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FACULTE DE MEDECINE

**ÉTUDE DU PHÉNOTYPE ET DE LA FONCTION DES CELLULES
RÉGULATRICES T CD4⁺CD25⁺ DANS LE SANG DE PATIENTS
TOLÉRANT SPONTANÉMENT
UNE GREFFE DE REIN**

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Abréviations

CD: Cluster de Différenciation

CMH: Complexe Majeur d'Histocompatibilité

CPA: Cellule Présentatrice d'Antigène

CR : Chronic Rejection

CsA: Cyclosporine A

DC: Dendritic Cell

DF-Tol : Drug-free tolerant

DTH : Réaction d'hypersensibilité retardée (Delayed-Type Hypersensitivity)

FOXP3: Forkhead bOX P3

HLA: Human Leucocyte Antigen

HO1: Heme Oxygenase 1

HV: Healthy Volunteers

Ig: Immunoglobuline

IL: Interleukine

LPS: lipopolysaccharide

LT: Lymphocyte T

LTS: Long-Term Survival

MMF: Mycophenolate MoFetil

NK: Natural Killer

NKT: Cellule T Natural Killer

NOD: Non Obese Diabetic

PBMC: Peripheral Blood Mononuclear Cells

PTLD: Post-Transplant Lymphoproliferative Disorders

Sta: Stable

TCR: T Cell Receptor

Th: T helper

TLR: Toll-like recepteur

Tr1: T regulatory 1

Treg: T régulateur

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Introduction

1- Transplantation et reconnaissance immune

En cas de défaillance fonctionnelle d'un organe, la transplantation reste le principal traitement pour lutter contre certaines pathologies (insuffisance rénale terminale, dysfonctionnement cardiaque, mucoviscidose etc.). La première greffe d'organe a été réalisée dans les années 1950, depuis des efforts considérables ont été faits pour améliorer le traitement et la qualité de vie des patients (Murray 2005). La transplantation d'organes est en effet associée à la prise de traitements immunosuppresseurs à long terme, qui peuvent avoir des effets secondaires importants (cancers, infections virales, complications métaboliques) (Dantal, Hourmant et al. 1998) (Hojo, Morimoto et al. 1999) (Soulillou and Giral 2001).

Un des obstacles majeurs est le rejet du greffon qui peut survenir malgré le traitement immunosuppresseur (Orosz 2002). En effet, lors d'une greffe, le système immunitaire rencontre alors des éléments étrangers considérés comme un « danger ». La réponse immunitaire du receveur à la greffe est déterminante pour la survie du greffon. Cependant, nous savons que d'autres paramètres tels que la nature (cadavérique ou vivante), l'âge du donneur et la qualité du greffon peuvent intervenir sur le devenir de la greffe.

Notre système immunitaire constamment en éveil, permet à notre organisme de lutter contre tous dangers et agressions éventuels. Il va permettre le rejet de tous éléments étrangers : le « non soi » ou « danger » et au contraire permettre une tolérance à ce qui lui est propre : le « soi » ou « non danger ».

Il existe 2 types de réponses immunitaires : innée et adaptative. La réponse innée est rapide et non spécifique. Elle fait intervenir plusieurs types cellulaires : les cellules dendritiques, les macrophages, les cellules Natural Killer (NK), les NKT, ainsi que les neutrophiles. Ces cellules et plus particulièrement les cellules présentatrices d'antigènes sont capables de reconnaître des motifs très conservés au cours du temps, issus de bactéries ou de virus, tels que les PAMPS (Pathogen-Associated Molecular Patterns), reconnus par des récepteurs tels que les Toll-Like Recepteurs (TLR) (Kawai and Akira 2005). Ce sont des PRRs (Pattern Recognition Receptors) qui, une fois liés à leurs ligands, sont à l'origine de la réponse inflammatoire. Des mécanismes de phagocytose et l'activation du complément vont également intervenir pour lutter contre les éléments étrangers.

Cependant, certains éléments dangereux pour notre organisme et ne portant pas ces motifs conservés se sont développés au cours de l'évolution (cellules tumorales, virus). Notre système immunitaire a acquis des mécanismes adaptatifs, spécifiques de l'antigène rencontré. Cette réponse adaptative plus lente que la précédente, fait intervenir principalement des

lymphocytes T spécifiques possédant une capacité de mémoire, ainsi que des lymphocytes B et des cellules présentatrices d'antigènes. Je décrirais brièvement le complexe majeur d'histocompatibilité et le TCR ainsi que les voies de présentation des antigènes, car ceci n'est pas le sujet principal de cette thèse, mais aide à la compréhension des mécanismes de la réponse immunitaire.

1-1 Allogénicité et Complexe Majeur d'Histocompatibilité

L'antigène (peptide) est présenté sur le CMH des cellules présentatrices (CPA) à des lymphocytes T spécifiques par l'intermédiaire de leurs récepteurs appelés TCR (T cell receptor) (Rudolph, Stanfield et al. 2006). Le phénomène de restriction du TCR découle des travaux de Zinkernagel et Doherty en 1974 qui montrèrent que des lymphocytes T spécifiques d'un virus lysent préférentiellement les cellules infectées qui présentent les mêmes molécules de CMH qu'eux. Les lymphocytes T peuvent être divisés en deux grandes populations: les lymphocytes T CD4⁺ qui reconnaissent classiquement les complexes CMH II – peptide, et les lymphocytes T CD8⁺ ou T cytotoxiques qui reconnaissent les complexes CMH I – peptide (Rock and Shen 2005; Porcelli and Hammerling 2006). Cependant, ce système n'est pas toujours aussi restrictif (voir chapitre présentation croisée). Les lymphocytes T reconnaissent les épitopes de peptides antigéniques présentés par les CPA. Ces dernières présentent des peptides endogènes ou exogènes apprêtés par leurs molécules du CMH de classe I ou de classe II respectivement (Trombetta and Mellman 2005). Chez l'homme ce complexe est appelé HLA pour Antigènes Leucocytaires Humains. Ce complexe représente la région génétique la plus polymorphique impliquée dans de nombreuses réponses biologiques (réponses immunes, régulation) et ayant un impact médical important (Charron 2005).

1-2-1 Les molécules de CMH de classe I

Les molécules du CMH de classe I correspondent aux Antigènes Leucocytaires Humains (HLA) HLA-A, -B, et -C. Ces molécules sont synthétisées dans le réticulum endoplasmique et sont présentes à la surface de la plupart des cellules nucléées (Lehner and Cresswell 1996). Le CMH de classe I est constitué de 2 chaînes associées de manière non covalente : une chaîne légère non polymorphique, la $\beta 2$ microglobuline et une chaîne lourde polymorphique α . La chaîne α comporte 3 domaines extramembranaires ($\alpha 1$, $\alpha 2$ et $\alpha 3$), une région transmembranaire et une région intracytoplasmique (**Figure 1**). Les domaines $\alpha 1$ et $\alpha 2$ forment le site de fixation au peptide (8 à 9 acides aminés). Le domaine $\alpha 3$ est impliqué dans l'association à la $\beta 2$ microglobuline et constitue le site de liaison du corécepteur CD8

(Bjorkman, Saper et al. 1987). Les molécules de CMH de classe I présentent classiquement les peptides issus de protéines endogènes, de virus ou de bactéries aux lymphocytes T CD8⁺ (Marusic-Galesic, Stephany et al. 1988).

1-2-2 Les molécules de CMH de classe II

Les molécules du CMH de classe II correspondent aux HLA -DR, -DQ, et -DP chez l'homme. Ces molécules sont formées par l'association d'une chaîne lourde β et d'une chaîne légère α , chacune comportant deux domaines extramembranaires, un domaine membranaire et un domaine intracellulaire (**Figure 1**). La chaîne α et la chaîne β participent toutes deux à la fixation du peptide (Stern, Brown et al. 1994). L'expression des molécules du CMH de classe II est principalement restreinte aux CPA telles que les DC, les lymphocytes B, les monocytes macrophages mais peut être induite sur certaines cellules en conditions inflammatoires (cellules endothéliales, épithéliales, kératinocytes et les T (Holling, Schooten et al. 2004). Ces molécules présentent classiquement des peptides exogènes captés de l'extérieur par voie endosomale, aux lymphocytes T CD4⁺.

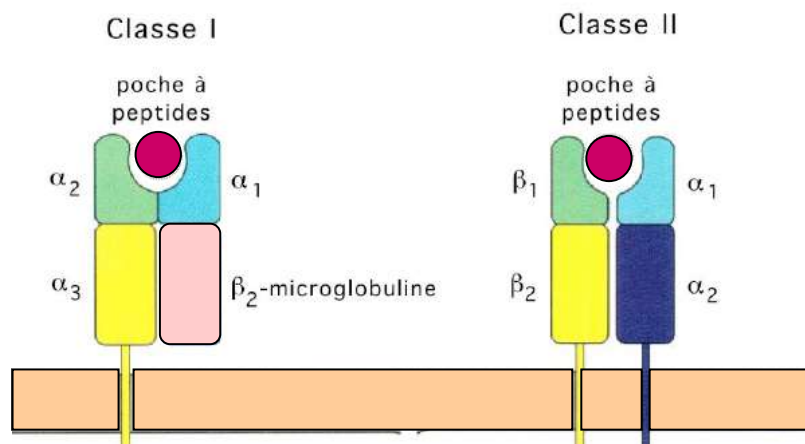


Figure 1: Structure schématique des molécules du CMH de classe I et II

1-2-3 La présentation croisée

La capacité pour les molécules de CMH de présentation croisée a été décrite en 1976 par Bevan (Bevan 1976). En effet, il a été démontré que les molécules de CMH de classe I ne se restreignent pas à présenter des peptides endogènes aux lymphocytes T CD8⁺ (Shen and Rock 2006). Ils peuvent également présenter des peptides exogènes aux lymphocytes T CD8⁺. De la même manière, les molécules de CMH de classe II ne se limitent pas à présenter des

peptides exogènes aux lymphocytes T CD4⁺, ils peuvent leur présenter des peptides endogènes (Rock and Shen 2005).

1-2-4 La présentation sur les molécules CD1

Les molécules CD1 sont des molécules présentatrices d'antigènes appelées molécules de CMH de classe I non classiques. Chez l'homme, 4 molécules CD1 ont été décrites: CD1a à d. Elles présentent des lipides, des glycolipides et lipopeptides d'antigènes exogènes ou endogènes (Moody, Zajonc et al. 2005). Il a également été montré qu'elles pouvaient présenter de petites molécules non lipidiques (Van Rhijn, Young et al. 2004). Elles contribuent à l'immunité mais sont également impliquées dans l'auto-immunité et les réponses anti-tumorales. La molécule CD1 est un marqueur essentiel des cellules dendritiques myéloïdes et les cellules de Langerhans, mais elle est également exprimée par les thymocytes et les cellules B (Moody, Zajonc et al. 2005). Les molécules CD1 sont classées en deux groupes, le premier rassemble le CD1a, le CD1b, le CD1c et le CD1e tandis que le second groupe est constitué que du CD1d du fait de ses caractéristiques structurales et de sa localisation cellulaire (essentiellement les NKT) (Calabi, Jarvis et al. 1989).

Les cellules T qui reconnaissent les lipides associés aux molécules CD1 peuvent être divisées en deux selon les deux groupes de CD1 : celles restreintes aux molécules du groupe I qui partagent les mêmes propriétés que les cellules T spécifiques d'un peptide (Grant, Degano et al. 1999) et celles restreintes au groupe II, c'est-à-dire le CD1d qui partagent plusieurs propriétés des cellules de l'immunité innée (Bendelac 1995) (Skold and Behar 2003). La population principale de ces cellules restreintes au CD1d, les cellules NKT, partagent plusieurs marqueurs des cellules NK et un récepteur des cellules T semi-invariant c'est-à-dire une chaîne α invariante (V α 14 chez la souris et V α 24 chez l'Homme) (Bendelac 1995).

Les cellules T restreintes au CD1 du groupe I ont plusieurs caractéristiques notamment celles d'être cytolytiques et de pouvoir lyser une variété de cellules cibles de façon antigène spécifique soit par un mécanisme FasL pour les T CD4⁻ CD8⁻, soit par la voie perforine/granzyme pour les T CD8⁺ (Stenger, Mazzaccaro et al. 1997). Chez l'homme, ces cellules sont aussi productrices de cytokines incluant l'IFN- γ et le TNF- α qui toutes deux ont des propriétés antimicrobiennes importantes basées sur leur capacité à activer les macrophages infectés et les DC à produire des médiateurs bactéricides tels que des radicaux libres et du NO mais aussi de l'IL-12. Par contre, les cellules T restreintes au CD1 du groupe II agissent différemment. Il semble que l'IL-12 soit un signal important dans l'activation des cellules NKT (Vincent, Leslie et al. 2002) (Brigl, Bry et al. 2003). De plus, ces cellules NKT ont aussi une

capacité cytolytique directe similaire aux cellules T CD8⁺ et γ/δ , qui nécessitent l'expression du CD1d par les cellules infectées (Gansert, Kiessler et al. 2003). Cette voie du CD1d serait aussi impliquée dans l'immunité anti-tumorale (Smyth, Thia et al. 2000) (Smyth, Crowe et al. 2002).

1-2 Le TCR et l'activation lymphocytaire T

Les lymphocytes T sont les principaux médiateurs de la réponse immunitaire spécifique. Ils expriment à leur surface un récepteur, le TCR, capable de reconnaître le complexe CMH – peptide (Rudolph, Stanfield et al. 2006). Le TCR est constitué de 2 chaînes polypeptidiques α et β (Garcia, Teyton et al. 1999). Il est associé à la molécule CD3, qui va permettre la transmission du signal. On distingue 2 grandes sous populations de lymphocytes T $\alpha\beta$ en fonctions de co-récepteurs qu'ils expriment, les lymphocytes T CD4⁺ et les lymphocytes T CD8⁺. Les lymphocytes T CD4⁺ sont appelés T helper, ils sont capables d'activer des cellules telles que les lymphocytes B, les macrophages et les lymphocytes T CD8⁺. En fonction de leur environnement, ils peuvent se différencier en lymphocyte Th1 ou Th2. Les cellules Th1 contrôlent la réponse cellulaire, telles que la réponse d'hypersensibilité retardée et certaines réponses humorales. Elles jouent également un rôle dans l'activation des macrophages et des cellules T CD8⁺ cytotoxiques. Les cellules Th2, de par leur capacité à produire de l'IL-4, favorisent la réponse humorale et les hypersensibilités allergiques (IgE).

L'activation du lymphocyte T implique trois signaux indépendants qui vont déterminer l'expansion clonale et la fonction effectrice de ce lymphocyte (**Figure 2**).

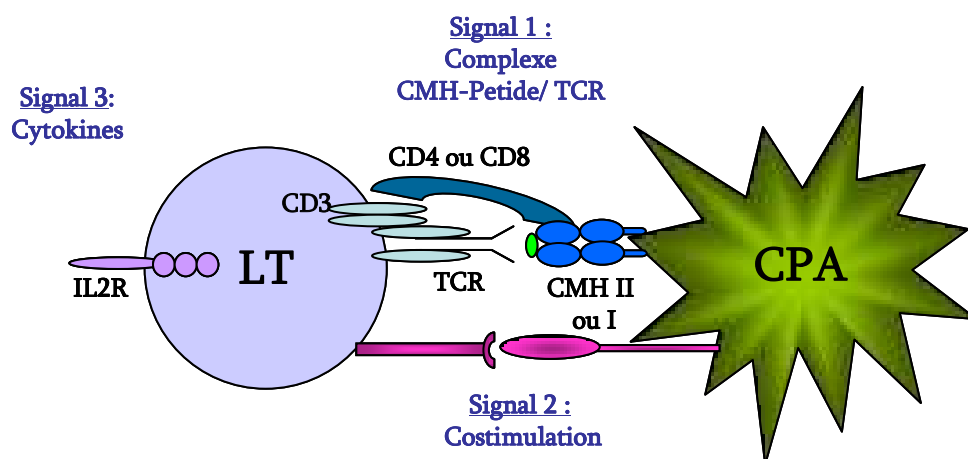


Figure 2: Les signaux d'activation des lymphocytes.

Dans un premier temps il y a formation d'une synapse immunologique entre le lymphocyte T et la cellule présentatrice d'antigène (Paul and Seder 1994) (Monks, Freiberg et

al. 1998). Le « signal 1 » est spécifique de l'antigène et consiste en la formation du complexe CMH-peptide/TCR qui va permettre la transduction d'un signal intracellulaire. Un second signal, dit de costimulation, augmente l'avidité de l'interaction de la cellule T avec la cellule présentant l'antigène. Il est non spécifique de l'antigène, et est fourni par des interactions récepteurs/ligands entre les CPA et les lymphocytes T. Ces voies de costimulation sont multiples et de nouvelles molécules sont sans cesse découvertes. Elles se répartissent entre la famille des immunoglobulines (CD28, ICOS, CD2...), la famille des récepteurs du TNF (CD40L, TRANCE, CD27, CD30, GITR...) et la famille des intégrines (LFA-1, VLA-4) (Watts 2005) (**Figure 3**). Un troisième signal est fourni par les cytokines agissant de manière autocrine et/ou paracrine. L'interleukine-2 (IL2) joue un rôle majeur sur la prolifération et la différenciation du lymphocyte. Par un mode autocrine et paracrine, l'interaction de l'IL2 avec son récepteur permet alors l'expansion clonale et la phase effectrice de la réponse immune. Néanmoins, d'autres cytokines, comme l'IL7 et l'IL15, peuvent aussi induire la prolifération de lymphocytes T naïfs (Geginat, Sallusto et al. 2001) (Schluns, Kieper et al. 2000). Enfin, la régulation de la réponse immune est primordiale. Elle fait intervenir l'expression sur le lymphocyte T activé de molécules inhibitrices comme CTLA4 (Cytotoxic T Lymphocyte Antigen 4) ou encore PD1 (Programmed Death 1), deux membres de la famille CD28 (Freeman, Long et al. 2000) (Zhang, Schwartz et al. 2004). Une fois les antigènes éliminés et la réponse inflammatoire arrêtée, la grande majorité des lymphocytes T activés meurent par apoptose, tandis qu'un petit contingent constituera un pool de cellules mémoires.

En l'absence de costimulation efficace (pas de signal 2), l'activation d'un lymphocyte T naïf est incomplète et il devient anergique (Powell 2006). Cet état se définit comme un état de non réponse du lymphocyte à un stimulus (Schwartz, Mueller et al. 1989), et il ne produit pas d'IL2. Cet état reste transitoire et peut être levé par l'ajout d'IL2 (Sykes, Harty et al. 1994).

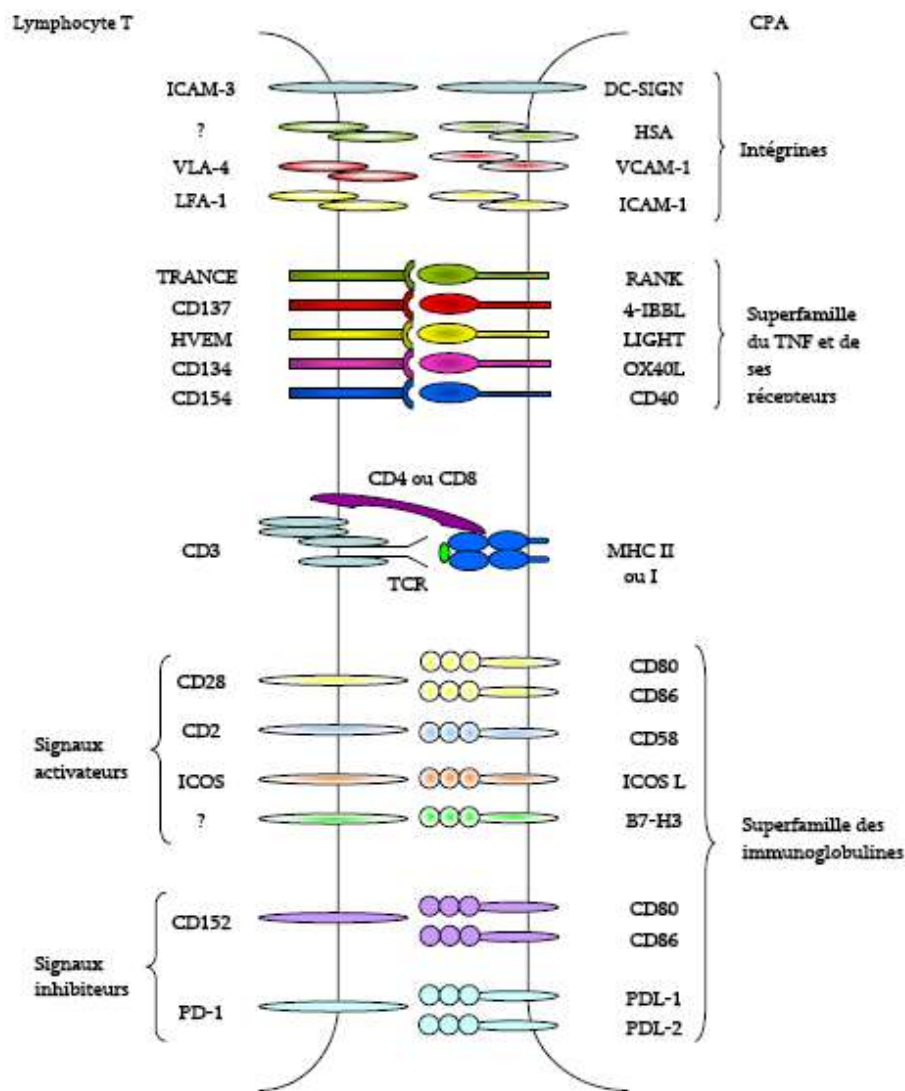


Figure 3 : Principales molécules impliquées dans l'activation lymphocytaire T

1-3 Les voies de présentation des alloantigènes

Les lymphocytes T peuvent interagir avec les alloantigènes par trois voies indépendantes (non exclusives), par l'intermédiaire d'une reconnaissance directe, indirecte, ou semi-directe. Les 2 voies les mieux décrites, directe et indirecte, ont été découvertes pour la première fois en 1982 par Lechler et al. (Lechler and Batchelor 1982) (Lechler, Lombardi et al. 1990) et impliquent différentes cellules présentatrices d'antigènes.

La voie de reconnaissance directe correspond à la présentation de l'antigène par la cellule présentatrice du donneur aux lymphocytes T du receveur. D'autre part, des fragments des antigènes peuvent être présentés par les cellules présentatrices d'antigènes du receveur, cette voie est appelée reconnaissance indirecte (**Figure 4**).

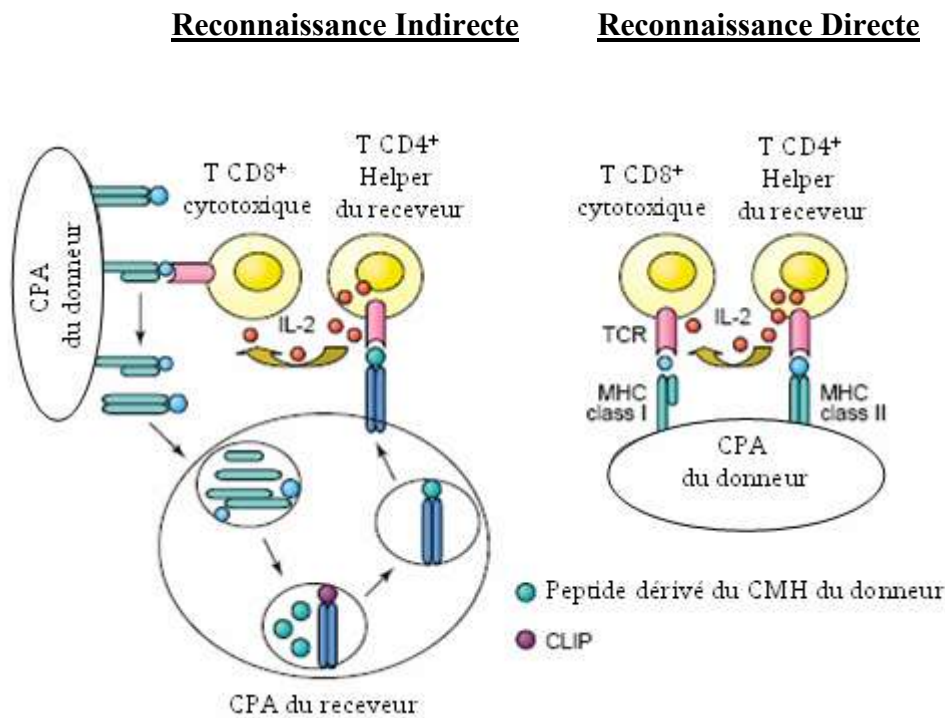


Figure 4: Voies de présentation des antigènes aux lymphocytes T
(adapté d'après (Jiang, Herrera et al. 2004))

La présentation directe est caractérisée par une réponse polyclonale, polyspécifique, mobilisant une part importante du répertoire lymphocytaire T. De 1 à 10% des cellules T naïves pourraient être activées par cette voie d'alloreconnaissance directe (Matis, Sorger et al. 1987) (Suchin, Langmuir et al. 2001). Notre laboratoire a mis en évidence que la reconnaissance par la voie de présentation directe entraînait une modification du répertoire des lymphocytes T (mobilisation de 30% du répertoire), caractérisée par une accumulation des transcrits V β et par une mobilisation polyclonale du répertoire (Sebille, Gagne et al. 2001).

