfert et le maintien de survie à long terme de greffon dans ce modèle est bien établie (Takayashiki et al. 2005), la façon dont ces cellules régulent les mécanismes effecteurs et protègent contre le rejet chronique n'est pas claire. Nous avons montré que les lymphocytes T spléniques, qui montrent un profil régulateur et transfèrent la survie à long terme d'une greffe n'empêchent pas le développement d'un rejet chronique. Plusieurs autres études ont montré que le rejet chronique se développe chez les animaux apparemment «tolérants» dans des modèles d'allogreffe cardiaque (Koshiba et al. 2003; Pirenne et al. 2005) ou rénales (Jablonski et al. 1995) alors que les greffes syngéniques ne montrent pas de signes de rejets chroniques (Koshiba et al. 2003). Le rejet chronique, représente la principale cause de dysfonction des greffons dans le temps. Il est multifactoriel et influencé par des facteurs dépendants et indépendants des allo-antigènes. Dans notre étude, les receveurs traités par TSD et les receveurs secondaires montrent des signes de rejet chronique. Cette observation suggère que les cellules potentiellement régulatrices sont probablement insuffisantes pour protéger la greffe du rejet chronique. Cependant, nous ne pouvons pas exclure que les cellules régulatrices qui sont capables d'empêcher le rejet aigu pourraient également avoir pour effet secondaire de

contribuer au rejet chronique. En effet, la tolérance s'est avérée être un phénomène actif lié aux cellules cytotoxiques dirigés contre les cellules du donneur (Bugeon et al. 1993). De plus, il a été également montré que la tolérance intervient dans le contexte d'une polarisation Th2 associée au développement des lésions de rejet chronique dans les greffes (Koshiba et al. 2003; Pirenne et al. 2005). Dans notre modèle, on n'a observé aucune déviation Th1/Th2 chez nos animaux traités par TSD, ce qui suggère une autre hypothèse: les cellules potentiellement régulatrices sont peut être en nombre insuffisant pour protéger la greffe contre le rejet chronique après l'induction de tolérance par la TSD.

En conclusion, nos données suggèrent que le sang n'est pas un réservoir de cellules régulatrices. Le répertoire clonal en particulier des cellules $TCD8^+$ suggère plutôt que ces cellules sont potentiellement cytotoxiques et non régulatrices. Le répertoire altéré du sang représente une caractéristique qui définit les receveurs traités par TSD comme le montrent les analyses statistiques. Nos observations constituent un nouvel exemple de la complexité qui résulte de l'induction de tolérance par la TSD.

Enfin, nos résultats montrent que le traitement par la TSD permet la différentiation de cellules régulatrices dans la rate mais également dans le compartiment des cellules non-T du sang qui transfèrent la survie à long terme d'une greffe chez des receveurs secondaires.

Conclusions et Perspectives

Les cellules régulatrices capables de prévenir le développement du rejet aigu de manière spécifique du donneur ne peuvent prévenir la survenue du rejet chronique comme le montre l'article 3 ou les travaux de Koshiba et al (Koshiba et al. 2003). Ces cellules régulatrices sont d'origine splénique et de phénotype TCD25$^-$ comme le montre l'article 2. Elles inhibent la prolifération des cellules effectrices naïves TCD25$^-$ *in vitro* en réponse à une stimulation par les cellules présentatrices d'antigène, et ce de manière spécifique du donneur. Le transfert de ces cellules TCD25$^-$ à des animaux receveurs irradiés non traités permet d'induire une survie à long terme du greffon, de manière spécifique des antigènes du donneur. L'origine de ces cellules et leurs mécanismes d'action demeurent encore inconnues et font l'objet de nombreuses recherches. Des études cinétiques sont nécessaires pour connaitre le moment d'apparition de ces cellules et en particulier si elles sont présentes dès la phase d'induction de la «tolérance» ou si elles apparaissent plus tard pendant la phase de maintien. Le rôle de l'IL-10 et de sa sur-régulation reste à élucider, une explication possible d'absence d'effet lors de l'addition d'anticorps bloquant l'IL-10 dans nos expériences se trouve peut être dans le fait que la quantité d'anticorps utilisée dans ces expériences n'est pas suffisante pour bloquer la production massive d'IL-10 de ces cellules. Il serait également intéressant de savoir de quelles sous-populations cellulaires CD4 ou CD8 sont issues ces cellules régulatrices. Il nous faut également tester des quantités d'anticorps variables et pas seulement celle employée dans cette étude. Nous avons également noté un fort niveau de transcrits codant pour l'IFN-γ et il serait intéressant de voir l'action d'un anti-IFN-γ dans nos cultures *in vitro*.

Puisque nous et d'autres (Chiffoleau et al. 2002; Jiang et al. 2003; Oluwole et al. 2003; Quezada et al. 2003; Pirenne et al. 2005) avons montré que les lymphocytes T jouaient un rôle important dans le devenir du greffon (rejet ou «tolérance»), une autre des perspectives de notre travail est d'identifier les peptides reconnus par les lymphocytes T dans différents compartiments (rate, sang etc…) du receveur au cours du rejet aigu de greffe, de la «tolérance» (à 100 jours) ou du rejet chronique (> à 250 jours) et après transfusion spécifique du donneur.

Notre laboratoire possède une expertise dans l'identification des peptides immuno-dominants comme l'attestent les travaux publiés en 2001 (van Denderen et al. 2001). Cette expertise se trouve renforcée par la disponibilité d'outils d'investigation, tels que l'ELISPOT.

Pour chaque groupe précité (rejet aigu, «tolérant», rejet chronique), les lymphocytes T seront stimulés par des peptides de 16 acides aminés issus des régions polymorphiques des molécules de classe I (domaines α1, α2 et α3 de la chaine α RT1.Au) et de classe II (domaines

β1 des chaines β de RT1Bu et RT1.Du). La culture sera faite pendant 24h (37°C, 5% CO2) dans des plaques ELISPOT 96 puits préalablement coatées avec un anticorps anti-IFN-γ (400 000 cellules/puits). La sécrétion d'IFN-γ constituera notre paramètre de sélection entre peptides immunodominants et peptides non-immunogènes. Brièvement, une fois sécrétée, la cytokine se fixe à l'anticorps primaire anti-IFN-γ. Après lavage des cellules, la présence de cette cytokine est révélée par ajout d'un anticorps secondaire couplé à une enzyme. L'ajout du substrat de l'enzyme induit l'apparition de spots. Un spot correspond à la production d'IFN-γ par une cellule. La fréquence des cellules produisant l'IFN-γ peut donc se déduire en rapportant le nombre de spots au nombre de cellules dans chaque puits. Après cette première série de test, les peptides candidats identifiés seront découpés en différents peptides de 9 acides aminés (taille des peptides présentés par les molécules du CMH) et testés de nouveau en ELISPOT afin d'identifier le ou les peptides immunodominants. Des expériences nous ont déjà permis d'identifier plusieurs peptides reconnus par les lymphocytes T isolés de la rate d'animaux en rejet aigu et d'animaux traités par TSD 7 jours après la transplantation. La Figure 6 présente les résultats obtenus à l'heure actuelle avec à chaque fois un profil représentatif de plusieurs expériences. La réponse vis-à-vis des contrôles (sans peptides) et des acides-aminés (1) 18-33, (2) 58-73, (3) 62-77 (4) 70-85 issus des molécules de CMH de classe II (chaîne β de la molécule RT1.D) est mesurée par ELISPOT. Les rats LEW.1A (RT1a) ayant reçus une greffe allogénique de cœur LEW.1W (RT1u) sans aucun traitement, répondent significativement (réponse ≥ à la moyenne des réponses observées pour l'ensemble des peptides testés ± 2 standard déviation) lors du rejet aigu 7 jours après la greffe contre les peptides 2 et 3 (n=3). Les animaux receveurs prétraités par TSD ne répondent plus contre ces peptides mais répondent 7 jours après la greffe contre les peptides 1 et 4 (n=2). Aucun des animaux ayant reçu une greffe syngénique (n=2) ou d'un donneur tierce partie BN (RT1n) (n=2) ne répondent contre les peptides suggérant une réponse spécifique du donneur.

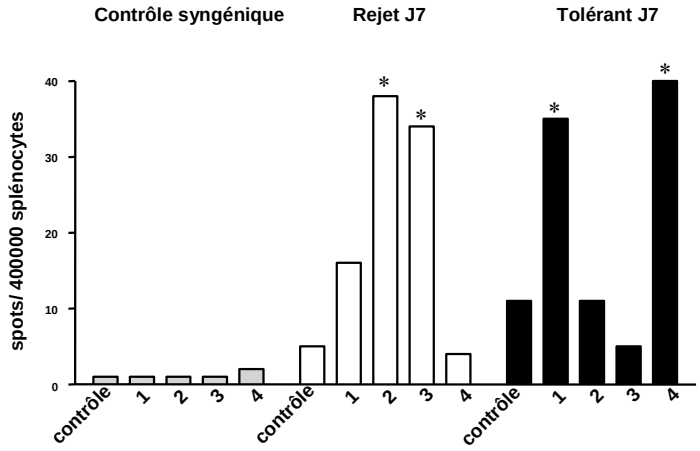


Figure 6: Fréquence des splénocytes produisant de l'IFN-γ après une stimulation avec des peptides issus des molécules de CMH de classe II (chaîne β de la molécule RT1.D).

Les peptides immunodominants identifiés nous permettront ensuite de caractériser les lymphocytes T les reconnaissant à l'aide de tétramères (Lair et al, en préparation). Les tétramères sont un outil puissant de quantification de la fréquence des cellules spécifiques pour un antigène d'intérêt. Ils permettent également d'isoler les cellules d'intérêts en vue de leur caractérisation phénotypique et fonctionnelle. Nous avons mis au point le premier tétramère dans un modèle expérimental d'infection par borna virus chez le rat LEWIS, en collaboration avec l'équipe du Dr Oliver Planz (Tübingen, Allemagne). Le modèle expérimental développé par ce laboratoire (Planz et al. 2001) est caractérisé par le développement d'une encéphalomyélite accompagnée par un infiltrat macrophagique et lymphocytaire ($TCD4^+$ et $TCD8^+$). Oliver Planz (Planz et al. 2001) a pu identifier et quantifier un peptide issu du Borna virus qui est présenté de manière naturelle par les molécules du CMH de classe I et qui est reconnu par les lymphocytes $TCD8^+$. Pour construire ce tétramère, en collaboration avec Étienne Joly et James Stevens (Stevens et al. 1998; Stevens et al. 2000), nous avons cloné et amplifié la séquence codant pour les molécules RT1A(av1) et β2m, via des méthodes classiques de biologie moléculaire. Ces séquences ont été insérées dans des vecteurs d'expression. Une fois les protéines synthétisées, la chaîne α du CMH de classe I et la chaine β2m ont été réassociées («refolding») en présence du peptide d'intérêt. Les monomères ainsi obtenus seront peptides spécifiques. L'ajout d'un résidu biotinylé à la chaîne α du CMH de classe I permet de former des tétramères par association de quatre monomères à une molécule de streptavidine.

Les résultats préliminaires montrent que ce tétramère permet d'isoler spécifiquement des lymphocytes TCD8$^+$ dans la phase pré-aigüe de la pathologie dans le compartiment splénique et lymphatique cervical. La fréquence de ces cellules semble croître avec le développement de la pathologie.

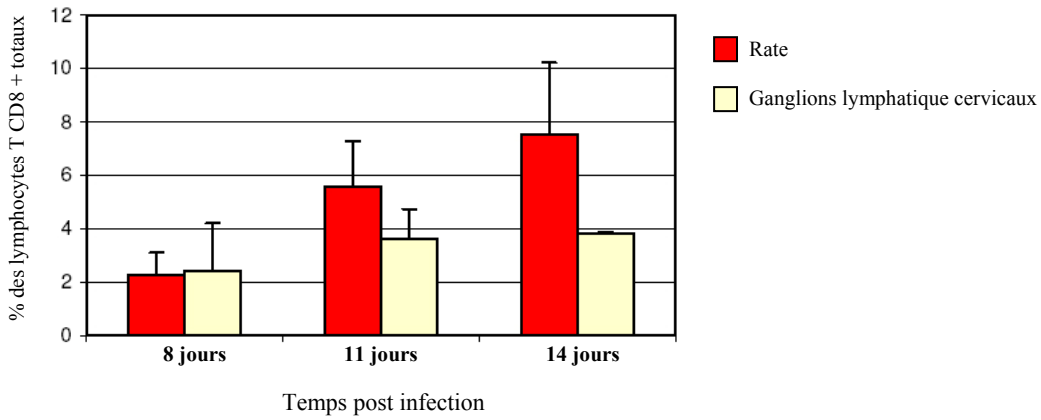


Figure 7: Pourcentages des lymphocytes CD8$^+$ spécifique du Borna Virus à différents temps et leurs localisations après l'infection.

Des études fonctionnelles de clones ainsi isolés sont en cours de réalisation. La validation de la procédure de production des tétramères par ces résultats est essentielle à la réussite du projet présenté.

En conclusion, la réalisation de ce projet devrait nous permettre de mieux comprendre les processus aboutissant à l'activation des clones lymphocytaires T «délétères» ou «bénéfiques» pour le devenir de la greffe dans le modèle d'induction de tolérance par transfusion spécifique du donneur.

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Annexes

I.

Annexe 1

Titre original:

Dominant tolerance to kidney allografts induced by Anti-donor MHC class II antibodies: Cooperation between T and non-T CD103+ cells

Nicolas Degauque, David Lair, Alexandre Dupont, Anne Moreau, Gwénaelle Roussey, Frédérique Moizant, François Xavier Hubert, Cédric Louvet, Marcelo Hill, Fabienne Haspot, Régis Josien, Claire Usal, Bernard Vanhove, Jean Paul Soulillou* and Sophie Brouard*.

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Dominant tolerance to kidney allografts induced by anti-donor MHC class II antibodies: cooperation between T and non-T CD103+ cells.

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Allograft acceptance can be induced in the rat by pretransplant infusion of donor blood or spleen cells. Although promoting long-term acceptance, this treatment is also associated with chronic rejection. In this study, we show that a single administration of anti-donor MHC class II alloimmune serum on the day of transplantation results in indefinite survival of a MHC-mismatched kidney graft. Long-term recipients accept a donor-type skin graft and display no histological evidence of chronic rejection. The kidney grafts of tolerant animals display an accumulation of TCR Cbeta, FoxP3, and IDO transcripts. Moreover, as compared with syngeneic recipients, tolerant recipients harbor a large infiltrate of MHC class II(+) cells and CD103(+) cells. In vitro, splenocytes from tolerant recipients exhibit decreased donor-specific proliferation, which is restored by depletion of non-T cells and partially restored by the blockade of IDO. Finally, splenocytes from tolerant recipients, but not purified T cell splenocytes, transfer donor-specific infectious tolerance without chronic rejection, after infusion into naive recipients, over two generations. However, splenocytes depleted of T cells or splenocytes depleted of CD103(+) cells fail to transfer tolerance. Collectively, these data show that a single administration of anti-donor MHC class II alloimmune serum induces a tolerant state characterized by an infiltration of the kidney graft by regulatory T cells and CD103(+) cells. These data also show that the transfer of tolerance requires the presence of both T cells and CD103(+) dendritic cells. The precise mechanism of cooperation of these two cell subsets remains to be defined.

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Dominant tolerance to kidney allografts induced by Anti-donor MHC class II antibodies: Cooperation between T and non-T CD103$^+$ cells [1].

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Running Title: T/non-T CD103$^+$ cell cooperation after anti-donor class II treatment.

Abstract

Allograft acceptance can be induced in the rat by pre-transplant infusion of donor blood or spleen cells. Although promoting long term acceptance, this treatment is also associated with chronic rejection. Here, we show that a single administration of anti-donor MHC class II alloimmune serum on the day of transplantation results in indefinite survival of an MHC mismatched kidney graft. Long-term recipients accept a donor type skin graft and display no histological evidence of chronic rejection. The kidney grafts of tolerant animals display an accumulation of TCR Cβ, FoxP3 and indeolamine 2,3-dioxygenase (IDO) transcripts. Moreover, as compared to syngeneic recipients, tolerant recipients harbor a large infiltrate of MHC class II^+ cells and $CD103^+$ cells. *In vitro*, splenocytes from tolerant recipients exhibit decreased donor-specific proliferation, which is restored by depletion of non-T cells and partially restored by the blockade of IDO. Finally, splenocytes from tolerant recipients, but not purified T cell splenocytes, transfer donor-specific infectious tolerance without chronic rejection, after infusion into naive recipients, over two generations. However, splenocytes depleted of T cells, or splenocytes depleted of $CD103^+$ cells fail to transfer tolerance. Collectively, these data show that a single administration of anti-donor MHC class II alloimmune serum induces a tolerant state, characterized by an infiltration of the kidney graft by regulatory T cells and $CD103^+$ cells. These data also show that the transfer of tolerance requires the presence of both T cells and $CD103^+$ dendritic cells. The precise mechanism of cooperation of these two cell subsets remains to be defined.

Introduction

Inducing and understanding the mechanism of donor-specific tolerance, defined as the indefinite survival of a well-functioning graft in an immunocompetent adult host in the absence of immunosuppression, remains the challenge of transplant immunology. Experimentally, a variety of maneuvers can induce donor-specific allograft tolerance, including pre-transplantation priming with donor MHC antigens (blood or splenocytes) (1-3), transfected cells expressing donor MHC antigens (4), soluble MHC molecules or allopeptides (5), MHC gene transfer (6, 7) or DNA vaccination (8). In addition, we have shown that injection of recipients with antibodies directed against donor class II MHC can also induce specific tolerance to renal vascularized allografts in adult rats (9, 10). This approach has two potential advantages over donor cell priming (1, 11); it works when the injection is administered at the time of surgery (9) and only donor determinants are targeted. In a model of heart transplantation in the MHC mismatched LEW.1W to LEW.1A combination, the administration of anti-donor class II antibodies induced substantial graft survival prolongation (10). In these experiments, injection of a blocking anti-TGFβ antibody inhibited the capacity of anti-donor class II antibodies to prolong graft survival. Graft survival was also shortened if the graft was depleted of dendritic cells before being transplanted (10). This observation suggested that the tolerance "inducing" effect of anti-donor antibodies was dependant on their interaction with class II-expressing graft resident dendritic cells.

Different hypotheses have been proposed to explain the mechanism of action of anti-donor MHC class II antibodies. Because the only MHC class II cells in normal rat hearts are DC (12-14), anti-donor antibodies could act in a passive way by inhibiting the direct recognition of LEW.1W DC by LEW.1A T cells. This hypothesis is supported by the absence of $CD4^+$ cells in the heart graft (10). Moreover, the interaction of host $CD4^+$ cells with donor MHC class II molecules has been reported as critical for the rejection of murine cardiac allografts (15). However, in the same strain combination, removing DC from donor hearts (by irradiation or cyclophosphamide treatment) prolonged survival to a limited extent only (*Roussey et al., Am. J. Transpl., In Press*). Thus, one can hypothesize that anti-donor MHC class II antibodies could act in an active manner by inducing a regulatory signal after interaction with DC resident within the heart. Anti-donor MHC class II antibodies could opsonize donor resident DC, and thus target them for phagocytosis by a specific subset of DC. These tolerogenic DC could lead to the anergy of allogeneic effector cells and/or the conversion of T effector cells into regulatory T cells. Several reports favor this hypothesis and

highlight the ability of certain subsets of DC to induce T cells with regulatory functions (for review, (16)).