La voie de reconnaissance indirecte est un concept démontré par Auchincloss et al. en 1993 (Auchincloss, Lee et al. 1993). Elle est plus tardive que la voie directe car elle nécessite au préalable un apprêtement des antigènes du donneur par les cellules du receveur.

Une voie semi-directe a été décrite récemment et soulève encore de nombreuses interrogations (Herrera, Golshayan et al. 2004). En effet, il semblerait que les DC puissent acquérir le complexe CMH-peptide intact à partir d'autres cellules (cellules endothéliales et dendritiques) et le présenter aux lymphocytes T. Cette voie pourrait faire le lien entre la reconnaissance directe et indirecte (Jiang, Herrera et al. 2004).

1-4 Rôle des voies directe et indirecte dans le rejet d'allogreffe

Lors d'une transplantation, la plupart des greffons contiennent des cellules présentatrices d'antigènes encore appelés « Leucocytes Passagers ». Après la greffe, ces cellules vont migrer dans les organes lymphoïdes secondaires (Saiki, Ezaki et al. 2001). Ainsi Saiki a montré dans un modèle d'allogreffe cardiaque que les DC du donneur migraient, dans les 4 premiers jours, principalement vers la rate et les ganglions lymphatiques hépatiques. Les lymphocytes T (LT) des organes lymphoïdes vont alors être activés directement par les CPA du donneur ou indirectement par les CPA du receveur et peuvent ensuite entraîner le rejet du greffon (Sayegh and Turka 1998).

La forte fréquence de LT avec une allospécificité directe et la faible fréquence de LT avec une allospécificité indirecte dans le répertoire T normal, a suggéré que l'alloréponse directe prédominait dans les phases précoces de la réponse immune après transplantation (rejet aigu) tandis que la voie indirecte jouait un rôle majoritaire pendant les phases tardives de l'alloréponse (rejet chronique). Benichou et al. ont établi l'importance des deux types de présentation au cours des premiers jours de la greffe. Au onzième jour de la greffe, plus de 90% des cellules alloréactives au niveau des organes lymphoïdes du receveur interagissent avec des molécules du CMH du donneur (voie directe) alors que seules 1 à 5% d'entre elles sont, à ce stade, dirigées contre des peptides du donneur présentés par des molécules HLA du soi (voie indirecte) (Benichou 1999).

Parmi les CPA, les cellules dendritiques jouent un rôle majoritaire dans la survenue du rejet par voie directe, ainsi une déplétion ou un parking des DC du donneur prolonge la survie du greffon (Roussey-Kesler, Brouard et al. 2005). Dans un modèle de greffe cardiaque, des rats tolérants après transfusion de sang du donneur développent un rejet si les DC du donneur sont déplétées par la cyclophosphamide (Josien, Heslan et al. 1998).

Pietra et al. ont établi pour la première fois en 2000 que des cellules T, avec des capacités de réponse directe contre le donneur, étaient capables d'induire un rejet de greffe (Pietra, Wiseman et al. 2000). Ils ont montré grâce à l'utilisation de souris immunodéficientes que la voie de reconnaissance directe via les cellules T CD4⁺ était nécessaire et suffisante pour induire un rejet d'allogreffe dans leur modèle.

Il est probable que la force de ces réponses directes anti-donneur diminue avec le temps puisque les cellules dendritiques sont éliminées après la transplantation (Baker, Hernandez-Fuentes et al. 2001). Lechler et al. ont montré que chez des patients transplantés avec des greffons rénaux ou cardiaques, la fréquence des cellules T anti-donneur avec des spécificités de réponse directe diminuait avec le temps (Hornick, Mason et al. 1998). Ce déclin est aussi

présent chez des patients montrant des signes de rejet chronique que chez ceux montrant une fonction rénale stable. Ces résultats suggèrent donc que la voie de présentation directe des alloantigènes n'est pas un mode de reconnaissance important du rejet chronique (Jiang, Herrera et al. 2004).

Auchincloss et al. ont démontré l'implication de la voie indirecte dans le rejet de greffe en 1993. Dans un modèle de greffe de peau, ils ont utilisé des souris donneuses déficientes en CMH de classe II, et ont montré que cette greffe était rejetée et qu'il y avait donc présentation des antigènes du donneur par le CMH du receveur. De plus, en déplétant les T CD8⁺ des receveurs, ils ont montré que les cellules T CD4⁺ des animaux receveurs étaient seules capables d'être stimulées indirectement par la reconnaissance des molécules du CMH de classe I du donneur. Récemment plusieurs études en transplantations rénales, cardiaques et pulmonaires chez l'homme ont montré l'importance de la reconnaissance indirecte dans le rejet chronique (Ballet, Giral et al. 2006). Ainsi, la fréquence des cellules alloréactives activées par voie indirecte augmente chez les sujets transplantés présentant un rejet chronique (Vella, Spadafora-Ferreira et al. 1997) (Suciu-Foca, Ciubotariu et al. 1998) (Lee, Yamada et al. 2001) (Stanford, Ahmed et al. 2003).

Ces différentes études suggèrent donc qu'après une transplantation, la présentation directe prédomine dans les phases précoces de la réponse immune et donc dans le rejet aigu, alors que la présentation indirecte intervient surtout dans les processus de rejet chronique.

2- Le rejet d'allogreffe

De nos jours, trois types de greffe sont possibles. Si le donneur est lui-même le receveur, il s'agit d'autogreffe. Elle concerne principalement les greffes de peau et de cellules hématopoïétiques (moelle osseuse). Si le donneur et le receveur sont deux individus différents mais de la même espèce, c'est une allogreffe. Enfin, si le receveur est d'une espèce différente du donneur, il s'agit d'une xéno greffe.

Dans ces deux derniers types de greffes, le greffon va alors être reconnu comme un « danger » et peut induire une réponse immunitaire entraînant le rejet de ce greffon. Les molécules qui sont reconnues comme étrangères sur les allogreffes sont appelées alloantigènes. Les lymphocytes et les anticorps qui reconnaissent les alloantigènes sont décrits comme étant alloréactifs.

On entend par rejet, les mécanismes qui entraînent la destruction partielle ou complète du greffon. La réponse alloréactive peut se diviser en plusieurs étapes successives:

premièrement, la reconnaissance des alloantigènes par les cellules T de l'hôte; deuxièmement, l'activation et la différenciation des cellules T alloréactives; et troisièmement, la phase effectrice, qui conduit à la perte de la fonction de la greffe.

Les mécanismes par lesquels l'allogreffe peut-être rejetée dépendent de nombreux facteurs impliquant un grand nombre de cellules et de molécules du système immunitaire (Cuturi, Blanco et al. 1994) (Orosz 2002). De façon schématique, les mécanismes qui se mettent en place à la suite d'une première stimulation antigénique sont dominés par la population T $CD4^+$ qui active différents mécanismes effecteurs du rejet: l'activation des macrophages, l'activation des lymphocytes T $CD8^+$ cytotoxiques, la différenciation des lymphocytes B en cellules sécrétrices d'anticorps, l'activation des NK et l'attraction des leucocytes (**Figure 5**).

Les lymphocytes T $CD4^+$ de type Th1, en produisant des cytokines telles que l'IL2 et l'IFN γ , sont généralement associés au développement de ce type de mécanismes effecteurs puisque ces cytokines sont retrouvées dans différents modèles d'allogreffe lors du rejet aigu (Holweg, Peeters et al. 2003) (Wang, Hosiawa et al. 2003).

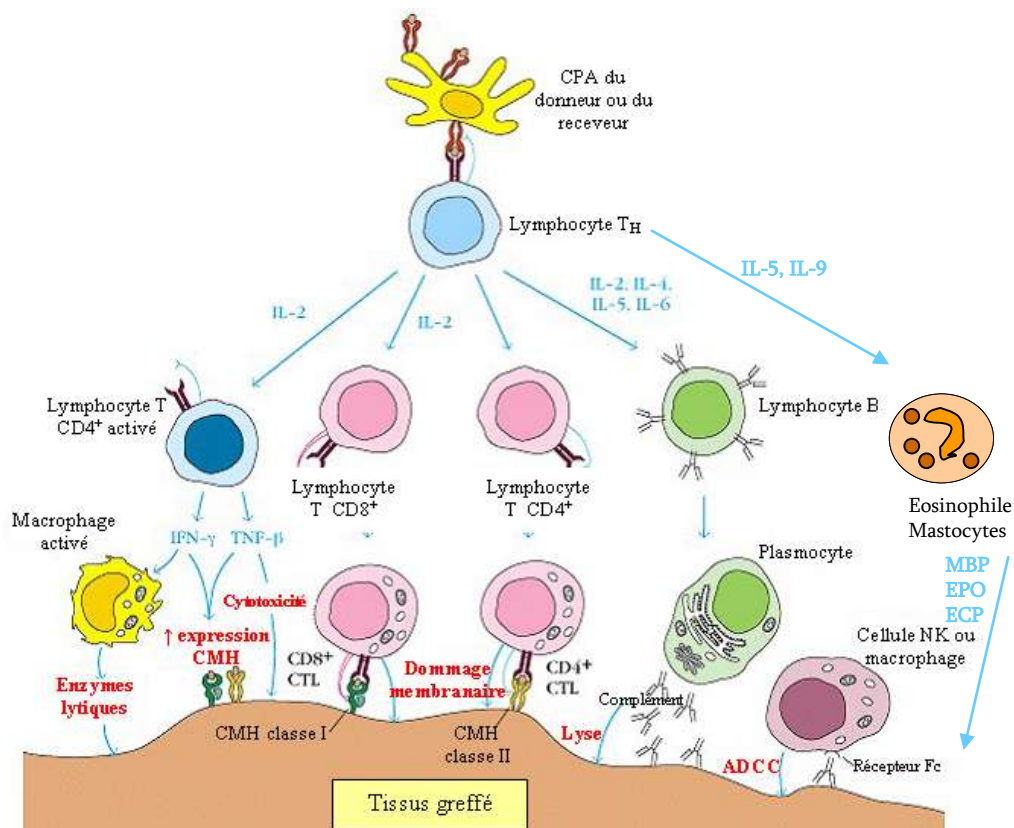


Figure 5: Représentation schématique des mécanismes de rejet de greffes
(adapté de Goldsby et al. 2000)

Trois types de rejets immunitaires peuvent survenir après une greffe: le rejet hyper aigu, le rejet aigu et le rejet chronique. Ils présentent des mécanismes et des cinétiques différents.

2-1 Le rejet hyperaigu

Le rejet hyperaigu apparaît dans les minutes suivant l'introduction du greffon dans l'organisme et concerne uniquement les greffes vascularisées. Ce rejet, très rapide, est caractérisé par une thrombose des vaisseaux qui provoque la nécrose du greffon. Le rejet hyperaigu est dû à la présence chez le receveur d'anticorps anti donneur préformés (Cai and Terasaki 2005) (Terasaki and Cai 2005). Il n'y a pas d'infiltration cellulaire dans ce rejet. Les anticorps préformés sont dirigés contre des antigènes présents sur le greffon qui se lient principalement aux antigènes de l'endothélium. Ils entraînent l'activation du complément, une activation de la cellule endothéliale qui va sécréter, entre autres, le facteur procoagulant Von Willbrand et provoquer l'adhésion et l'agrégation des plaquettes. Cette série de réactions va alors engendrer une thrombose intravasculaire aboutissant à la perte du greffon (Cai and Terasaki 2005). Ces anticorps préformés sont naturels (comme les anticorps anti-A et B des groupes sanguins) ou acquis lors de situations immunogènes antérieures (transfusions, grossesses, greffes). Les anticorps acquis reconnaissent notamment des déterminants du CMH (anticorps anti-HLA).

De nos jours, ce type de rejet est évité dans la majorité des cas par l'analyse des compatibilités HLA entre le donneur et le receveur et par la recherche de tels anticorps dirigés contre le donneur avant toute transplantation. L'objectif des médecins est de déceler la présence ou non de ces anticorps avant la greffe. Ils procèdent ainsi à un test dit "*cross match*" juste avant la transplantation: quelques cellules du donneur sont prélevées et mélangées au sérum du receveur. Si les cellules sont détruites à l'issue du mélange, cela révèle la présence d'anticorps et il y a donc impossibilité de greffer.

2-2 Le rejet aigu

Le rejet aigu est dû à la réaction du système immunitaire contre le greffon et peut survenir une semaine à des mois après la transplantation. Le rejet aigu cellulaire nécessite une immunisation et met donc plusieurs jours à survenir. Il est inéluctable en l'absence de traitement immunosuppresseur et reste un problème sérieux de nos jours en transplantation. Il est diagnostiqué par une biopsie de l'organe greffé et les lésions observées font l'objet de classifications internationales (classification de Banff pour le rein) (Marks and Finke 2006). C'est pourquoi des traitements immunosuppresseurs sont prescrits. Actuellement, grâce aux

traitements immunosuppresseurs, les épisodes de rejet aigu surviennent dans moins de 15% des transplantations (Port, Dykstra et al. 2004). Ils limitent la réaction immunitaire et permettent d'éviter le rejet. Dans les premiers temps après la greffe, il peut cependant y avoir un rejet aigu. Il s'agit alors de réadapter le traitement immunosuppresseur. Traité à temps, il peut être vite jugulé.

Le rejet aigu est le résultat de 2 mécanismes immunologiques, un processus T dépendant et un processus B dépendant qui agissent seul ou ensemble (Marks and Finke 2006). Le rejet aigu cellulaire est dû à la reconnaissance par les lymphocytes T du receveur des antigènes allogéniques du donneur dans un contexte CMH. Les cellules dendritiques du donneur vont migrer vers les organes lymphoïdes secondaires du receveur où elles vont activer les cellules T du receveur par présentation directe et indirecte. Les lymphocytes T ainsi activés prolifèrent puis migrent vers le greffon. Ils sont attirés par des molécules d'adhésion exprimées par l'endothélium devenu inflammatoire.

D'un point de vue histologique, le rejet aigu est caractérisé par un fort infiltrat interstitiel de macrophages et de lymphocytes T. Une fois dans le greffon, les lymphocytes T $CD4^+$ et T $CD8^+$ vont mettre en place des mécanismes aboutissant à la destruction du greffon. Les lymphocytes T $CD4^+$ Th1 déclenchent une réponse dite d'hypersensibilité retardée (DTH). En transplantation, plusieurs études ont montré que la présence d'une réaction d'hypersensibilité retardée était associée à la perte du greffon (Sirak, Orosz et al. 1997), tandis qu'une survie à long terme d'un greffon cardiaque était corrélée à l'absence de DTH (Orosz, Wakely et al. 1997) (VanBuskirk, Wakely et al. 1998). Lors de la DTH, les lymphocytes T $CD4^+$ Th1 vont sécréter des cytokines pro-inflammatoires telles que l'IL2, le $TNF\alpha$ et l' $IFN\gamma$ (Wu, Lovett et al. 1992; Morgan, Pelletier et al. 1993) attirant ainsi les macrophages dans le greffon. Cette activation des macrophages entraîne une augmentation de la production de chimiokines, de monoxyde d'azote (NO), et d'autres facteurs solubles qui activent l'endothélium et entretiennent la réponse inflammatoire (Le Moine, Goldman et al. 2002). D'autre part, les lymphocytes T cytotoxiques $CD8^+$ éliminent les cellules du greffon par contact direct impliquant la voie Fas-FasL et par la sécrétion de granzyme et perforine (Graziotto, Del Prete et al. 2006).

Les cytokines semblent donc jouer un rôle très important dans les mécanismes de rejet (Dai and Lakkis 1999). De plus, elles permettent également la différenciation des lymphocytes B en plasmocytes. Ils produisent alors des anticorps dirigés contre les antigènes du donneur et jouent ainsi un rôle dans le rejet aigu (Le Moine, Goldman et al. 2002). Les IgG et IgM produites par les plasmocytes recrutés sur le site inflammatoire vont alors se fixer sur les

cellules endothéliales du greffon et entraîner l'activation de la cascade du complément et la destruction de l'endothélium.

2-3 Le rejet chronique

Les traitements immunosuppresseurs, bien qu'efficaces sur le rejet aigu, ne peuvent pas empêcher le développement d'un rejet chronique. Ainsi, le rejet chronique reste la principale cause de perte de greffon à long terme (Joosten, van Kooten et al. 2003) (Ballet, Giral et al. 2006) malgré l'amélioration du taux de transplants fonctionnels (Kasiske, Gaston et al. 2005).

Le rejet chronique est caractérisé histologiquement par une vasculopathie, avec un épaissement intimal et une prolifération des cellules musculaires lisses, et par une fibrose interstitielle. Dans le cas de la greffe rénale, il existe également une glomérulopathie qui est un dédoublement des membranes basales et une atrophie tubulaire (Nankivell, Borrows et al. 2003) (Nankivell and Chapman 2006). Le rejet vasculaire chronique peut s'accompagner d'un dépôt de la molécule du complément C4d au niveau des capillaires péri-tubulaires du greffon (Vargha, Mueller et al. 2006), dû à la présence d'anticorps anti-donneur. Le greffon peut également présenter des lésions dues à une toxicité des anti-calcineurines (ciclosporine A, tacrolimus) caractérisée par des dépôts hyalins nodulaires à la périphérie des artérioles. Ces altérations sont classées selon le score de Banff qui permet de graduer l'importance et le type des lésions (Racusen, Halloran et al. 2004).

Des facteurs aussi bien immunologiques que non immunologiques sont impliqués dans le rejet chronique (Massy, Guijarro et al. 1996). Parmi les facteurs non immunologiques, la mort cérébrale du donneur et les phénomènes d'ischémie reperfusion activent l'endothélium et augmentent l'expression des molécules du CMH et des molécules d'adhésion (Szabo and Heemann 1998). D'autre part, il a été établi que la présence d'alloanticorps (Susal and Opelz 2002) (Hourmant, Cesbron-Gautier et al. 2005), le nombre d'épisodes de rejets aigus (Matas, Humar et al. 1999), le nombre d'incompatibilités HLA (Terasaki and Ozawa 2004), le retard de démarrage du greffon (Giral-Classe, Hourmant et al. 1998) (Perico, Cattaneo et al. 2004), l'âge du donneur (Oppenheimer, Aljama et al. 2004), ainsi que le poids du greffon (Giral, Nguyen et al. 2005) favorisaient le développement du rejet chronique.

Il a également été montré qu'une orientation préférentielle Th2, avec production d'IL4, IL5, IL10 et de TGF β , facteur pro-fibrosant, contribuait à l'augmentation de la production matricielle donc à la fibrose et par conséquent au rejet chronique (Shirwan 1999). De nombreuses études, à la fois chez l'homme et l'animal ont démontré le rôle du TGF β dans le développement du rejet chronique (Jain, Furness et al. 2000; Bedard, Jiang et al. 2006)

(Zegarska, Paczek et al. 2006). Nicholson et al ont montré dans des biopsies rénales une corrélation entre le niveau d'ARNm de TGF β et le taux de fibrose (Nicholson, Waller et al. 2002). D'autre part, Densem et al. ont montré qu'un polymorphisme du TGF β entraînait une accélération de la vasculopathie des greffons cardiaques (Densem, Hutchinson et al. 2000). Enfin, récemment, Bedard et al ont montré dans un modèle de greffe rénale chez le rat que l'injection de SERP-1, une protéine virale anti-inflammatoire, diminuait le niveau d'ARNm du TGF β et diminuait les signes de rejet chronique (Bedard, Jiang et al. 2006).

D'autre part, l'IFN γ semble être impliqué dans la survenue de lésions vasculaires chroniques, en diminuant l'activité de certains médiateurs (comme le NO) et l'épaississement de l'intima au niveau de l'endothélium (Koh, Wang et al. 2004). Récemment, Yi et al. ont montré dans un modèle de greffe d'artère humaine chez la souris, que des traitements associant le sirolimus et la ciclosporine A, diminuaient le taux d'IFN γ et ainsi les lésions d'artériopathie des vaisseaux (Yi, Cuchara et al. 2006).

Les traitements immunosuppresseurs entraînant des effets secondaires néfastes sur le long terme et leur efficacité sur le développement du rejet chronique étant limitée, l'obtention d'une tolérance spécifique du greffon sans immunosuppression globale prolongée est le but ultime en transplantation.

3- La tolérance et ses mécanismes

3-1 Tolérance *opérationnelle* et Tolérance *partielle (prope)*

La tolérance immunitaire peut être définie en transplantation comme un état de paralysie immunitaire spécifique d'un antigène donné (le tolérogène) coexistant avec le maintien d'une immunocompétence normale vis à vis des autres antigènes, sans destruction du greffon (Bach 2006). Cette acceptation indéfinie du greffon s'accompagne d'une absence de signes de rejet aigu et chronique et d'un maintien de la fonction du transplant sans aucun traitement immunosuppresseur. Chez l'homme, on distingue plusieurs types de tolérance.

La tolérance *opérationnelle* : elle correspond à une survie prolongée d'une allogreffe d'organe avec une fonction stable en l'absence de traitement immunosuppresseur chez un receveur parfaitement immunocompétent (Monaco 2004) (Ansari and Sayegh 2004). Certains critères sont parfois retenus pour compléter la définition de tolérance : l'absence d'anticorps anti-donneur, l'absence d'infiltration du greffon par des lymphocytes et une faible réponse dirigée contre le donneur *in vitro*, alors que la réponse contre les cellules d'une autre personne est conservée (Fehr and Sykes 2004). Le terme de tolérance opérationnelle ne signifie pas

forcément aucune réponse du système immunitaire contre les cellules du donneur, mais se réfère plutôt à une réponse immunitaire n'entraînant pas la destruction du greffon, malgré la présence de capacités de réponse immune du receveur (Reding, Gras et al. 2006).

Le foie est l'organe pour lequel une tolérance s'observe le plus souvent. Li et al. ont étudié les cellules T $CD4^+CD25^{high}$ chez des enfants tolérant leur greffe de foie sans traitement. En effet, le foie semble être un site privilégié où une tolérance opérationnelle, sans traitement immunosuppresseur, peut s'établir chez 38% des patients greffés (Takatsuki, Uemoto et al. 2001) mais cette tolérance est plus rarement décrite dans d'autres greffes d'organes (Strober, Benike et al. 2000) (Burlingham, Grailer et al. 1995) (Fischer, Schobel et al. 1996) (Christensen, Grunnet et al. 1998). Certaines équipes mettent même en place une diminution progressive des traitements immunosuppresseurs chez des patients greffés depuis plus de 2 ans et sans épisode de rejet aigu dans l'année précédente. Ce protocole permet de mettre en évidence qu'environ un tiers de ces patients est tolérant à leur greffon (Devlin, Doherty et al. 1998) (Takatsuki, Uemoto et al. 2001). Quand un rejet survient au cours de la décroissance thérapeutique, il est dans la plupart des cas réversible avec la reprise du traitement immunosuppresseur ou avec des bolus de corticostéroïdes (Takatsuki, Uemoto et al. 2001). Un chimérisme est retrouvé chez 39% des patients, mais sans corrélation claire avec l'état de tolérance (Devlin, Doherty et al. 1998).

Il existe quelques rares descriptions dans la littérature de patients qui tolèrent spontanément une greffe rénale (**Tableau 1**) (Roussey-Kesler, Giral et al. 2006) (annexe 5). Dans les années 1960, parmi 49 patients greffés traités initialement par azathioprine, 15% ont conservé une bonne fonction rénale 3 à 38 ans après l'arrêt de l'immunosuppression (Starzl, Murase et al. 2004). Lors de l'autopsie de l'un de ces patients, le greffon avait un aspect histologique normal (Starzl, Murase et al. 2004). Zoller et al. ont rapporté une cohorte de 48 patients (parmi 6000 greffés) qui avaient arrêté leur traitement immunosuppresseur depuis plus de 5 jours. Parmi eux, 23 (soit 43%) ont dégradé leur fonction rénale dans les semaines suivant l'arrêt de l'immunosuppression. Seuls 6 patients conservent une fonction rénale stable en l'absence d'immunosuppresseurs depuis plus de 3 ans. Tous étaient greffés à partir d'un donneur vivant apparenté (Zoller, Cho et al. 1980). D'autres cas anecdotiques ont été rapportés dans les années 1970 (Uehling, Hussey et al. 1976) (Owens, Maxwell et al. 1975).