In this paper, using the same LEW.1W to LEW.1A genetic combination but a kidney graft model in which anti-class II antibodies induce indefinite graft survival, we further characterize the mechanisms involved in the maintenance phase of tolerance (100 days after transplantation). We show that anti-donor class II antibodies induce a donor-specific tolerance, which could be transfered to naive recipients. The grafts of tolerant recipients are characterized by an infiltration of regulatory T cells, suggesting that regulatory T cells may be instrumental within the graft environment. However, we show that transfer of tolerance in this model cannot be achieved by large numbers of purified T cells but require the presence of both non-T $CD103^+$ dendritic cells and T cell splenocytes. The cooperative mechanisms between these two cell subsets remains to be defined.

Materials and Methods

Animals and surgical procedures: Inbred adult rats (males) (200-250g) of the LEW.1A ($RT1^a$) and LEW.1W ($RT1^u$) congeneic strains were purchased from Janvier (Savigny/Orge, France). All animals were maintained under standard conditions according to European and Institutional Guidelines. Orthotopic kidney transplantations were performed aseptically, as previously described (9). Recipients underwent an initial nephrectomy on the day of transplantation and a second nephrectomy was performed seven days later. Rejection, indicated by the death of the binephrectomized rat, was confirmed by histology. In parallel, modifications in renal function were monitored in the urine (total protein and creatinine) and serum (urea, creatinine). The protein/creatinine ratio was considered normal when below 0.2 and pathological (proteinuria) when above this level. Tolerant recipients (>100 days) were challenged with donor (LEW.1W), third-party (BN) or recipient (LEW.1A) MHC-type expressing skin grafts. Skin grafts were transplanted onto the lateral thoracic wall and were inspected daily for rejection. Graft function was monitored by measuring the blood urea (mmol/l) and creatinemia (µmol/l) levels and the urine protein (g/l)/creatinine (mmol/l) ratios (proteinuria, g/mmol) in tolerant recipients (n=5) compared to naive rats (n=4).

Experimental groups and adoptive transfer experiments: Group I consisted of unmodified LEW.1A recipients of LEW.1W kidneys (n=6). In group II LEW.1A recipients were injected with 0.5ml (IV) of anti-LEW.1W class II alloimmune serum (n=15) at the end of the surgical procedure (9) (table 1). Additional groups of recipients were used to test the effect of cell transfer from tolerant recipients to naive non-irradiated recipients of a LEW.1W kidney. Adoptive transfers were performed intravenously on the day of transplantation. Group III received $8x10^7$ splenocytes from tolerant recipients (n=12). Group IV received $8x10^7$ purified T cells from the spleens of tolerant recipients (n=5). Group V received 5 to $8x10^7$ T cell-depleted splenocytes from tolerant recipients (n=6). Group VI received $8x10^7$ $CD103^+$ cell-depleted splenocytes from tolerant recipients (n=5). Group VII received $8x10^7$ splenocytes from naive LEW.1A rats (n=3). Group VIII received $8x10^7$ T cell-depleted splenocytes from naive LEW.1A rats (n=2).

Preparation of anti-donor MHC class II alloimmune serum and depletion of anti-class I antibodies: LEW.1A rats (n=10) were immunized with LEW.1W skin grafts and injected (IV) three days later and biweekly for two months with 10^8 mononuclear cells purified from LEW.1W spleen cells. Depletion of anti-class I antibodies was performed as described elsewhere (10).

Cell purification: *APC purification:* APC from tolerant recipients, naive LEW.1A, LEW.1W or BN rats were enriched from spleens digested with collagenase D (2mg/ml; Boehringer Mannheim, Mannheim, Germany) for 20 minutes at 37°C. 10 μM EDTA was added for 5 minutes and cells were washed and resuspended in 5μM EDTA/PBS/2% FCS. The resulting suspension was deposited on a Nicodenz gradient (14.5%) (Nycomed Pharma, Roskilde, Denmark), centrifuged and adjusted to the appropriate concentration. *Spleen and blood cell purification:* One hundred days after transplantation, spleen cells from tolerant recipients and naive LEW.1A rats were isolated by passing spleen tissue through a stainless steel mesh and depleting erythrocytes by osmotic shock. The splenocytes isolated were then washed twice in PBS. One portion was enriched for T cells using rat T cell enrichment columns (R&D systems) and another portion was depleted of T cells using an anti-CD3 antibody followed by Dynal beads. Cell purifications and depletions were systematically checked by flow-cytometric analysis (>95%). Peripheral blood lymphocytes (PBL) from tolerant recipients and naive LEW.1A rats were enriched by a ficoll gradient procedure and washed twice in PBS. *$CD103^+$ splenocyte depletion*: $CD103^+$ splenocytes were depleted by positive selection of anti-αE-chain integrin-expressing cells ($CD103^+$) using OX62 mAb followed by incubation with Pan Mouse IgG Dynabeads® according to the manufacture's recommendations (Dynal Biotech, Oslo, Norway); cell purity was systematically assessed using flow cytometry (>95%). *Graft infiltrating cell extraction:* Kidneys were digested with collagenase D (2mg/ml; Boehringer Mannheim, Mannheim, Germany) twice for 10 minutes at 37°C. Cells were then extracted by passing kidney tissue through a stainless steel mesh. The resulting suspension was deposited on a Ficoll gradient, centrifuged and depleted of erythrocytes by osmotic shock. The kidney infiltrating cells isolated were then washed twice in PBS. One part was used for flow-cytometry analysis and the other for MLR.

Mixed lymphocyte reactions and inhibition assays: Standard one-way mixed lymphocyte reactions (MLR) were performed. Splenocytes were isolated from naive rats and tolerant

recipients 100 days after transplantation. Donor-type LEW.1W and third-party Brown Norway (BN) enriched antigen presenting cells (APC) were irradiated. Triplicate samples of responder (10^5 cells/well) and stimulator cells ($2x10^4$ cells/wells) were plated in 96-well round-bottom plates in a final volume of 200µl of RPMI 1640 medium supplemented with 2mM L-glutamine, $5x10^{-5}$M 2-ME, 1mM sodium pyruvate (Life technologies), 1% non essential amino acids, 100U/ml penicillin, 0.1mg/ml streptomycin and 10% heat-inactivated FCS (Life Technologies). The cells were then cultured at 37°C in 5% CO_2. Proliferation of the responder population was assessed on day 3 and/or 5 by measuring the incorporation of [^{3}H] TdR (0.5 µCi per well; Amersham, Les Ulis, France) during the final 8 hours of culture. Cells were then harvested onto glass fiber filters and [^{3}H] TdR incorporation was measured by standard scintillation procedures (Packard Instruments, Meriden, CT). In certain experiments, MLR were performed in the presence of the Indeolamine 2,3-dioxygenase (IDO) antagonist 1-Methyltriptophan (500µg/well; Sigma). The results were expressed as specific c.p.m.

To test the suppressive activity of TCRαβ$^-$CD103$^+$ dendritic cells from tolerant recipients, $2x10^4$ enriched dendritic cell suspensions from LEW.1W rats were cultured in triplicate in U-bottom 96-well plates (0.2mL) with $1x10^5$ T cells purified from naive LEW.1A and decreasing numbers of enriched TCRαβ$^-$CD103$^+$ dendritic cells from tolerant anti-class II-treated LEW.1A rats irradiated (used as control) or not for 5 days at 37°C/7% CO_2. Cultures were pulsed with [^{3}H] T for the last 8 hours of culture. To test the suppressive activity of T cells from tolerant recipients, $1x10^5$ splenocytes or whole T cells purified from naive or tolerant LEW.1A rats were cultured in triplicate in U-bottom 96-well plates (0.2mL) with $2x10^4$ enriched dendritic cell suspensions from either LEW.1W or BN rats for 5 days at 37°C/7% CO_2. For inhibition assessment, increasing numbers of splenocytes purified from naive or tolerant LEW.1A rats were added to the MLR using naive T cells as responders. Cultures were pulsed with [^{3}H] T for the last 12 hours of culture. The results were expressed as specific c.p.m.

Antibodies and Flow-cytometric analysis: Splenocytes and GIC were isolated and prepared as previously described. Analysis was performed using CellQuest software (BD Biosciences, Mountain view, CA). The following hybridomas were obtained from the ATCC and produced by Bioatlantic (Nantes, France), or in our own laboratory and were used to phenotype rat leukocytes from the spleen and blood of naive LEW.1A rats and anti-donor class II-treated recipients 100 days after transplantation : W3/25 (anti-CD4), OX1 and OX30 (anti-CD45),

OX3 (anti-RT1.u), OX6 (anti-RT1.u/ RT1.a), OX7 (anti-Thy 1-1), OX8 (anti-CD8), OX19 (anti-CD5), OX22 (anti-CD45RC), OX26 (anti-CD71), OX33 (anti-CD45 present on B cells), OX34 (anti-CD2), OX35 (anti-CD4), OX39 (anti-CD25), 0X41 (anti-αGRP), OX62 (anti-α1-chain integrin; CD103), OX85 (anti-CD62L), WT1 (anti-CD11A), WT5 (anti-CD11B), 8A2 (anti-CD11C), JJ319 (anti-CD28), anti-CD80 (anti-B7.1, 3H5), anti-CD86 (anti-B7.2, 24F) 3.2.3 (anti-NKRP1), and R7.3 (anti-TCR). *Dendritic cell phenotype analysis:* Kidneys were minced and digested in 2 mg/ml collagenase D (Roche, Meylan, France) in RPMI 1640/1% FCS for 15 min at 37°C. EDTA (10 mM) was added for the last 5 min and the cell suspension was then pipetted up and down several times and filtered. Cells were washed once in PBS/2 mM EDTA/1% FCS, and mononuclear cells were isolated by centrifugation over Ficoll-Hypaque (Amersham, Les Ulis, France). For cytofluorometric analyses, $1x10^6$ cells were incubated for 20 min at 4°C with R7.3-PhycoErythrine (PE), CD4-PE-Cyanin 7 (PC7), OX62-AlloPhycoCyanin (APC) or OX6-APC-Cyanin 7 (APC-Cy7) mAb along with different FITC-conjugated mAb: TCRαβ, TCRγδ, CD25, CD161, CD11b, CD62L, CD172a, CD45RC or CD8. Cells were washed twice and analyzed on a FACSAria (BD Biosciences). CD161a and CD172a were used to discriminate pDC (OX62+ CD4+ DC) and OX62+CD4- DC subsets (17). Data were analyzed using FlowJo software (Treestar, Ashland, OR).

Histology and immunohistology: Kidney tissue samples were placed in 10% formol. Hematoxylin phloxin Safran (H&S) staining was performed on paraffin embedded sections. Vascular lesions (percentage of obstruction, leukocyte infiltration and medium lesions) were analyzed in at least 10 medium size vessels. Snap-frozen graft sections from tolerant recipients (>100 days) embedded in Tissue Tek (OCT compound; Bayer Diagnostics, Puteaux, France) were cut into 5-µm sections and fixed in acetone for 10 min at room temperature for immunochemistry. The cell infiltrate was measured by immunochemistry using a three-step indirect immunoperoxidase technique with primary antibody and the corresponding biotin-conjugated anti-mouse Ig-Ab, HRP-conjugated streptavidin and VIP substrate. The area of each immunoperoxidase-labeled tissue section infiltrated by cells was determined by quantitative morphometric analysis, as previously described (18). Briefly, the number of positively stained cells on each slide was counted by morphometric analysis using the point-counting technique, with a 121 intersection squared grid. The number of positively stained cells under the grid intersection was counted in a minimum of 10 adjacent fields (at a magnification of ×400). The percentage area of each section occupied by cells of a particular

phenotype was calculated as follows: [number of positive cells under grid intersection/(total number of grid intersections = 121)] × 100.

The results were expressed as the mean ± SD of positive cells per area of the tissue section. The following primary antibodies were used: Ox1/Ox30 (anti-CD45), ED2 (anti-CD68 like), R7-3 (anti-TCRαβ), OX62 (anti-α1-chain integrin), OX6 (anti-RT1.u/ RT1.a), W3/25 (anti-CD4), OX8 (anti-CD8) and 3.2.3 (anti-NKRP-1). Anti-donor-specific IgM and IgG isotypes were examined in the kidney grafts of tolerant recipients (>100 days post-transplantation) and syngeneic rats. Rejected grafts from untreated recipients harvested on day 7 were used as a positive control. Deposits of the different isotypes of rat Ig in the kidneys were studied by immunochemistry. Non-transplanted kidneys from LEW.1W rats were used as controls. Sections were incubated for 45 minutes with FITC-conjugated mouse monoclonal antibodies directed against rat IgM (MARM-4) (1/100) and IgG (MARG1) (1/100) (University of Louvain, Brussels, Belgium) and rinsed 3 times with PBS.

mRNA transcript analysis: Cβ, IDO, HO-1, FOXP3 and cytokine (IL2, IFNγ, IL13, TGFβ1, IL10, IL4 and TNFα) transcript analysis was performed by real-time PCR as described elsewhere (19) on kidney grafts from tolerant recipients and syngeneic LEW.1A grafts on day 100 after transplantation, and on kidneys from naive non-transplanted LEW.1W rats. HPRT was used as an endogenous control to normalize for RNA levels. The results were expressed as the intra-sample target / HPRT mRNA copy number ratio.

Statistical analysis: Data were analyzed using the Mann-Whitney test (analysis of two groups) or the Kruskal-Wallis test followed by a Dunn's post-hoc test (analysis of more than two groups). A two-way ANOVA followed by a Bonferonni post-hoc test was used to analyze the effect of the addition of 1-MT and the effect of anti-donor class II treatment. Survival curves were analyzed using the Kaplan-Meier test. Differences were defined as statistically significant when $p < 0.05$ (*) and $p < 0.01$ (**).

Results

Anti-donor class II antibodies induce tolerance of kidney grafts in recipients that reject third party skin grafts: MHC mismatched LEW.1W kidney graft survival in untreated LEW.1A recipients was 11 ± 1 days (n=6) (Table 1). Administration of a single dose of 0.5 ml of anti-donor class II alloimmune serum (Table 1) induced long-term graft survival in all LEW.1A recipients (n=15). The same serum, however, did not prolong the survival of BN ($RT1^n$) kidneys (data not shown). Furthermore, long-term surviving kidney allograft recipients accepted a LEW.1W skin graft but rejected a third party BN skin graft (Figure 1A). Tolerated kidney allografts display no histological or clinical signs of chronic rejection (Figure 1B). Finally, these results were supported by normal 100 day protein/creatinine ratios in urine samples (0.16 ± 0.02 g/mmol) (n=5) and by low and stable blood urea (6.42 ± 2.6 mmol/l) and creatinemia (35.4 ± 7 µmol/l) levels (naive rats urea: 5.5 ± 0.3mmol/l, creatinemia: 27.5 ± 4.4 µmol/l, protein/creatinine ratio<0.2 g/mmol) (Figure 1C). Kidneys from tolerant recipients displayed only barely detectable levels of IgM and IgG deposition, similar to that observed in syngeneic grafts harvested 100 days after transplantation (Figure 1D).

The grafts of anti-donor class II-treated tolerant recipients displayed an increased number of MHC-II and α1-integrin+ ($CD103^+$) infiltrating cells: One hundred days after transplantation, the grafts of tolerant rats exhibited a larger infiltrate than those from syngeneic recipients (81 ± 46 vs. 12.5 ± 2.9 $CD45^+$ cells per graft surface area in tolerant rat kidneys and syngeneic kidneys respectively) ($p<0.05$) or normal LEW.1W rats (9.7 $CD45^+$ cells per graft surface area) (Figure 2). As assessed by immunochemistry, the grafts from tolerant rats were mainly infiltrated by MHC class II^+ cells (66 ± 27 vs 15 ± 10 cells per graft surface area in tolerant rat kidneys and syngeneic kidneys respectively) and $CD103^+$ cells (27.8 ± 8.3 vs 4.2 ± 0.5 cells per graft surface area in tolerant rat kidneys and syngeneic kidneys respectively) ($p<0.05$). Grafts from tolerant recipients presented a T cell infiltrate ($CD4^+$ or $CD8^+$) similar to that observed in the grafts of syngeneic recipients (Figure 2). Graft infiltrating cells from tolerant rats were then analyzed by 5-color flow-cytometry to characterize the $CD103^+$ cells in more detail. Fitting with the immunochemistry analysis, the graft infiltrating cells contained relatively high numbers of $CD103^+$ cells compared to naive animals (Figure 3). Eighty percent (78% ± 4.8) of these $CD103^+$ cells co-expressed TCRαβ,

among which about 76% ± 3.5 were $CD8^+$ with a $CD45RC^{-/+}$ $CD62L^{low}$ $CD25^-$ phenotype, 19% ± 2 were $CD4^+$ T cells characterized by a $CD25^{low}$ $CD45RC^-$ $CD62L^-$ phenotype (Figure 3) and 7% ± 5 were double positive $CD4^+CD8^+$ cells. One third of the remaining 20% ± 4 .2 of the $CD103^+$ $TCR\alpha\beta^-$ cells could be further divided into MHC II^+ $CD4^+$ (45% ± 3) and MHC II^+ $CD4^-$ (55% ± 3) DC (20), and two thirds were MHC II^- , among which 59% ± 3.1 were γδ T cells with a $CD25^+$ phenotype. These data suggest that tolerated grafts harbor various types of infiltrating cells, including $CD8^+$ T cells with a $CD103^+$ $CD45RC^-$ $CD62L^{low}$ phenotype, a population that has also been described as being potentially regulatory/activated T cells (21, 22).

Tolerated kidneys from anti-donor class II-treated tolerant rats display high levels of Cβ, FOXP3 and IDO transcripts: Transcript levels of Cβ, HO-1, FOXP3, IDO and the cytokines IFNγ, IL2, IL4, IL13, IL10, TNFα and TGFβ were measured in the grafts of tolerant rats and compared to those in syngeneic rats 100 days after transplantation and in normal kidneys. Kidney grafts from tolerant recipients displayed significantly higher levels of IDO transcripts (>8 fold, $p<0.05$) and FOXP3 (> 100 fold, $p<0.05$). The level of Cβ was > 30 fold ($p<0.05$) that measured in syngeneic grafts and normal kidneys (Figure 4). No difference was found for TGFβ1, HO-1 or for Th1 and Th2 cytokines in kidneys from the three groups (*data not shown*).

Non T cells from anti-donor class II-treated recipients (>100 days) inhibit the donor-specific response of purified T cells from anti-donor class II-treated recipients: Splenocytes and purified spleen T cells from naive and tolerant (>100 days) LEW.1A rats were stimulated for 5 days with donor LEW.1W or with BN third-party APC-enriched populations. Splenocytes from tolerant recipients displayed a 5 fold decrease in their donor-specific proliferative response to LEW.1W APC ($p<0.05$) whereas their response to third party BN-enriched APC was unmodified (Figure 5A). In contrast, purified T cells from the spleens of naive LEW.1A rats or tolerant recipients (>100d) proliferated similarly when stimulated with donor LEW.1W APC (Figure 5B).

These experiments strongly suggest the control of the T cell alloresponse of tolerant recipients by the non-T cells of the same tolerant recipient. However, the cells transferred from tolerant recipients to secondary hosts should also affect the alloreactive T cells from naive host by

exerting a dominant tolerance effect. To test this possibility, we first investigated the ability of tolerant rat T cells to inhibit the response of naive LEW.1A T cells to donor LEW.1W APC. When added in increasing amounts, T cells from tolerant recipients did not modify the proliferative response of naive LEW.1A T cells against donor antigen APC (Figure 6A). Interestingly, whereas T cells from tolerant recipients did not exhibit any regulatory function, $TCR\alpha\beta^{-}CD103^{+}$ cells from tolerant recipients were able to reduce the response of naive LEW.1A T cells co-cultured with enriched donor antigen APC (Figure 6B).