Burlingham et al. ont décrit le cas d'un patient greffé avec le rein de sa mère et présentant une bonne fonction rénale en l'absence de traitement immunosuppresseur. Une biopsie réalisée au moment de l'arrêt de l'immunosuppression a montré un infiltrat focal lymphocytaire, des zones de fibrose interstitielle, une hyperplasie intinale artériolaire. Les

tubes et les glomérules étaient normaux (Burlingham, Grailer et al. 1995). Un microchimérisme a été retrouvé au niveau des lymphocytes sanguins et de la peau. Des cellules régulatrices spécifiques et une anergie semblent en jeu dans le maintien de cette tolérance puisqu'il existe une faible réactivité contre le donneur *in vitro*, pouvant être restaurée par l'adjonction d'IL2 et de CPA du donneur (Burlingham, Grailer et al. 1995).

Christensen et al. rapportent le cas d'un autre patient, greffé une première fois avec un greffon d'origine maternelle, puis une deuxième fois avec un greffon d'origine paternelle. Après cette deuxième greffe, le traitement immunosuppresseur a été arrêté en raison d'un syndrome lymphoprolifératif. Le patient a néanmoins maintenu une bonne fonction rénale. Les études *in vitro* ont révélé une faible réactivité en MLR contre les cellules du père (et de la mère) avec une diminution de la production des cytokines de type Th1 (IL2, IFN β) (Christensen, Grunnet et al. 1998). Il existe encore quelques cas isolés, comme celui de cette patiente qui a arrêté son traitement immunosuppresseur après 9 ans de greffe et a conservé par la suite une fonction rénale correcte (Fischer, Schobel et al. 1996). Récemment, Fudaba et al. ont observé 3 cas de tolérance opérationnelle chez des patients ayant reçu simultanément une greffe de rein et de cellules hématopoïétiques de moelle osseuse (Fudaba, Spitzer et al. 2006). Ces patients présentent un chimérisme transitoire, sans réponse anti-donneur spécifique en culture lymphocytaire mixte.

Nous avons étudié au laboratoire une petite cohorte de patients greffés rénaux opérationnellement tolérants (10 patients) dont nous avons décrit les caractéristiques cliniques (Roussey-Kesler, Giral et al. 2006) (annexe 5) (voir méthodologie, **tableau 3**). L'analyse de ces patients a permis de constater que certains patients opérationnellement tolérants semblaient conserver une réactivité contre certains antigènes, illustrée par la présence d'anticorps anti-classe II spécifiques du donneur. Ils ont probablement une réponse alloréactive plus faible puisque l'immunisation anti-HLA avant la greffe était aussi globalement faible malgré le nombre de transfusions sanguines administrées (9+/-3.9% de PRA pour 6+/-7.3 transfusions). D'autre part, ces patients ne présentent pas de signes cliniques d'immunodépression, puisque depuis l'arrêt des traitements immunosuppresseurs, ils n'ont pas eu d'infections sévères. Le jeune âge des donneurs et une reprise de fonction du greffon pas ou peu retardée, associé à l'arrêt progressif de l'immunosuppression (jusqu'à 4ans), constituent des éléments favorisant potentiellement la survenue d'un état de tolérance opérationnelle. Plusieurs études ont été menées sur ces patients dans le but de trouver des caractéristiques qui leur sont propres par rapport à d'autres groupes de transplantés rénaux, et ceci notamment au niveau sanguin. Les analyses transcriptionnelles du répertoire des lymphocytes T sanguins nous ont révélé que ces

patients, comme les patients en rejet chronique, présentaient des altérations de la distribution des longueurs de CDR3 (Brouard, Dupont et al. 2005)(annexe 4). Des résultats similaires ont été obtenus par Alvarez et al. (Alvarez, Opelz et al. 2005). De plus, les lymphocytes T correspondant à ces altérations chez les patients opérationnellement tolérants ne montrent que peu ou pas de transcrits de cytokines Th1 et Th2 (IL13, IL10, IL2, IFN γ), suggérant une diminution de leur réponse immunitaire par rapport aux patients en rejet chronique (Brouard, Dupont et al. 2005). Les altérations de la distribution des longueurs de CDR3 étant présentes majoritairement dans le compartiment CD8, une étude plus approfondie des cellules T CD8⁺ a été menée (Baeten, Louis et al. 2006).

Dans une autre étude, nous nous sommes également intéressés au phénotype des cellules sanguines de ces patients. Nous avons ainsi montré une augmentation des lymphocytes T CD8⁺CD28⁻ cytotoxiques (CD27⁻CD57⁺perforine⁺granzymeA⁺) chez les patients en rejet chronique par rapport aux patients opérationnellement tolérants et aux individus sains (Baeten, Louis et al. 2006) (Annexe 2).

Tableau 1 : Tolérance opérationnelle en transplantation rénale chez l'homme

Patients	1	6	7	1	1	1	2	1	3/5/10	3
Donneur	Apparenté	Apparenté	Apparenté	Apparenté	Apparenté	Cadavérique	1 apparenté 1 cadavérique			Apparenté
Incompatibilités HLA	?	0	?	3 ? (haplo-identique)	3 ? (haplo-identique)	6	2 pour l'un 0 pour l'autre	1 (classII non déterminé)		0
Traitements			AZA+(CS si rejet aigu)	DST puis CsA+AZA+CS		Irradiation lymphoïde totale +ATG+CS	OKT3 en induction pour 1, rien pour le 2ième	AZA+CS		Greffé de moelle
Cause de l'arrêt	Non compliance	Non compliance		Non compliance	Syndrome lymphoprolifératif	Protocole	Non compliance	Non compliance	Non compliance Syndrome lymphoprolifératif	Protocole
Période de tolérance	?	3-8 ans	3-38 ans		> 3ans	12 ans	5-57 ans	9 ans	> 3ans	1-7 ans
Histologie			normale	Infiltrat lymphocytaire, fibrose interstitielle hyperplasie intima artériolaire						
Mécanismes				Microchimérisme, régulation, anergie	Faible réactivité anti-donneur	Pas de microchimérisme, réactivité anti-donneur	Régulation		Altération du répertoire T	Chimérisme, pas de réactivité anti-donneur
Références	(Uehling, Hussey et al. 1976)	(Zoller, Cho et al. 1980)	(Starzl, Murase et al. 2004)	(Burlingham, Grailer et al. 1995)	(Christensen, Grunnet et al. 1998)	(Strober, Lowsky et al. 2004)	(VanBuskirk, Burlingham et al. 2000)	(Fischer, Schobel et al. 1996)	(Alvarez, Opelz et al. 2005) / (Brouard, Dupont et al. 2005) / (Roussey-Kesler, Giral et al. 2006)	(Fudaba, Spitzer et al. 2006)

La tolérance partielle ou propre: Les risques d'un conditionnement, même non-myéloablatif, sont loin d'être négligeables et les cellules de la moelle osseuse du donneur ne sont pas toujours disponibles. La difficulté de ces protocoles a amené certains auteurs à rechercher, non pas un état de tolérance stricte, mais une fonction du greffon satisfaisante avec un traitement immunosuppresseur minimal, diminuant ainsi le risque de toxicité médicamenteuse et les complications infectieuses et tumorales. Calne et al. ont ainsi développé le concept de *propre* tolérance qui correspond à une survie prolongée du greffon avec une fonction stable chez un receveur parfaitement immunocompétent sous une dose minimale, non toxique, d'immunosuppresseurs (Calne 2004). Cette tolérance a également été appelée *tolérance partielle* (Cortesini and Suciu-Foca 2004). Calne l'a appliqué pour 31 patients

transplantés rénaux. Le traitement d'induction reposait sur l'administration d'anticorps anti-CD52 précédée d'une injection de prednisone. Une monothérapie par ciclosporine A a été ensuite maintenue à faible dose (Calne, Friend et al. 1998). Six rejets aigus ont été observés, tous réversibles avec la majoration du traitement immunosuppresseur. Deux infections opportunistes sont survenues. Avec un recul de 5 ans, 93% des patients avaient une fonction rénale satisfaisante, 90% d'entre eux sont toujours sous ciclosporine seule à faible dose (Calne, Moffatt et al. 1999) (Calne 2004). Cette même stratégie utilisant la rapamycine à la place de la ciclosporine s'est cependant accompagnée d'un fort taux de rejet (27%), en particulier de rejet humoral (17%) (Knechtle, Pirsch et al. 2003). Avec un recul de 6 à 12 mois, 72% des sujets ont une bonne fonction rénale, sans signes histologiques de rejet, et il n'y eut pour aucun d'eux d'infections systémiques sévères ou de pathologies tumorales (Knechtle, Pirsch et al. 2003). Les taux de rejet observés sont cependant supérieurs à ceux rencontrés actuellement avec une immunosuppression conventionnelle. Enfin, l'association d'un traitement par ATG puis par sirolimus en monothérapie a été étudiée chez 12 patients : ils présentent tous une fonction du greffon satisfaisante (malgré 3 rejets aigus), mais le recul est encore limité (Swanson, Hale et al. 2002).

Cette recherche de tolérance « a minima » a également été appliquée chez une cohorte plus importante de patients par l'équipe de Starzl. Quatre-vingt deux patients ayant reçu une greffe d'organe (rein, foie, pancréas ou intestin) ont été traités par de l'ATG puis par tacrolimus en monothérapie. Des cellules hématopoïétiques (moelle osseuse) du donneur ont été injectées dans certains cas. Après 4 mois de greffe, l'administration de tacrolimus est progressivement espacée si aucun rejet n'est observé. A 1 an, les survies des patients et des greffons sont respectivement de 95% et 89%. 60% des patients reçoivent des doses réduites de tacrolimus (Starzl, Murase et al. 2003). La même équipe a traité 150 patients après une transplantation rénale par ATG (et corticostéroïdes initialement) puis par tacrolimus seul en traitement d'entretien (Shapiro, Jordan et al. 2003). La prise de celui-ci est progressivement espacée au bout de 4 mois en l'absence de complication. Des épisodes de rejets aigus ont été observés dans 37% des cas, ce qui n'est pas négligeable. La survie du greffon à 1 an est cependant satisfaisante (92%) avec seulement 7% des patients recevant plus d'un immunosuppresseur, et 63% des patients ayant des prises espacées de tacrolimus. Ces résultats rejoignent donc ceux donnés précédemment même en l'absence d'injection de cellules du donneur.

Récemment l'équipe de Faendrich a analysé 20 patients transplantés rénaux, suivis pendant 2 ans, sans immunosuppresseurs pendant 72h après la transplantation puis sous très

faibles doses d'immunosuppresseurs (Dresske, Haendschke et al. 2006). Le rationnel de l'étude, est de dégager une fenêtre d'opportunité pour un engagement immunologique non pathogène (WOFIE, window of opportunity for immunological engagement). La reconstitution immunologique va se faire dans un contexte dénué de signaux de danger (loin des effets de l'ischémie et du bistouri du chirurgien) et le contact avec les alloantigènes induit un état de tolérance dont l'immunosuppresseur assurera le maintien. Ils ont alors constaté une diminution de l'incidence du rejet aigu et une meilleure fonction rénale (taux de créatinine faible) que les patients avec un traitement immunosuppresseur classique. Ces patients présentent également une augmentation des lymphocytes T $CD4^+CD25^+FOXP3^+$ suggérant un rôle potentiel de ces cellules dans la *propre* tolérance (Dresske, Haendschke et al. 2006).

Ces études sont donc encourageantes pour envisager une réduction progressive des traitements immunosuppresseurs, pour des patients à faible risque immunologique. Cependant il n'existe pas actuellement de marqueur prédictif de réussite lors de la réduction ou l'arrêt du traitement, comme nous le verrons plus loin.

Différents mécanismes sont mis en jeu dans l'induction et le maintien d'un état de tolérance en transplantation: la délétion centrale (souvent associée à un macrochimérisme) et périphérique, l'anergie, l'ignorance et la suppression (par des cellules régulatrices). La tolérance centrale correspond à l'élimination des lymphocytes T autoréactifs dans le thymus (sélection négative) et la tolérance périphérique correspond à une inactivation ou une délétion des lymphocytes T.

3-2 Tolérance centrale

Lors de leur différenciation dans le thymus, les lymphocytes T subissent une sélection positive suivie d'une sélection négative. Ces étapes vont respectivement permettre l'expansion sélective des cellules T capables de reconnaître le CMH du soi et l'apoptose des thymocytes potentiellement autoréactifs interagissant avec une trop forte affinité avec les complexes CMH/peptide du soi (Starr, Jameson et al. 2003). Au cours de la sélection positive, les DC ou les cellules épithéliales du cortex thymique présentent des complexes CMH/peptide aux cellules T, et fournissent un signal de survie aux cellules capables d'interagir avec les molécules du CMH du soi. Les cellules T qui reconnaissent les molécules du CMH du soi de manière trop faible meurent par apoptose dans le cortex thymique. Lors de la sélection négative, les cellules T qui ne reconnaissent pas les antigènes du soi survivent et poursuivent leur maturation, tandis que les thymocytes avec un TCR de haute affinité pour les antigènes du soi sont éliminés (Palmer 2003). Ainsi, la tolérance centrale joue un rôle essentiel dans la

tolérance au soi, en éliminant une grande majorité des cellules T autoréactives (Kyewski and Klein 2006).

La tolérance centrale peut également être observée après une greffe et plus particulièrement après une greffe de cellules hématopoïétiques de moelle osseuse. Ainsi, l'induction d'un macrochimérisme par la réalisation d'une greffe de cellules hématopoïétiques de moelle osseuse allogénique permet d'observer la tolérance ultérieure de greffes d'organes (ou de peau) à partir du même donneur (Ildstad and Sachs 1984). Les lignées hématopoïétiques du donneur produisent des cellules qui vont coloniser le thymus du receveur. Ceci est également à l'origine d'une délétion centrale des lymphocytes reconnaissant les antigènes du donneur par le processus de sélection négative (Sykes 2001) (Cosimi and Sachs 2004).

Dans des modèles animaux, la délétion thymique a également été observée après injections intrathymiques de peptides du CMH du donneur ou même de cellules (Koksoy, Elpek et al. 2005) (Jones, Fluck et al. 1998). La délétion des lymphocytes alloréactifs qui s'en suit est transitoire et est dépendante de la persistance dans le thymus des peptides du CMH.

3-3 Tolérance périphérique

Malgré les sélections intra thymiques, certains lymphocytes autoréactifs peuvent s'échapper et générer des maladies autoimmunes telles que le diabète de type I. Dans le cadre de la transplantation, la réponse immunitaire doit être contrôlée si on veut éviter le rejet du greffon. Les lymphocytes T alloréactifs doivent donc être stoppés. L'organisme a développé des mécanismes périphériques pour contrôler ces lymphocytes potentiellement dangereux et ainsi se protéger. Bien que tous les mécanismes de la tolérance ne soient pas encore totalement connus, on peut distinguer parmi ceux-ci l'anergie (Jones, Chin et al. 1990), la délétion clonale (Wells, Li et al. 2001) (Li, Li et al. 1999), l'ignorance (Lakkis, Arakelov et al. 2000) et la suppression ou régulation (Wood and Sakaguchi 2003).

3-3-1 Anergie

Comme décrit plus haut, l'activation complète d'un lymphocyte nécessite plusieurs signaux. L'anergie peut faire suite au blocage de la costimulation alors que le lymphocyte T a rencontré un antigène spécifique présenté par les molécules du CMH. Lorsqu'un lymphocyte rencontre un antigène présenté sur une CPA, mais qu'il n'a pas été activé avec les 3 signaux, il devient incapable de répondre à la stimulation. Il a également été montré que la dose d'antigènes était importante pour entraîner une anergie des LT CD8⁺ (Redmond, Marincek et al. 2005). Les LT sont devenus anergiques (Powell 2006) et leur capacité proliférative est alors

abolie. Ils ne peuvent plus synthétiser de l'IL2 (Jenkins, Pardoll et al. 1987). L'anergie peut cependant être levée par l'ajout d'IL2 (Beverly, Kang et al. 1992). Cependant, les cellules anergiques peuvent, malgré leur incapacité à proliférer, conserver leur capacité fonctionnelle. Ainsi des cellules régulatrices telles que les lymphocytes T $CD4^+CD25^+$ sont anergiques mais leur propriétés suppressives restent intactes (Li, Godfrey et al. 2005).

3-3-2 Délétion clonale

Nous avons vu que la délétion centrale dans le thymus joue un grand rôle dans la sélection des lymphocytes. Il existe également un phénomène de délétion en périphérie pour contrôler les réponses lymphocytaires T non appropriées et éviter les phénomènes d'autoimmunité et de rejet de greffe. La délétion clonale entraîne l'élimination par apoptose des lymphocytes.

Deux formes de mort par apoptose peuvent conduire à l'élimination des LT (Van Parijs, Ibraghimov et al. 1996). On parle de mort « passive » lorsque les LT meurent par privation de facteurs de croissance mais que la mort peut être bloquée par les protéines anti-apoptotiques Bcl-2 et Bcl-xL (Van Parijs, Ibraghimov et al. 1996). Le blocage des voies de costimulation, stratégie souvent utilisée pour induire une tolérance (Sayegh and Turka 1998), entraîne une mort passive puisque l'expression de Bcl-xL et d'IL2 diminue. La mort « active » intervient lorsqu'il y a stimulation répétée du TCR du LT et fait intervenir les voies de Fas/FasL et du TNF récepteur (van Parijs, Perez et al. 1998). Ce phénomène est dépendant de la production d'IL2 mais indépendant de l'expression de Bcl-2 et Bcl-xL.

Ainsi, une activation incomplète des lymphocytes T par manque de signaux de costimulation peut entraîner une délétion de ces lymphocytes (Ferber, Schonrich et al. 1994) (Li, Li et al. 1999). Plusieurs études ont également établi l'importance de la dose de l'antigène dans l'induction d'une délétion ou d'une anergie (Faria and Weiner 2005) (Redmond, Marincek et al. 2005), mais les résultats restent encore contradictoires. D'autre part, les leucocytes passagers présents dans le greffon au moment de la transplantation (microchimérisme) permettraient d'entraîner une délétion périphérique des clones alloréactifs par AICD et mort cellulaire passive (Wekerle, Kurtz et al. 2001).

3-3-3 Ignorance

L'ignorance est un phénomène décrit pour la première fois par Lakkis et al. (Lakkis, Arakelov et al. 2000). Ils ont démontré que des souris sans organes lymphoïdes secondaires acceptaient définitivement une greffe de peau ou de cœur. Cette absence de rejet résulte dans

l'incapacité des LT à être activés par les antigènes du donneur en dehors des organes lymphoïdes secondaires. L'ignorance peut être induite par plusieurs mécanismes: le niveau d'antigènes peut être en dessous du seuil requis ou bien les antigènes peuvent être physiquement séparés des cellules T (par exemple dans le cas d'organes privilégiés comme le cerveau, la cornée...) (Simpson 2006).

3-3-4 Suppression et régulation

Le concept des cellules suppressives puis ensuite régulatrices est apparu à la fin des années 70 (Gershon 1975). Ce sont les travaux de Hall et al. qui ont permis de mettre en évidence, par transfert adoptif, le rôle des lymphocytes T $CD4^+$ dans la tolérance à une allogreffe cardiaque chez le rat (Hall, Jelbart et al. 1985). Après plusieurs années d'expériences peu convaincantes les cellules régulatrices furent décrites plus précisément par Sakaguchi et al. dans les années 90 (Sakaguchi, Sakaguchi et al. 1995). Depuis, de très nombreuses études ont démontré l'existence de phénomènes régulateurs/suppresseurs par différents sous types cellulaires. Je détaillerais dans le chapitre suivant les différentes sous populations décrites à ce jour et leur rôle dans la transplantation.

4- Les cellules régulatrices

Ces dernières années, de nombreux chercheurs ont montré l'énorme potentiel des cellules régulatrices dans les maladies auto-immunes (Chatila 2005) (Baecher-Allan and Hafler 2006) (Tang and Bluestone 2006), en transplantation (Waldmann, Chen et al. 2006) ou encore dans la maladie du greffon contre l'hôte (Chatenoud, Salomon et al. 2001) mais également dans la tolérance au soi (Sakaguchi 2005). Plusieurs types de cellules régulatrices ont été décrites à ce jour (Beissert, Schwarz et al. 2006) (Becker, Stoll et al. 2006) (Beissert, Schwarz et al. 2006) et sont résumées dans le **tableau 2**. Les cellules T $CD4^+CD25^+$ sont les cellules régulatrices les plus étudiées. On distingue les LT $CD4^+CD25^+$ naturelles (Dieckmann, Plottner et al. 2001) (Toda and Piccirillo 2006) et induites: Tr1 (Groux, O'Garra et al. 1997) (Grazia Roncarolo, Gregori et al. 2006) et Th3 (Weiner 2001) (Weiner 2001). Ces cellules T régulatrices (Treg) représentent des populations très hétérogènes de part leur génération (cellules induites ou naturelles), leur phénotype, leur fonction et leurs mécanismes d'action.

Tableau 2: Présentation non exhaustive des sous types de cellules régulatrices naturelles et induites

	<i>Sous types de Treg</i>	<i>Phénotype</i>	<i>Mode d'action</i>
<i>T CD4⁺CD25⁺</i>	Naturelles	FOXP3, GITR, CD127 CTLA-4, CD103, CD62L, CD45, CD122	Inhibition contact dépendant Synthèse d'IL10 Inhibent la synthèse d'IL2
	Tr1 (induites)	CTLA-4	Synthèse d'IL10 ⁺⁺ , TGFβ, IL5, IFNγ Inhibent la synthèse d'IL2
	Th3 (induites)	CTLA-4	Synthèse de TGFβ ⁺⁺ , d'IL10, Inhibent la synthèse d'IL2
<i>Autres</i>	T CD8 ⁺ CD28 ⁻ (induites)	FOXP3	Induction de CPA tolérogènes exprimant ILT3 et ILT4 Inhibition contact dépendant
	NKT (induites)	CD3 ⁺ Vα24 ⁺ Vβ11 ⁺	Synthèse de IL4, TGFβ, d'IL10, Cytotoxicité
	NK (induites)	CD56 ^{high}	Synthèse d'IL10, inhibition contact dépendant
	Doubles négatives (induites)	CD3 ⁺ CD4 ⁻ CD8 ⁻	Cytotoxicité, synthèse d'IFNγ
	T CD4 ⁺ CD25 ⁻ (induites)	CD3 ⁺ CD4 ⁺ CD25 ⁻	??
	T CD8 ⁺ CD45RC ^{low} (naturelles)	FOXP3, CTLA-4	Inhibition contact dépendant
	Thymocytes CD8 ⁺ CD25 ⁺ (naturelles)	FOXP3, CTLA-4, GITR, CCR8	Inhibition contact dépendant Synthèse de TGF β

4-1 Les cellules régulatrices naturelles T CD4⁺CD25⁺

4-1-1 Origine et caractéristiques phénotypiques

Ce sont Sakaguchi et al. qui ont décrit les fonctions régulatrices des lymphocytes T CD4⁺CD25⁺. Ils ont montré que les cellules T CD4⁺CD25⁺ participaient au maintien de la tolérance au soi en neutralisant les réponses immunes dirigées contre des antigènes du soi et du non-soi. Ainsi, l'élimination des cellules CD25⁺ parmi une suspension de cellules CD4⁺ de souris BALB/c entraîne le développement de maladies autoimmunes lorsque cette suspension de cellules T CD4⁺CD25⁻ est injectée à des souris BALB/c nude (Sakaguchi et al., 1995). Ces souris reconstituées avec des cellules T CD4⁺CD25⁻ présentent des réponses immunes beaucoup plus fortes lors du rejet de peau allogénique, l'ajout de cellules T CD4⁺CD25⁺ permettant de rétablir des réponses immunes. Depuis ces travaux, ces cellules ont suscité un

grand intérêt et font l'objet d'études très poussées pour comprendre leur genèse, fonctions et mécanismes d'action (Toda and Piccirillo 2006).

Les cellules T régulatrices naturelles $CD4^+CD25^+$ sont générées dans le thymus (Stephens, Mottet et al. 2001; Annunziato, Cosmi et al. 2002; Fehervari and Sakaguchi 2004) et représentent 5 à 10% des thymocytes matures $CD4^+$ et 2 à 5% des PBMC (Bacchetta, Gregori et al. 2005). Ces cellules sont sélectionnées positivement pour leur reconnaissance avec le CMH du soi sur les cellules épithéliales corticales et ont une affinité moyenne envers le complexe CMH-antigène du soi (Jonuleit and Schmitt 2003) (Jordan, Boesteanu et al. 2001) (Cupedo, Nagasawa et al. 2005) (Cabarrocas, Cassan et al. 2006). Chez l'homme, elles apparaissent dès la 13^{ième} semaine de gestation (Darrasse-Jeze, Marodon et al. 2005). On les trouve dans le thymus, dans le sang de cordon, mais également en périphérie (sang, organes lymphoïdes secondaires) ou elles peuvent être induites (Godfrey, Spoden et al. 2005)

Leur évolution avec l'âge reste encore controversée. Le thymus régressant avec l'âge, certains chercheurs ont montré que leur nombre mais également leur fonction diminuaient (Dejaco, Duftner et al. 2006) (Tsaknaridis, Spencer et al. 2003). Cependant une étude a montré que leur nombre augmentait avec l'âge (Gregg, Smith et al. 2005). Récemment, une autre étude a démontré que leur nombre était identique quelque soit l'âge (Vukmanovic-Stejc, Zhang et al. 2006).

Plusieurs études ont montré l'implication des molécules de costimulation B7/CD28 dans la génération et l'activité des T régulateurs. (Tang, Henriksen et al. 2003) (Lyddane, Gajewska et al. 2006). Le signal fournit par le CD28 serait critique pour leur génération dans le thymus mais également pour leur genèse et leur survie en périphérie. En effet le nombre de lymphocytes T $CD4^+CD25^+$ est très réduit chez les souris déficientes en B7 et CD28 (Tang, Henriksen et al. 2003) (Salomon, Lenschow et al. 2000) (Takahashi, Tagami et al. 2000). De manière similaire, l'administration d'anticorps anti-B7 ou de CTLA4-Ig diminue le nombre de ces cellules régulatrices dans le thymus et en périphérie (Salomon, Lenschow et al. 2000). Salomon et al. ont établi que la liaison B7/CD28 était essentielle au développement et à l'homéostasie des cellules régulatrices naturelles T $CD4^+CD25^+$ (Salomon, Lenschow et al. 2000). Ainsi, les souris NOD déficientes pour les molécules B7 ou pour les molécules CD28 présentent un diabète exacerbé dont la survenue s'explique par un déficit en cellules régulatrices naturelles T $CD4^+CD25^+$. Ce diabète peut être contrôlé par l'injection de LT régulateurs. Récemment, il a été montré que les LT régulateurs avaient une durée de vie courte due à des télomères courts (Vukmanovic-Stejc, Zhang et al. 2006). Ils présentent aussi une sensibilité à l'apoptose due à une diminution de l'expression des molécules Bcl-2 et Bcl-x_L (Li, Godfrey et al. 2005)..

L'IL2 semble être une cytokine importante dans l'homéostasie et la fonction des LT régulateurs (Setoguchi, Hori et al. 2005). En effet, son rôle a été suggéré dans les désordres lymphoprolifératifs se produisant chez les souris déficientes en IL2 (Wolf, Schimpl et al. 2001), en CD25 (IL2R α sous unité de basse affinité) (Willerford, Chen et al. 1995), et IL2R β (sous unité de haute affinité) (Suzuki, Kundig et al. 1995) (Malek, Porter et al. 2000). Ces souris déficientes en IL2, avec de nombreux désordres autoimmuns, sont aussi déficientes en LT régulateurs, mais le transfert de LT régulateurs, issus de souris normales, contrôle les phénomènes d'autoimmunité. L'IL2 serait indispensable à l'homéostasie mais aussi à la survie des LT CD4⁺CD25⁺ (Nelson 2004) (Toda and Piccirillo 2006) (**Figure 6**). En effet, ces souris déficients en IL2 ont très peu de CD4⁺CD25⁺ mais ces cellules CD4⁺CD25⁺FOXP3⁺ possèdent une activité suppressive normale, suggérant que l'IL2 pourrait intervenir dans la survie ou l'expansion des Treg et non dans leur fonction (Klein, Khazaie et al. 2003) (Fontenot, Rasmussen et al. 2005) (Fontenot and Rudensky 2005) (D'Cruz and Klein 2005). Par opposition, Thornton et al. ont montré que la production transitoire d'IL2 par les cellules effectrices était nécessaire à la fonction suppressive des LT CD4⁺CD25⁺ (Thornton, Donovan et al. 2004). Chez les patients atteints d'IPEX (décrits plus loin), c'est-à-dire portant une mutation de FOXP3, le défaut de production d'IL2 par les PBMC n'affecte pas la différenciation thymique des T régulateurs (Bacchetta, Passerini et al. 2006), mais serait responsable de leur défaut de fonction/expansion en périphérie. L'IL2 activerait deux signaux pouvant intervenir dans la prolifération et la survie des T CD4⁺CD25⁺, le facteur de transcription STAT5 et le facteur anti-apoptotique Bcl-2 (Nelson 2004) (Van Parijs, Refaeli et al. 1999) (Moriggl, Topham et al. 1999). Les souris STAT5A/B^{-/-} développent une maladie autoimmune similaire aux souris déficientes en IL2 et IL2R, et présentent une diminution des cellules régulatrices naturelles (Snow, Abraham et al. 2003). De plus, les souris transgéniques qui surexpriment STAT5 ont une augmentation de leurs cellules régulatrices. Almeida et al. ont démontré récemment un lien entre le nombre de LT CD4⁺CD25⁺ et le nombre de cellules produisant de l'IL2, à l'origine d'un équilibre entre les populations lymphocytaires palliant toute dérégulation (Almeida, Zaragoza et al. 2006). Ainsi tous ces résultats confirment le rôle important de l'IL2 dans l'homéostasie des cellules régulatrices naturelles (Antov, Yang et al. 2003) (Snow, Abraham et al. 2003) (Burchill, Goetz et al. 2003) (Kagami, Nakajima et al. 2001).

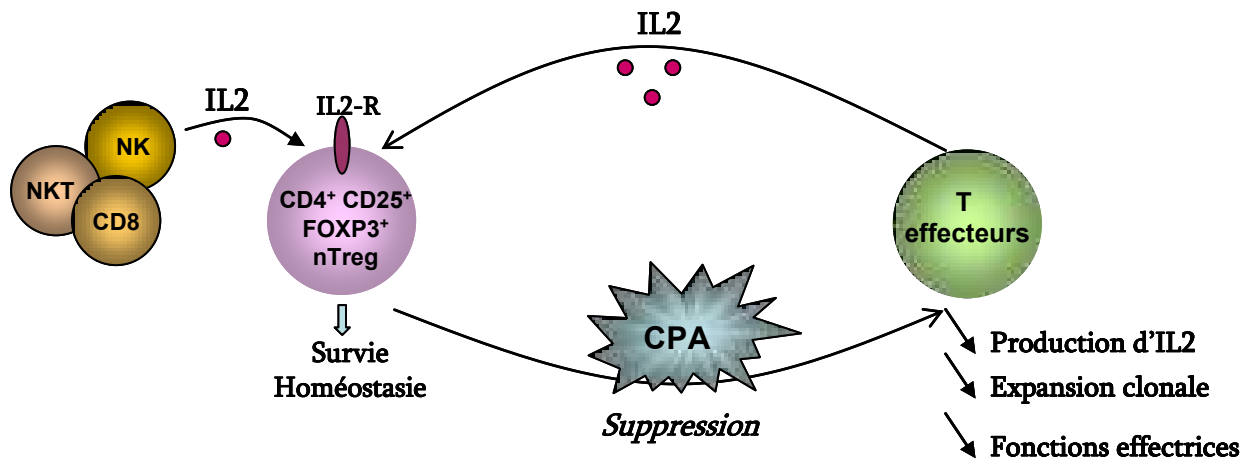


Figure 6: L'IL2 régule l'homéostasie périphérique des cellules régulatrices naturelles

L'IL2 produite par les lymphocytes T effecteurs, les LT CD8, NKT et NK, maintient l'homéostasie et permet la survie des Treg naturels, qui en retour exercent leurs effets suppresseurs, par l'intermédiaire des cellules présentatrices d'antigènes ou pas, sur les LT effecteurs. D'après (Sakaguchi, Ono et al. 2006) (Toda and Piccirillo 2006)

Les cellules T CD4⁺CD25⁺ naturelles n'ont pas un phénotype unique et clairement défini. Cependant, il est maintenant bien établi qu'elles expriment de manière constitutive la chaîne α du récepteur à l'IL-2 (Sakaguchi, Sakaguchi et al. 1995). Chez l'homme, seules les cellules exprimant fortement l'IL2R α (CD25^{high}) possèderaient des propriétés suppressives (Baecher-Allan, Brown et al. 2001), alors qu'un niveau d'expression intermédiaire correspondrait aux cellules activées, mais cette distinction n'est pas absolue puisque des cellules régulatrices CD25^{low} ont également été décrites (Tsaknaridis, Spencer et al. 2003). La liste des marqueurs décrivant les cellules régulatrices grandit tous les jours mais aucun marqueur ne remplit les critères d'exclusivité et de stabilité. Plusieurs marqueurs ont été associés à ces LT régulateurs: le CD45RO (Baecher-Allan, Brown et al. 2001), CD122 (chaîne β du récepteur de l'IL2) (Wing, Ekmark et al. 2002), CD62L (L-sélectine) (Thornton and Shevach 2000) (Ermann, Hoffmann et al. 2005), CD103 (intégrine $\alpha_E\beta_7$) (Lehmann, Huehn et al. 2002). Ils sont caractérisés également par une expression constitutive de CTLA-4 (Birebent, Lorho et al. 2004) et de GITR (Glucocorticoid-Induced-TNF-R) (Takahashi, Tagami et al. 2000) (Tsaknaridis, Spencer et al. 2003) (McHugh, Whitters et al. 2002). Récemment Seddiki et al. ont montré que les LT régulateurs CD4⁺CD25⁺ humains peuvent être distingués des LT activés par une faible expression du CD127, la chaîne α du récepteur à l'IL7 (Seddiki, Santner-Nanan et al. 2006).

Le facteur de transcription FOXP3 semble être un marqueur essentiel pour les cellules régulatrices. Il servirait de régulateur du développement (Hori, Nomura et al. 2003) (Kim and Rudensky 2006) et de la fonction des LT suppresseurs (Fontenot, Gavin et al. 2003)

(Sakaguchi, Ono et al. 2006). Les souris déficientes en FOXP3 (scurfy) n'ont pas de LT régulateurs naturels et développent une maladie autoimmune lymphoproliférative fatale conduisant à leur mort en 4 à 5 semaines (Brunkow, Jeffery et al. 2001). L'injection de LT $CD4^+CD25^+FOXP3^+$ fait régresser la maladie chez les souris scurfy (Nishimura, Sakihama et al. 2004). De manière similaire chez l'homme, l'IPEX (immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) est une maladie fatale rare, liée au chromosome X, caractérisée par une mutation du gène FOXP3 (Wildin, Ramsdell et al. 2001) (Bennett, Christie et al. 2001), un défaut de fonction des cellules T $CD4^+CD25^+$ régulatrices et un défaut de production par les PBMC d' $IFN\gamma$ et d'IL2 après activation du TCR (Bacchetta, Passerini et al. 2006). Le rôle de FOXP3 dans la genèse des LT régulateurs a été démontré grâce à l'analyse de souris chimériques. Des souris irradiées de manière létale ont été reconstituées avec un mélange de cellules hématopoïétiques de moelle osseuse de souris normales et des souris déficientes en FOXP3. Un marquage des cellules de chaque moelle a permis d'étudier leur contribution dans la reconstitution du système immunitaire de la souris. Seule les cellules hématopoïétiques de la moelle osseuse de la souris normale contribuent au développement des LT $CD4^+CD25^+$ démontrant ainsi le rôle de FOXP3 dans la génération de lymphocytes T régulateurs (Fontenot, Gavin et al. 2003)

Chez la souris, FOXP3 est exclusivement exprimé par les cellules régulatrices, une expression ectopique par transfert de gène dans les LT $CD4^+CD25^-$ leur confèrent des capacités suppressives (Hori, Nomura et al. 2003) (Khattri, Cox et al. 2003). Des travaux ont suggéré un rôle thérapeutique potentiel de lymphocytes exprimant FOXP3 de manière ectopique. Ainsi des lymphocytes T $CD4^+$ transgéniques pour un TCR transduit avec un rétrovirus codant pour FOXP3 sont capables de stabiliser voir enrayer le diabète lorsqu'ils sont injectés chez les souris NOD (Jaeckel, von Boehmer et al. 2005). Cependant le transfert de LT $CD4^+$ polyclonaux transduits avec le rétrovirus codant pour FOXP3 de souris NOD n'a aucun effet. Ceci suggère que la fréquence des T régulateurs spécifiques d'antigène est un facteur important dans leur capacité à contrôler l'autoimmunité. Il a également été démontré que les LT $CD8^+$ chez les souris transgéniques FOXP3 possédaient une faible capacité régulatrice démontrant que l'expression de FOXP3 dans les cellules non $CD4^+$ entraîne une conversion phénotypique (Khattri, Cox et al. 2003).

Chez l'homme, des travaux montrent que l'expression de FOXP3 n'est pas limitée aux seules cellules régulatrices $CD4^+CD25^+$, mais son expression peut être induite sur les LT $CD4^+CD25^-$ frais ou sur des clones par l'activation du TCR (Walker, Kasprowicz et al. 2003) (Allan, Passerini et al. 2005; Morgan, van Bilsen et al. 2005; Walker, Carson et al. 2005) ou par le

TGF β (Rao, Petrone et al. 2005). Récemment, Wang et al. ont montré, chez l'homme et la souris, que l'INF γ pouvait également convertir les LT CD4⁺CD25⁻ en LT régulateurs FOXP3⁺ (Wang, Hong et al. 2006). Zorn et al. ont récemment montré que l'IL2 augmentait l'expression de FOXP3 sur les CD4⁺CD25⁺ humains en faisant intervenir les protéines STAT3 et STAT5 (Zorn, Nelson et al. 2006).

Fisson et al. ont décrit, chez la souris, plusieurs types de LT régulateurs en fonction de leur état d'activation (Fisson, Darrasse-Jeze et al. 2003). Grâce à des expériences de prolifération (BrdU), ils ont confirmé l'existence de 2 sous populations de LT régulateurs, une avec un turn-over rapide caractérisée par un phénotype CD44^{high} et une autre sous population, à l'état quiescent, caractérisée par un phénotype CD44^{int}. Ainsi il existerait des cellules régulatrices autoréactives (20 à 30%) qui seraient en cycle continu et exprimeraient de multiples marqueurs d'activation (CD69, CD134). D'autres LT régulateurs, majoritaires, seraient quiescents et n'exprimeraient que peu de marqueurs d'activation (Fisson, Darrasse-Jeze et al. 2003). De part leurs expériences, ils ont proposé un modèle d'homéostasie des LT régulateurs. En permanence, les antigènes du soi sont présentés aux cellules T. A partir du pool de régulateurs quiescents, les cellules fortement autoréactives qui rencontrent un antigène du soi, se divisent rapidement et acquièrent un phénotype de Treg activés. Par la suite, ces cellules activées meurent ou recirculent. Ainsi cette hypothèse expliquerait l'effet bystander non spécifique des Treg une fois activés. Ces Treg activés exerceraient une suppression basale et permanente de l'activation cellulaire T (Fisson, Darrasse-Jeze et al. 2003).

4-1-2 Mécanismes d'action

Les mécanismes d'immunosuppression des cellules T régulatrices naturelles et les molécules impliquées dans cette suppression bien que largement étudiés sont toutefois encore mal connus (von Boehmer 2005). Cependant, il est clairement établi maintenant que les cellules régulatrices naturelles sont activées par leur TCR (Sanchez-Fueyo, Sandner et al. 2006), sont anergiques et ne produisent pas d'IL2 (Thornton and Shevach 1998; Shevach 2002) (Thornton, Donovan et al. 2004). Cette anergie peut être levée par ajout d'IL2 (Dieckmann, Plottner et al. 2001) ou par une forte stimulation de la molécule CD28 (Baecher-Allan, Brown et al. 2001). Les cellules régulatrices sont bloquées dans leur cycle cellulaire, elles ont une diminution des cyclines E et A (Li, Godfrey et al. 2005). Elles présentent une diminution d'activation des protéine kinases MEK1/2 et Erk1/2. La présence exogène d'IL2, augmente le niveau de cyclines E et A ainsi que des protéines kinases, ce qui engendre la survie et la prolifération des LT régulateurs (Li, Godfrey et al. 2005). Une fois activée par un antigène, leur

action suppressive n'est plus dépendante de l'antigène qui les a stimulées (Thornton and Shevach 2000). Thornton et al. ont montré, grâce à des modèles de souris transgéniques, que les lymphocytes régulateurs $CD4^+CD25^+$ avaient besoin d'être activés par l'intermédiaire de leur TCR pour devenir suppresseurs, mais une fois activés leur fonction régulatrice est non spécifique (Thornton and Shevach 2000).

Elles sont alors capables d'inhiber la prolifération et la production de cytokines des lymphocytes T $CD4^+CD25^-$, des T $CD8^+$ (Suvas, Kumaraguru et al. 2003) (Camara, Seville et al. 2003) et elles sont capables d'inhiber la différenciation Th1/Th2 (Xu, Liu et al. 2003) (Toda and Piccirillo 2006). L'action régulatrice des cellules naturelles $CD4^+CD25^+$ sur les $CD4^+CD25^-$ s'exerce en inhibant la transcription d'IL2 et nécessite *in vitro* un contact cellulaire (Thornton and Shevach 1998) (Dieckmann, Plottner et al. 2001). Cette activité régulatrice semble CPA indépendante puisque leur fonction suppressive s'exerce dans des cultures sans CPA (Shevach 2002).

Après leur activation, les cellules régulatrices produisent du TGF β (Levings, Sangregorio et al. 2002), un peu d'IL10 et d'IL4 (Dieckmann, Plottner et al. 2001) (Stephens, Mottet et al. 2001) mais la suppression semble indépendante de ces cytokines *in vitro* comme le montre le blocage de ces cytokines par des anticorps ou l'utilisation de souris déficientes en IL10 ou TGF β (Trzonkowski, Szmit et al. 2004) (Piccirillo, Letterio et al. 2002) (Shevach 2002). Donc, bien que l'IL10 ne soit pas essentielle *in vitro* pour la suppression, elle est une molécule clé de la régulation qui mérite que son rôle *in vivo* soit plus étudié. En 1998, il a été démontré qu'une stimulation antigénique chronique *in vivo* entraînait l'apparition de LT $CD4^+$ anergiques *in vitro* capables de sécréter de l'IL10 (Buer, Lanoue et al. 1998). Les lymphocytes T $CD4^+CD25^+$ sont capables de sécréter de l'IL10 *in vivo* (Klein, Khazaie et al. 2003) (Annacker, Pimenta-Araujo et al. 2001) et dans certaines maladies autoimmunes comme la colite, les cellules T $CD4^+CD25^+$ contrôlent la réponse immune par production d'IL10 (Asseman, Mauze et al. 1999) (Suri-Payer and Cantor 2001) tandis que d'autres phénomènes autoimmuns (gastrite) sont indépendants de cette cytokine.

La cytotoxicité est également un des mécanismes supposés des T régulateurs naturels. Grossman et al. ont montré que les lymphocytes T $CD4^+CD25^+FOXP3^+$ activés exprimaient granzyme A et étaient capables de tuer les lymphocytes T $CD4^+$ et $CD8^+$ par un mécanisme perforine dépendant et Fas/FasL indépendant (Grossman, Verbsky et al. 2004). D'autre part, Gondek et al. ont décrit un rôle de granzyme B, mais perforine indépendant, dans l'activité suppressive des T régulateurs, grâce aux souris déficientes en granzyme B et ayant des T

régulateurs avec de faibles capacités suppressives (Gondek, Lu et al. 2005). Ces cellules régulatrices granzyme B⁺ induiraient l'apoptose des cellules effectrices T CD4⁺CD25⁻.

D'autres mécanismes et molécules de régulation des T régulateurs naturels ont été décrits mais restent encore mal connus. Un lien entre FOXP3 et l'hème oxygénase-1 (HO1) a été démontré par Choi et al., puisque FOXP3 induirait l'expression de HO1, enzyme nécessaire au mécanisme de suppression des lymphocytes T CD4⁺CD25⁺ (Choi, Pae et al. 2005). L'implication de CTLA-4, GITR ou du TGFβ membranaire est très controversée (Jonuleit and Schmitt 2003) (Piccirillo and Thornton 2004) (Taams, Vukmanovic-Stejic et al. 2003). Toutefois, ces résultats contradictoires pourraient refléter le fait que les mécanismes des cellules T CD4⁺CD25⁺ puissent être différents *in vitro* et *in vivo* (Jonuleit, Schmitt et al. 2002).

Une étude particulière de Caramalho et al. (Caramalho, Lopes-Carvalho et al. 2003) a montré que la stimulation *in vitro* des lymphocytes T régulateurs naturels CD4⁺CD25⁺ par de fortes doses de LPS, via son ligand le TLR4, induisait leur prolifération et augmentait leur capacité suppressive, même sans CPA. Il est également intéressant de noter que la voie du LPS/TLR4 peut induire la production d'IL6 par les DC immatures ainsi qu'un autre facteur encore inconnu, et bloque la fonction suppressive des T régulateurs (Pasare and Medzhitov 2003). Par conséquent, il semble que les signaux spécifiques d'antigènes (soi ou non soi) ainsi que des signaux non spécifiques des TLRs, puissent moduler l'activation et la fonction de cellules régulatrices naturelles. Il a également été montré récemment que la protéine heat shock 60 pouvait stimuler la capacité suppressive des lymphocytes T CD4⁺CD25^{high} via le TLR2 (Zanin-Zhorov, Cahalon et al. 2006).

Les lymphocytes T régulateurs CD4⁺CD25⁺ peuvent également inhiber la prolifération et la fonction des cellules NK chez l'homme de manière contact dépendant (Trzonkowski, Szmit et al. 2004). Ils peuvent également supprimer, de manière contact-dépendant, la prolifération des cellules NKT et leur production de cytokines telles que l'IFNγ et l'IL4. Les lymphocytes T régulateurs CD4⁺CD25⁺ peuvent également inhiber l'activité cytotoxique des cellules NKT contre certaines cellules tumorales, de manière contact dépendant (Azuma, Takahashi et al. 2003). Mais les NKT peuvent inversement contrôler les T régulateurs humains. Jiang et al. ont montré que les NKT pouvaient sécréter de l'IL2 et entraîner ainsi la prolifération des LT CD4⁺CD25⁺ (Jiang, Game et al. 2005; Cava, Kaer et al. 2006). Les lymphocytes T régulateurs CD4⁺CD25⁺ peuvent également moduler la fonction pro-inflammatoire des monocytes/macrophages en inhibant la production de cytokines telles que le TNF et l'IL6 et en diminuant l'expression de leurs molécules de costimulation (CD80, CD40) (Taams, van Amelsfort et al. 2005).

Récemment, Zhao et al. ont montré que les T régulateurs $CD4^+CD25^+$ pouvait induire la mort des lymphocytes B activés de manière contact dépendant et perforine granzyme B dépendant (Zhao, Thornton et al. 2006)

De plus, les lymphocytes T régulateurs $CD4^+CD25^+$ peuvent agir sur les cellules dendritiques, en leur faisant sécréter de l'IL10 et en induisant à leur surface la molécule B7-H4 (récepteur qui régule négativement la réponse lymphocytaire) et en les rendant ainsi immunosuppressive (Kryczek, Wei et al. 2006). Ils peuvent également agir sur leur maturation et les rendre peu efficaces comme cellules présentatrices (diminution des molécules de costimulation) (Misra, Bayry et al. 2004). Les mécanismes mis en jeu dans ces expériences restent encore à éclaircir.

Ainsi ces différentes observations suggèrent que les lymphocytes T régulateurs naturels possèdent plusieurs mécanismes d'action à leur disposition selon le microenvironnement où ils se trouvent activés et selon les pathologies rencontrées.