Donor-specific inhibition of the proliferative responses of splenocytes from tolerant recipients is partly dependant on IDO: Because IDO, which was found to be over-expressed in tolerated grafts, has also been shown to be involved in several regulatory effects of non T cells and particularly dendritic cells, we further investigated its potential role *in vitro* in controlling the tolerant T cell response to donor APC. Splenocytes from tolerant recipients were tested in MLR in the presence or absence of a specific IDO antagonist (1-MethylTriptophan; 1-MT). As earlier, splenocytes from tolerant recipients proliferated significantly less (5 fold) than naive splenocytes ($p<0.05$; Figure 5A and 6C). Addition of 1-MT significantly restored the proliferation of splenocytes from tolerant recipients stimulated with LEW.1W donor APC ($p<0.01$; Figure 6C), suggesting a role for this molecule in this model.

***Transfer of splenocytes from anti-donor class II-treated recipients induces donor-specific tolerance of LEW.1W kidney grafts in secondary immunocompetent LEW.1A naive rats*:** To test the hypothesis of regulatory cell involvement in the maintenance phase of tolerance, we performed different series of cell transfers from tolerant recipients (>100 days) into secondary immunocompetent, non-irradiated, naive LEW.1A recipients of LEW.1W kidney grafts. Splenocytes harvested from tolerant recipients and administered intravenously to secondary LEW.1A recipients consistently induced long-term survival of LEW.1W kidneys (Table 1, Figure 7A) but not of third-party BN kidneys (data not shown). As a control, splenocytes from naive LEW.1A rats did not prolong survival of LEW.1W kidneys (mean survival time 11 ± 1 days; Table 1, Figure 7A). Secondary hosts of a MHC-disparate LEW.1W kidney graft that had received an injection of splenocytes from anti-donor class II-treated recipients displayed a robust and dominant tolerance without histological or clinical signs of chronic rejection (Figure 7B). The absence of chronic rejection in secondary hosts

was also supported by normal protein/creatinine ratios in the urine and normal levels of blood urea and creatinine on day 100 following adoptive transfer (data not shown). Finally, this tolerant state was transferable over a second generation of naive recipients when splenocytes from secondary tolerant recipients were transferred into a third immunocompetent naive LEW.1A recipient of a LEW.1W kidney graft (data not shown).

TCRαβ CD103$^+$ spleen cells from tolerant rats are instrumental in transferring tolerance to a second LEW.1W kidney graft: Because splenocytes from tolerant recipients consistently induced tolerance in secondary non-irradiated LEW.1A hosts and because CD4$^+$CD25$^+$ regulatory T cells have been shown to play an important role in other models of tolerance in the same LEW.1W/LEW.1A strain combination (23) (*and D. Lair et al, Submitted*), we first tested the capacity of purified T cells to transfer tolerance. However, even at high doses ($8x10^7$), purified spleen T cells from tolerant recipients had no effect on the survival of subsequent LEW.1W kidney allografts placed into secondary LEW.1A hosts (n=5) (Table 1 and Figure 7A). These data are in agreement with the *in vitro* findings (Figure 6A) and demonstrate the inability of the T cell population from tolerant recipients to control the alloresponse of naive T cells.

Because unseparated splenocytes but not T cells induced tolerance, we then tested the effect of non-T cell splenocyte transfer. T cell-depleted splenocytes ($5x10^7$ to $8x10^7$) prolonged survival of subsequent LEW.1W kidney allografts in 3 out of 6 naive secondary recipients without inducing long term acceptance or tolerance (Table 1, Figure 7A). The effect of this transfer was not dose-dependent (3 recipients injected with $5x10^7$ cells rejected their graft on days 14, 21 and 21 and 3 recipients injected with $8x10^7$ cells still rejected on days 12, 12 and 50). T cell-depleted splenocytes ($8x10^7$ cells) from naive LEW.1A rats used as controls had no effect on graft survival (Table I).

Collectively, these data strongly suggest that whereas T cells alone are unable to transfer tolerance, they are instrumental for the full effect of non T cells. We thus tried to further investigate the non-T cell compartment which could cooperate with T cells in transferring full tolerance in this model. Kidney grafts from tolerant recipients are mainly infiltrated by MHC class II$^+$ cells (Figure 2). As DC is the main population expressing MHC class II molecules, we investigated their ability to cooperate with T cells in order to achieve tolerance transfer. Most of the dendritic cells present in the spleen are recognized by the OX62$^+$ antibody specific for the α1 integrin (CD103$^+$) marker (24). Interestingly, $8x10^7$ CD103$^+$ depleted

splenocytes from tolerant recipients were no longer able to transfer prolongation of graft survival of subsequent LEW.1W kidney allografts placed in secondary LEW.1A hosts (n=5) (Table 1 and Figure 7A). These data, which are also in agreement with the *in vitro* findings (Figure 6B), strongly suggest that $CD103^+$ non-T cells from tolerant rats are not only able to educate naive host T cells when transferred *in vivo* but could also cooperate with T cells from the spleen to transfer full tolerance in this model.

Discussion

The capacity of anti-donor MHC alloantibodies to prolong survival or induce tolerance to allografts is well documented. We previously showed that a single administration of anti-donor MHC class II alloimmune serum at the time of transplantation induces significant prolongation of heart graft survival (10). Here, using the same strain combination, we show that anti-donor class II antibodies induced a full state of tolerance to a kidney graft i.e. without functional or histological signs of chronic rejection, that could be transferred to immunocompetent secondary recipients over two generations. This contrasts with the tolerance induced by donor cell priming in which severe chronic rejection co-exists with the capacity to transfer long term survival to naive secondary recipients (25) (*and our similar observations in the LEW.1W/LEW.1A combination*).

The ability to transfer tolerance to unmanipulated recipients by cell transfer over multiple generations is a well known phenomenon referred to as "infectious tolerance" where the regulatory properties of cells from tolerant recipients can be transferred to a naive cell population thus converting them into regulatory cells. Since regulatory $CD4^+CD25^+$ T cells have been shown to be capable of converting naive effector T cells into regulatory T cells (26), we initially set out to determine whether regulatory T cells were responsible for the infectious tolerance in this model.

The normal numbers of spleen $CD4^+CD25^+$ T cells in tolerant animals (*data not shown*), the lack of suppressive activity of T cells from tolerant animals when tested *in vitro*, together with the inability of purified T cells from tolerant recipients to transfer tolerance to naive secondary hosts, suggested that $CD4^+CD25^+$ regulatory T cells alone could not explain the ability of splenocytes to transfer tolerance. Along the same lines, contrasting with the majority of tolerance transfer protocols including our own observations in the same strain combination (23) (Lair *et* al., *Submitted*), irradiation of the secondary recipient was not required. This suggests that the classical homeostatic expansion of $CD4^+CD25^+$ regulatory T cells in a secondary recipient where empty space has been made is not necessary here. The lack of effect of a large number of purified T cells in transferring long-term graft survival here contrasts with the reproducible effect of T cell transfer in the same strain combination in two other protocols of tolerance; DST-induced tolerance (25) (Lair *et* al., *Submitted*) and desoxyspergualine derivative-induced tolerance (23), again indicating that T cells were not directly instrumental in transferring tolerance in this model.

We therefore addressed the possibility that non T cells and in particular, DC, were responsible for tolerance transfer, as observed in other models (3, 10, 23, 27, 28). This was possible given our findings that proliferation of donor-reactive T cells from tolerant recipients in MLR was inhibited by the presence of non-T cells and that TCRαβ$^-$CD103$^+$ cells from tolerant recipients were able to suppress the proliferation of naive host T cells *in vitro*. However, the possibility of tolerance transfer being mediated solely by non-T cells or DC was also excluded by the finding that neither non-T cells nor splenocytes depleted of CD103 (a marker specific for spleen DC) were able to transfer tolerance.

Thus, our data show that both T cells and CD103$^+$ cells have to be present to transfer tolerance. Such a hypothesis could be validated by a "mixing approach" demonstrating the need for both T lymphocytes and CD103$^+$ APC to transfer tolerance. However, to our knowledge, no study in the literature has reported this type of experiment. This may be explained by the difficulties encountered when attempting to reconstitute a mixed cell population *in vivo* after an *in vitro* cell purification. Here, negative selection was chosen in order to avoid any possible alteration of the selected population resulting from positive selection through antibody binding. Using this approach we were able to identify the different cell populations required for the transfer of tolerance and to minimize the procedures necessary to obtain a pure cell population.

With regards to the mechanisms of the maintenance of tolerance in this model, several lines of evidence point towards a role for regulatory T cells: high intragraft accumulation of IDO and FOXP3 and large numbers of infiltrating TCRαβ$^+$CD103$^+$ cells, a cell type previously described as having regulatory properties (21, 29). In fact, CD103$^+$ has also been shown to be present on distinct regulatory T cell populations at different sites and its expression may be regulated locally (21, 30, 31). These cells were, however, not detected in the spleen (*data not shown*). The strong accumulation of Cβ transcripts contrasting with the normal TCRαβ$^+$ infiltrating cells compared to syngeneic rats gives further credence to the hypothesis that T cells from the tolerant rats had acquired a regulatory/activated phenotype. Moreover, several models have described the existence of dendritic cells that function by inducing or enhancing the suppressive efficacy of different lineages of regulatory T cells (32, 33) as well as the induction of FOXP3 expression by T cells that have acquired regulatory function following stimulation by autologous mature DC (34, 35).

The finding of IDO transcript accumulation in kidney grafts from tolerant recipients and that a specific IDO antagonist partially restored the proliferative anti-donor response of splenocytes

from tolerant recipients *in vitro* suggests a role for this molecule in our model. IDO is expressed by non-T cells, and particularly by DC (36-38) and can act directly on T cells, or indirectly on non-T cells (37, 39) Its mechanism of action here remains to be explored further. The incomplete restoration of the proliferative response of splenocytes from tolerant recipients by the IDO-inhibitor 1-MT may be related to the high sensitivity of activated T cells to inhibition by tryptophan metabolites (40). Moreover, the increased proliferation of naive responding cells in MLR during IDO blockade suggests that it may exert a "natural" immunosuppressive effect. This is in accordance with the fact that T cells have a regulatory checkpoint within their cell cycle that is sensitive to the level of free tryptophan (41). This effect may be intensified in tolerant recipients. However, the lack of IDO transcripts in the spleens of tolerant recipients, suggests that this enzyme is mostly produced in the graft. Nevertheless, given that IDO was measured in whole splenocytes, it is possible that the dendritic cells from the spleen (1-2% of the total cells) may not be in sufficient numbers to allow differences with spleens from syngeneic grafts to be detected.

Altogether, these data show that following tolerance induction by anti-donor class II antibody administration, T cells alone are not sufficient to transfer a state of dominant tolerance but require the presence of TCR$\alpha\beta^{-}$CD103^{+} dendritic cells. We suggest that these cells act in concert with T cells from tolerant recipients to educate naive host T cells. Finally, we suggest that the indeolamine 2,3-dioxygenase pathway plays a role in this effect.

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2. Senior authors contributed equally to this project.
3. Abbreviations used in this paper: APC-Cy7: AlloPhycoCyanin Cyanin-7; BN: Brown Norway; CD: Cluster of Differentiation ; DC: Dendritic Cell; HPRT: Hypoxanthine Guanine Phosphoribosyl Transferase; 1-MT:1-Methyl Tryptophane; LEW: Lewis ; PC7: PE-cyanin 7; Tol:tolerant.

Legends

Table 1: *Experimental groups*.

Figure 1: *Anti-donor class II antibodies induce donor-specific tolerance.* A) Long-term surviving LEW.1A recipients of a kidney allograft accepted a donor-antigen (LEW.1W) skin graft but rejected a third party (BN) skin graft. Tolerant recipients accepted a donor-matched skin graft. B) Kidney grafts from tolerant recipients did not show pathological changes indicative of chronic rejection, but only slight local fibrosis and lymphoid infiltration, only moderate acute tubular necrosis and no vascular or glomerular lesions. C) Blood urea and creatinine and urine protein/creatinine ratios were measured in tolerant recipients one year post transplantation and compared to normal rats. D) Kidney grafts from tolerant recipients displayed barely detectable levels of IgM (a) and IgG (d) deposition, similar to that observed in syngeneic grafts (b and e respectively) unlike rejected grafts from untreated recipients harvested on day 7 after transplantation and used as a positive control (c and f respectively).

Figure 2: *Phenotype of cells infiltrating the grafts of tolerant and syngeneic recipients 100 days after transplantation*. Graft infiltrating cells from tolerant (black bar) or syngeneic (white bar) recipients were characterized by immunochemistry. The phenotype of graft infiltrating cells is displayed as mean ± SD of positive cells per area of tissue section, as previously described (18).

Figure 3: *Phenotype of $CD103^+$ graft infiltrating cells from tolerant recipients 100 days after transplantation*. Graft infiltrating cells (see Materials and Methods for isolation process) of 3 tolerant recipients were stained with a mixture of TCRαβ-PE, OX35-PC7, CD103-APC and MHC Cl.II-APC-Cy7 mAbs together with different FITC-conjugated mAbs directed against TCRαβ, TCRγδ, CD25, CD161, CD11b, CD62L, CD172a, CD45RC or CD8, and analyzed on a FACS Aria. Cells were first separated, based on the expression of CD103 and TCRαβ, into $CD103^+$ $TCR\alpha\beta^-$, $CD103^+$ $TCR\alpha\beta^+$ and $CD103^-$ $TCR\alpha\beta^+$ cells (top right panel). The phenotype of these subsets was then analyzed using different markers as indicated. Figure 3 shows data obtained in graft infiltrating cells of 1 tolerant recipient, representative of 3. The expression of CD103 was tested without or with collagenase digestion and no significant differences were observed (*data not shown*).

Figure 4: *Kidneys from tolerant recipients display a higher level of FOXP3, Cβ and IDO transcripts.* IDO (A), Cβ (B) and FOXP3 (C) transcript levels were measured by RT-PCR in

kidneys from tolerant recipients and compared to those in normal kidneys and syngeneic grafts 100 days post transplantation.

Figure 5: *Splenocytes but not spleen T cells from tolerant recipients exhibit donor-specific hyporesponse.* Total spleen cells and purified spleen T cells from naive LEW.1A rats and tolerant recipients were stimulated for 5 days with donor antigen (LEW.1W) or third party (BN) enriched APC populations. A) Spleen cells from tolerant recipients displayed a decreased proliferative response to donor antigen APC in MLR ($p<0.05$; a) but not to third-party APC (b). B) Purified T cells from tolerant recipients proliferated similarly to T cells isolated from naive recipients when stimulated with donor antigen.

Figure 6: *$CD103^+$ non-T cells, but not T cells, from tolerant recipients exhibit regulatory properties.* A). The ability of purified T cells from tolerant recipients to inhibit proliferation of naive LEW.1A T cells against donor-antigen APC was assessed in a coculture system by adding increasing numbers of T cells from tolerant recipients. B). Suppressive activity of $TCR\alpha\beta^-CD103^+$ cells from tolerant recipients when added to cocultures of naive LEW.1A T cells stimulated with LEW.1W donor APC. C). Effect of the inhibition of IDO production, by addition of 1-MT, on the proliferative response of splenocytes from tolerant recipients and naive rats in MLR.

Figure 7: *Tolerance induced by anti-donor class II administration is infectious.* A) Kidney graft survival in untreated LEW.1A recipients was 11 ± 1 days (n=6). Splenocytes ($8x10^7$) from tolerant recipients (n=12) induced long-term survival of secondary untreated and immunocompetent LEW.1W kidney grafts. T cell-depleted splenocytes (5 to $8x10^7$) from tolerant recipients (n=6) only moderately prolonged graft survival. $8x10^7$ $CD103^+$-depleted splenocytes from tolerant recipients had no effect on graft survival prolongation (n=5). B) The kidneys of secondary recipients that had received spleen cells from tolerant recipients did not show any pathological changes classically associated with chronic rejection.

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Groups	Treatments	N	Graft survival (days)	p
I	Untreated	6	11+/- 1	
II	Anti-donor MHC class II alloimmune serum	15	> 100	< 0.01
III	$8x10^7$ splenocytes from Tol.	12	> 100	< 0.01
IV	$8x10^7$ spleen T cells from Tol.	5	10, 11, 11, 11, 11	N.S
V	5 to $8x10^7$ T depleted splenocytes from Tol.	6	12, 12, 14, 21, 21, 50	N.S
VI	$8x10^7$ CD103 depleted splenocytes from Tol.	5	11, 11, 11, 11, 11	N.S
VII	$8x10^7$ splenocytes from naive LEW.1A rats	3	10, 11, 11	N.S
VIII	$8x10^7$ T depleted splenocytes from naive LEW.1A rats	3	11, 11, 11	N.S

Table 1

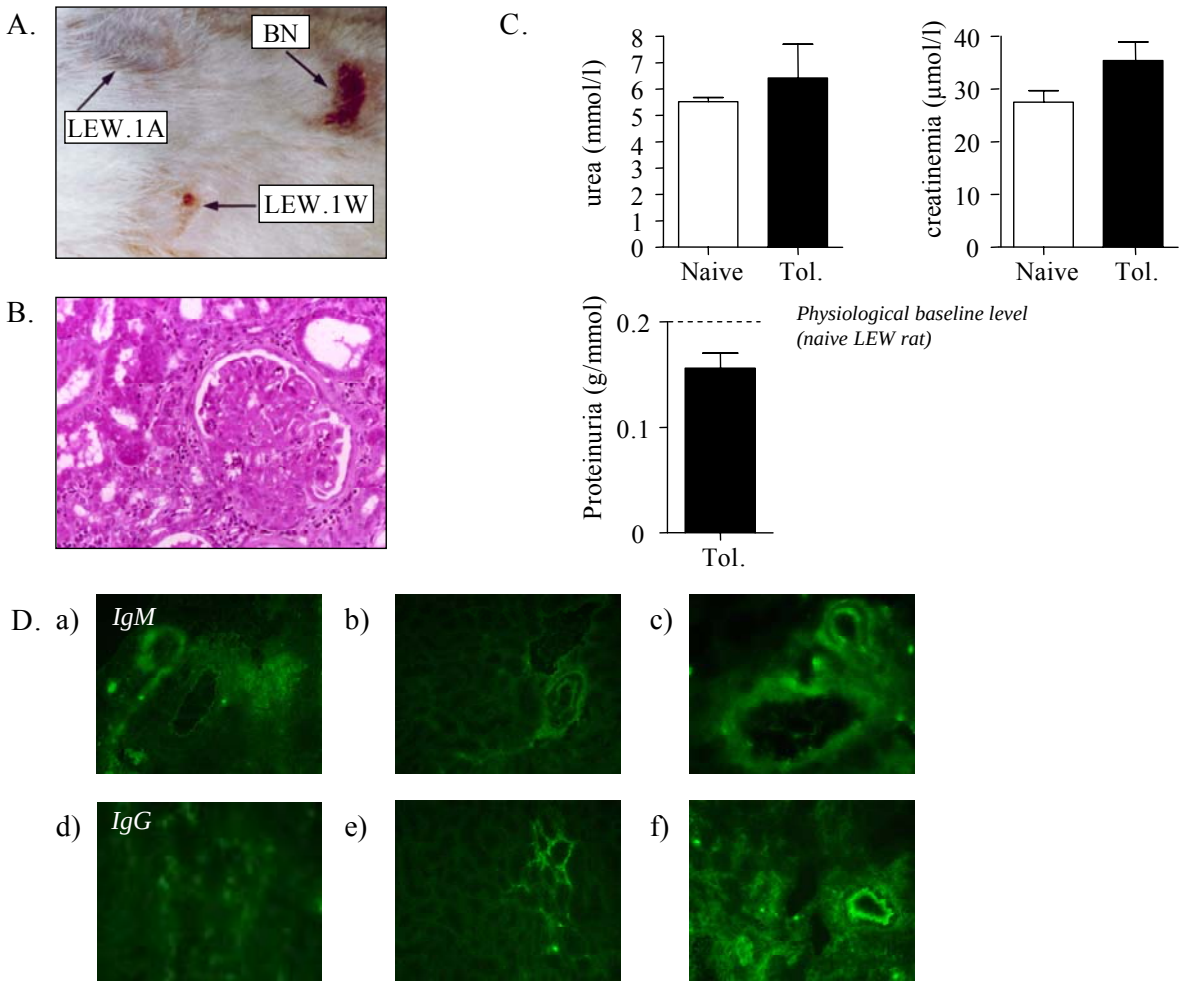

A.
BN
LEW.1A
LEW.1W
B.
C.
urea (mmol/l)
0
1
2
3
4
5
6
7
8
Naive
Tol.
creatinemia (µmol/l)
0
10
20
30
40
Naive
Tol.
Proteinuria (g/mmol)
0
0.1
0.2
Tol.
Physiological baseline level
(naive LEW rat)
D. a)
IgM
b)
c)
d)
IgG
e)
f)