4-1-3 Rôle en transplantation

Le rôle de ces cellules régulatrices en transplantation a été suggéré pour la première fois en 1985 quand une sous population lymphocytaire W3/25⁺ avec des capacités régulatrices, est mise en évidence après une greffe cardiaque chez le rat sous ciclosporine (Hall, Jelbart et al. 1985). Taylor et al. ont par la suite établi le rôle des cellules suppressives dans des modèles de GVHD ou maladie du greffon contre l'hôte. Alors que le transfert de cellules naïves allogéniques $CD4^+CD25^-$ ou de cellules T effectrices entraîne une GVHD, le co-transfert de T régulateurs $CD4^+CD25^+$ purifiés avec les cellules T $CD4^+CD25^-$ retarde significativement la survenue de la GVHD (Taylor, Noelle et al. 2001). Un modèle expérimental de GVHD proche de la clinique a été étudié dans les laboratoires de Blazar (Taylor, Noelle et al. 2001), Salomon (Cohen, Trenado et al. 2002) et Strober (Hoffmann, Ermann et al. 2002). Lorsque des souris sont irradiées et reconstituées avec des cellules de moelle osseuse et des lymphocytes T, une GVHD fatale se développe. La déplétion des LT $CD4^+CD25^+$ accélère la GVHD (Taylor, Noelle et al. 2001) (Hoffmann, Ermann et al. 2002) (Cohen, Trenado et al. 2002) tandis que l'injection de LT $CD4^+CD25^+$ la retarde (Edinger, Hoffmann et al. 2003) (Cohen, Trenado et al. 2002) (Hoffmann, Ermann et al. 2002) (Trenado, Sudres et al. 2006)

Par la suite, différents travaux ont montré que les cellules T $CD4^+CD25^+$ étaient indispensables pour l'induction et/ou le maintien de la tolérance aux alloantigènes dans certains modèles chez les rongeurs (Chiffolleau, Beriou et al. 2002) (Kingsley, Karim et al. 2002) (Wood and Sakaguchi 2003). Ainsi, dans des modèles de transplantation d'organes ou de tissus, le co-

transfert de LT régulateurs $CD4^+CD25^+$ et de $CD4^+CD25^-$ chez des souris déficientes en cellules T empêche le rejet de greffes de peau allogéniques (Hara, Kingsley et al. 2001) (Graca, Cobbold 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, Cobbold et al. 2002). Darrasse-Jeze et al. ont récemment montré le rôle des T régulateurs $CD4^+CD25^+$ dans la tolérance aux alloantigènes paternels lors de la gestation chez la souris. En effet une déplétion de ces cellules régulatrices entraînent un rejet du fœtus allogénique mais pas syngénique (Darrasse-Jeze, Klatzmann et al. 2006).

Chez l'homme, Salama et al. ont fortement suggéré le rôle des cellules suppressives dans la régulation de la voie indirecte de présentation des antigènes, puisqu'ils ont montré que 40% des patients greffés rénaux stables, qui n'avaient jamais fait de rejet aigu, avaient une régulation dépendante des cellules $CD25^+$ contre les peptides du CMH de classe II du donneur (Salama, Najafian et al. 2003). Mais seulement 12,5% des patients qui avaient présenté un rejet aigu présentaient une telle régulation. Cette régulation apparaît au troisième mois après la greffe et persiste malgré l'immunosuppression. La génération *in vitro* de telles cellules régulatrices spécifiques d'un peptide correspondant à un CMH du donneur a été envisagée pour induire une tolérance. En effet, Jiang et al. ont montré que ce type de cellules pouvaient non seulement inhiber la prolifération contre ce peptide, mais aussi par «suppression liée» («linked suppression») contre un autre peptide présenté sur la même CPA (Jiang and Lechler 2003). Au contraire, la voie directe de présentation ne semble pas être contrôlée par les lymphocytes T $CD4^+CD25^+$ puisqu'une étude menée chez 12 patients greffés rénaux stables a montré que la déplétion en cellules T $CD4^+CD25^+$ ne modifiait pas la réponse directe (tests Elispot, LDA) contre le donneur *in vitro* (Game, Hernandez-Fuentes et al. 2003).

Li et al. ont montré une augmentation des LT $CD4^+CD25^{high}$ régulateurs dans le sang des patients opérationnellement tolérants comparativement aux patients stables suggérant un rôle potentiel de ces cellules dans la tolérance opérationnelle (Li, Koshiba et al. 2004). Ils n'ont pas décrit de différence entre les patients tolérants et les sujets sains. Aucune étude de la fonctionnalité de ces cellules potentiellement régulatrices n'a été réalisée dans ces travaux. Demirkiran et al. ont récemment montré qu'après une greffe de foie, les patients présentaient une diminution transitoire de cellules T régulatrices $CD4^+CD25^+ CD45RO^+ CTLA4^+$ à 3 mois post-greffe, due aux immunosuppresseurs (Demirkiran, Kok et al. 2005). Par la suite, ils ont montré que les patients faisant un rejet aigu, avaient une diminution de ces cellules régulatrices et de FOXP3, à 1 an post-greffe, comparativement à ceux qui ne font pas de rejet. Cependant,

malgré la diminution du nombre de cellules régulatrices chez les patients en rejet aigu, leurs cellules CD4⁺CD25⁺ ont une activité suppressive comparable aux sujets sans rejet et aux individus sains (Demirkiran, Kok et al. 2006).

D'autre part, Meloni et al. ont montré qu'une diminution de la fréquence des lymphocytes T CD4⁺CD25^{high} régulateurs périphériques pouvait être associée au développement d'un rejet chronique (bronchiolite oblitérante) chez les patients greffés du poumon comparativement à des sujets stables ou des individus normaux (Meloni, Vitulo et al. 2004). Ces cellules T CD4⁺CD25⁺ des patients en rejet chronique ont une fonction suppressive, vis-à-vis des LT CD4⁺CD25⁻ autologues, comparable à celle des sujets sains, et sécrètent de grande quantité d'IL10. Les auteurs n'ont cependant pas montré de lien direct entre la sécrétion d'IL10 et l'effet suppresseur des T CD4⁺CD25⁺ (Meloni, Vitulo et al. 2004). Dans une autre étude, Alvarez et al. ont observé une augmentation du pourcentage des lymphocytes T CD4⁺CD25⁺CD69⁺ chez des patients greffés rénaux avec une fonction stable sous immunosuppression, comparativement aux sujets sains (Alvarez, Paris et al. 2004). Aucune différence n'a été observée avec les patients en rejet chronique. Les auteurs concluent que cette augmentation de LT CD4⁺CD25⁺CD69⁺ chez des patients greffés rénaux avec une fonction stable sous immunosuppression, suggère que les mécanismes régulateurs sont des phénomènes actifs (Alvarez, Paris et al. 2004).

Ainsi ces expériences suggèrent qu'un faible nombre de LT CD4⁺CD25⁺ régulateurs ne serait pas suffisant pour contrebalancer l'activité des cellules effectrices chez ces patients et contribuerait ainsi au rejet de greffe.

D'autre part, Muthukumar et al. ont montré qu'un faible taux de transcrits FOXP3 dans les urines était prédictif d'un rejet aigu irréversible et de dommages du greffon (Muthukumar, Dadhania et al. 2005) chez des patients greffés rénaux.

Les immunosuppresseurs peuvent influencer le phénotype et la fonction des cellules régulatrices. En effet, une étude de Baan et al. a montré une diminution de l'induction de FOXP3 en présence d'inhibiteurs de calcineurine, l'anticorps anti-CD25, en culture lymphocytaire mixte (Baan, van der Mast et al. 2005). La rapamycine n'a aucun effet sur l'induction de FOXP3 (Baan, van der Mast et al. 2005) ni sur l'activité des cellules régulatrices (Coenen, Koenen et al. 2006). Cependant ces résultats sont encore controversés. En effet, une étude de Tian et al. a montré dans un modèle de greffe cardiaque chez le rat, que la rapamycine entraînait une augmentation des cellules T CD4⁺CD25⁺. Battaglia et al. ont trouvé des résultats similaires chez la souris (Battaglia, Stabilini et al. 2005). De même chez l'homme, Valmori et al. ont décrit que la rapamycine entraînait une augmentation de l'activité

suppressives des T régulateurs et permettait à des cellules CD4⁺ conventionnelles d'acquérir des capacités régulatrices (Valmori, Tosello et al. 2006).

Ces résultats confortent le rôle important de l'IL2 pour les cellules régulatrices et certains auteurs suggèrent que des traitements immunosuppresseurs comme les inhibiteurs de la calcineurine pourraient empêcher l'induction d'une tolérance opérationnelle chez les patients traités (Baan, van der Mast et al. 2005). Des résultats similaires ont été obtenus chez le rat et montrent que la CsA affecte la fonction des T régulateurs, mais pas la rapamycine ni le mycophénolate mofetil (MMF) (Zeiser, Nguyen et al. 2006). Une autre étude a montré que l'anticorps anti-CD25 en traitement d'induction post-greffe n'influçait pas le nombre de T régulateurs CD4⁺CD25⁺ chez les patients greffés du foie (Demirkiran, Kok et al. 2006). En revanche, le traitement du rejet aigu avec la méthyprednisolone entraîne une diminution de ces cellules suppressives dans le sang de ces patients, probablement par apoptose des cellules T régulatrices (Demirkiran, Kok et al. 2006).

Ces résultats suggèrent donc que les traitements immunosuppresseurs peuvent affecter l'homéostasie et la fonction des cellules régulatrices, mais une meilleure caractérisation de ces effets sont nécessaires avant d'envisager une minimisation ou une modification des traitements chez les transplantés.

La conversion des LT régulateurs :

Jusqu'à présent, parmi les LT CD4⁺CD25⁺ chez l'homme, 2 grandes sous-populations de LT régulateurs ont été décrites en fonction de leur origine et leurs mécanismes d'action : les LT naturels issus du thymus et les LT CD4⁺CD25⁺ induits qui sont des LT dont les fonctions régulatrices sont induites par différents stimuli.

Les LT CD4⁺CD25⁺ naturels sont des cellules issues de la sélection thymique qui expriment le marqueur CD25 directement à l'origine dans le thymus puis passent en périphérie où elles conservent le marqueur CD25. Elles agissent de manière contact dépendant (voir ci-dessus).

Deux populations de LT CD4⁺CD25⁺ induits ont été largement décrites chez l'homme dans la littérature, les Tr1 (Groux, O'Garra et al. 1997) et les Th3 (Chen, Kuchroo et al. 1994) . Ces cellules sont issues de la conversion en périphérie de LT CD4⁺CD25⁻ en LT CD4⁺CD25⁺. Plusieurs stimuli peuvent induire ces cellules régulatrices, pour les lymphocytes Tr1 : l'IL2 (Groux, O'Garra et al. 1997), la vitamine D3 et le dexaméthasone (Barrat, Cua et al. 2002), ou encore la rapamycine associé à l'IL10 (Battaglia, Gregori et al. 2006) (voir ci-dessous) ; pour les Th3 : le TGFβ, IL10, IL4 et IL12 (Weiner 2001) (voir ci-dessous).

Récemment, il a été suggéré que des LT régulateurs $CD4^+CD25^+$ pourraient émerger en périphérie, après stimulation antigénique, du pool de cellules mémoires $CD4^+CD45RO^+CD25^-$. Ces cellules auraient un répertoire réduit et auraient la même origine que des cellules mémoires (Vukmanovic-Stejic, Zhang et al. 2006). Les auteurs de cet article n'ont cependant pas démontré leurs mécanismes d'action. L'existence de cellules régulatrices induites $CD4^+CD25^+$ ni Tr1 ni Th3 n'est donc pas à exclure. La genèse de LT régulateurs $CD4^+CD25^+$ induits en périphérie par conversion pourraient expliquer le fait que leur nombre varie peu avec l'âge, malgré l'involution du thymus (Vukmanovic-Stejic, Zhang et al. 2006). De plus, cela permettrait à l'organisme de générer en périphérie des LT régulateurs spécifiques de nouveaux antigènes qui ne sont pas présents dans le thymus.

4-2 Les cellules régulatrices T $CD4^+CD25^+$ Tr1

Les lymphocytes T $CD4^+$ régulateurs de type 1 (Tr1) ont été identifiés à l'origine uniquement par leur production de cytokines (Groux, O'Garra et al. 1997). C'est le groupe de Roncarolo qui a montré, pour la première fois, que l'activation *in vitro* des LT $CD4^+$ humains ou murins avec des fortes doses d'IL10 permettait la génération de clones T produisant des cytokines distinctes de celles produites par les lymphocytes Th1 et Th2 (Battaglia, Gregori et al. 2006) (Grazia Roncarolo, Gregori et al. 2006). De plus, ces clones sont comparables à ceux isolés à partir de patients atteints d'immunodéficience sévère combinée (SCID) qui produisent de forte quantité d'IL10. Les cellules Tr1, après activation via le TCR, produisent de l'IL10, du TGF β , de l'IL5 mais peu d'IFN γ et d'IL2 et pas d'IL4 (Groux, O'Garra et al. 1997).

4-2-1 Origine et caractéristiques phénotypiques

Les cellules Tr1 sont des cellules régulatrices inductibles et sont donc générées, *in vitro* comme *in vivo*, à partir de précurseurs périphériques naïfs $CD4^+$. L'IL10 permet la génération des Tr1, comme l'ont montré les expériences de Groux et al., dans lesquelles, les Tr1 humains et murins sont induites *ex vivo* par une stimulation répétée du TCR et de forte dose d'IL10 (Groux, O'Garra et al. 1997). Elles peuvent être générés chez l'homme et la souris en présence de vitamines D3 et de dexaméthasone. Le blocage de l'IL10 dans ces cultures inhibent le développement des Tr1 (Barrat, Cua et al. 2002). Cependant, pour la génération des Tr1 humains, l'IL10 semble nécessaire mais ne semble pas suffisante. En effet, l'IFN α , cytokine importante dans la lutte contre une infection virale chez l'homme, permet d'induire *in vitro* des Tr1 en présence d'IL10 (Levings, Sangregorio et al. 2001), en revanche, Wakkach et al. ont montré, *in vitro*, que des Tr1 pouvaient être induits via la costimulation par CD2,

indépendamment de l'IL10 (Wakkach, Cottrez et al. 2001). De plus, le groupe d'Atkinson a montré, chez l'homme, que le CD46 était impliqué dans la génération de Tr1 qui exercent leur capacités suppressives via l'IL10 et un mécanisme granzyme B/perforine dépendant (Kemper, Chan et al. 2003) (Grossman, Verbsky et al. 2004). *Streptococcus pyogenes* est capable de se lier au CD46 et d'induire des cellules T CD4⁺ avec un phénotype comparable aux Tr1 chez l'homme (IL10⁺, IL2⁻) (Price, Schaumburg et al. 2005). Le rôle des cellules dendritiques (DC) dans cette génération des Tr1 est encore peu clair. Levings et al. ont récemment montré que des lymphocytes T CD4⁺ stimulés avec des cellules dendritiques allogéniques immatures entraînaient la différenciation de Tr1 (Levings, Gregori et al. 2005). Récemment, Battaglia et al. ont montré, que *in vivo*, un traitement avec la rapamycine et de l'IL10 induisait des Tr1 chez la souris, et induisait une tolérance à allogreffe d'îlots (Battaglia, Gregori et al. 2006). Il n'a pas encore été défini chez l'homme si cette thérapie entraîne la différenciation de Tr1.

Chez la souris, des DC immatures CD45RB^{high} produisant de l'IL10, peuvent induire des Tr1 *ex vivo* et *in vivo* (Wakkach, Fournier et al. 2003). Différents protocoles utilisant des microorganismes (*bordetella pertussis*, *cholera*, *staphylococcal enterotoxin B*) chez la souris ont permis également d'induire des Tr1 (McGuirk, McCann et al. 2002; Zuany-Amorim, Sawicka et al. 2002) (Lavelle, McNeela et al. 2003) (Mahic, Yaqub et al. 2006). La voie Notch a également été décrite comme impliquée dans leur genèse chez la souris et l'homme (Vigouroux, Yvon et al. 2003).

Les Tr1 prolifèrent très peu après activation du TCR, bien qu'elles puissent proliférer avec de l'IL2 et l'IL15. Cette anergie est en partie due à une production autocrine d'IL10 (Bacchetta, Sartirana et al. 2002).

Les Tr1 ne possèdent pas à ce jour de marqueurs phénotypiques spécifiques. Ils expriment des molécules telles que CXCR3, GATA3, CCR3, CCR4, CCR5, CCR8, mais ces molécules sont aussi exprimées par les Th1 et/ou Th2 (Sebastiani, Allavena et al. 2001; Cobbold, Nolan et al. 2003). Les Tr1 n'expriment pas de manière constitutive le marqueur FOXP3, mais celui-ci peut être induit après activation par des DC immatures chez l'homme (Levings, Gregori et al. 2005).

4-2-2 Mécanismes d'action

Les cellules régulatrices Tr1 exercent leur capacité suppressive par l'intermédiaire des cytokines qu'elles produisent. L'IL10, et le TGFβ dans une moindre mesure, sont les principales cytokines mises en jeu (Groux, O'Garra et al. 1997). Cependant, il a également été décrit un mécanisme contact dépendant (Hawrylowicz and O'Garra 2005). D'autre part, Mahic et al. ont récemment montré que les CD4⁺CD25⁺ exprimaient la cyclooxygénase-2 et

exerçaient leur pouvoir supprimeur par l'intermédiaire de la prostaglandine E2 (Mahic, Yaqub et al. 2006) Les lymphocytes Tr1 sont inductibles, spécifiques de l'antigène, et doivent être activés via leur TCR pour exercer leur capacité suppressive. Une fois activés, les lymphocytes Tr1 deviennent des cellules suppressives non spécifiques agissant par effet « bystander » c'est-à-dire que les cellules régulatrices semblent pouvoir induire elles-mêmes d'autres cellules régulatrices (Groux, O'Garra et al. 1997). Ainsi, des clones T alloréactifs pourraient être régulés par des cellules T régulatrices de même spécificité si l'alloantigène en question était présenté par la même cellule présentatrice d'antigènes (Davies, Leong et al. 1996) (Walsh, Taylor et al. 2004). Ils deviennent alors, à leur tour eux-mêmes régulateurs, via l'action de cytokines comme l'IL10 ou le TGF β . Les cellules régulatrices peuvent donc «transmettre» leurs propriétés régulatrices; ce phénomène est appelé la «linked immunosuppression» ou «bystander suppression». C'est probablement un des mécanismes mis en jeu dans la «tolérance infectieuse» 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, Cobbold et al. 1993).

Plusieurs études ont montré que l'IL10 agissait sur les CPA en inhibant la sécrétion de médiateurs pro-inflammatoires (IL1 β , IL6, IL12, TNF α), en diminuant les molécules de costimulation et en favorisant l'expression de molécules inhibitrices telles que ILT4 (Vlad, Piazza et al. 2003) (Wakkach, Cottrez et al. 2001). De même, l'IL10 inhibe la voie intracellulaire NF- κ B responsable de la production d'IL2 dans les lymphocytes T CD4⁺ (Vigouroux, Yvon et al. 2004).

4-2-3 Rôle en transplantation

En 1994, les expériences de Bachetta et al. ont suggéré que les Tr1 étaient impliqués *in vivo* dans la tolérance périphérique chez des patients avec une immunodéficience combinée sévère et transplantés avec des cellules souches hématopoïétiques (Bacchetta, Bigler et al. 1994). Dans le cadre de la transplantation de cellules hématopoïétiques de moelle osseuse chez l'homme, une augmentation de la production d'IL10 par les PBMC des receveurs est associée à une faible incidence de la GVHD et de la mortalité (Baker, Roncarolo et al. 1999) (Holler, Roncarolo et al. 2000). De même, une forte fréquence de cellules du donneur synthétisant de l'IL10 en réponse aux alloantigènes du receveur, est corrélée à une absence de GVHD aigüe après transplantation de cellules hématopoïétiques de moelle osseuse. A l'opposé, une faible fréquence est associée au développement d'une GVHD sévère chez l'homme (Weston, Geczy et al. 2006). De plus, des études chez l'homme ont montré une corrélation entre un

polymorphisme au niveau du promoteur de l'IL10 et le risque de GVHD après transplantation de cellules hématopoïétiques de moelle osseuse (Lin, Storer et al. 2003) (Lin, Storer et al. 2005). Des études chez des patients développant spontanément une tolérance à une greffe de rein ou de foie, révèlent la présence de LT $CD4^+$ capables de supprimer les réponses de T naïfs (test de DTH) en produisant de l'IL10 et du TGF β (VanBuskirk, Burlingham et al. 2000). Chez la souris, Kingsley et al. ont montré que le blocage de l'IL10 inhibait la suppression du rejet de greffe allogénique de peau par les cellules régulatrices (Kingsley, Karim et al. 2002). Récemment, il a été montré que le traitement *in vivo* rapamycine + IL10 était capable d'empêcher le rejet d'allogreffe dans un modèle de transplantation d'îlots chez la souris (Battaglia, Stabilini et al. 2006).

Ainsi toutes ces expériences indiquent que les Tr1 peuvent contrôler les réponses immunes et induire une tolérance aux alloantigènes *in vivo* dans le cadre de transplantation de cellules hématopoïétiques de moelle osseuse ou de transplantation d'organes.

4-3 Les cellules régulatrices T $CD4^+CD25^+$ Th3

Les cellules Th3 ont été identifiées dans un modèle d'EAE (Encéphalite Allergique Expérimentale) (Chen, Kuchroo et al. 1994) après injection de la MBP (Myelin Basic Protein) pour immuniser des souris. Lorsque la MBP est administrée par voie orale, des cellules T capables d'inhiber les réactions inflammatoires sont générés (Faria and Weiner 2005). La suppression des symptômes cliniques de l'EAE est bloquée par l'injection d'anticorps anti-TGF β (Chen, Kuchroo et al. 1994). En effet, les cellules Th3 produisent de grande quantité de TGF β et une quantité modérée d'IL10 (Weiner 1997) (Kitani, Chua et al. 2000).

In vitro, les cellules régulatrices Th3 peuvent être générées à partir de précurseurs Th0 en présence de TGF β , IL10, IL4 et IL12 (Weiner 2001). *Ex vivo* des lymphocytes Th3 spécifiques du donneur pourraient être induits par stimulation, dépendante de Notch, de cellules T naïves d'un receveur de greffe de cellules hématopoïétiques de moelle osseuse (Yvon, Vigouroux et al. 2003).

L'expression et le rôle de FOXP3 dans les Th3 ne sont toujours pas clairs. Il a été montré que le TGF β produit par les Th3 entraînait l'expression de FOXP3 à l'origine de la conversion des LT $CD4^+CD25^-$ non régulatrices en T $CD4^+CD25^+$ régulatrices (Chen, Jin et al. 2003). Très récemment, une étude publiée dans un modèle murin a démontré que l'expression de FOXP3, la taille du compartiment en Treg et l'activité suppressive étaient dépendants de signaux induits par le TGF β dans les T régulateurs (Marie, Letterio et al. 2005).

Les Th3 agissent, contrairement aux cellules régulatrices naturelles, par l'intermédiaire de la sécrétion de cytokines immunosuppressives le TGFβ et l'IL10 (Weiner 2001). Ainsi, elles sont capables d'inhiber le développement de phénomènes autoimmuns dans plusieurs modèles animaux (Weiner 1997) et inhibent en particulier la prolifération cellulaire et la synthèse de cytokines de clones Th1 et Th2 (Weiner 1997) (Beissert, Schwarz et al. 2006). Elles agissent de manière non spécifique de l'antigène et par effet bystander via surtout la sécrétion de TGFβ. La fixation de cette cytokine sur son récepteur active le complexe intracellulaire Smad qui est alors transloqué vers le noyau entraînant l'inhibition de la prolifération et de la différenciation des cellules T ainsi que le blocage de la maturation des cellules dendritiques (Vigouroux, Yvon et al. 2004). Des souris déficientes en TGFβ développent des infiltrats inflammatoires multi organes et ont une prolifération T incontrôlée (Gorelik and Flavell 2000) (Leveen, Larsson et al. 2002). Le TGFβ a été trouvé directement dans les greffons ce qui suggère que les T régulateurs ont un effet tolérogène directement au niveau des greffons (Josien, Heslan et al. 1998).

4-4 Les cellules régulatrices T CD4⁺CD25⁻

L'existence de cellules T CD4⁺CD25⁻ régulatrices a été évoquée dans plusieurs études (Stephens and Mason 2000) (Graca, Cobbold et al. 2002) (Degauque, Lair et al. annexe 1). Ainsi, il a été montré que le transfert de cellules T CD4⁺CD45RC⁻CD25⁻ mémoires prélevées en périphérie (rate et ganglions) permettaient à elles seules de diminuer l'incidence du diabète autoimmun chez le rat (Stephens and Mason 2000). Cependant, ces cellules seraient 10 fois moins efficaces que les CD4⁺CD25⁺ (Graca et al. 2002). Ainsi, dans un modèle de transplantation entre individus différents pour leurs molécules du complexe mineur d'histocompatibilité, le transfert de cellules CD4⁺CD25⁻ provenant de la rate de souris tolérant une greffe de peau permet l'acceptation d'une greffe de peau chez une souris non traitée. Afin d'obtenir le même effet régulateur qu'avec les cellules CD4⁺CD25⁺ de la rate, il est cependant nécessaire d'employer 10 fois plus de cellules CD4⁺CD25⁻ (Graca, Thompson et al. 2002).

Notre laboratoire a montré que des cellules T CD25⁻ (Degauque, Lair et al. annexe 1) 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. Ces cellules régulatrices auraient une action suppressive de manière contact dépendant et cytokines dépendantes (IL10, IFNγ) (Degauque, Lair et al. annexe 1). La perte du marqueur CD25 par les cellules régulatrices TCD25⁺ a été rapporté dans un autre modèle de souris transgéniques où le transfert de cellules CD4⁺CD25⁺ issues de la rate

et des ganglions lymphatiques de souris B6 à des souris lymphopéniques Rag-1^{-/-} se traduit par une forte expansion de ces cellules qui perdent le marqueur CD25 après cinq divisions (Gavin, Clarke et al. 2002). Les mécanismes d'action de ces cellules restent encore à élucider.

4-5 Les cellules suppressives T CD8⁺

Plusieurs types de cellules T CD8⁺ suppressives ont été décrits à ce jour, les LT CD8⁺CD45RC^{low}, les LT CD8⁺CD28⁻ et les LT CD8⁺CD103⁺. Récemment, notre laboratoire a montré que le blocage de la voie CD40/CD40L permettait la génération de cellules régulatrices T CD8⁺CD45RC^{low} capables de tolérance infectieuse dans un modèle d'allogreffe cardiaque chez le rat (Guillonnet et al, en révision). Auparavant, les travaux de Saoudi et al. ont démontré, pour la première fois chez le rat, l'existence d'une sous population de lymphocytes T CD8⁺ avec des capacités régulatrices (Xystrakis, Dejean et al. 2004) (Xystrakis, Cavaillès et al. 2004). L'expression de CD45RC a permis de distinguer 2 types de sous populations chez le rat: CD45RC^{high} et CD45RC^{low}. Ils ont démontré que les cellules CD8⁺CD45RC^{low} fraîchement isolées ont des propriétés régulatrices, ne sont pas cytotoxiques, produisent des cytokines de type Th2 (IL4, IL10 et IL13), expriment sélectivement FOXP3 et CTLA-4 et inhibent les réponses immunitaires alloréactives à la fois *in vitro* et *in vivo*. Les fonctions régulatrices et les mécanismes d'action de ces cellules sont liés à une inhibition de l'expansion des cellules effectrices CD4⁺ Th1. De plus, cette régulation requiert des contacts cellulaires et non une action dépendante des cytokines (Xystrakis, Dejean et al. 2004).

D'autre part, chez l'homme, Cosmi et al. ont caractérisé une population de thymocytes CD8⁺CD25⁺ avec un phénotype, des caractéristiques fonctionnelles et un mécanisme d'action équivalent aux cellules T CD4⁺CD25⁺ (Cosmi, Liotta et al. 2003). Des lymphocytes CD8⁺CD25⁺ FOXP3⁺ régulateurs ont également été caractérisés en périphérie dans le sang de patients diabétiques traités avec un anti- CD3 (Bisikirska, Colgan et al. 2005).

Plusieurs travaux de l'équipe de Suci-Foca et Cortesini ont montré l'existence de LT supresseurs CD8⁺CD28⁻ notamment dans le cadre de la transplantation d'organes, cœur, foie et rein (Liu, Tugulea et al. 1998) (Cortesini, LeMaoult et al. 2001) (Cortesini, Renna-Molajoni et al. 2002) (Suci-Foca, Manavalan et al. 2003). L'effet supresseur des cellules T CD8⁺CD28⁻ passe par une tolérogénisation des CPA professionnelles (cellules dendritiques) et semi-professionnelles (cellules endothéliales) qui induisent ensuite l'anergie des lymphocytes T helper (Chang, Ciubotariu et al. 2002) (Manavalan, Kim-Schulze et al. 2004). La tripartite CPA/TCD8⁺CD28⁻/Thelper est obligatoire. En effet, le lymphocyte T supresseurs CD8⁺CD28⁻ en reconnaissant des molécules de CMH de classe I sur la CPA bloque la voie de signalisation

NF- κ B dépendante de CD40 (Li et al., 1999). Ces cellules T CD8⁺CD28⁻, spécifiques des molécules de CMH de classe I, suppriment de façon spécifique de l'antigène les réponses lymphocytaires T CD4⁺ helper en empêchant la production d'IL2 et la surexpression de la molécule CD40L (Liu, Tugulea et al. 1998). Ces LT CD8⁺CD28⁻ inhibent l'expression de molécules costimulatrices et augmentent l'expression de molécules inhibitrices ILT3 et ILT4 (Immunoglobulin Like Transcript) sur les CPA professionnelles (cellules dendritiques) et non professionnelles (cellules endothéliales) du donneur (Chang, Ciubotariu et al. 2002) (Kim-Schulze, Seki et al. 2006) (**Figure 7**). Ainsi, ces CPA ILT3^{high} et ILT4^{high} devenues tolérogènes, induisent l'anergie des cellules T helper et stimulent la génération de Treg spécifiques des alloantigènes avec les caractéristiques phénotypiques CD4⁺CD25⁺CD45RO⁺FOXP3⁺ et de nouvelles générations de LT suppresseurs CD8⁺CD28⁻FOXP3⁺ (Manavalan, Rossi et al. 2003) (Manavalan, Kim-Schulze 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, Mirza et al. 2003). De ce mode d'action a été mise au point une méthode de détection des cellules T suppressives dans le sang de patients greffés (cœur, rein, foie) (Colovai, Mirza et al. 2003) (Cortesini, Renna-Molajoni et al. 2002) (Ciubotariu, Liu et al. 1998). Cette méthode a permis de montrer, chez des patients greffés rénaux et hépatiques, que la présence de cellules T dans les premiers mois qui suivent la transplantation, est inversement corrélée à une alloréactivité contre les peptides du CMH du donneur et donc au rejet chronique (Ciubotariu, Liu et al. 1998) (Ciubotariu, Vasilescu et al. 2001) (Cortesini, Renna-Molajoni et al. 2002).

Au laboratoire, l'étude réalisée chez les patients tolérants ou présentant un rejet chronique après une transplantation rénale, a permis de montrer une augmentation des lymphocytes T CD8⁺CD28⁻ chez les patients développant un rejet chronique. Ces cellules T CD8⁺CD28⁻, FOXP3 négatives, capables de proliférer, présentent un phénotype cytotoxique (perforine⁺/GranzymeA⁺) et ne seraient donc pas des cellules suppressives régulatrices mais des cellules cytotoxiques (Baeten, Louis et al. 2006) (annexe 2).

Certaines études suggèrent que l'inhibition des réponses des cellules T CD4⁺ activées par certains T suppresseurs CD8⁺ dépendrait de l'expression par les cellules T CD4⁺ de la molécule de CMH de classe Ib Qa-1 (Jiang and Chess 2000) (Panoutsakopoulou, Huster et al. 2004) (Sarantopoulos, Lu et al. 2004).

Des cellules suppressives $CD8^+CD28^-FOXP3^+$ ont également été retrouvées après transfusions spécifiques du donneur, dans un modèle d'allogreffe cardiaque chez le rat (Liu, Liu et al. 2004).

Enfin, récemment des cellules $CD8^+CD103^+$ régulatrices ont été décrites chez l'homme (Uss, Rowshani et al. 2006). Elles ont de faible capacité cytotoxique, de faible capacité de prolifération, produisent beaucoup d'IL10 et peu d'IFN γ . En coculture, elles peuvent inhiber la prolifération de cellules alloreactives de manière contact dépendant (Uss, Rowshani et al. 2006).

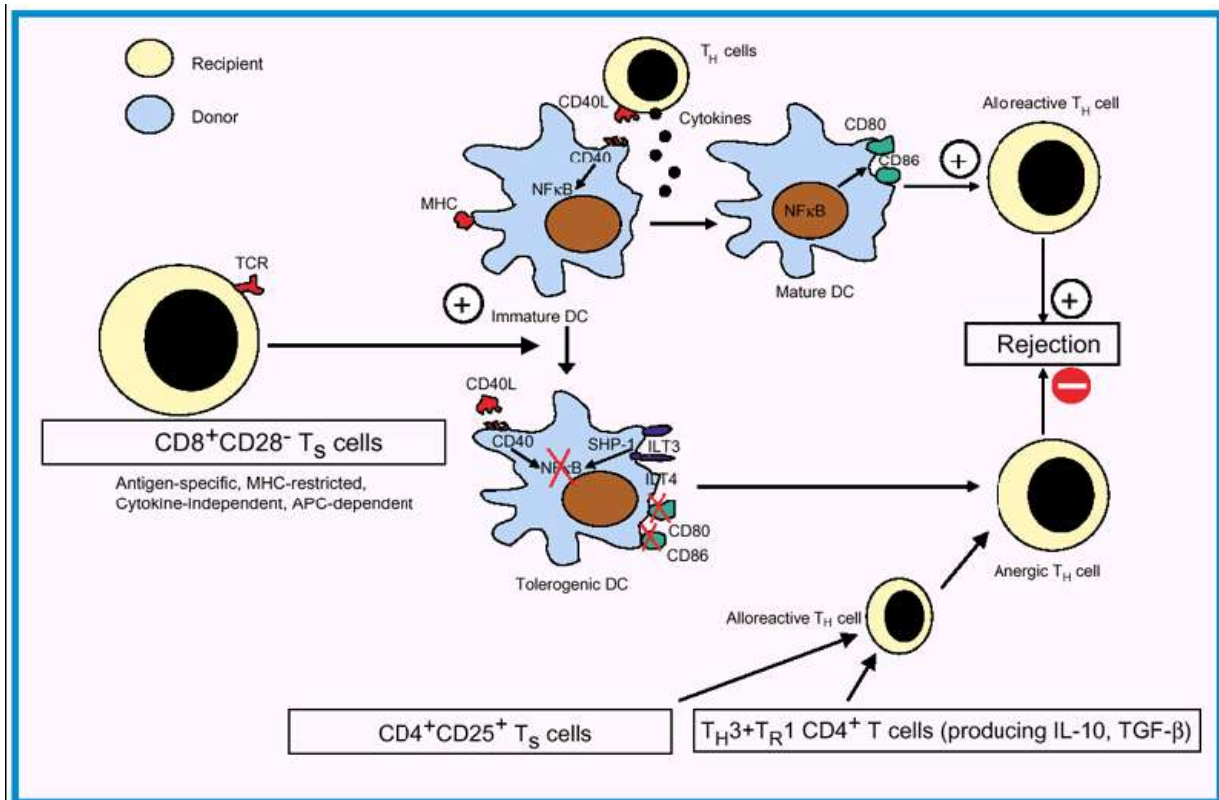


Figure 7: Mode d'action des LT $CD8^+CD28^-$ supresseurs lors d'une greffe

Les lymphocytes T $CD8^+CD28^-$ supresseurs du receveur inhibent l'activation de NF- κ B dans les cellules dendritiques du donneur qui deviennent alors tolérogènes (ILT3+ ILT4+ CD80-CD86-). Ces dernières anergisent les lymphocytes Th alloreactifs du receveur. D'après (Feinberg and Silvestri 2002).

4-6 Les cellules régulatrices NKT

Les cellules NKT constituent une population hétérogène, avec des caractéristiques des cellules NK et des lymphocytes T (van der Vliet, Pinedo et al. 2002) (Cava, Kaer et al. 2006). Elles expriment un TCR invariant constitué des segments V α 24 et V β 11 et des marqueurs NK tels que CD161 ou NKR-P1. Bien que la majorité des NKT soient $CD4^+$, la plupart des cellules restantes sont $CD4^-CD8^-$. Une petite portion de NKT est $CD8^+$ chez l'homme. Elles reconnaissent des CMH de classe I non classique CD1d qui présentent des glycolipides

(Godfrey, Hammond et al. 2000). Les cellules NKT sont divisées en deux groupes fonctionnellement distincts: la sous population $CD4^-CD8^-$ qui produit essentiellement des cytokines Th1 et qui possède une activité cytotoxique et la sous population $CD4^+$ qui sécrète des cytokines Th1 et Th2 en grande quantité (Lee, Benlagha et al. 2002).

Les NKT possèdent des caractéristiques communes avec les T régulateurs. En effet, elles peuvent inhiber la prolifération cellulaire et la production de cytokines (Terabe and Berzofsky 2004). Elles peuvent également inhiber la génération des lymphocytes Th1 (Miyamoto, Miyake et al. 2001) (Moodycliffe, Nghiem et al. 2000) (Beaudoin, Laloux et al. 2002) et $CD8^+$ (Terabe and Berzofsky 2004) (Hammond and Kronenberg 2003).

Elles semblent impliquées dans des modèles de tolérance en allo-transplantation, notamment dans la tolérance néonatale où leur nombre est augmenté (Kawamura, Kameyama et al. 2002), dans un modèle de souris où après le blocage de la voie de costimulation CD28/B7, les NKT sont indispensables à l'établissement d'une tolérance à une allogreffe cardiaque (Seino, Fukao et al. 2001) ou dans un modèle de greffe cardiaque avec une greffe de cellules hématopoïétiques de moelle osseuse chez la souris où la tolérance ne peut s'établir chez des souris déficientes en NKT (Higuchi, Zeng et al. 2002). Contrairement à ces travaux, Li et al. ont montré une diminution des NKT chez des patients pédiatriques, greffés du foie, opérationnellement tolérants (Li, Koshiba et al. 2004). Les mécanismes par lesquels les NKT régulent l'induction de tolérance sont complexes et peuvent impliquer des interactions avec les cellules régulatrices (Cava, Kaer et al. 2006).

4-7 Les cellules régulatrices doubles négatives $CD4^-CD8^-$

Les cellules T doubles négatives (DN) $TCR\alpha\beta^+CD4^-CD8^-$ représentent 1 à 3% des lymphocytes TCD3⁺ (Zhang, Yang et al. 2000). Elles sont NK1.1⁻ et expriment CXCR5 (Lee, Chen et al. 2006). Récemment, les DN ont été caractérisées chez l'homme et elles peuvent réguler l'action de cellules T spécifiques d'un antigène donné (Fischer, Voelkl et al. 2005). En transplantation, elles sont impliquées dans la tolérance après transfusion spécifique du donneur dans des modèles de greffe de peau (Young, Yang et al. 2002) et de cœur (Chen, Ford et al. 2003) (Lee, Chen et al. 2006) chez la souris en entraînant la délétion de clones $CD8^+$ allo-réactifs. *In vitro*, les Treg DN de souris tolérantes peuvent spécifiquement inhiber et tuer les cellules T $CD4^+$ et $CD8^+$ syngéniques dirigées contre le donneur (Ford, Young et al. 2002) (Lee, Mansfield et al. 2005). L'injection de Treg DN permet de prolonger la survie d'une greffe de peau allogénique chez des souris (Zhang, Yang et al. 2000) (Ford, Young et al. 2002). Cette population DN est cytotoxique contre les cellules T $CD8^+$, produit de fort taux d'IFN γ (Lee,

Mansfield et al. 2005) et peut aussi tuer par l'intermédiaire de la voie Fas/FasL (Priatel, Utting et al. 2001) (Zhang, Yang et al. 2000) (Ford, Young et al. 2002).

4-8 Les cellules régulatrices NK

Plusieurs travaux ont récemment suggéré un rôle régulateur des NK chez l'homme (Li, Lim et al. 2005) (Bielekova, Catalfamo et al. 2006). Li et al. ont montré que le traitement avec un anti-IL2R (Daclizumad) était efficace pour traiter les patients atteints d'uvéïte en induisant une forte expansion des cellules NK CD56^{high} régulatrices, qui agiraient en produisant de forte quantité d'IL10 et diminueraient ainsi les phénomènes inflammatoires (Li, Lim et al. 2005). D'autre part, des cellules régulatrices NK CD56^{high} ont également été décrites chez des patients atteints de sclérose en plaque et traités avec un anti-IL2R (Daclizumad). Ces cellules seraient différentes de celles décrites précédemment par Li et al. puisqu'elles agiraient en inhibant la survie des lymphocytes T de manière contact dépendant (Bielekova, Catalfamo et al. 2006).

Méthodologie

1- Objectif de l'étude

Les mécanismes immunologiques impliqués dans le devenir d'une greffe rénale restent encore mal compris. Tandis que le rejet chronique demeure malheureusement la principale cause de perte du greffon, la tolérance opérationnelle reste un phénomène très rare. Peu de choses ont été décrites sur la tolérance opérationnelle due à la rareté des patients et à l'absence de marqueur de détection de la tolérance. Les observations faites jusqu'à présent sont assez disparates et souvent contradictoires, ceci étant probablement dû à la difficulté d'étudier ces types de patients. De plus, il est très difficile de savoir si ces observations sont la cause ou la conséquence de l'état du greffon.

Nous disposons d'une petite cohorte de patients transplantés rénaux, opérationnellement tolérants, ayant arrêté totalement leurs traitements immunosuppresseurs et n'ayant aucun signe de rejet aigu ou chronique. J'ai étudié les cellules du sang périphérique (analyse phénotypique, transcriptionnelle et fonctionnelle) chez ces patients opérationnellement tolérants par rapport à des patients avec un rejet chronique, des patients avec une fonction rénale stable sous immunosuppresseurs et des volontaires sains non greffés.

Nous avons montré, pour la première fois, l'existence de cellules $CD4^+CD25^+FOXP3^+$ chez ces patients. Nous avons décrit une diminution du nombre et de la fonction de ces cellules chez les patients qui développent un rejet chronique par rapport aux patients spontanément tolérants.

2- Description des patients

Mon travail de thèse a consisté à analyser et comparer le phénotype et la fonction des cellules de différents groupes de patients transplantés rénaux :

1- Patients présentant une tolérance opérationnelle après une transplantation rénale. Il s'agit de patients recrutés avec la participation de nombreux centres français de transplantation rénale. Ces patients sont très rares, et les études portant sur les mécanismes impliqués dans la survenue de cet état de tolérance opérationnelle sont donc limitées. Notre laboratoire a publié récemment les détails cliniques de ces patients (Roussey-Kesler, Giral et al. 2006) (**tableau 3**) (annexe 5). Ce sont des patients qui ont arrêté tout traitement immunosuppresseur depuis au moins 1 an, qui ont une fonction rénale stable avec une clairance > 40 ml/min et une protéinurie < 1 g/j. Ces patients ne présentent pas de signes cliniques d'immunosuppression, puisque depuis l'arrêt des traitements immunosuppresseurs, ils n'ont pas eu d'infections

sévères. Ballet et al. ont montré que certains patients présentaient une faible réponse humorale, similaire à celle de patients stables sous traitement immunosuppresseur, tandis que d'autres avaient une réponse forte similaire à celle de sujets sains (Ballet, Roussey-Kesler et al. 2006). Ces patients sont très hétérogènes d'un point de vue de leurs maladies initiales et de leurs thérapies d'induction. Par contre, ils ont pour la plupart reçu plusieurs transfusions sanguines, 6 ± 7.3 transfusions, mais présentent cependant, une immunisation anti-HLA avant la greffe globalement faible $9 \pm 3.9\%$ de PRA. Le jeune âge des donneurs et une reprise de fonction du greffon pas ou peu retardée reflètent par ailleurs probablement la bonne qualité des greffons rénaux. Ce paramètre, associé à l'arrêt progressif de l'immunosuppression, constitueraient des éléments favorisant potentiellement la survenue d'un état de tolérance opérationnelle (Roussey-Kesler, Giral et al. 2006) (annexe 5).

Tableau 3:Caractéristiques cliniques des patients opérationnellement tolérants

Patients	Tol1	Tol2	Tol3	Tol4	Tol5	Tol6	Tol7	Tol8	Tol9	Tol10	Tol11
Sexe	H	H	H	H	H	F	F	H	H	H	F
Date de greffe	1973	1981	1987	1987	1991	1993	1994	1998	1980	1987	2001
Maladie rénale initiale	Glomérulopathie membrano-proliférative	Uropathie obstructive	Glomérulopathie d'origine indéterminée	Uropathie obstructive	Uropathie obstructive	Néphropathie interstitielle	Hypoplasie rénale	Néphropathie interstitielle	Glomérulopathie Hypocomplémentaire	Glomérulopathie d'origine indéterminée	Reflux
Age du donneur	25	7	16	ND	21	39	30	28	17	30	ND
Age du receveur	42	15	41	19	13	64	27	27	43	52	38
Transfusions Pré-greffe	ND	6	4	ND	2	24	5	2	2	4	ND
PRA pré-greffe	0	7%	0	ND	12%	9%	15%	0	7%	4%	ND
Incompatibilités HLA	1	3	4	4	3	1	4	0	3	3	4
Nombre de greffe	2	1	1	1	1	1	1	1	1	1	2
Thérapie d'induction	Cyclo-phosphamide	non	ATG	ATG	ATG	non	ATG	non	non	Anti-IL2R	ND
Rejet aigu	non prouvé	oui	non	oui	oui	oui	non	non	non	non	oui
Infections	HCV	HCV	non	ND	non	non	non	non	non	non	non
Raison de l'arrêt des IS	Non compliance	Non compliance	Non compliance	Non compliance	PTLD	Rein non fonctionnel	Non compliance	Non compliance	Non compliance	PTLD	Non compliance
Anti-HLA après l'arrêt des IS	non	non	non	non	non	Anti-classe II DR11	Anti-classe II DR04	non	non	Suspecté Anti-classe II	ND
Période sans IS	17	15	6	10	7	12	2	6	13	9	3
Devenir	Toujours tolérant	Toujours tolérant	Toujours tolérant	Toujours tolérant	Toujours tolérant	Toujours Tolérant	Dysfonction Chronique	Toujours Tolérant	Dysfonction Chronique Décédé	Dysfonction Chronique	Toujours Tolérant

Les données du tableau 3 représentent des constatations (PRA, nombre de transfusions, âge du donneur...). Elles ne sont pas intervenues dans les critères de choix pour la sélection des patients. Seule une bonne fonctionnalité du greffon (clairance > 40 ml/min et une protéinurie < 1g/j) chez des patients sans traitement immunosuppresseurs depuis plus d'un an a permis la sélection des patients. La plupart des patients ont arrêté leur traitement de leur plein gré (non compliance) et 2 pour désordre lymphoprolifératif post-transplantation (PTLD). Seuls 2 patients présentent une infection virale (hépatite C). Neuf patients sur onze ont reçu une seule greffe et 2 ont dû être greffés. Après la transplantation, 2/11 patients ont développé des anticorps anti HLA du donneur. Trois patients ont développé récemment une dysfonction chronique et ont été exclus de l'étude. L'un d'entre eux est récemment décédé.

Dans l'article n°1 ont été inclus les patients Tol 1, 2, 3, 4, 5, 8, 9, 10. Dans l'article n°2 ont été inclus pour le phénotype les patients Tol 1, 2, 3, 5, 6, 8, 11, et pour les expériences de coculture (nécessité d'avoir beaucoup de cellules) les patients Tol 2, 3, 5, 6, 8. Ce choix a été réalisé en fonction du nombre de cellules disponibles.

2- Patients greffés présentant une dégradation progressive de leur fonction rénale (protéinurie > 1g/j) et des signes histologiques de rejet vasculaire chronique ou néphropathie chronique du transplant (endartérite, glomérulopathie, $\pm$ C4d). Parmi eux, certains ne recevaient plus de traitement immunosuppresseur au moment du prélèvement et étaient sous hémodialyse.

3- Patients greffés ayant une fonction rénale stable, sous immunosuppression standard.

4- L'analyse a aussi porté sur des patients sous immunosuppression minimale qui ont les mêmes critères de stabilité de la fonction rénale que les tolérants mais qui sont sous faible dose de corticostéroïdes (<10mg par jour) depuis 7.1 ± 6.3 ans.

5- Enfin, des individus sains non greffés sans infection connue depuis au moins 6 mois avant le prélèvement ont servi de groupe contrôle.

Résultats

1- Article I

*Profil d'expression des cellules T CD4⁺CD25^{high}
différent entre les patients greffés rénaux en rejet
chronique et les patients « opérationnellement tolérants »*

Par: **Braudeau C.** *, Louis S. *, Giral M., Dupont A., Moizant F., Robillard N., Soulillou JP.
and Brouard S.

** Les auteurs ont contribué de manière égale à ce travail*

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Titre Original: *Contrasting CD25^{hi}CD4⁺T cells/FOXP3 patterns in chronic rejection and
operational drug-free tolerance*

Contrasting CD25^{hi}CD4⁺T Cells/FOXP3 Patterns in Chronic Rejection and Operational Drug-Free Tolerance

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Background. Although immunosuppression withdrawal in kidney recipients usually leads to rejection, in some patients it does not, leading to a state of clinical operational tolerance.

Methods. We compared these highly contrasted situations by analyzing blood cell phenotype and transcriptional patterns in drug-free spontaneously tolerant kidney recipients, recipients with chronic rejection, recipients with stable graft function under standard or minimal immunosuppression and healthy individuals

Results. The blood cell phenotype of clinically tolerant patients did not differ from that of healthy individuals. In contrast, recipients with chronic rejection had significantly less CD25^{hi}CD4⁺T cells and lower levels of FOXP3 transcripts compared with clinically tolerant recipients. Patients with chronic rejection also displayed CD25^{hi}CD4⁺T cells expressing NKG2D⁺CD94⁺ and CD57⁺CD27⁺CD28⁺ cytotoxic-associated markers ($P < 0.05$).