Figure 1

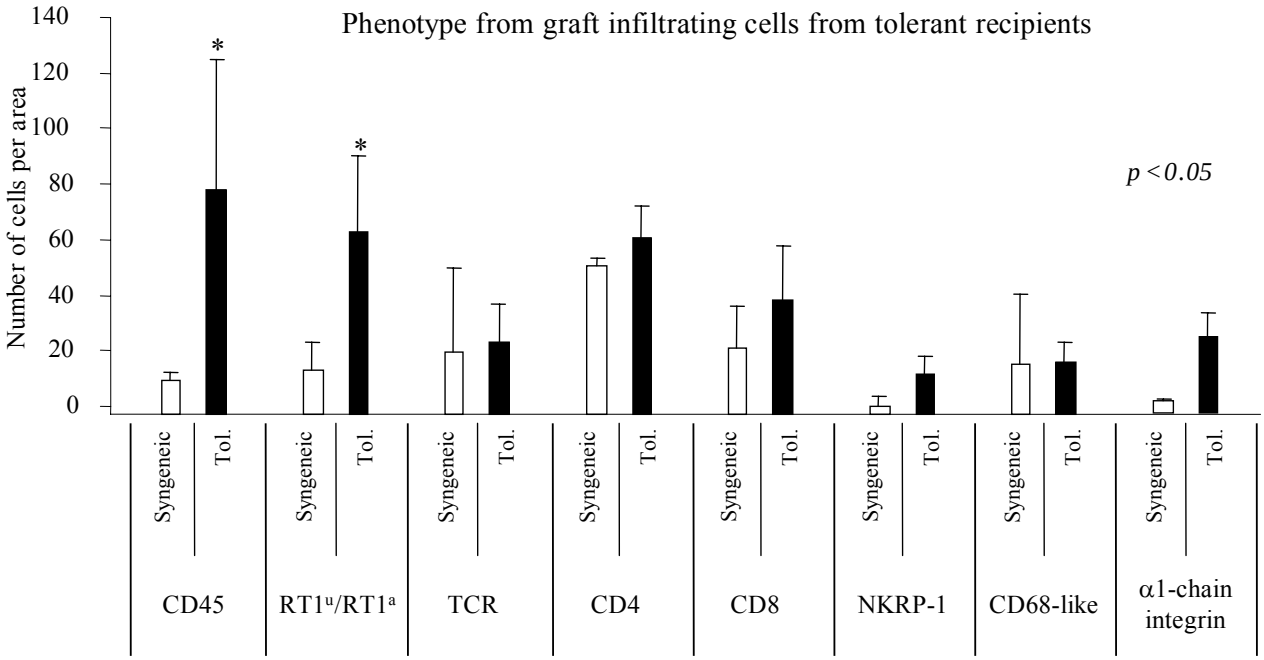

Phenotype from graft infiltrating cells from tolerant recipients
Number of cells per area
140
120
100
80
60
40
20
0
*
*
p < 0.05
Syngeneic
Tol.
CD45
RT1u/RT1a
TCR
CD4
CD8
NKRP-1
CD68-like
α1-chain integrin

Figure 2

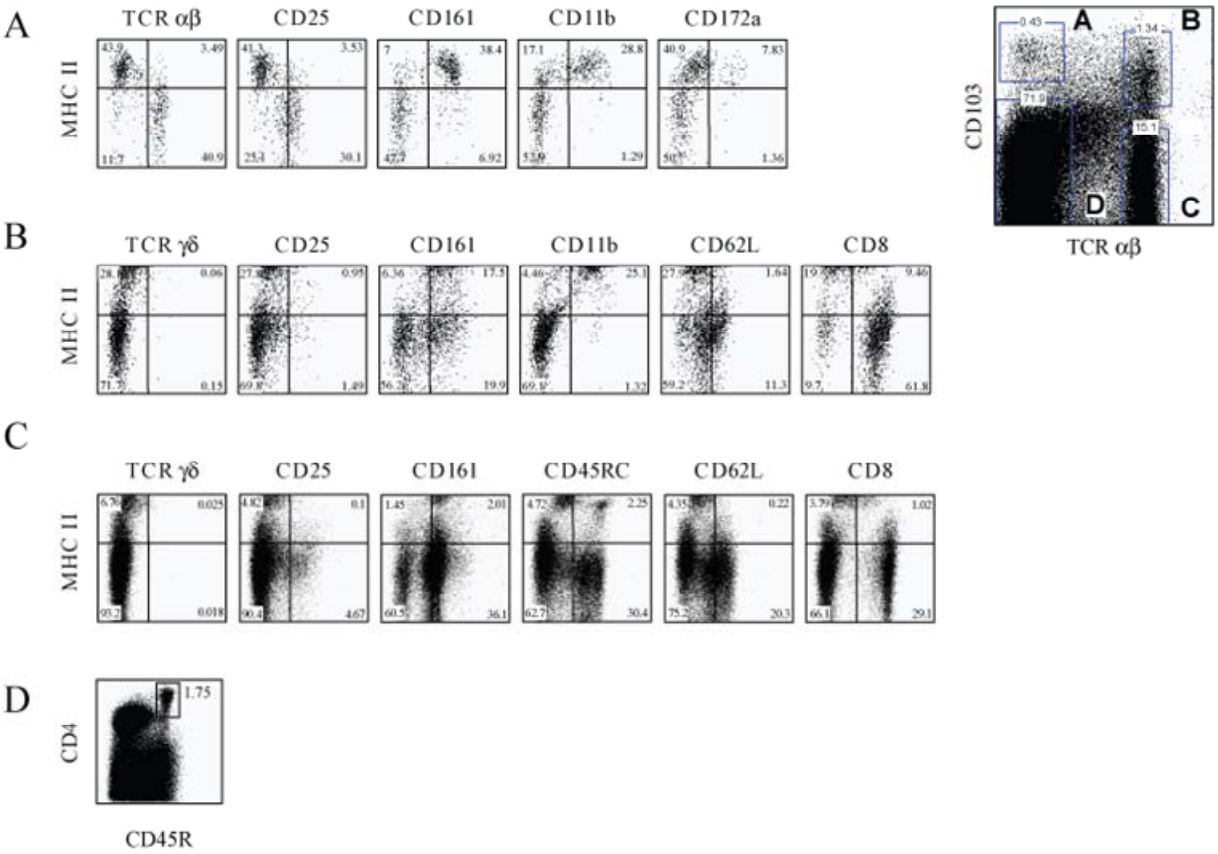
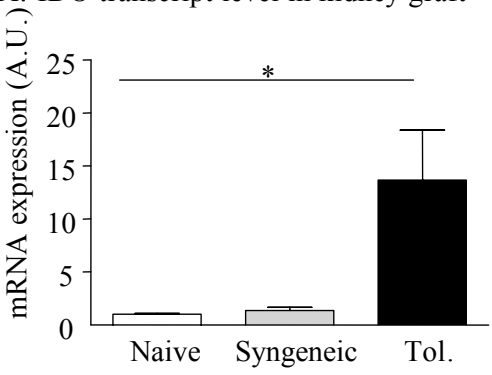
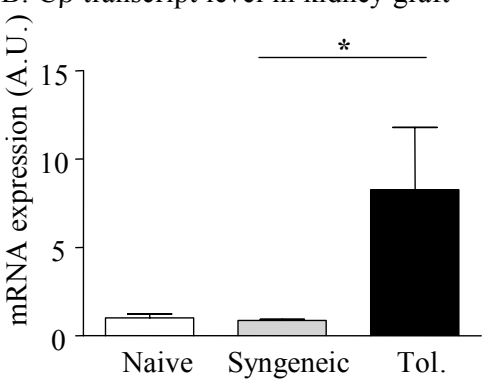

A
TCR αβ
CD25
CD161
CD11b
CD172a
MHC II
B
TCR γδ
CD25
CD161
CD11b
CD62L
CD8
MHC II
C
TCR γδ
CD25
CD161
CD45RC
CD62L
CD8
MHC II
D
CD4
CD45R
1.75
CD103
TCR αβ
A
B
C
D

Figure 3

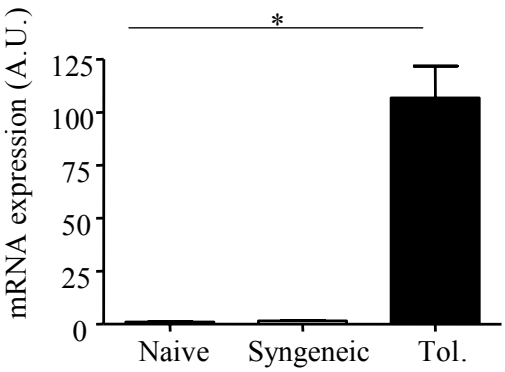
A. IDO transcript level in kidney graft



B. Cβ transcript level in kidney graft



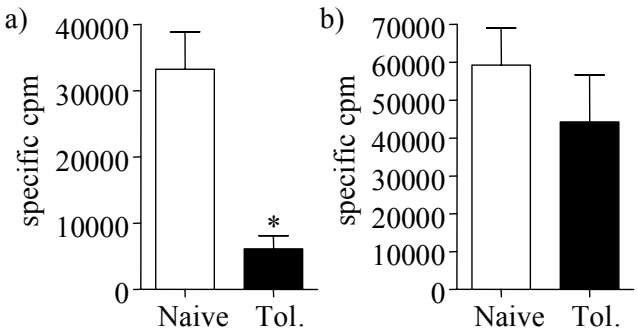
C. FOXP3 transcript level in kidney graft



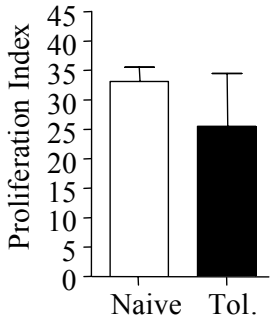
** p < 0.05*

Figure 4

A.



B.



** p < 0.05*

Figure 5

In vitro suppressive activity of purified T cells and non-T cells from tolerant recipients

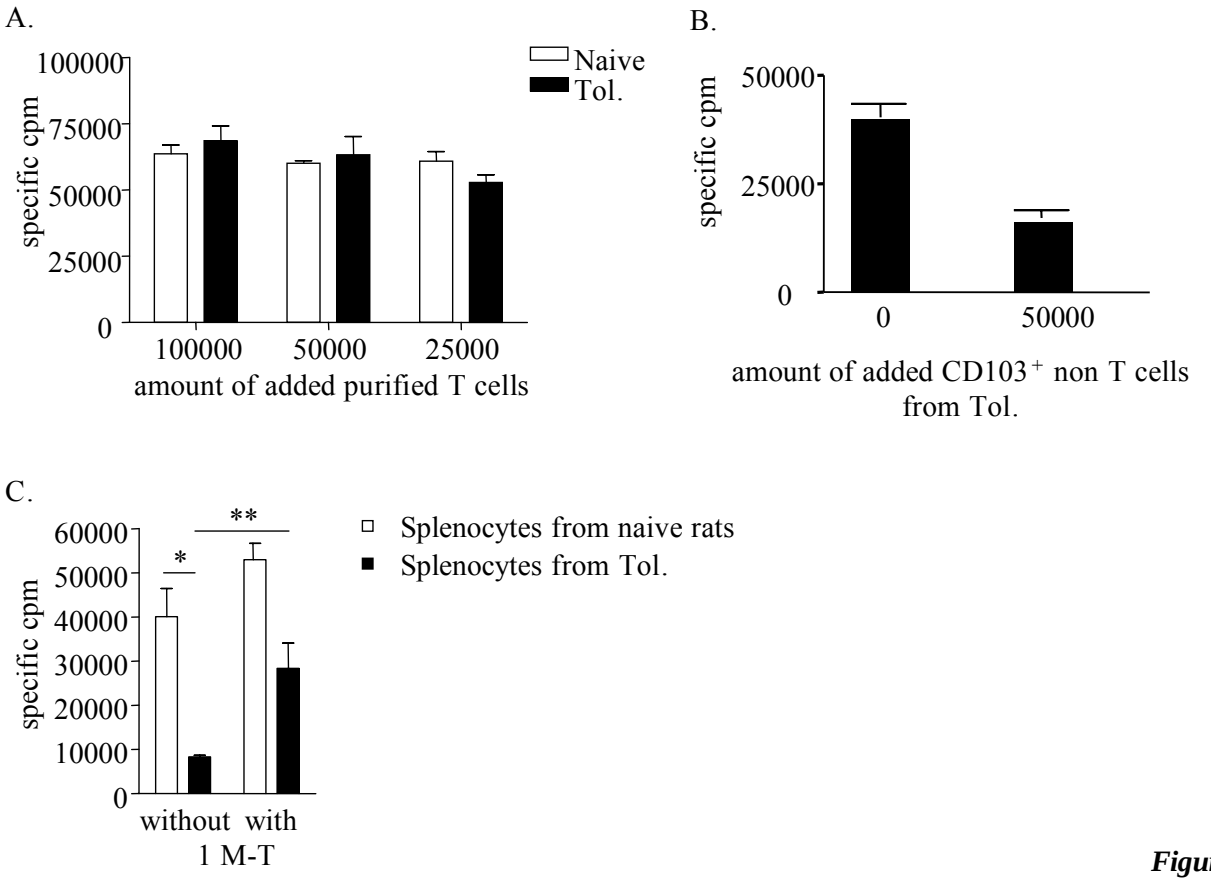
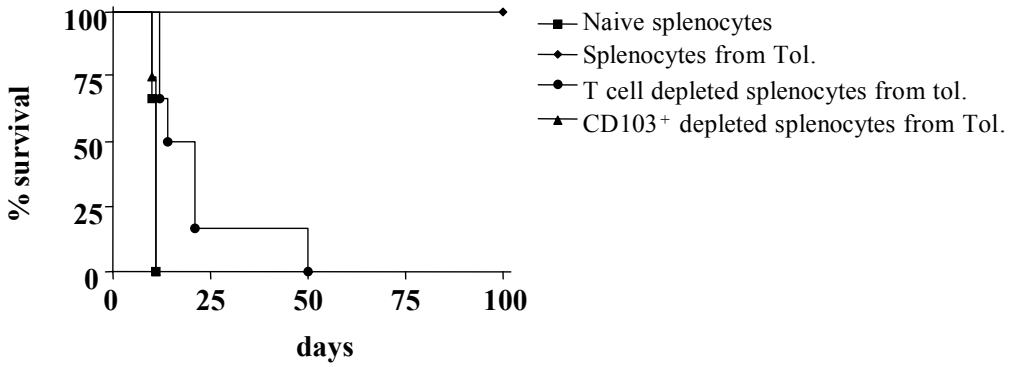


Figure 6

A.



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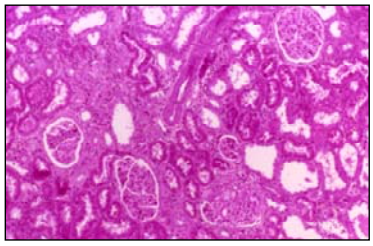

Figure 7

II.

Annexe 2

Titre original:

Operationally tolerant and minimally immunosuppressed kidney recipients display strongly altered blood T-cell clonal regulation.

Sophie Brouard, Alexandre Dupont, Magali Giral, Stéphanie Louis, David Lair, Cécile Braudeau, Nicolas Degauque, Frédérique Moizant, Annaick Pallier, Catherine Ruiz, Marina Guillet, David Laplaud and Jean-Paul Soulillou

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III.

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Operationally Tolerant and Minimally Immunosuppressed Kidney Recipients Display Strongly Altered Blood T-Cell Clonal Regulation

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Most kidney transplant recipients who discontinue immunosuppression reject their graft. Nevertheless, a small number do not, suggesting that allogeneic tolerance state (referred to operational tolerance) is achievable in humans. So far, however, the rarity of such patients has limited their study. Because operational tolerance could be linked to anergy, ignorance or to an active regulatory mechanism, we analyzed the blood T-cell repertoire usage of these patients. We report on comparison of T-cell selection in drug-free operationally tolerant kidney recipients (or with minimal immunosuppression), recipients with stable graft function, chronic rejection and healthy individuals. The blood T cells of operationally tolerant patients display two major characteristics: an unexpected strongly altered T-cell receptor (TCR) Vβ usage and high TCR transcript accumulation in selected T cells. The cytokine transcriptional patterns of sorted T cells with altered TCR usage show no accumulation of cytokine transcripts (IL10, IL2, IL13, IFN-γ), suggesting a state of hyporesponsiveness in these patients. Identification of such a potential surrogate pattern of operational tolerance in transplant recipients under life-long immunosuppression may provide a new basis and rationale for exploration of tolerance state. However, these data obtained in a limited number of patients require further confirmation on larger series.

Key words: Kidney transplantation, tolerance, T cells, T-cell receptor

Abbreviations: N^L, normal individual; DF-Tol, drug-free operationally tolerant patient; Ster, steroid; CR, chronic rejection; Sta, stable graft function; CNI, CalciNeurin inhibitor; PBMC, peripheral blood mononuclear cells; APC, antigen presenting cell; CDR3-LD, complementary determining region 3-length distribution; HPRT, hypoxanthine phosphoribosyl transferase; ALG, anti lymphocyte globulins; PCR, polymerase chain reaction; TCR, T-cell receptor; Ab, antibody; PE, phycoerythrine; FITC, Fluorescein isothiocyanate; PRA, panel reactive antibody; HLA, human leukocyte antigen; CMV, cytomegalovirus; HCV, hepatitis C virus; PTLD, post-transplant lymphoproliferative disorder; ATG, anti-thymocyte globulin; Manova, multivariate statistical analysis of variance; EBV, Epstein Barr virus; HSV, herpes simplex virus; IS, immunosuppressant; Inc, incompatibilities.

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Introduction

Kidney transplantation is the reference treatment for patients with end-stage renal disease (1). However, the rate of progressive graft loss 1 year after transplantation has been little influenced by increasingly efficient immunosuppressants (2,3), suggesting that immunologically driven chronic graft rejection might not be the primary mechanism involved in graft loss and that chronic dysfunction is also fundamental (2,4). In addition, a link between an increased incidence of skin cancer and lymphoma and long-term exposure to immunosuppression has been well documented (5). Consequently, the possibility of decreasing or withdrawing immunosuppression in kidney recipients, particularly in the long term, would be a major advance.