Conclusion. These data show that whereas clinically tolerant recipients displayed normal levels of CD25^{hi}CD4⁺T cells and FOXP3 transcripts, chronic rejection is associated with a decrease in CD25^{hi}CD4⁺T cells and FOXP3 transcripts, suggesting that clinically "operational tolerance" may be due to a maintained phenomenon of natural tolerance that is lacking in patients with chronic rejection.

Keywords: Chronic rejection, Kidney, Tolerance, Phenotype.

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Continuous improvements in immunosuppression and the clinical management of transplant recipients have contributed to increasing graft survival and to limiting the risks of rejection in kidney transplantation (1). However, such lifelong immunosuppression, which poorly influences long-term chronic transplant dysfunction (2), may promote tumor growth by a direct effect on tumoral cells (3) and may also decrease recipient immune responses to pathogens, including oncogenic viruses (4, 5). In humans, immunosuppression withdrawal leads, in most cases, to transplant rejection. Nevertheless, certain rare patients maintain stable graft function despite the absence of treatment, suggesting that a state of nonresponsiveness can be achieved in clinical transplantation (6). This phenomenon, which has been particularly described in liver transplantation (7, 8), can also occur in kidney recipients (6). Numerous studies have investigated the mechanisms involved in transplantation tolerance. Data

from various experimental models suggest that suppressor/regulatory T cells may be involved in the induction and maintenance of tolerance (9, 10). These cells have been shown to display different phenotypes including CD28⁺CD8⁺T cells, natural killer T cells (NKT), IL10-producing Type 1 regulatory cells, "natural" CD25⁺CD4⁺T cells, TGF β -producing T helper 3 cells, CD4⁺CD8⁺CD3⁺T cells and anergic CD4⁺T cells (11). Interestingly, some studies have also shown that a decrease in the number of natural regulatory CD25⁺CD4⁺T cells may be involved in certain suspected cases of abnormal immune regulation (12, 13). A lack of regulation by CD25^{hi}CD4⁺ cells has also been suspected in several clinical conditions such as rheumatoid arthritis (14), multiple sclerosis (15) and graft-versus-host disease (GVHD) (16). Due to their rarity, few studies have been done on operationally tolerant graft recipients. Recently, Li et al. reported phenotypic changes in patients tolerant to liver transplants (17). However, to date, no extensive studies of phenotype or transcriptional patterns have been performed on the blood of kidney recipients with such a highly contrasted clinical state as that between drug-free clinical tolerance and chronic rejection. Furthermore, no indication is available as to whether "operational tolerance" is the result of an active regulatory process that is lacking in chronic rejection.

In this paper, we analyzed, for the first time, the blood mononuclear cell phenotype in such patients and extended our study to investigating key factors that have been implicated in regulatory processes (such as FOXP3, CTLA4 and GITR). We report that drug-free clinically tolerant patients have normal numbers of CD25^{hi}CD4⁺T cells and FOXP3 transcripts. In contrast, this phenotype was found to be significantly altered in patients with chronic rejection. These data suggest that the state of clinically "operational tolerance" may resemble that of self-tolerance and that chronic rejection

S.L., C.B., J.P.S., and S.B. contributed equally to this work.

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may be favored by a reduction in CD25^{hi}CD4⁺ cells and low-levels of FOXP3 transcription.

PATIENTS, MATERIALS AND METHODS

A total of eight drug-free clinically tolerant (DF-Tol) recipients, 11 minimally immunosuppressed (MIS) recipients, 17 chronic rejection (CR) patients, 15 stable (Sta) patients, and 14 healthy individuals were included in the present study. Because of the rarity of DF-Tol patients and because the experiments performed span several years, the different analyses were not performed on all patients.

Drug-Free Clinically Tolerant Recipients

DF-Tol recipients ($n=8$; mean age: 46.5; range: 25–73) were patients with a stable kidney graft function in the absence of immunosuppression for at least 2 years (range: 2–17), except for one who was drug-free for 6 months (18). Seven of the eight patients were cadaveric graft recipients. These patients had a creatinemia inferior to 160 $\mu\text{mol/L}$ and a proteinuria below 0.5 g/day, as indicated in Figure 1. Biopsies were not taken from these patients due to recommendations by the University Hospital Ethical Committee and the Commit for the Protection of Patients from Biological Risks. Their clinical history is summarized in Table 1.

Minimally Immunosuppressed Recipients

MIS patients ($n=11$; mean age: 58; range: 28–83) received low doses (≤ 10 mg/day) of prednisone monotherapy and exhibited a creatinemia inferior to 160 $\mu\text{mol/L}$ and a proteinuria below 0.5 g/day. All other immunosuppressive drugs had been stopped for 7.1 ± 6.3 years due to medical reasons. As for the DF-Tol patients, due to ethical reasons, no biopsy was taken from these patients with normal graft function.

Chronic Rejection Group

CR patients ($n=17$; mean age: 53.1 years; range 27–74) exhibited a progressive degradation of their renal function (Fig. 1). In all cases, a biopsy confirmed signs of chronic vascular rejection which, in nine cases (53%), was associated with an allograft glomerulopathy. Eleven of the 17 patients with chronic rejection were under immunosuppression (treated CR). Six patients with CR, who had returned to dialysis and had been off immunosuppressive treatment for at least one year (DF-CR), were also studied in an attempt to identify immunosuppressive drug-related patterns. In order to take the effects of renal insufficiency into account, patients with non immunological kidney diseases (polycytosis or uterine reflux) with a moderate-to-severe level of chronic renal failure (creatinemia: 455 $\mu\text{mol/L}$, range: 200–800) (RI, $n=5$) were also included. Finally, nine patients who had not received a transplant but were on chronic dialysis were also studied (Dialysis).

Stable Group

The stable group ($n=15$; mean age 50.5 years; range 22–78) had a creatinemia lower than 160 $\mu\text{mol/L}$ and a proteinuria below 0.5 g/day (Fig. 1) under standard immunosuppression (calcineurin inhibitor and azathioprine or mycophenolate mofetil and/or steroids).

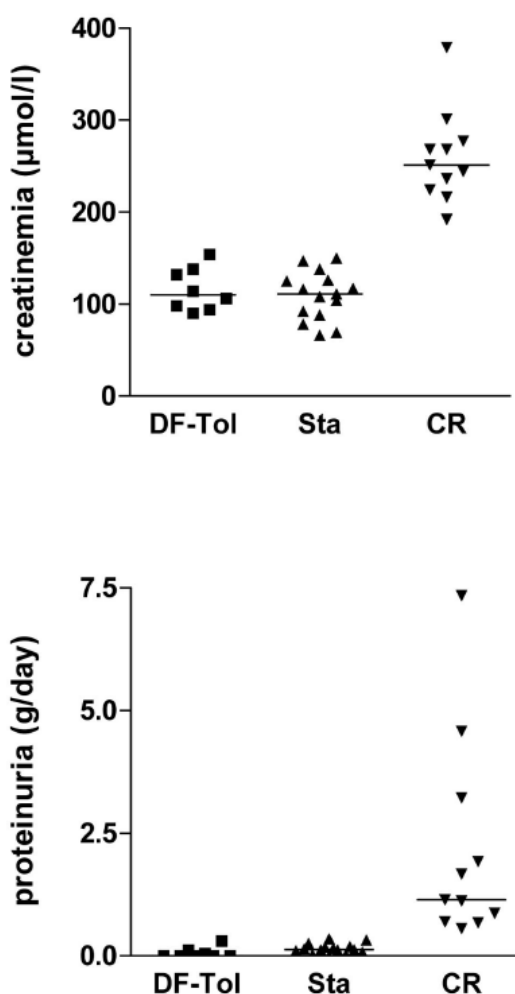


FIGURE 1. Graft function according to each patient group. Creatinemia and daily proteinuria in the DF-Tol (■), Sta (▲) or CR (▼) patient groups. DF-Tol and Sta patients display the same low level of each parameter, significantly lower than in the CR group.

Healthy Individuals

The healthy subjects (N^L ; $n=14$; mean age 47.9 years; range 25–60) included individuals with a normal blood formula and no infectious pathology for at least 6 months prior to the study.

Antibodies

Antibodies were purchased from Beckman Coulter (Marseille, France) (anti-CD45 FITC, anti-CD3 PC5 or PC7, anti-CD4 APC, anti-CD8 APC or PE, anti-CD16 PE, anti-CD56 PE, anti-CD19 PE, anti-CD28 FITC, anti-CD160 PE, anti-CD45RA FITC, anti-CD62L PE, anti-TCR V α 24 PE, anti-TCR $\gamma\delta$ FITC, anti-TCR $\alpha\beta$ PE and anti-CD40L PE), from

TABLE 1. Clinical history of drug-free clinically tolerant (DF-Tol) recipients

Patient	1	2	3	4	5	6	7	8
Age at study (years)	37	73	71	24	38	58	36	38
Sex	Male	Female	Male	Male	Male	Male	Male	Male
Number of grafts	1	1	2	1	1	1	1	1
Year of graft	1998	1993	1974	1991	2000	1987	1987	1981
Induction therapy (duration)	No	No	No	ATG (6 days)	anti-IL2 mAb	ATG (15 days)	ND	No
HLA -A-B-DR ^a	0 (living related donor)	3S	ND	3	3	4	ND	2 (HLA-DR typing not performed)
Pregraft anti-T PRA (%)	0	0	ND	0	ND	0	ND	0
Acute rejection episode	No	2	No	No	No	No	1	1
CMV infection	No	No	No	No	No	No	ND	No
Other viral infections	No	No	HCV	No	No	Herpes	ND	HCV
Reason of immunosuppression interruption	uncompliance	CNI toxicity	uncompliance	PTLD	uncompliance	uncompliance	uncompliance	uncompliance
Time drug-free	3 years	8 years	12 years	3 years	6 months	3 years	7 years	17 years

Patient 3 received a second graft. The mean HLA-A, -B and -DR incompatibility was 2.6. Patients 5, 6, and 7 received an induction therapy after transplantation with a 10-day course of an anti-IL2- α antibody, a 15-day therapy of ATG, and a 6-day therapy of ATG, respectively. Patient 2, 7 and 8 underwent acute rejection episodes. No patient in this group had a CMV disease during their follow-up. Patients 3 and 8 were chronically infected by the hepatitis C virus.

^a S indicates typing by serology.

BD Biosciences (Mountain View, CA) (anti-HLA-DR PE, anti-CD25 PE or FITC, anti-CD4 APC, anti-CCR4 PE, anti-CCR5 PE and anti-CD103 PE), from Santa Cruz Biotechnology (Santa Cruz, CA) (anti-TLR4 PE) and from R&D Systems (Minneapolis, MN) (anti-GITR PE, anti-CXCR3 PE, anti-CCR7 PE and anti-CCR9 PE).

Purification of Blood Cell Subsets from PBMC and Surface Antigen Analysis

Peripheral blood mononuclear cells (PBMC) separated on a Ficoll layer (Eurobio, Les Ulis, France) were used directly or conserved in a medium consisting of PBS/50% autologous serum /10% DMSO and frozen in liquid nitrogen. For RNA extraction, PBMC were frozen in TRIzol reagent (Invitrogen, Cergy Pontoise, France). Negative selection of CD4⁺/CD8⁺ T cells was performed using MACS human CD4⁺ or CD8⁺ T cell isolation kits II (Miltenyi Biotec, Germany). Purity was routinely >85%. Extracellular four-color staining was performed either on whole blood or on PBMC. For intracellular staining, cells were permeabilized with PBS/0.5% saponin. Four-color staining was analyzed using a FACS Calibur with Cellquest Pro software (BD Biosciences, Mountain View, CA).

Quantitative Reverse-Transcriptase Polymerase Chain Reaction

Messenger RNA was reverse transcribed using the M-MLV reverse-transcriptase kit (Invitrogen, Cergy Pontoise, France) for PBMC and the RT Omniscript kit (Qiagen, Courtaboeuf, France) for CD4⁺ and CD8⁺ T cells. Real-time

quantitative polymerase chain reaction (PCR) was performed using labeled TaqMan probes. GAPDH, HPRT, FOXP3, CTLA4, TGF β 1, IL-2, PD1 and GITR quantitative analyses were performed using Assay on Demand products (Applied Biosystems). HPRT and GAPDH were used as endogenous controls to normalize RNA amounts. Transcript levels were calculated according to the $2^{-\Delta\Delta C_t}$ method (ABI PRISM 7900 user bulletin, PE Applied Biosystems, Foster City, CA).

Polyclonal Stimulation and Cytokine Production by CD4⁺ and CD8⁺ T Cell Subsets

Microplates (96-well flat-bottom) were coated with 0.1 μ g/ml of anti-CD3 antibody (Orthoclone OKT3[®], Janssen-Cilag, Germany) for 2 hr at 37°C. Next, 5×10^4 CD4⁺ or CD8⁺ T cells were distributed per well in duplicate in a total volume of 0.2 ml of culture medium consisting of RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1% nonessential aminoacids, 1 mM sodium pyruvate and 10% of heat inactivated FCS. An anti-CD28 antibody (BD Biosciences) and IL2 (Proleukin, Chiron Corporation, Emeryville, CA) were added at the respective concentrations of 1 μ g/ml and 100 UI/ml. After 72 h at 37°C in 5% CO₂, [³H]TdR incorporation over a 16-h period was measured using standard scintillation procedures (Packard Institute, Meriden, CT). Data were expressed as an index corresponding to (cpm anti-CD3 + anti-CD28) $\times$ 100 / (cpm anti-CD3 + anti-CD28 + IL2). After 72 hr, supernatants from the prior stimulation experiments were harvested and cytokines were quantified using the Th1/Th2 cytokine kit II (BD Biosciences, Mountain View, CA).

Statistical Analysis

The Mann-Whitney and Kruskal-Wallis nonparametric tests were performed in the appropriate conditions. A Wilcoxon paired test was used for the longitudinal study. Differences were defined as statistically significant when $P < 0.05$.

RESULTS

CR Recipients Display a Decrease in Circulating B Cells

No significant difference was observed for the CD45RA and CD62L markers for CD4⁺ and CD8⁺ T cells between DF-Tol, CR recipients and healthy individuals (Supplemental Ta-

ble 1, available at <http://www.transplantjournal.com>). CR recipients displayed a significantly lower absolute number of B cells than DF-Tol recipients ($P < 0.05$; Fig. 2A) but these numbers were not significantly different to those of healthy individuals. Moreover, drug-free and immunosuppressed CR patients as well as patients on dialysis and patients with renal insufficiency displayed less B cells than DF-Tol recipients ($P < 0.05$; Fig. 2A). Taken together, this suggests that the difference in blood B cell counts between CR and DF-Tol patients may be more related to renal failure than to chronic rejection per se. Finally, no significant difference was found for NK cells (Supplemental Table 1, available at <http://www.transplantjournal.com>).

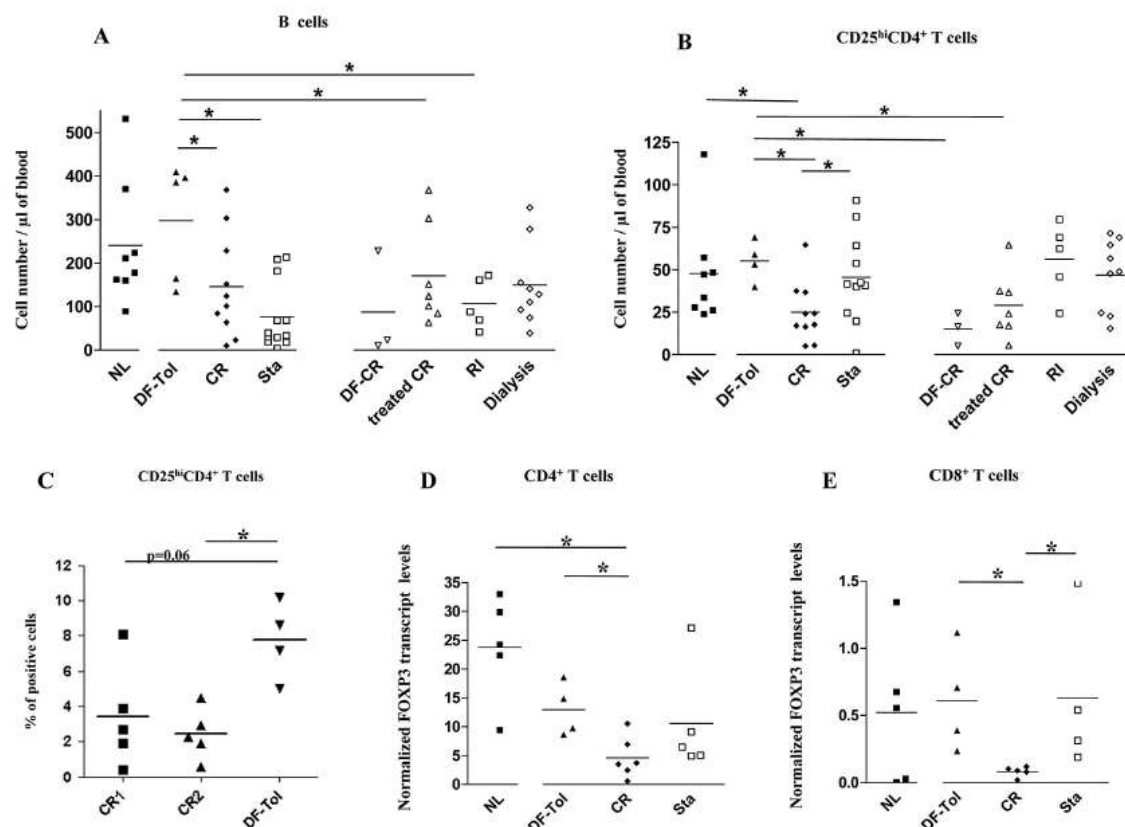


FIGURE 2. Cell populations in the different patient groups. (A) Number of circulating B cells (in cell number per microliter of blood) were analyzed by direct whole blood staining and compared between DF-Tol ($n=5$), CR ($n=10$), Sta ($n=12$) recipients and N^L individuals ($n=9$). To test the influence of immunosuppression, dialysis and renal insufficiency, four control groups were also compared (DF-CR, treated CR, dialysis and RI). (B) "Potentially regulatory" CD25^{hi}CD4⁺ T cells in DF-Tol and CR recipients. Peripheral CD25^{hi}CD4⁺ T cells from DF-Tol ($n=4$), CR ($n=10$), Sta ($n=12$) recipients and N^L individuals ($n=9$) were analyzed by direct whole blood staining. To test the influence of immunosuppression, dialysis and renal insufficiency, four control groups were also compared (DF-CR, treated CR, dialysis and RI). The results are expressed as cell number per microliter of blood. (C) Stability of CD25^{hi}CD4⁺ T cell numbers over time. The percentage of CD25^{hi}CD4⁺ T cells was analyzed longitudinally in five CR recipients and compared with DF-Tol patients. (D, E) FOXP3 transcript analysis in CD4⁺ and CD8⁺ T cells from different groups. Real-time PCR analysis at the FOXP3 mRNA level (normalized with HPRT) were performed on peripheral CD4⁺ (D) and CD8⁺ (E) T cells from DF-Tol ($n=4$), CR ($n=6$) and Sta ($n=5$) kidney recipients and healthy individuals ($n=5$). *Significant difference ($P < 0.05$).

CR Recipients Have Less CD25^{hi}CD4⁺T Cells than DF-Tol Patients and Healthy Individuals and Lack FOXP3 Transcripts

We next looked for markers that have been associated with regulatory T cells in various experimental and clinical situations (9, 19, 20) in purified CD4⁺ and CD8⁺ blood T cells from the various groups. TCR $\alpha\beta$ ⁺CD8[−]CD4[−]T cell and CD28[−]CD8⁺T cell numbers did not differ between DF-Tol patients, CR patients and healthy individuals (Supplemental Table 1, available at <http://www.transplantjournal.com>). Interestingly, CR patients displayed significantly less CD25^{hi}CD4⁺T cells than DF-Tol recipients and healthy individuals, both in absolute value (Fig. 2B) and in percentage (data not shown; $P < 0.05$). This reduction was observed in both the treated CR patients and in the DF-CR recipients ($P < 0.05$; Fig. 2B), and coincided with the normal average values observed in immunosuppressed stable patients (45.4 ± 26.3 CD25^{hi}CD4⁺T cells/ μ l), suggesting that this decrease was not related to immunosuppression. It was not due to the initial ATG induction treatment either (data not shown). Moreover, the values of CD25^{hi}CD4⁺T cells in patients on dialysis or with RI were normal (46.7 ± 21.2 cells/ μ l and 54.6 ± 24.7 cells/ μ l respectively, $P = \text{NS}$; Fig. 2B), indicating that the decrease in CD25^{hi}CD4⁺T cells was not related to dialysis and/or renal insufficiency. The stability of the percentage of CD25^{hi}CD4⁺T cells over time was analyzed longitudinally in five CR patients (at 2 to 41 month intervals) (Fig. 2C). No statistical differences were observed between the first and second samples in these five patients (respectively 3.4% and 2.4%, $P = 1.0$), who had significantly lower CD25^{hi}CD4⁺T cell numbers than DF-Tol patients (7.8%).

FOXP3 transcript levels were analyzed in CD4⁺ and CD8⁺T cells from CR patients, DF-Tol recipients and healthy individuals. Two house keeping genes (HPRT and GAPDH) were used to normalize the FOXP3 transcript levels. Interestingly, a significant decrease in FOXP3/HPRT (and FOXP3/GAPDH, data not shown) transcript levels was observed in the CD4⁺ and CD8⁺T cells of CR patients compared to those of DF-Tol recipients and healthy individuals ($P < 0.05$) (Fig. 2, D and E). CTLA4, TGF β 1, IL-2, PD1 and GITR transcript levels did not differ between the different groups (data not shown). Taken together, these data suggest a deficit in CD25^{hi}CD4⁺T cells and in FOXP3 transcripts in CR patients, rather than an accumulation of these subsets and transcripts in DF-Tol recipients, who display a cell profile similar to healthy individuals.

Recipients Under Standard Immunosuppression With Stable Graft Function Display a Composite Blood Phenotype, Analogous to That of Either DF-Tol or CR Patients

Interestingly, some Sta patients shared characteristics with DF-Tol and/or with CR recipients. As for the DF-Tol group, a significantly higher number of CD25^{hi}CD4⁺T cells was detected compared with CR recipients ($P < 0.05$; Fig. 2B). The levels of FOXP3 and IL2 transcripts were higher in CD8⁺/CD4⁺T cells from Sta patients than in those from CR recipients ($P < 0.05$; Fig. 2, D and E). They had significantly less B cells ($P < 0.05$; Fig. 2A), a lower level of TGF β transcripts in their CD4⁺T cell compartment and a lower level of

PD1 transcripts in their CD8⁺T cell compartment (data not shown) compared with DF-Tol patients ($P < 0.05$). Taken together, these results suggest this group of patients to be highly heterogeneous.

The CD25[−]CD4⁺T Cell Subset of CR Patients Expresses Molecules Associated With a Cytotoxic-Effector Cell Phenotype (CD27[−]NKG2D⁺CD94⁺CD57⁺CD28[−])

We subsequently focused on the expression of chemokine receptors (CCR4, CCR8, CXCR3, CCR5, CCR9 and CCR7), clusters of molecules classically associated with cytotoxic cells (CD28, CD27, CD57, perforin, CD94, NKG2D and KIR-NKAT2), activation/memory markers (CTLA4, CD40L, CD95, CD62L, CD45RO, CD69 and CD127) and regulatory markers (GITR, CD103 and TLR4). For all the molecules tested, the CD25⁺CD4⁺ and CD25^{hi}CD4⁺T cell populations displayed the same phenotypic profile (data not shown), i.e. they expressed significantly more CCR4, GITR, CD103 and intracellular CTLA4 than their CD25[−]CD4⁺T cell counterparts ($P < 0.05$; Fig. 3A). Moreover, CR patients were characterized by a CD25[−]CD4⁺T cell population expressing more NK associated molecules, NKG2D, CD94 and CD57 and less CD27/CD28 ($P < 0.05$; Fig. 3B and data not shown) than their CD25⁺CD4⁺T cell counterparts, a pattern that has been associated with cytotoxic-effector cells (21). No difference was observed for the expression of the chemokine receptors (CXCR3, CCR5, CCR7 and CCR8), activation/memory cell markers (CD40L, CD95 and CD45RO) and other molecules associated with cytotoxic-effector cells (intracellular perforin and KIR-NKAT2) (data not shown).