Anecdotal cases of kidney recipients having interrupted immunosuppression without subsequent rejection (6–9) have raised the possibility that some patients with well-functioning grafts may have become operationally tolerant. Certain graft recipients with stable function have been shown to develop circulating T cells exhibiting regulatory properties *in vitro* for autologous pre-graft T cells

stimulated by donor leukocytes, whereas patients with chronic rejection more frequently develop effector cells against donor cell determinants (10,11). Nevertheless, there is currently no established procedure to determine who, among stable immunosuppressed recipients, may be operationally tolerant and thus could be withdrawn from chronic immunosuppression.

Because operational tolerance could be linked to clonal deletion, anergy, ignorance or to an active regulation, we analyzed T-cell selection and activation in the blood of kidney recipients who, many years after transplantation, are 'operationally tolerant' to their graft. We show that drug-free operationally tolerant patients have previously unobserved peripheral T-cell clonal alteration. In addition, sorted T-cell populations using selected T-cell receptors (TCR) from drug-free operationally tolerant recipients are mainly $CD8^+$, characterized by an absence of cytokine transcript accumulation. Finally, minimally immunosuppressed kidney recipients display the same TCR alteration patterns as drug-free operationally tolerant recipients, suggesting they may be operationally tolerant too. These new observations open up the possibility that a decrease in the diversity of the $CD8^+$ TCR repertoire in these patients may contribute to the maintenance of their operationally tolerant state. Taken together, our data also suggest that altered clonal distribution in the blood of operationally tolerant recipients may act as a marker, which could help to identify a state of operational tolerance in recipients under chronic immunosuppression with stable graft function.

Patients, Materials and Methods

Patients

The protocol was approved by the University Hospital Ethical Committee and the Committee for the Protection of Patients from Biological Risks. All patients were recruited at the Nantes Institute for Transplantation (except for one who was recruited at the St-Louis Hospital in Paris). All patients and normal individuals were informed of the protocol and gave informed consent. The University Ethical Committee recommended against performing biopsies in long-term recipients with normal stable function.

***Drug-free operationally tolerant (DF-Tol) group*:** DF-Tol recipients with stable kidney graft function had not taken any immunosuppressive drugs for at least 3 years before analysis ($n = 5$) (Table 1). These patients were the only ones identified as being drug-free and operationally tolerant among an estimated 25 000 or more kidney transplant recipients in France. Figures 1A (left panel) and 1B show the renal function (serum creatinine) of these drug-free tolerant patients, which remained remarkably stable and within the limit of 200 μM of blood creatinine and 1.5 g/24 h of proteinuria. The mean drug-free time period was 8 ± 3.3 years (range 3–12).

***Minimally immunosuppressed kidney recipients*:** Another group of patients received low doses (≤10 mg/day) of prednisone or prednisolone as the only immunosuppressive drug for at least 3 years before analysis (9.8 ± 4.4 years (range 4–15)). These minimally immunosuppressed patients are referred to as the Ster group ($n = 5$)(Table 1). Figure 1A (right panel) shows the creatinemia levels before and after the onset of monotherapy in each of these patients.

***Patients with chronic rejection, patients with stable graft function under an immunosuppressive maintenance regimen and normal, healthy individuals*:** Because there were several possible rationales for the most appropriate control group (based on having a stable graft function or an absence of immunosuppression), several clinical situations were studied. The stable function group (Sta) included kidney recipients with long-term stable graft function who were under a dual-therapy immunosuppressive regimen ($n = 7$) including a calcineurin inhibitor (CNI^+; $n = 4$) or not (CNI^-; $n = 3$). The chronic rejection group included patients with a degradation of renal function and histological chronic rejection lesions (CR group; $n = 7$) associated or not with an allograft glomerulopathy. In 4 patients, immunosuppressive treatment at the time of the study consisted of a combination of tacrolimus and azathioprine or prednisone. Three CR patients were on dialysis following allograft nephrectomies and had been free of immunosuppressive drug treatment (DF-CR) for at least 1 year. Figure 1B summarizes the kinetics of graft function in these groups. The apparent late decrease in creatinemia in the chronic rejection group is due to the fact that the values of the patients who had reverted to dialysis were not taken into account. Normal individuals, 25–60 years of age, included healthy individuals (N^L group, $n = 15$) with normal blood results and no known infectious pathology for at least 6 months prior to the study.

Blood samples

Blood was collected in EDTA tubes. Ionogram, white blood cells (WBC), formula and calcineurin inhibitor (CNI) blood levels were performed on each sample. peripheral blood mononuclear cells (PBMC) were separated on a Ficoll layer (Eurobio, Les Ulis, France) and frozen in TRIzol® reagent (Invitrogen, Life technologies, CA) for RNA extraction. Negative selection of $CD4^+/CD8^+$ T cells was performed using MACS® human cell isolation kits (Miltenyi Biotec, Germany). Purity was >90%.

TCR repertoire analysis

Diversity and T-cell selection were assessed by analysis of TCR (β chain) usage biases. In an immunologically quiescent state, the length distribution of the TCR-β chain complementary determining region 3 (CDR3) hypervariable region is gaussian. Alteration of this gaussian distribution is the hallmark of an activation phenomenon. Total mRNA was reverse-transcribed using a cDNA synthesis kit (Boehringer Mannheim, Indianapolis, IN) and complementary DNA was amplified by PCR, elongated, loaded onto a 6% acrylamide, 8-M urea gel and seperated by electrophoresis as previously described (12). Analysis of CDR3-length distribution (LD) was performed using Immunoscope® software (Institut Pasteur, Paris, France) (12–15) that provides distribution profiles of CDR3 lengths in amino acids. The CDR3 length profiles were compared with those from 15 normal individuals used as controls (15) and defining non-disturbed gaussian profiles. The average of these normal distributions, for each Vβ family separately, was then used as a control distribution for analysis of the other samples. All perturbations, including those of the controls, were assessed by comparison with the control distribution for each individual Vβ family individually. The alteration per Vβ family is thus defined as the sum of the absolute values of difference for all CDR3 lengths in that profile. No alteration gives a difference of 0%. A difference of 50% represents a very significant alteration. The average of the alterations in all Vβ families in one sample gives the global percentage of alteration for each individual. To assess the magnitude of the Vβ transcript accumulation, the level of Vβ RNA was measured by real-time quantitative PCR. The data are displayed as a tridimentional TcLandscape® (Nantes Cedex 01, France) of the entire blood T-cell repertoire for each patient analyzed. A topview of the TcLandscape allows an easier assessment of CDR3-LD alterations. Percentages of CDR3-LD alterations are represented by a color code, ranging from deep blue (−50%) to dark red (50%). The X-axis displays the 24 Vβ families in humans. Correspondence between numbers indicated on the X axis and the different Vβ families is given in Figure 2. The Y-axis gives the 13 CDR3 lengths and the Z-axis gives the amount of each Vβ concerned,

Table 1: Clinical history of DF-Tol and Ster patients

	DF-Tol 1	DF-Tol 2	DF-Tol 3	DF-Tol 4	DF-Tol 5	Ster 1	Ster 2	Ster 3	Ster 4	Ster 5
Patient age at time of study	67	65	73	71	24	76	83	29	66	54
Patient sex	Male	Male	Female	Male	Male	Male	Male	Female	Female	Female
Number of transplants	1	1	1	2	1	1	2	1	1	1
Year of transplantation	1987	1980	1993	1974	1991	1997	1990	1998	1983	1980
Induction therapy (duration)	Anti RIL2 mAb (10 days)	No	No	No	ATG (6 days)	ATG (11 days)	ATG (17 days)	ATG (11 days)	ATG (19 days)	No No
HLA-A-B-DR inc.*	3[s]	3[s]	3[s]	ND	3	3[mb]	0[s]	5[mb]	4[s]	2[s]
Anti-T PRA prior to transplantation (%)	0	20	0	ND	0	0	72	0	60	20
Anti-T PRA at time of study (%)	0	0	0	ND	0	ND	0	0	0	0
Acute rejection episode	No	No	2	No	No	No	No	No	No	1
CMV infection	No	No	No	No	No	No	No	No	Yes (1987)	No
Other viral infections	No	No	No	HCV	No	No	HSV (1991)	Zona (1992) EBV (1996) Zona (1997) HCV (2000)	No	No
Reason for IS interruption	PTLD	Non-compliance	CNI toxicity	Non-compliance	PTLD	Recurrent serious bacterial infections	Large bowel carcinoma	PTLD	Vocal cord epithelioma	Cutaneous epithelioma
Time period : drug-free or low dose	8 years	>10 years	8 years	12 years	3 years	5 years	4 years	6 years	15 years	9 years

s: Indicates typing by serology.

mb: Indicates typing by molecular biology.

Note: Table 1 summarizes the clinical history of DF-Tol (n = 5) and Ster (n = 5) patients. One patient (patient DF-Tol 4) received a second graft. The mean HLA-A, -B and -DR incompatibility was 3. Two patients received an induction therapy after transplantation with a 10-day course of an anti-IL2-R-α antibody (DF-Tol 1) and a 6-day therapy of ATG (DF-Tol 5). One patient (DF-Tol 3) underwent two acute rejection episodes 14 days and 7 months after transplantation, which were both treated with steroid boluses. No patient in this group had a CMV disease during their follow-up. Patient DF-Tol 4 was chronically infected by the hepatitis C virus. One patient (Ster 2) received a second graft. The mean HLA-A, -B and -DR incompatibility was 2.8 $\pm$ 1.9. The mean pre-graft anti-T PRA was 30 $\pm$ 33%. Four patients received ATG induction therapy for 14 $\pm$ 4 days. Patient Ster 5 received azathioprine and steroids, and underwent one episode of acute rejection just after transplantation, which was treated with a 10-day course of ALG. Patient Ster 4 had a CMV disease 4 years after transplantation, which was treated with gancyclovir. Patient Ster 3 suffered from serial virus infections, that is, two herpes zoster virus reactivation at 4 and 9 years, EBV at 8 years and hepatitis C infection at 12 years post-transplantation.

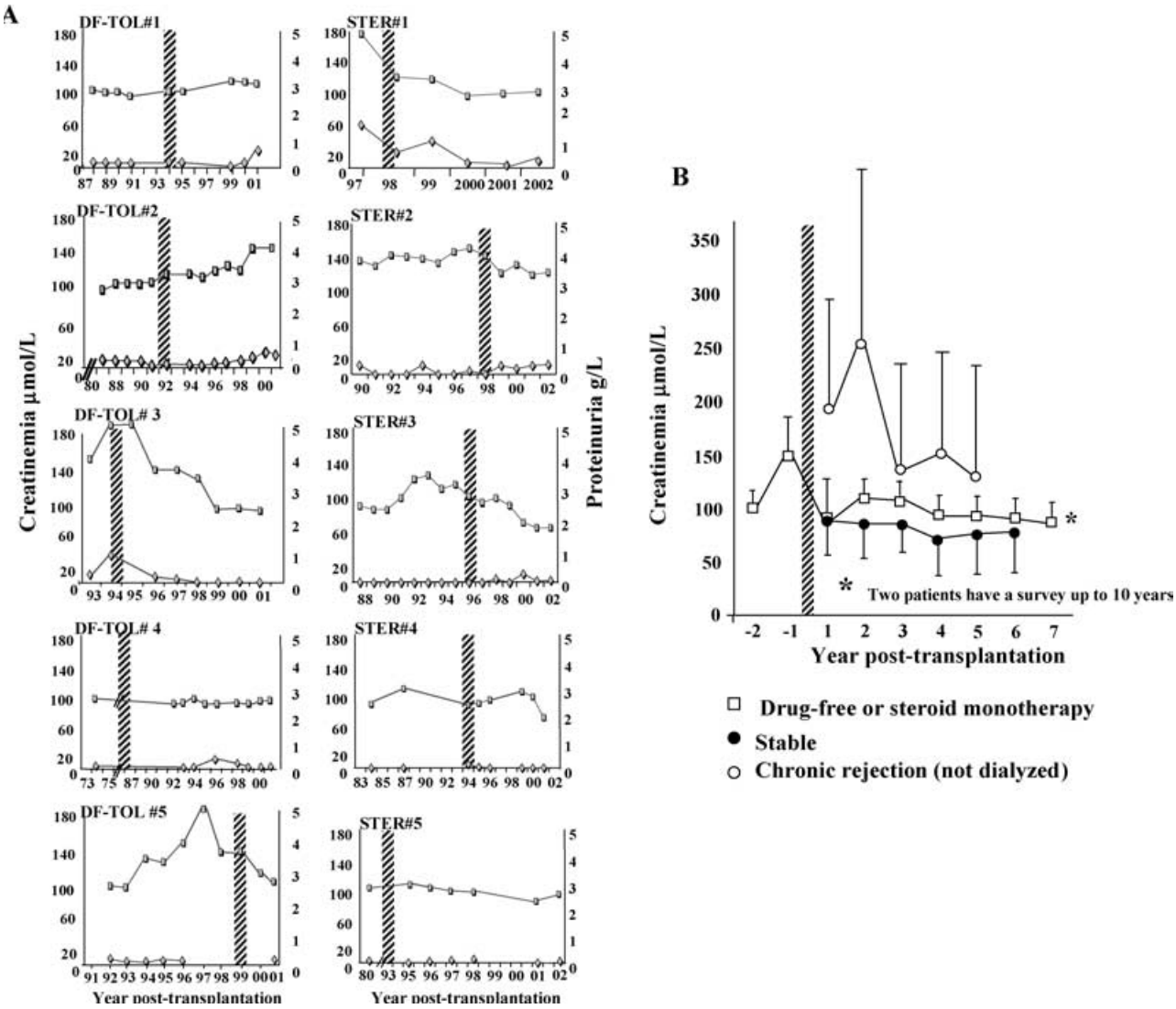

Figure 1: Graft function in the various kidney recipient groups. (A) The left-hand panel shows the creatinemia (■) and proteinuria levels (♦) for each drug-free patient (DF-Tol group) before and after withdrawal of immunosuppressive treatment until the time of analysis. The corresponding data for patients under low-dose corticosteroid monotherapy (Ster group) are displayed on the right-hand panel. The vertical black bars indicate the time of immunosuppression interruption or the initiation of the very low-dose immunosuppressive regimen. Patients DF-Tol 1 and 5 presented a lymphoma, 52 and 96 months following transplantation without recurrence when tested at 7 and 3 years, respectively. Patient DF-Tol 3 stopped immunosuppressive treatment due to kidney dysfunction 6 months after transplantation. He returned to chronic dialysis, his graft function progressively recovered after Cyclosporine A (CsA) interruption and his dialysis was interrupted 1 year later. Patients DF-Tol 2 and 4 stopped immunosuppressive treatment voluntarily without any medical reason. (B) The mean blood creatinemia values for 'operationally tolerant' patients (DF-Tol and Ster) before and after immunosuppression interruption and for patients with stable function under immunosuppression and with chronic rejection are shown. In the group of patients with chronic rejection (CR group), the values for patients under chronic dialysis are not indicated (resulting in the apparent delayed decreased in mean creatinine levels).

normalized against levels of the housekeeping gene HPRT (Vβ/HPRT ratio) (16,17).

IL2-R-α , perforin and cytokine transcript quantification

IL2-R-α and cytokine transcript measurement were performed on RNA from PBMC, $CD4^+/CD8^+$ subpopulations and Vβ families positively selected using MACS® anti-PE micro-beads and a MS^+ type separation column (Miltenyi Biotec, Germany). RNA was prepared using the SMART kit (Boehringer Mannheim, Indianapolis, IN). Real-time quantitative PCR was performed for IL2-R-α, IL2, IFN-γ, IL8, IL10, TGF-β, IL13 and perforin and normalized against the HPRT transcript level (16).

T-cell Vβ surface staining

Two color labeling was performed on PBMC using FITC-conjugated anti-CD3, -CD4 and -CD8 antibodies (Abs) (Immunotech, Coulter Company,

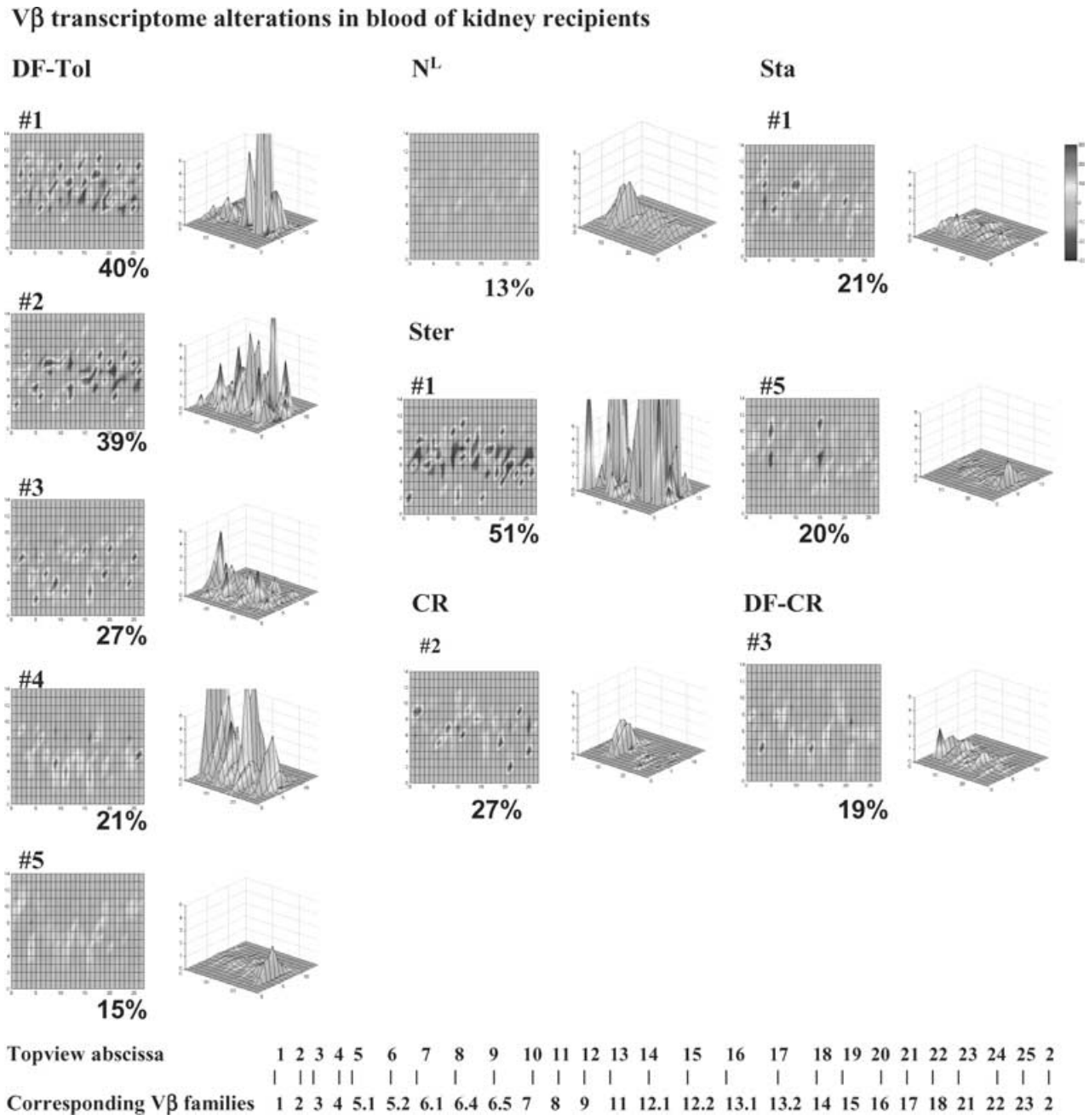

Figure 2: TcLandscape representation of the T-cell Vβ transcriptome in blood of transplanted patients. The five TcLandscape from drug-free tolerant kidney recipients (DF-Tol), 1 out of 15 healthy individuals (N^L); 2 out of 5 minimally immunosuppressed patients under low dose of steroids (Ster), 1 out of 7 patients with stable graft function (StaCNI); 2 out of 7 patients with chronic rejection (CR).

Marseille, France), and the 24 available PE-conjugated anti-Vβ Abs (Immunotech, Coulter Company, Marseille, France) or isotype-matched control Abs (IgG-FITC and IgG-PE). The staining for each Vβ family was expressed as a percentage of the total staining.

Statistical analysis

The different groups were compared on the basis of CDR3-LD and Vβ/HPRT transcript ratio values, considering their possible correlation (Manova), with a global test (F-test) and the related pairwise comparisons. After checking for normal distribution, Manova was performed with SAS© software (Cary, NC) and the 'Mixed' procedure. The influence of age, HLA mismatch, graft number, PRA (%), previous acute rejection, CMV, lymphoma, cancer, corticosteroids and the different immunosuppressive drugs used on the CDR3-LD and Vβ/HPRT transcript ratio values was evaluated using Manova with a global test (F-test) and the related pairwise comparisons. Comparison was performed between the different groups: drug-free patients (DF-Tol),

minimally immunosuppressed patients (Ster), patients with stable graft function (Sta), patients with chronic rejection (CR) and normal individuals. Due to the low number of patients in each group, patients considered clinically as operationally tolerant (i.e. drug-free patients (DF-Tol) and minimally immunosuppressed patients (Ster)) were also pooled for CDR3-LD and Vβ/HPRT transcript ratio analyses. Statistical analyses of cytokine transcript accumulation were performed using a non-parametric Kruskal–Wallis test. The results were considered significant when $p < 0.05$.

Results

Vβ transcriptome analysis and the blood T-cell repertoire of normal individuals

Our new method provides an integrating vision of *in vivo* T-cell clonal distribution where a green landscape indicated unselected T cells and red spots indicated strong T-cell selection. CDR3-LD and Vβ/HPRT ratios were also statistically compared (see Patients, Materials and Methods for technical details). A representative TcLandscape topology of the 15 normal individuals are displayed in Figure 2 and online Figure 1 (see http://www.blackwell-science.com/products/journals/suppmat/AJT/AJT700/AJT700sm.htm for online figures). Normal individuals exhibited only slightly altered CDR3-LD with 93% of Vβ families displaying <30% CDR3-LD alteration and about 1% of Vβ families displaying more than 50% CDR3-LD alteration. Compared to the mean CDR3-LD alteration observed in the 15 healthy individuals, global CDR3-LD alteration for each normal individual (sum of alteration of the 24 Vβ families for each individual) remained within 10.9 ± 2.9%. In addition, in normal individuals, 83% of Vβ families had a Vβ/HPRT transcript ratio <5 whereas only 1% had ratios >15. The TCR patterns were found to be roughly similar in time when tested every 2 months for 8 months (M. Guillet et al., unpublished). In the following section, the patterns for each recipient group are described. The statistical differences between groups are detailed in a separate section.

The blood T cells of drug-free operationally tolerant patients display altered CDR3-LD patterns together with high Vβ /HPRT transcript ratios

Figure 1A shows the graft function (creatinemia and proteinuria) of drug-free operationally tolerant patients (DF-Tol) (left panel) before and after withdrawal of classical immunosuppressive treatment until the time of testing. Figure 1B shows the graft function of the recipient groups. A summary of the clinical history of each patient of the DF-Tol group is provided in Table 1 (left-hand part). Detailed CDR3-LD values of DF-Tol patients are displayed in Table 2 and TcLandscape topologies (Figure 2 left-hand panel). In DF-Tol recipients, 9% of Vβ families showed more than 50% CDR3-LD alteration (Table 2) whereas the global CDR3-LD alteration was 28.3 ± 11%. In addition, these recipients showed high Vβ/HPRT transcript ratios with approximately 8% more than 15 (Table 2). These ratios corresponded to a strong accumulation of Vβ transcripts since HPRT levels were unchanged in operationally tolerant patients compared to the other groups (data not shown). This phenomenon was not associated with deletion of the remaining Vβ transcripts since no decrease was observed in operationally tolerant compared to stable and chronic rejection patients, nor did it correlate to significant changes in the percentage of staining for the corresponding anti-Vβ antibodies. For example, the transcripts of the Vβ1, 4 and 11 families represented 29, 6.5 and 12%, respectively, of the whole Vβ transcriptome in one representative patient (DF-Tol 4 patient) whereas the percentages of T-cells staining for the corresponding Vβ protein were only 6.9, 0.2 and 0.5%, respectively, (Figure 3C) and did not differ from the values of normal individuals.

Table 2: CDR3-LD alteration and Vβ/HPRT transcript ratios in normal individuals and kidney recipients

Alterations:	<30%	30–50%	>50%	Mean (%)
N^L	93*	6	1	10.9 ± 2.9
DF-Tol	59	32	9	28.3 ± 11
Ster	32	30	38	42 ± 15
Sta	81	16	3	20.6 ± 3.7
CR	68	26	6	23.9 ± 5.5
Vβ/HPRT (%)	0–5	5–15	>15	
N^L	90	9	1	
DF-Tol	74	18	8	
Ster	69	22	8	
Sta	92	8	0	
CR	94	6	0	

*% of Vβ families in the alteration interval.

Note: (a) the distribution of Vβ families according to the percentage of CDR3-LD alteration, global CDR3-LD alterations and (b) the Vβ/HPRT transcript ratios are given for the different clinical groups of kidney recipients tested (drug-free (DF-Tol) and those under low-dose steroids (Ster); Stable = stable with (CNI^+) and without (CNI^-) a calcineurin inhibitor; CR = chronic rejection with (CR) or without (DF-CR) treatment) and healthy age-mached individuals.

CDR3-LD analysis was performed on purified $CD4^+$ and $CD8^+$ T-cell populations in 4 DF-Tol patients. Most of the alterations were found within the $CD8^+$ T-cell population (Figure 3A) (corresponding to at least 80% of $CD8^+$ T cells as measured by flow-cytometry) whereas the $CD4^+$ T-cell population displayed a gaussian CDR3-LD pattern. CDR3-LD analysis performed over a 6-month interval in 2 DF-Tol patients (DF-Tol 2 and 4) revealed patterns roughly stable in time (Figure 3B).

Patients under low-dose steroid monotherapy (Ster) exhibit a TCR Vβ pattern, which resembled that observed in drug-free operationally tolerant patients

An analysis of the blood TCR repertoire in Ster patients also revealed highly altered CDR3-LD patterns (Figure 2 and online Figure 2): 38% of Vβ families showed more than 50% CDR3-LD alteration and the global CDR3-LD alteration was extremely high (42 ± 15%) (Table 2). Furthermore, these patients had high Vβ/HPRT transcript ratios with 8% of Vβ families having a Vβ/HPRT ratio >15 (Table 2). Thus, drug-free patients and those under low-dose steroid

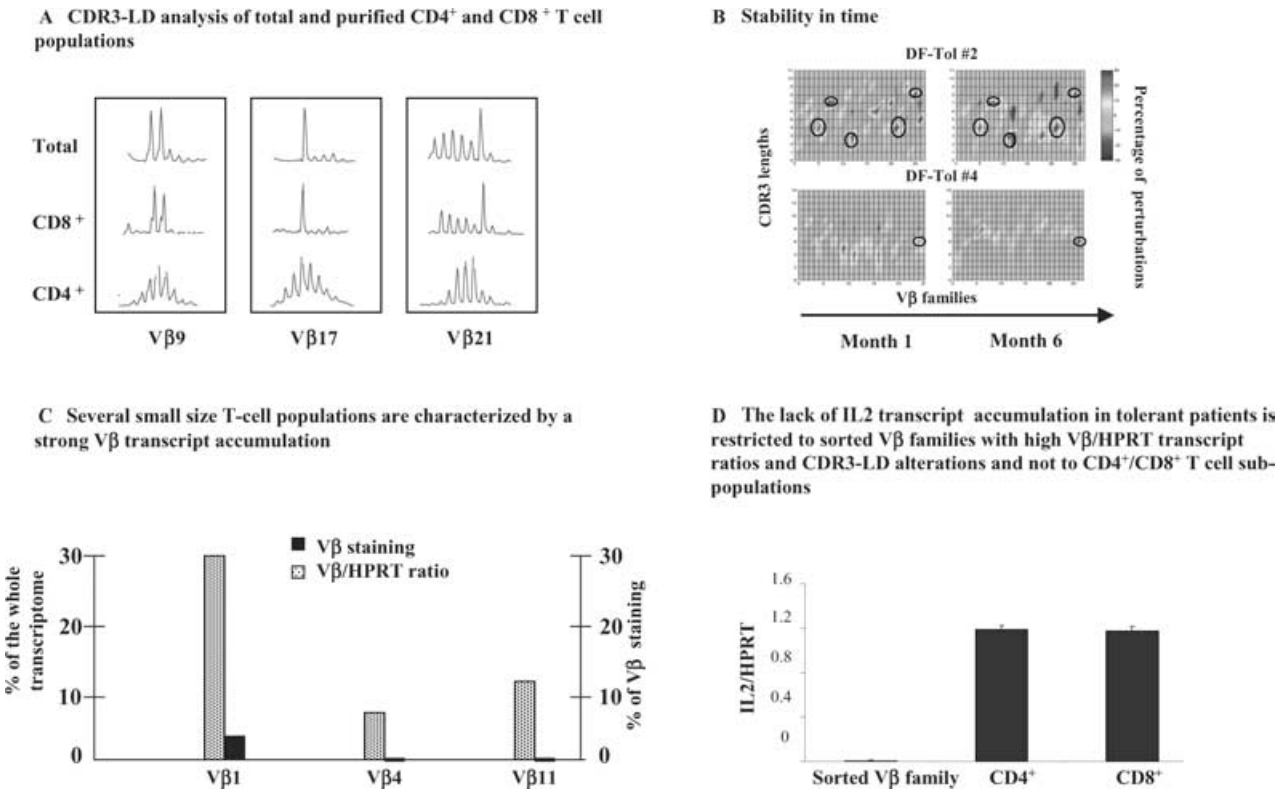

Figure 3: (A) CDR3-LD analysis was first performed on purified $CD4^+$ and $CD8^+$ T-cell populations in 4 DF-Tol patients. Figure 3A gives a representative example of a CDR3-LD analysis performed on three families sorted from patient DF-Tol 2. The alterations were mainly found to correspond to a $CD8^+$ T-cell population (>3/4 of the Vβ families analyzed). The $CD4^+$ T-cell population displayed a gaussian CDR3-LD pattern. (B) CDR3-LD analyses performed at 6-month intervals in 2 DF-Tol patients (patients DF-Tol 2 and 4) showed roughly stable patterns in time. (C) Two color labeling using anti-Vβ and anti-CD3 antibodies was performed on PBL from DF-Tol and Ster group patients. Vβ staining (■) was expressed as a percentage of the total staining. Similarly, the Vβ/HPRT ratio (▨) was expressed as the percentage of Vβ RNA for the 19 corresponding Vβ families identified by available antibodies. This figure gives a representative example of patient DF-Tol 4 where several small size (low-percentage-specific Vβ positive staining) T-cell populations were characterized by a strong Vβ transcript accumulation. Vβ1, 4 and 11 represented 29, 6.5 and 12%, respectively, of the whole Vβ transcriptome whereas cells staining for the corresponding Vβ protein represented only 6.9, 0.2 and 0.5%, respectively, and did not differ from normal individual values. (D) Cytokine transcript measurement was performed on purified $CD4^+$ and $CD8^+$ T-cell populations and sorted Vβ families from 4 DF-Tol patients. The lack of IL2 transcript accumulation was restricted to sorted Vβ families with high Vβ/HPRT transcript ratios and CDR3-LD alterations and not to $CD4^+/CD8^+$ T-cell sub-populations.

monotherapy (Ster) were both characterized by highly altered CDR3-LD patterns and strong Vβ/HPRT transcript ratios for several Vβ families.

The blood T cells of recipients with stable graft function or with chronic rejection exhibit moderate CDR3-LD alterations and low Vβ /HPRT transcript ratios

The TCR repertoire of patients with stable graft function (Sta) under calcineurin inhibitor (CNI) treatment displayed few CDR3-LD alterations and low Vβ/HPRT transcript ratios (Figure 2 and online Figure 3A) (Table 2). More than 62% of the Vβ families of $StaCNI^+$ patients displayed less than 30% CDR3-LD alterations and their global CDR3-LD alteration was $20.6 \pm 3.7\%$. In these patients, low Vβ/HPRT transcript ratios (<5) were observed for 84% of the Vβ families, whereas only 1% of families had a ratio >15 (Table 2). Three patients without CNI displayed the same pattern (online Figure 3A) (Table 2). Thus, patients with stable kidney graft function, with or without calcineurin inhibitor treatment, had CDR3-LD and Vβ/HPRT ratios within the range of normal individuals. Slightly more altered patterns were observed in the blood of patients with chronic rejection (CR) (Figure 2 and online Figure 3A): 54% of Vβ families showed less than 30% CDR3-LD alteration but 11% showed >50% alteration (Table 2). The global CDR3-LD alteration was $27 \pm 6.3\%$. Whereas the CDR3-LD showed more alterations in CR patients than in normal individuals, Vβ/HPRT transcript ratios in CR patients remained low, with 90% of Vβ families having a Vβ/HPRT ratio <5 and only 1% of them having a ratio >15 (Table 2). As chronic immunosuppressive drug intake could modify the magnitude of the alterations,

drug-free CR patients (DF-CR) were also analyzed (online Figure 3B). In these individuals, 59% of Vβ families showed CDR3-LD alterations below 30% whereas only 1% of families displayed alterations >50% (Table 2) and their global CDR3-LD alteration was 20.9 ± 4.8%. These patients also displayed low Vβ/HPRT transcript ratios with 98% of Vβ families having ratios <5 and no ratio >15 (Table 2).

Global statistical correlations

Whereas drug-free operationally tolerant patients and minimally immunosuppressed patients (low-dose steroid monotherapy) did not significantly differ, neither for CDR3-LD alteration nor for Vβ/HPRT transcript ratios, statistical analysis (Manova) showed that both of these groups displayed significantly stronger CDR3-LD alteration and higher Vβ/HPRT transcript ratios than any of the other groups analyzed: recipients with stable graft function ($p < 0.01$ and $p = 0.01$, respectively) or chronic rejection ($p = 0.01$ and $p < 0.01$, respectively) and normal individuals ($p < 0.01$ and $p < 0.01$, respectively). Finally, all recipient groups had significantly more altered CDR3-LD ($p < 0.01$) than normal individuals whereas patients with stable graft function and patients with chronic rejection did not significantly differ from each other in their CDR3-LD. Because specific clinical parameters could have influenced TCR patterns, special attention was paid to age, HLA mismatch, graft numbers, PRA %, previous acute rejection, CMV, malignancies and specific treatments (presence of steroids, type of immunosuppressive treatment). Two of 5 drug-free patients had a history of malignancies (see Table 1). These malignancies (2 post-transplant lymphoproliferative disorders (PTLD) and no other cancers) were considered to be totally cured and the corresponding patients were tested 7 (for DF-Tol 1) and 2 (for DF-Tol 5) years after diagnosis. Of importance is that these patients were not the most characteristic in terms of TCR alteration. For instance, DF-Tol 2, without a history of malignancy, had the second strongest global CDR3-LD alteration and the highest Vβ/HPRT ratio. Finally, the listed clinical parameters were also analyzed for their possible influence on CDR3-LD and Vβ/HPRT transcript ratio in drug-free and minimally immunosuppressed patients, either considered together or separately, using Manova with a global *t*-test (F-test) and related pairwise comparison (see Patients, Materials and Methods). None of these clinical parameters, and particularly age, lymphoma, cancer and corticosteroid therapy, was a determining factor in significantly shaping the blood T-cell alterations in each group.

Sorted T cells from families with strong CDR3-LD alterations in drug-free operationally tolerant recipients accumulate lower levels of cytokine transcripts and higher levels of IL2-R-α than those from patients with chronic rejection

Because operationally tolerant patients displayed strongly biased blood clonal distribution, we investigated the possibility that their blood T cells could have a particular transcriptional pattern for a panel of representative cytokines (IL2, IL10, IL13, TGF-β, IFN-γ and IL8), perforin and IL2-R-α. First, we tested the transcripts of unseparated PBMC from operationally tolerant patients, minimally immunosuppressed patients, patients with chronic rejection and normal individuals and found no significant differences (data not shown). However, because any differences may only concern the selected T-cell populations, we specifically analyzed purified T cells from sorted Vβ families with strong CDR3-LD alteration in DF-Tol patients, in recipients with chronic rejection (Figure 4A) and normal individuals. Whereas most of the sorted T-cell populations from chronic rejection patients accumulated high levels of cytokine transcripts, the corresponding T cells from operationally tolerant recipients did not, particularly for IL2 ($p < 0.05$), IL8, IL13 ($p < 0.05$), TGF-β, IFN-γ and perforin (Figure 4B). In this context, the pattern observed in operationally tolerant patients was much closer to that observed in normal individuals than that of chronically rejecting patients. Interestingly, sorted T cells from operationally tolerant recipients accumulated higher levels of IL2-R-α transcripts than the corresponding T cells from chronic rejection patients ($p < 0.05$). Figure 3D shows that this decreased transcript accumulation (particularly IL2, $p < 0.05$) was not observed when purified global $CD4^+$ or $CD8^+$ T-cell populations were tested, fitting with the patterns obtained in PBMC and restricting the difference to the T cells having engaged in clonal selection. Thus, despite a strongly selected TCR usage, sorted T cells from operationally tolerant patients had only low but detectable levels of cytokine transcripts (IL2 ($p < 0.05$), IL13 ($p < 0.05$), IFN-γ, IL8, perforin, TGF-β and IL10) (Figure 4B) and increased levels of IL2-R-α ($p < 0.05$) transcripts as compared to CR patients.

Discussion

The identification and characterization of patients who have developed operational tolerance to an allotransplant may make it possible to identify other transplant recipients who may no longer require 'life long' immunosuppression. A number of isolated drug-free operationally tolerant recipients have been reported with normal or decreased anti-donor T-cell responses (6–9). In human graft recipients, key tests (such as the rejection of a third-party allograft) that have been established in rodents (18,19), cannot be performed. Furthermore, no prospective studies of a possible tolerance-associated pattern can be carried out. Indeed, the 5 drug-free operationally tolerant patients described in this report are the only ones identified in France and there is currently no a priori indication that would enable the screening of a population of 'potentially tolerant' patients. However, it is also necessary to consider that immune tolerance has not been characterized in humans up to now and therefore no a priori theory should be accepted in terms of the possible mechanisms involved in such a phenomenon. In this article, we analyzed the blood TCR repertoire and T-cell selection and activation in such operationally

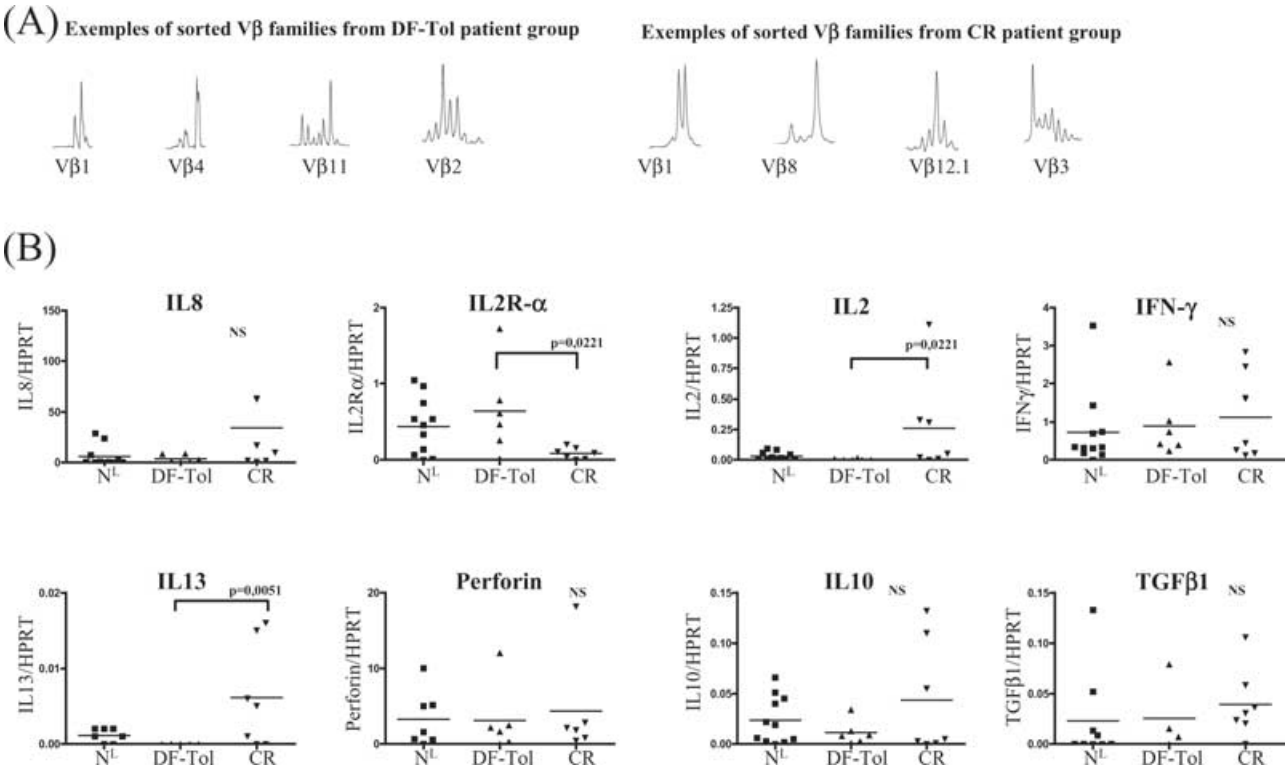

Figure 4: Cytokine transcript accumulation in sorted Vβ families from drug-free operationally tolerant patients and recipients with chronic rejection. (A) CDR3-LD of T cells in sorted Vβ families analyzed for cytokine transcript accumulation. (B) Cytokine transcript measurements were performed on sorted families with altered CDR3-LD from DF-Tol patients (n = 7 to 11), CR patients (n = 7) and normal individuals (n = 7–11).

tolerant patients with stable graft function many years after immunosuppression interruption. We show that these patients have a unique blood TCR pattern combining a restricted CDR3-LD and abnormal Vβ/HPRT transcript ratios. Furthermore, these peripheral oligoclonal T-cell selection expansions, which are the cellular basis of these findings, were mostly characterized by a lack of cytokine transcript accumulation, contrasting with those from chronically rejecting patients and suggesting a state of hyporesponsiveness. However, these 5 patients did not present symptoms of unusual immunoincompetence against specific human viral antigens after their treatment interruption (or while under monotherapy). In fact, the DF-Tol patients had the lowest incidence of infection (1 HCV and 1 bacterial infection) despite the post-transplant survey often spanning a decade, suggesting that they are specifically tolerant to their grafts.

Despite reports of certain T-cell selections in animal models of tolerance (20–22), including our own work (12,23), we did not expect to find such highly altered CDR3-LD in the blood, years after transplantation, given that previous studies have found TCR alterations in tolerated graft lymphocytes (20,23). To our knowledge, the blood was not studied in these reports. It is possible that the selected T cells found in the blood of operationally tolerant patients are no longer able to enter the graft after such a long time and in the absence of local inflammation. It is also possible that these selected T-cell populations have a specific distribution of cell surface molecules involved in lymphocyte homing. Given that TCR-β chain transcript accumulation is not observed before the onset of responding cell proliferation when T cells are stimulated *in vitro* by allogeneic APC (N. Degauque et al., unpublished), the high Vβ/HPRT ratios probably developed progressively, in parallel with T-cell expansion, rather than reflecting a short-term activation. In addition, these ratios were not related to an increased percentage of lymphocytes in DF-Tol and Ster blood as compared to CR and stable recipients or normal individuals. In fact, whereas the $CD4^+/CD8^+$ ratio remained unchanged, the number of T cells was even lower in the low-dose steroid group of patients (792 ± 551 lymphocytes/μL blood, $p < 0.05$). Taking this parameter into account would even further increase the already extremely high Vβ/HPRT ratios in such patients. The cause and significance of this TCR transcript accumulation in T cells with restricted CDR3-LD is still unknown but could be linked to memory T-cell clones, fitting with their predominant $CD8^+$ phenotype. However, only 2 recipients (DF-Tol 4, Ster 3) out of 10 drug-free or minimally immunosuppressed

patients demonstrated a clinical history of chronic HCV and CMV diseases and the non-compliant DF-Tol 2 patient, who had no chronic viral infection, displayed the second most altered CDR3-LD pattern and the highest Vβ/HPRT ratio. The fact that patients under low-dose steroid monotherapy exhibited a similar pattern to drug-free operationally tolerant patients and that multivariate analysis did not link this pattern to steroids, strongly suggests that the former patients are also operationally tolerant, fitting with their clinical condition. Moreover, multivariate statistical analyses showed an independence between TCR alterations and Vβ/HPRT ratios, lymphoma and cancer history. In this context, these data provide a rationale for the progressive weaning of these patients from this low steroid intake.

It is interesting to note that T cells sorted from families with strongly altered TCR from DF-Tol patients did not accumulate Th1 (IL2, IFN-γ) or Th2 (IL10, IL13) cytokine transcripts. Therefore, on this basis, operationally tolerant recipients display a profile that does not differ from normal individual contrasting with the situation of chronic rejection where the families are bearing selected T cells that seem activated memory T cells. Considering their concomitantly selected CDR3-LD pattern and high Vβ/HPRT transcript ratios, this is compatible with a state of anergy (24) rather than ignorance. The stability of these patterns in time also suggests a continuous selection pressure. In addition, altered CDR3-LD were found to be mostly made up of $CD8^+$ T cells. $CD8^+$ T-cell subsets have been shown to have regulatory properties in several animal models as well as in humans (12,25,26). However, there was also a significantly higher level of $CD4^+CD25^+$ T cells ($p < 0.05$), another T-cell population with putative regulatory properties, in the blood of DF-Tol and Ster patients compared to patients with chronic rejection ($20.5 \pm 7.3\%$ vs. $10.6 \pm 5.2\%$) (S. Louis et al., submitted for publication). Determining such regulatory activity would however require functional studies using donor cells. Given the unavailability of frozen donor cells from transplantations, the majority of which were performed more than a decade ago and for whom most of the donor typings were performed by serology, this could not be done.

The moderate clonal selection observed in the blood of CR patients, which gives a partial overlap in terms of magnitude with operationally tolerant patients, also mostly concerning $CD8^+$ cells, may be due to the presence of circulating alloreactive cells restricted to donor determinants, as previously reported in chronic rejection (11,27). This possibility is sustained by the accumulation of cytokine transcripts in $CD8^+$ T cell from families with altered CDR3-LD in this group. In contrast, no accumulation was observed in families with altered CDR3-LD from operationally tolerant patients. Interestingly, 1 of 7 patients of the Sta group ($StaCNI^+$ 3) displayed a pattern that could fit with the profile observed in operationally tolerant recipients. Thus, theoretically speaking, this group is not expected to be homogeneous.

Our data also address the question of the relationship of these altered T-cell clonal patterns with the $CD8^+$ or $CD4^+$ clonal expansions observed in elderly individuals. Indeed, ageing has been shown to be associated with persisting blood $CD8^+$ T-cell expansions that are hyporesponsive or anergic (28–34). Nevertheless, the strong CDR3-LD alteration observed in the drug-free and low-dose steroid groups could not be explained by the age of the patients in the multivariate analysis. It is nevertheless possible that operationally tolerant recipients are also those who developed the most prominent clonal expansions because of a unique recruitment of naive T cells into the memory pools, combined with a lack of replacement of naive T cells from the thymus (35), a process that is age-dependent. This gives grounds for the hypothesis that the mechanisms involved in this 'naturally' occurring tolerance are much more complex than a pure active suppression and may also be related to a state of homeostatic competition where amplified clones with 'simplified' CDR3-LD induce a reduction of the $CD8^+$ repertoire and a decreased alloreactivity. Interestingly, $CD8^+$ T cells have been suggested to play a role in chronic rejection (36). This mechanism does not therefore exclude the possible presence of regulatory cells in the $CD4^+$ population, as suggested by the higher numbers of $CD25^+CD4^+$ cells in these patients compared to patients with chronic rejection. Our data thus open up a new field of exploration in kidney recipients with stable function. However, we are conscious that the small number of 'tolerant' patients require confirmation of the data. This confirmation, in a larger study, of patterns emulating those observed in operationally tolerant patients, may serve, together with other biological markers (10,37), as a possible surrogate indicator of tolerance in these patients.

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Annexe 3

Titre original:

Different patterns of TCR β chain regulation following allo- and xeno-transplantation

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Different patterns of TCR β chain regulation following allo- and xeno-transplantation

Guillet M, Gagne K, Lair D, Heslan J-M, Doré J-C, Soulillou J-P, Brouard S. Different patterns of TCR β chain regulation following allo- and xeno-transplantation.
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Abstract: Background: In the concordant hamster-to-rat cardiac xenograft model, recipients treated with cobra venom factor for the first 10 days following transplantation and daily with Cyclosporine A (CsA) do not reject their grafts. However, when CsA is withdrawn on day 40, an acute cellular rejection occurs within 4 ± 1 days. Allografts performed in the same conditions are rejected within 18 ± 4 days.
Methods: In this model, we have compared graft infiltrating T cells through both a quantitative (number of Vβ transcripts) and qualitative (CDR3 length distribution) assessment of the T cell receptor (TCR) β chain transcriptome in allo- and xeno-transplantations.
Results: We report striking differences in TCR usage at day 15 following allo- and xeno-transplantation as well as during rejection following CsA withdrawal. The number of Vβ transcripts was high in both rejected allo- and xenografts. However, whereas in xenografts acute rejection occurred without skewing of Vβ CDR3 length distribution, T cells infiltrating allografts during rejection after CsA interruption had a highly altered CDR3 length distribution pattern. In addition, using a correspondence factor analysis of the β chain transcriptome, we show that some families can clusterize and can discriminate allo- or xeno-patterns at the level of both the number of Vβ transcripts and the CDR3 length distribution.
Conclusions: Our data show that, in vivo, even in the hamster-to-rat concordant combination, the anti-xenograft T cell response is strong and will likely represent another challenge for xenotransplantation.

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Key words: allograft – T cells – TCR – xenograft

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Introduction

In order to study the involvement of T cells in xenograft rejection, we used a model of cellular rejection occurring in previously ''accommodated'' grafts [1]. In this concordant hamster-to-rat model of cardiac xenotransplantation, recipients are treated for the first 10 days post-transplantation with cobra venom factor (CVF) and daily with cyclosporine A (CsA) [2]. When CsA treatment is withdrawn on day 40, an acute cellular rejection occurs within 4 ± 1 days. Allografts, performed using the same strain recipient and similarly treated undergo rejection, later, 18 ± 4 days after withdrawal of CsA [1]. During CsA treatment, only a few infiltrating cells, mainly macrophages with no sign of T cell activation, are found in both allo- and xenografts. At rejection, despite a much more rapid kinetics of cellular infiltration in xenografts, the infiltrates are similar in allo- and xenografts, involving about 40% of activated T cells [1]. Likewise, the profile of cytokine production (RNA accumulation) at the time of rejection is also similar in both models, with the presence of Th1 type cytokines, suggesting an involvement of T cells in the rejection process [3]. The role of T cells in this acute xenograft rejection, likely involving a dominant direct pathway component, has been further demonstrated by experiments showing that purified CD4+ve T cells from naive rats transferred into T-cell-depleted recipients of accommodated heart xenografts induce an acute rejection of

xenografts within 5 days but not of allografts [3]. To further characterize the differences in the T cell activation elicited by allo- and xenografts, we previously studied the alterations of T cell repertoire usage at the level of the complementarity determining region 3 (CDR3) of the TCR β chain mRNA and found differential patterns. Unexpectedly, alterations of the TCR Vβ repertoire were restricted to rejected allografts [1] whereas the CDR3 length distributions (CDR3-LD) of T cells infiltrating rejected xenografts remained Gaussian, suggesting a polyclonal activation.

In the present report, we performed a quantitative study of the amount of each Vβ family mRNA (TcLandscape) in cells infiltrating allo- or xenografts placed in the same LEW.1A recipients. This allowed us to revisit the fine regulation of both CDR3-LD and the corresponding amounts of Vβ transcripts in the two models. We report striking qualitative (CDR3-LD patterns) and quantitative (Vβ transcript amounts) differences in TCR usage both at day 15 following transplantation and during rejection following CsA withdrawal in allo- and xenografts. Moreover, using a correspondence factor analysis of the Vβ transcriptome, we show that various Vβ families clustered according to the allo- or xeno-nature of the graft. Finally, we provide evidence that, in vivo, the anti-xenograft T cell response is strong and may represent a second major barrier for xenotransplantation.

Materials and methods

Animals and surgical procedures

Adult male rats (200 to 250 g) of the LEW.1W ($RT.1^u$) and LEW.1A ($RT.1^a$) congeneic strains and inbred male adult Syrian hamsters (100 to 150 g) were purchased from Janvier (Savigny/Orge, France). Two types of cardiac transplantation were studied: xenografts using hamsters as donors and LEW.1A rats as recipients and allografts with LEW.1W rats as donors and LEW.1A rats as recipients. Heterotopic cardiac transplantations were performed as previously described [4]. Graft function was evaluated daily by abdominal palpation.

Immunosuppressive protocol

In both types of graft (xeno and allo), recipients received 0.25 mg/kg i.m. of purified CVF (Quidel, San Diego, CA, USA) every 2 days for 10 days and 10 mg/kg CsA (Sandimmun, Novartis, Basel, Switzerland) per os daily for 40 days. After withdrawal of CsA, xenograft rejection occurred within 4 ± 1 days whereas allograft rejection occurred within 18 ± 4 days [1].

Experimental groups

Recipients of allo- and xenografts harvested on days 15 and 40 post-transplantation were studied. Another group consisted of allo- and xenografts harvested 44 days post-transplantation, which corresponded to day 4 ± 1 after CsA withdrawal, when xenografts are rejected. Additional groups were performed for allografts harvested 8 days after the withdrawal of CsA and at the time of rejection (18 ± 4 days after CsA withdrawal).

Qualitative analysis of the CDR3 region

Total RNA from hearts was isolated by the guanidinium isothiocyanate procedure and purified on a cesium chloride gradient [5]. Ten micrograms of RNA was then reverse-transcribed and diluted to a final volume of 100 µl. cDNA was amplified by PCR using a Cβ primer and one of the 20 Vβ-specific primers, in a 9600 Perkin-Elmer Automate (Applied Biosystems, Foster City, CA, USA) as previously described [6]. Amplification products were rendered fluorescent by an elongation reaction using a FAM-labeled Cβ primer, covering the CDR3 region and then loaded onto a 6% acrylamide-8 M urea gel and electrophoresed for 5 h using a 373 DNA Sequencer Applied Biosystems (Perkin-Elmer®). Fluorescent PCR products were resolved as sets of various peaks expressing CDR3 lengths as distribution profiles with Immunoscope® software [7].

Quantification of Cβ and Vβ transcripts

Real time quantitative PCR using the Cβ primer together with each Vβ primer was used to detect and measure fluorescence. The amplification products were carried out using ABI PRISM 7700 Sequence Detection system software (Applied Biosystems®) with SYBRGreen chemistry. For each Vβ and Cβ PCR prepared, the amount of target copies was determined using samples known to contain T cells, assuming that the number of copies was directly proportional to the amount and length of DNA as described elsewhere [8]. Vβ transcript distribution was highly representative of the Cβ mRNA accumulation in each sample as shown by calculation of the correlation coefficient between the sum of the amount of Vβ transcripts and the Cβ transcript accumulation (0.9854) (data not shown).

Graphical representation of integrated qualitative (CDR3-LD) and quantitative (Vβ transcript levels) TCR β chain assessments

For each animal, the CDR3 length profiles obtained from the Immunoscope® analysis were normalized, so that the total area was equal to 1. The profiles from three naive rats used as controls were determined and the mean profiles of each of the 20 Vβ families were used as references. For each CDR3 length profile experiment, the normalized profile was compared with the controls, and the difference plotted on a landscape according to the method used by Gorochov et al [9]. MatLab® software was used to compute and display the qualitative and quantitative TaqMan® data, as an integrated three-dimensional landscape, referred to as TcLandscape (for T cell landscape)®. Percentages of CDR3-LD alterations are represented as a color code on the landscapes ranging from deep blue (value −50%) to dark red (50%). The *x*-axis displays the 21 Vβ families in rat. The *y*-axis gives the Vβ/HPRT transcript ratios. The *z*-axis gives the 10 CDR3 lengths [10].

Statistical analysis of TcLandscape patterns

Correspondence factor analysis (CFA) was used to compare allo- and xenograft samples at different time points. The use of CFA to analyze a relatively small number of samples was valid because the method is based on χ^2 metrics, unlike principal components analysis based on covariance [11–13]. For analysis of the quantitative data, a matrix [three conditions × 20 Vβ] was used to analyze the quantity of mRNA of each Vβ family in allo- and xenografts, regardless of the harvesting time. For analysis of the qualitative data, another matrix [three conditions × 20 Vβ × 10 CDR3 lengths] was used to analyze the variation of each Vβ family in allo- and xenografts, regardless of the harvesting time. Splenocytes of LEW.1A naive rats were used as the normal Gaussian reference. The coordinates of the matrix were then displayed on a Cartesian bi-dimensional diagram. The main factorial axis (φ1) discriminated the different Vβ families and the second factorial axis (φ2) discriminated allo- from xenografted animals. In addition, comparisons between the different groups were performed using a MANOVA test and paired *t*-test on CDR3-LD alterations and on Vβ transcript values, following the MIXED procedure and using SAS software.

Results

TCR β chain transcript amounts in xeno- and allografts during CsA treatment and at rejection

As shown in Fig. 1, in allografts under CsA treatment, Cβ transcript amount were minimal whereas they were strong and significantly increased in xenografts on day 15 ($P < 0.05$). This was in keeping with the kinetics of T cell infiltration being almost absent at the beginning and reaching a peak on day 15 post-transplantation in this model and elsewhere [1]. As shown in Fig. 1, after withdrawal of CsA, Cβ transcript amount increased slowly in allografts and took 18 ± 4 days to reach a maximum, whereas the same magnitude of Cβ transcript amount was observed as soon as day ± 1 in xenografts.

Xenografts elicit a pattern of Vβ transcript accumulation different from that of allografts when recipients are treated with CsA

Figure 2A shows the kinetics of Vβ transcript accumulation in "accommodated" xenografts at day 15 and 40 post-transplantation, under CsA therapy. Two representative examples (out of four) are shown for each time point (left and right panels, at the left-hand side of the figure). During accommodation, Vβ transcript amounts for some families reached a peak on day 15, whereas most Vβ family mRNA accumulation had disappeared on day 40, except for two families (Vβ10 and 20) with Gaussian CDR3-LD. This day 15 pattern may

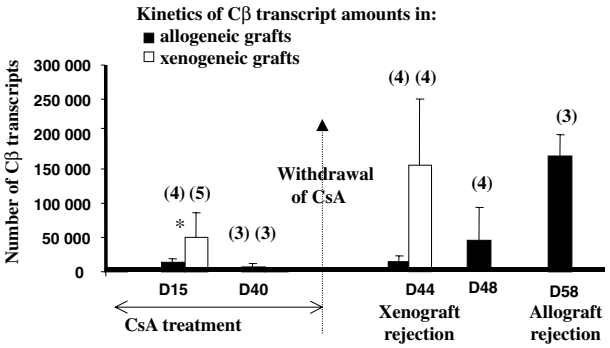

Fig. 1. Kinetics of the Cβ transcript amounts in T cells infiltrating allo- and xenografts. It shows the kinetics of Cβ transcript amounts in T cells infiltrating CsA-treated allografts (■) compared with those observed in accommodated xenografts (□). The number of graft analyzed at each time point is mentioned. The Cβ transcript amounts in allografts were significantly lower than in xenografts on day 15 after grafting despite recipients being under CsA therapy in both situations. At day 40, the amounts of Cβ mRNA were similarly low in accommodated allo- and xenografts. After CsA withdrawal, Cβ transcript amount peaked to reach a maximum in both situations at the time of rejection. This strong accumulation was slower in allografts than in xenografts.

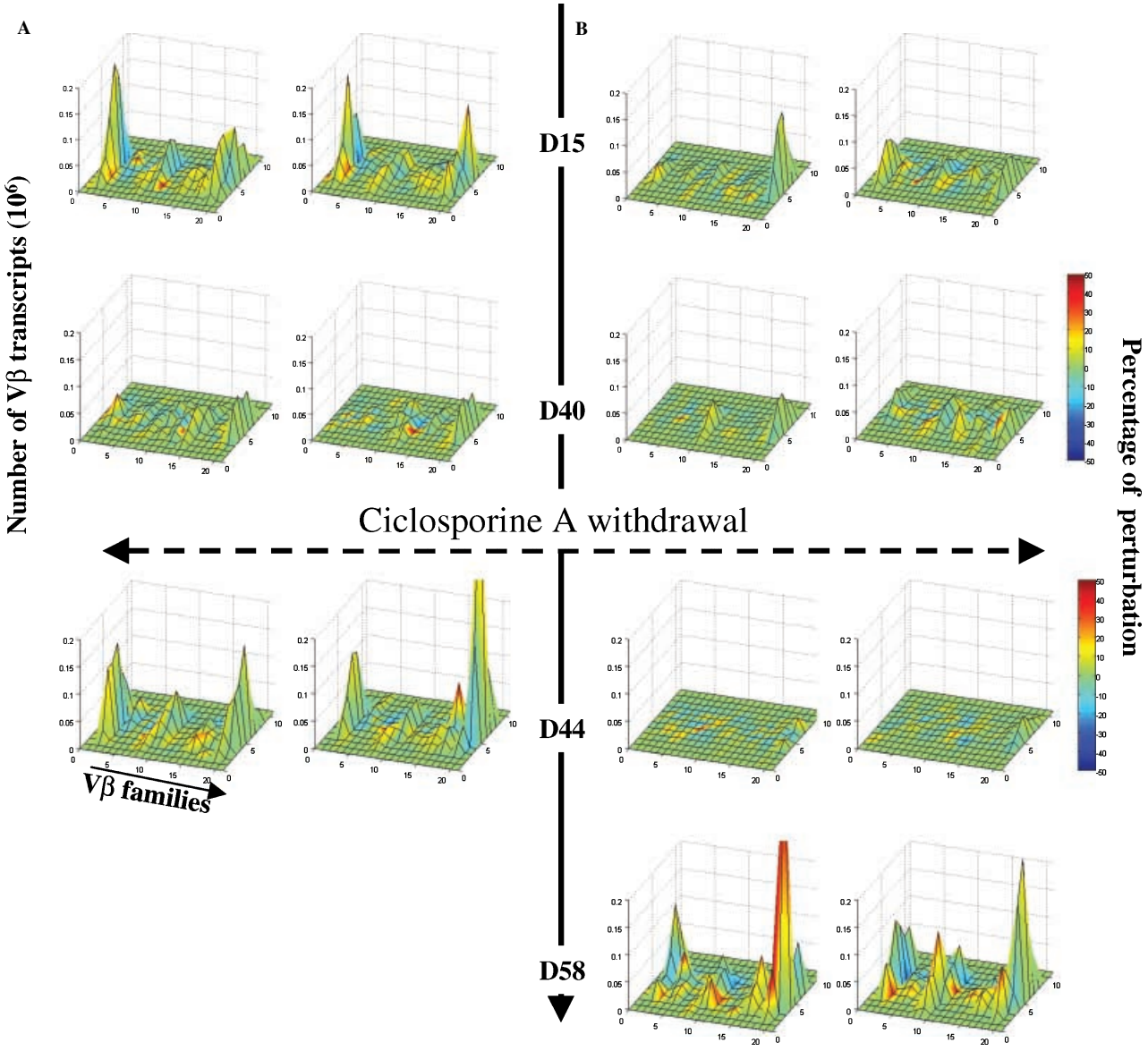

Fig. 2. Combined qualitative and quantitative analysis (TcLandscape) of accommodated and rejected xenografts (A) and allografts (B). Two representative examples are given, one on the left-hand side of each section of the figure and the other on the right-hand side. Analysis of CDR3-LD alteration was performed using Immunoscope® software. The level of Vβ/HPRT transcript ratio was measured by real-time quantitative PCR. Data were displayed in the conventional tridimensional TcLandscape®. Percentages of CDR3-LD alterations are represented as a color code on the landscapes ranging from deep blue (value −50%) to dark red (50%). The *x*-axis displays the 21 Vβ families in rat. The *y*-axis gives the Vβ/HPRT transcript ratios. The *z*-axis gives the 10 CDR3 lengths [10]. The TcLandscapes were obtained on days 15 and 40 when the recipients were under CsA therapy. According to the amount of Cβ mRNA, the Vβ transcript accumulation was lower in allografts than in xenografts. At rejection, in xenografts, the strong increase in Vβ transcript amount occurred without skewing of the CDR3-LD. In contrast, in allografts, an altered pattern was observed. This strong increase of the number in some Vβ family transcripts occurred more slowly in allografts than in xenografts.

reflect an abortive rejection. Indeed, xenograft rejection occurs in 40% of recipients in the second week following surgery [14].

Figure 2B shows the kinetics of Vβ transcript accumulation in allografts at days 15 and 40 post-transplantation in similar LEW.1A recipients. As for the amount of Cβ mRNA, Vβ transcript accumulations were low when compared with xenografts at early time points ($P < 0.05$ on day 15). In addition, allografts harvested on days 15 and 40 exhibited a Gaussian CDR3-LD, as for xenografts.

Contrasting Vβ transcriptome patterns in xeno and allografts at rejection following withdrawal of CsA

TcLandscapes obtained from xeno- and allografts harvested 4 days after CsA withdrawal (at the time

of xenograft rejection) are presented in Fig. 2. A strong Vβ transcript accumulation was observed as soon as day 4 ± 1 after CsA withdrawal in xenografts whereas, at the same time, no Vβ transcript accumulation was observed in allografts ($P < 0.01$). Despite these strong mRNA amounts, the CDR3-LD patterns remained remarkably Gaussian in rejected xenografts (only a few altered Vβ families were observed, Fig. 2A) ($P < 0.01$). In sharp contrast, whereas the Vβ transcript amounts was always stronger in xenografts compared with allograft ($P < 0.01$), strong CDR3-LD alterations were observed in allografts at the time of rejection ($P < 0.01$) (Fig. 2B).

Vβ family clustering according to the allo- or xenograft setting

Figure 3A shows the clustered organization of the various Vβ families with the magnitude of mRNA according to the allo- or xenograft situation. As assessed by the CFA, the Vβ2, 15, 17, 18 and 19 families and the Vβ9 and 16 families were found to be involved in the xeno- and allo-situation (Fig. 3A). This statistical analysis also identified the Vβ20 family as being highly expressed, irrespective of the allo- or xeno-stimulation. Finally, most of the remaining Vβ families had no increase in the amount of Vβ transcripts whatever the type of the graft.

As over-represented Vβ family mRNA did not necessarily involve altered CDR3-LD, the presence of Vβ family clusters was also investigated on the basis of CDR3-LD (Fig. 3B). When the covariance factor analysis was based on qualitative data (CDR3-LD), clusters of altered Vβ mRNA also discriminated the three allo-, xeno- and naive settings in the same way as for the quantitative data analysis (Fig. 3A). The Vβ8 family and the Vβ4, 6, 7, 10, 14, 17, 18 and 19 families were found to be involved in the xeno- and allo-settings whereas (Fig. 3B) the Vβ1, 13 and 20 families were found to be similarly involved in both situations. However, these clusters did not necessarily overlap those found in the analysis performed on the basis of the magnitude of mRNA accumulation presented above (Fig. 3A).

Discussion

In this study, we showed that cardiac xeno- and allografts performed in similar LEW.1A rat recipients elicited contrasted patterns of T cell activation as assessed by their T cell repertoire usage (CDR3 LD) and their amounts of Vβ transcripts. After CsA withdrawal, the TCR Vβ profile in xenografts quickly recovered a Gaussian profile with a rapid expansion of large T cell number. In contrast, allografts harbored T cells with a restricted TCR repertoire and were less vigorously rejected. Differences were observed both during the early post-transplantation period as well as during acute rejection, a long time after surgery, in terms of Vβ transcript amount ($P < 0.05$ on day 15, $P < 0.01$ at rejection). At the CDR3-LD level,

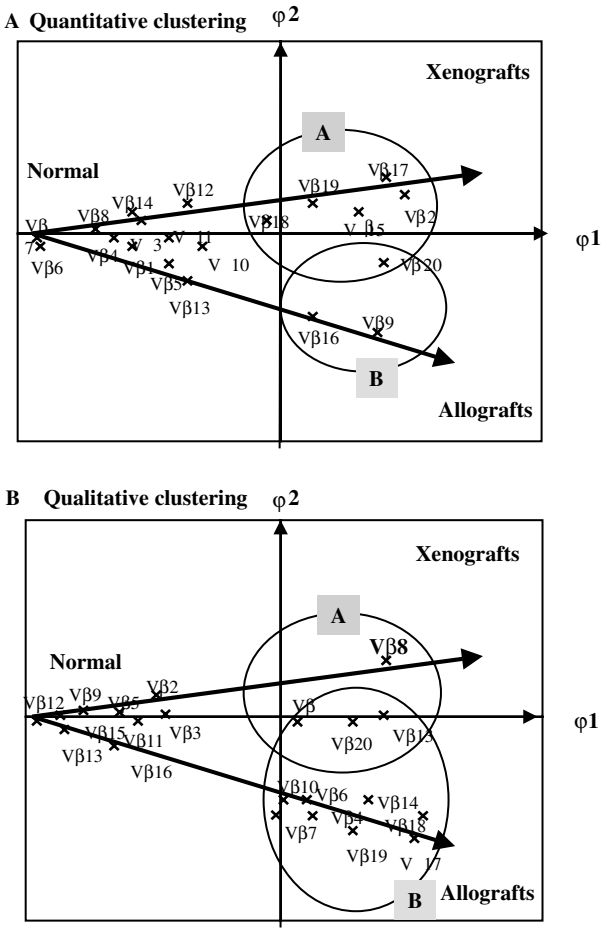

Fig. 3. Correlation between Vβ family usage and the allo- or xenograft setting at the level of Vβ mRNA amount (A) and CDR3-LD (B). For analysis of the quantitative data, a matrix [three conditions × 20 Vβ] was used to analyze the quantity of mRNA of each Vβ family in allo- and xenografts, regardless of the harvesting time. For analysis of the qualitative data, another matrix [three conditions × 20 Vβ × 10 CDR3 lengths] was used to analyze the variation of each Vβ family in allo- and xenografts, regardless of the harvesting time. Splenocytes of LEW.1A naive rats were used as the normal Gaussian reference. The coordinates of the matrix were then displayed on a Cartesian bi-dimensional diagram. The main factorial axis ($\varphi 1$) discriminated the different Vβ families and the second factorial axis ($\varphi 2$) discriminated allo- from xenografted animals. At the quantitative level (Vβ transcript accumulation), some family transcripts (Vβ9, Vβ16) were associated with allografts whereas others (Vβ2, 15, 17, 18 and 19) were associated with xenografts (A). Vβ20 family mRNA was found in both allo- and xenografts. At the qualitative level (CDR3-LD), the Vβ family clusters were different from those obtained from the quantitative data (B).

such differences, that were not observed at day 15, were also present at rejection ($P < 0.01$). In addition, we show that a different preferential clustering of some Vβ families could be observed in T cells infiltrating rejected allo- and xenografts. Taken together, and fitting with a high expression of class II and IL2-Rα activation markers [1] in xenografts, our data not only support the concept that the cellular response against xenografts is considerably more vigorous and rapid than in allografts, but also that this response proceeds through different conditions of T cell activation.

During CsA therapy, on day 15, the Vβ transcript amounts of most of the Vβ families were much lower in allografts than in xenografts. As explained previously, 60% of heart xenografts attained long-term survival (>40 days) whereas the remaining 40% of hearts were rejected on day 8.2 ± 2.5. Day 15 was chosen on the basis that a graft would have a 100% chance of LTS if it beat for longer than the mean + 2SD (>13.2 days) of treated rejected hearts (8.2 ± 2.3 days) [1]. Thus, one could hypothesize that day 15 corresponds to an edge time period for rejection [2,15].

Alternatively, the day 15 time point may be linked to a peak of anti-donor antibodies with a return of normal complement function following CVF withdrawal, which may create conditions of non-specific inflammation with bystander T cell accumulation [16].

After CsA withdrawal, a strong and rapid accumulation of Vβ mRNA with Gaussian CDR3-LD was observed in xenograft rejection whereas the same increase was associated with a strong skewing of CDR3-LD in allografts. Such a Gaussian TCR pattern has also been observed in direct-type mixte lymphocyte reaction (MLR), when pure T cells from naive animals were co-cultured with xenogeneic antigen presenting cells (APC) in vitro [10]. The direct pathway of recognition of xeno-determinants has been well documented [17] and the TCR cross-reactivity [18], that induces a minimum of 1 to 5% of T cells to proliferate in the presence of allogeneic APCs through the "direct" recognition pathway [19,20], also operates when T cells are confronted with xenogeneic APCs [10,18,21–23]. A roughly similar estimation of the proliferative precursor frequency has been made in xenogeneic and allogeneic direct-type MLR in our laboratory, using carboxyfluoroscein diacetate succinimidyl ester (CFSE)-based experiments [10]. Interestingly, the Gaussian CDR3-LD observed in late acute cellular rejection of accommodated xenografts also characterized TCR usage by graft infiltrating cells during acute rejection of fully major histocompatibility complex (MHC)-mismatched grafts [24]. However, a Gaussian CDR3-LD profile was not observed in the late rejection of allografts occurring after CsA withdrawal (Fig. 2B). In this context, late xenograft rejection could be related to the additional class II MHC stimuli provided by the graft endothelial cells of accommodated xenografts which are lacking in allografts under CsA treatment [25]. Such an inflammatory graft endothelium has been reported in concordant xenograft models [26–30]. This fits with our observation that the transfer of pure $CD4^{+ve}$ T cells from naive LEW.1A rats can induce an acute rejection in irradiated LEW.1A hosts harboring accommodated hamster hearts on day 40 [3]. In contrast, in agreement with the kinetics of the rejection process and the highly altered pattern, the "indirect" self-MHC restricted pathway of recognition is likely to be involved in late allograft rejection following CsA withdrawal. The same altered pattern was observed in the draining lymph nodes when allogeneic T cell antigens were injected into the footpad of an LEW.1A rat [10].

However, our data do not enable us to rule out the possibility that the accumulation of Vβ transcripts with unaltered CDR3-LD in xenografts at rejection was also related to the combined effect of a strong diversity and large quantity of foreign peptides provided by the xenograft [31–33] resulting in a polyclonal Gaussian type of self-restricted ("indirect") response. The persistence of anti-donor antibodies in accommodated recipients [34] may further increase the strength of self-restricted presentation of xeno-peptides by B cells and other APC [35].

In this report, we performed a CFA of TCR Vβ usage. We showed that the number of Vβ families specifically involved in xenografts is higher than in allografts, fitting with the hypothesis that the cellular response against xenografts is more vigorous than that against allografts. The fact that altered Vβ families clustered differently in xeno- and allografts suggests the involvement of xenoreactive T cells. In addition, T cells accumulated mRNA of the Vβ20 family irrespective of the allo- or xenogeneic nature of the antigens. This common pattern may result from some structural antigeneic similarities between allo- and xeno-determinants or alternatively from uncommitted bystander T cells which likely represent a strong proportion of T cells infiltrating rejected grafts [36]. However, this is not likely to be the case, as only the Vβ20 family, was found accumulated in both situations (see Fig. 3A).

In summary, our data suggest that late allograft rejection likely involves the indirect pathway of recognition (more CDR3-LD alterations, rejection

at later time points), in agreement with previous studies. In contrast, the late xenogeneic cellular response likely reflects more complex and probably multiple mechanisms involving either a direct recognition of activated endothelial cells or the recognition of a large variety of self-restricted xenopeptides.

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Contribution à l'analyse des mécanismes impliqués dans le maintien de la tolérance induite par transfusion spécifique de sang du donneur.

Mots clefs :

Tolérance / Cellules régulatrices / Transplantation / Transfusion spécifique du donneur (TSD) / Rat

Résumé de thèse:

Nous avons analysé les mécanismes impliqués dans le maintien et le transfert de tolérance après transfusion spécifique de sang du donneur dans un modèle de greffe cardiaque allogénique chez le rat. Nous avons étudié la localisation et l'activité des cellules régulatrices et/ou cytotoxiques, ainsi que le caractère « infectieux » de cette tolérance à distance de la greffe. Nos observations suggèrent une compartimentation des cellules impliquées dans le maintien et le transfert de la tolérance. Alors que la rate apparaît comme un réservoir de cellules ayant des propriétés régulatrices et capables de transférer la tolérance, le sang présente des cellules aux propriétés cytotoxiques incapables de transférer la tolérance. Enfin, la régulation mise en place lors du protocole de transfusion spécifique de sang du donneur ne suffit pas à empêcher la survenue d'un rejet chronique dans ce modèle.

RESUME DE LA THESE EN ANGLAIS

We have analyzed the mechanisms implied in the maintenance and the transfer of tolerance after Donor Specific blood Transfusion in a model of allogenic cardiac transplantation in the rat. We studied the localization and the activity of the regulatory and/or cytotoxic cells, as well as the "infectious" character of this tolerance at a time interval following transplantation.

Our observations suggest a compartmentalization of the cells implicated in the maintenance and the transfer of tolerance. Whereas the spleen seems to be a "reservoir" of cells that have regulatory properties and are able to transfer tolerance, the blood present cells with cytotoxic properties unable to transfer tolerance. Lastly, the regulation that occurs after the protocol of Donor Specific blood Transfusion is not enough to prevent chronic rejection in this model.

TITRE DE LA THESE EN ANGLAIS

Analysis of mechanisms implied in the maintenance of tolerance induced by Donor Specific blood Transfusion