Minimally Immunosuppressed Recipients Under Low-Dose Corticosteroids are Lymphopenic but Display the Same Marker Distribution as DF-Tol Recipients

The fact that the MIS recipients displayed a good and stable graft function for years following minor immunosuppressive drug treatment suggests that they may also be clinically "operationally" tolerant. We thus compared their blood cell phenotype with that of DF-Tol recipients. MIS patients were significantly lymphopenic ($P < 0.05$), displaying less B cells ($P < 0.01$) and consistently less T cells than DF-Tol recipients (TCR $\alpha\beta$ ⁺, CD4⁺, CD25⁺CD4⁺, CD25^{hi}CD4⁺ (Fig. 4), as well as less naïve CD45RA⁺CD62L⁺CD8⁺/CD4⁺, central CD45RA[−]CD62L⁺CD4⁺ and effector memory CD45RA[−]CD62L[−]CD4⁺T cell subsets ($P < 0.05$; Supplement Table 1 available at <http://www.transplantjournal.com>). Nevertheless, they exhibited a percentage of CD25⁺CD4⁺, CD25^{hi}CD4⁺T cells and B cells similar to that of DF-Tol recipients (Fig. 4). Taken together, our results show that, although MIS patients had significantly lower absolute B and T cell numbers probably due to a combination of chronic steroid intake (22), a past history of chemotherapy for malignancies and/or ATG induction treatment (23), they displayed percentages of CD25^{hi}CD4⁺T cells and B cells similar to DF-Tol recipients.

CD4⁺ and CD8⁺T Cells From DF-Tol Recipients Respond to Polyclonal Stimulation

Because no donor cells were available, as a preliminary approach, CD4⁺ and CD8⁺T cells from DF-Tol and CR re-

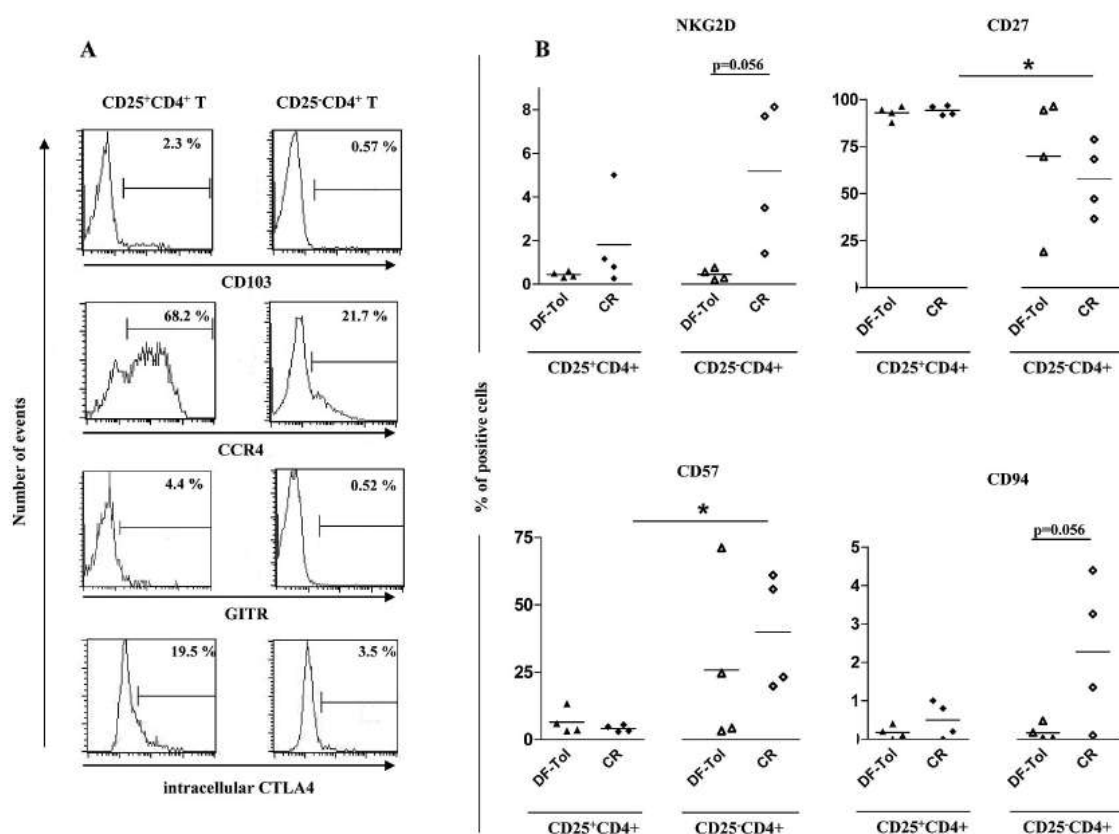


FIGURE 3. Expression of molecules in the CD25⁺CD4⁺ vs. the CD25⁻CD4⁺T cells. (A) "Potentially regulatory" molecules were compared in the CD25⁺CD4⁺ and CD25⁻CD4⁺T cell subsets from DF-Tol and CR patients and healthy individuals. Representative dot plots of differentially expressed molecules between the CD25⁺CD4⁺ (left panel) and CD25⁻CD4⁺ (right panel) T cell subsets in DF-Tol recipients are shown with the corresponding mean percentage. (B) Comparison of the expression of cytotoxic molecules in the CD25⁺CD4⁺ and CD25⁻CD4⁺T cell subsets between DF-Tol and CR recipients. The results are expressed as the percentage of positive cells amongst the gated CD25⁺CD4⁺ and CD25⁻CD4⁺T cells of the DF-Tol (n=4) and CR (n=4) patients. *Significant difference ($P < 0.05$).

recipients and healthy individuals were stimulated with a combination of anti-CD3 and anti-CD28 antibodies with or without IL2. Proliferation and production of a panel of Th1/Th2 cytokines (IFN γ , TNF α , IL2, IL4, IL6 and IL10) was examined after 3 days of stimulation. Despite inter-individual variations in cytokine production between the CR and DF-Tol patients, no significant difference was observed in terms of proliferation and cytokine production by the CD4⁺ and CD8⁺T cells between the three groups (Fig. 5). Taken together, CD4⁺ and CD8⁺T cells from DF-Tol recipients proliferate following anti-CD3/anti-CD28 stimulation and produce as much Th1 and Th2 cytokines as healthy individuals and CR patients.

DISCUSSION

In this study, we analyzed blood cell phenotype and transcriptional patterns in kidney recipients presenting vari-

ous clinical statuses: clinically operational tolerance (drug-free or under minimal immunosuppression), chronic rejection and stable function under standard maintenance immunosuppression. These patterns were also compared with those of healthy individuals. The existence of such drug-free patients with a perfectly stable renal function suggests that tolerance, tentatively defined as "a state of quiescence of the transplanted organ, functioning without a destructive immune response" (24), can occur in clinical transplantation. Clinically tolerant kidney recipients are extremely rare and the time interval between engraftment and testing often exceeds a decade. Recently, we showed that some of these recipients displayed altered blood V β families with low accumulation of IL10, IL2, IL13, and IFN γ transcripts compared with chronically rejecting patients (18) suggesting hyporesponsiveness. The subsequent lack of viable donor cells (all involved a cadaveric donor) meant that we were unable to di-

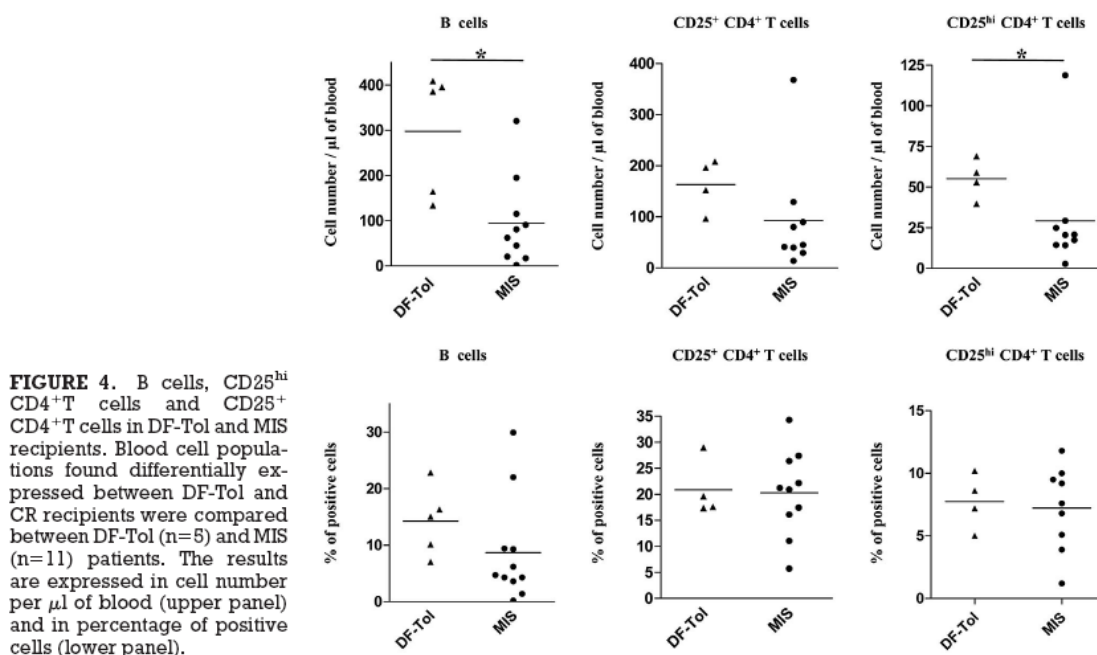


FIGURE 4. B cells, CD25^{hi} CD4⁺ T cells and CD25⁺ CD4⁺ T cells in DF-Tol and MIS recipients. Blood cell populations found differentially expressed between DF-Tol and CR recipients were compared between DF-Tol (n=5) and MIS (n=11) patients. The results are expressed in cell number per µl of blood (upper panel) and in percentage of positive cells (lower panel).

rectly test their anti-donor response. Nevertheless, T cells from these recipients do proliferate following anti-CD3/anti-CD28 or allogeneic stimulation (data not shown) and, when stimulated polyclonally, their T cells produced normal quantities of Th1 and Th2 cytokines. Despite the necessity of an analysis of the reactivity of these patients to surrogate donors or specific peptides (which has not yet been described in the literature), these primary data suggest that the hypothesis of hyporesponsiveness may not explain the tolerant state of these patients.

A possible mechanism that may explain the clinically tolerant status observed in these patients is the generation of regulatory cells, an active process that has been shown to contribute to the maintenance of established graft tolerance (9, 10) as well as self-tolerance (25) in various experimental models. However, to date, no specific phenotype or transcriptional pattern has been described in the blood of recipients with highly contrasted clinical situations (drug-free clinical tolerance or recipients with chronic rejection) and finally, no indication is available concerning an active regulatory process in "tolerance" or a lack of it in chronic rejection. In humans, regulatory CD25⁺CD4⁺ T cells, described in the blood (26, 27) and thymus (28) of healthy individuals, have been suggested to be involved in various pathological situations associated with abnormal self regulation (13, 15, 29–31). Although drug-free clinically tolerant patients displayed numbers of peripheral CD25^{hi}CD4⁺ T cells expressing intracellular CTLA4 (26), GITR (32), CCR4 (33), and CD103 (34) similar to healthy individuals, patients with chronic rejection exhibited a profound reduction in CD25^{hi}CD4⁺ T cells. This reduction was not due to immunosuppressive therapy, dial-

ysis or the level of renal insufficiency, as shown using appropriate controls, but seems to be related to chronic rejection.

The decrease in the numbers of CD25^{hi}CD4⁺ T cells observed in the CR recipients could have several other explanations. Firstly, CR recipients could have had low pretransplant CD25^{hi}CD4⁺ T cell numbers. This is unfortunately impossible to test due to the lack of availability of cells taken pretransplantation. Secondly, it has been suggested that such a decline may appear after transplantation due to a combination of drug-induced depletion and to a subsequent slow homeostasis of CD25^{hi}CD4⁺ T cells, which may be partly explained by a low availability of IL2 in the presence of calcineurin inhibitors (35, 36). We tested this hypothesis in patients with chronic rejection. However, no significant difference between the different groups was found for CD25^{hi}CD4⁺ and CD25⁺CD4⁺ T cell values. Recent studies have also demonstrated some variations in CD25⁺CD4⁺ T cell frequency in HIV (37) or HCV (12) infected patients. Nevertheless, in our study, viral infections do not seem to be responsible for the increased number of CD25^{hi}CD4⁺ T cells, as only two of the four DF-Tol recipients had experienced a viral infection (HCV and Herpes Virus) and both had CD25^{hi}CD4⁺ T cell numbers similar to those in healthy individuals. Unfortunately, the putative regulatory function of CD25^{hi}CD4⁺ T cells (16, 38) could not be assessed further in this study because of the limited availability of blood from these patients. A recent study in a model of lung transplantation (39) also suggests that a decrease in absolute CD25^{hi}CD4⁺ T cell number may contribute to the development of chronic rejection. Our results are also in accordance with the recent study by Li et al., who reported that tolerant

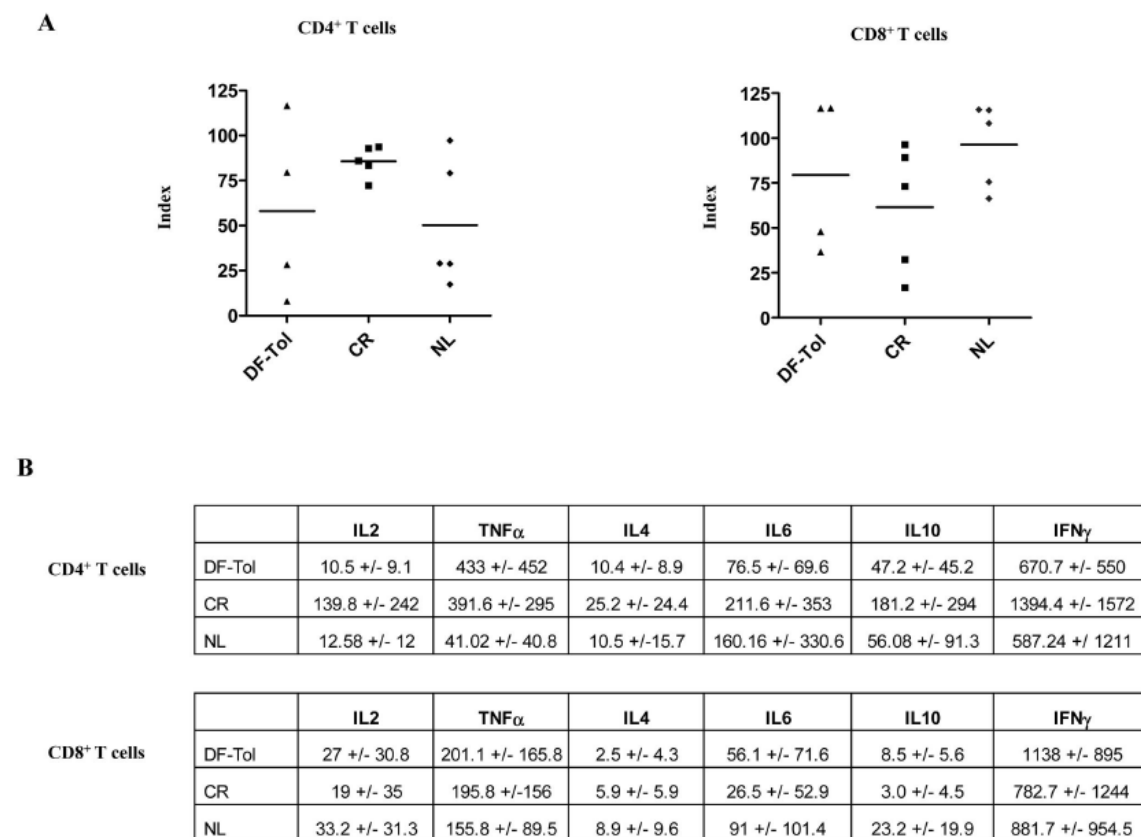


FIGURE 5. Response of polyclonally stimulated CD4⁺ and CD8⁺ T cells. Purified CD4⁺ and CD8⁺ T cells from DF-Tol (n=4) and CR (n=5) kidney recipients were stimulated with anti-CD3 and anti-CD28 antibodies with or without additional IL2. Proliferative responses (A) and Th1/Th2 cytokine production (B) were measured. Proliferation data are shown as an index corresponding to (cpm anti-CD3 + anti-CD28) * 100 / (cpm anti-CD3 + anti-CD28 + IL2). The results for IL2, IL4, IL10, IL6 and TNF α cytokines are expressed as pg/ml whereas values for IFN γ correspond to the mean of fluorescence.

liver recipients display as many CD25^{hi}CD4⁺ T cells (in absolute number) as healthy volunteers (17), whereas in the latter study, no comparison was made to patients with chronic rejection and CD25^{hi}CD4⁺ T cells were not characterized. In another study, Alvarez et al. observed, in patients with long-term surviving kidneys under immunosuppression (and not drug-free as in our study), an increase in CD25^{hi}CD4⁺ T cells compared with healthy volunteers, but not with chronic rejection recipients (40).

We also made the original observation that FOXP3 transcripts were strongly decreased in the CD4⁺ and CD8⁺ T cell subsets from chronically rejecting recipients compared with drug-free clinically tolerant patients and healthy individuals. FOXP3 has recently been proposed as one of the most relevant markers for CD25^{hi}CD4⁺ regulatory T cells in mice (41) and humans (42), and its decrease has been correlated with pathological disorders such as the development of GVHD (43) or autoimmune Myasthenia Gravis (44). Because of the low quantity of blood available for these patients, FOXP3 analysis was not performed directly in purified

CD25^{hi}CD4⁺ T cells but a positive correlation between CD25 expression and FOXP3 transcript levels was observed in the CD4⁺ T cells of the drug-free tolerant patients ($r=0.74$), whereas these molecules were negatively correlated in the chronic rejection group ($r=-0.78$). However, it remains to be determined whether this FOXP3 level is due to an increase in the amount of FOXP3 per CD25^{hi}CD4⁺ T cell or simply to the higher CD4⁺CD25⁺ cell number in the clinically operationally tolerant patients compared with patients with chronic rejection. In any case, both alternatives point towards a significant lack of CD25^{hi}CD4⁺ T cells and FOXP3 in patients with chronic rejection.

Suciu-Foca and co-workers reported the presence of suppressive CD28⁻CD8⁺ T cells that were able to inhibit the up-regulation of the costimulatory molecules CD80 and CD86 on donor antigen presenting cells in liver and kidney recipients with stable graft function (45). Neither the drug-free tolerant patients nor the immunosuppressed patients with a stable renal function from our own study displayed more blood CD28⁻CD8⁺ T cells than CR recipients. These

different results may be related to the fact that, in Suciu-Foca's work, the analysis was not performed in clinically tolerant patients but in stable patients under immunosuppression. In addition, this potentially suppressive population was only found in 40% of patients and only in the first few months following transplantation (46). Our patients (clinically tolerant and stable) were tested at a much later time point post-transplantation.

Surprisingly, CD25⁺CD4⁺T cells with a cytotoxic-associated phenotype (CD94⁺, NKG2D⁺, CD57⁺, CD27⁺, CD28⁺) (47, 48) were more abundant in patients with chronic rejection compared with drug-free clinically tolerant patients. Based on data from experimental animal studies, two hypotheses may explain this finding. Firstly, such cells may be induced directly against donor alloantigens and induce graft rejection (49). Secondly, they may originate from preexisting cells cytotoxic to viruses and cross-reactive with donor cells (50). Interestingly, a similar cytotoxic-associated phenotype was recently found in CD8⁺T cells from these patients with chronic rejection (51) and, as suggested by Zheng (52), chronic rejection might be supported by an imbalance between expanding cytotoxic/alloreactive T cells and decreasing regulatory T cells. Finally, recipients with chronic rejection displayed a reduced number of B cells in comparison with drug-free clinically tolerant patients and healthy individuals, a finding already reported for tolerant liver recipients (17). However, this feature seems to be related to a combination of several factors not associated with chronic rejection. We observed the same decrease in B cell counts in immunosuppressed patients with stable renal function. As mentioned in the paper by Li et al. (17), "it is not clear whether operational tolerance necessarily protects against development of chronic rejection to which humoral response to donor antigen may contribute". Particularly, in our study, there was no correlation in the stable patients between the presence of alloantibodies (donor-specific or not) and the number of blood B cells, as previously described in a study from our laboratory (53). Similarly, no correlation was found between B cell counts and alloantibodies in the CR recipients. Thus, this observation, which has been made in both liver and kidney transplant recipients, is not yet well understood.

Finally, our data also show that, despite a significant lymphopenia, probably due to ATG (23), chemotherapy and chronic steroid intake (22), minimally immunosuppressed patients displayed a percentage of CD25^{hi}CD4⁺T cells similar to drug-free clinically tolerant recipients. Thus, on the basis of their clinical status (i.e. stable long-term renal function with minimal immunosuppression), T cell phenotype and repertoire (18), this pattern suggests that minimally immunosuppressed patients may also be tolerant.

Taken collectively, our data suggest that clinically "operational" tolerance is not primarily due to an increased CD25^{hi}CD4⁺T cell pool. In addition, we show, for the first time, a reduction in the blood CD25^{hi}CD4⁺ pool and FOXP3 transcripts in patients with chronic rejection, suggesting that clinically "operational tolerance" may be due to a maintained phenomenon of natural tolerance that is lacking in patients with chronic rejection. We suggest that this newly described defect may contribute to the development of chronic rejection and should be studied longitudinally following transplantation to discriminate clinically tolerant patients from

patients who may develop chronic rejection in the context of reduced immunosuppressive therapy.

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2- Article II

Les patients transplantés rénaux en rejet chronique ont des cellules régulatrices $CD4^+CD25^+$ fonctionnellement différentes de celles de patients acceptant leur greffon à long terme

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Soumis

Titre Original: *A distinct pattern of $CD4^+CD25^+$ T cell regulatory function in patients with long term kidney graft survival and chronic rejection*

**A DISTINCT PATTERN OF CD4⁺CD25⁺ T CELL REGULATORY FUNCTION IN
PATIENTS WITH LONG TERM KIDNEY GRAFT SURVIVAL AND CHRONIC
REJECTION**

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ABBREVIATIONS

CR: Chronic Rejection

DF-Tol: Drug-Free Tolerant

FOXP3: Forkhead Box P3

HV: Healthy volunteers

LTS: Long term graft survival

PBMC: Peripheral Blood Mononuclear Cells

MLR: Mixed Lymphocyte Reaction

mAb: Monoclonal Antibody

ABSTRACT

Although novel immunosuppressive drugs have been shown to be effective in preventing acute rejection of solid organs, chronic rejection remains a major cause of graft loss in the long term. We have previously shown that patients with chronic rejection display fewer CD4⁺CD25^{high} blood T cells than patients with long term graft survival and healthy individuals. In this study, we analyzed the functional activity of these CD4⁺CD25^{high} cells in recipients with chronic rejection, long-term graft survival and healthy individuals. We assessed their ability to suppress the CD4⁺CD25⁻ cell response (proliferation/cytokine production) upon activation with anti-CD3mAb and autologous PBMC. CD4⁺ cells from chronic rejecters lacked CD25/FOXP3 expression and CD4⁺CD25^{high} cells displayed a decrease in their capacity to regulate stimulated CD4⁺CD25⁻ cells (53.1%±17) compared with patients with long-term graft survival (73.8%±10.2, $p<0.03$). In contrast, CD4⁺CD25^{high} cells from patients with long term graft survival, including operationally tolerant patients, showed a suppressive activity similar to healthy individuals (65.4%±10.9). Activated CD4⁺CD25^{high} cells from all patient groups were anergic and their suppressive activity was abrogated by the addition of IL2 and did not depend on IL10 or TGFβ. Our results indicate that chronic rejection is not only associated with a defect in the number of CD4⁺CD25^{high}FOXP3⁺ cells but also with an impaired capacity of CD4⁺CD25^{high} cells to suppress the proliferation of autologous CD4⁺CD25⁻ T cells. This “modulation” of activation was independent of IL10 and TGFβ.

INTRODUCTION

Despite improvement of immunosuppressive therapy over the last ten years, chronic rejection, characterized by progressive renal dysfunction with glomerulopathy, chronic interstitial fibrosis, tubular atrophy, vascular occlusive changes and glomerulosclerosis (Racusen, Halloran et al. 2004; Nankivell and Chapman 2006), remains a major cause of graft loss in the long term (Nankivell, Borrows et al. 2003; Sayegh and Carpenter 2004; Ballet, Giral et al. 2006). Moreover, long term exposure to immunosuppression leads to post-transplant infections (Fishman and Rubin 1998), malignancies and lymphoproliferative diseases (Dantal, Hourmant et al. 1998; Hojo, Morimoto et al. 1999) which contribute to the high level of morbidity and mortality observed in transplant patients (Soulillou and Giral 2001).

Compelling evidence indicates a crucial role for immunoregulatory $CD4^+CD25^+$ T cells in experimental rodent models of tolerance (Wood and Sakaguchi 2003). In humans, regulatory T cells may also be involved in the control of autoimmunity (Bluestone and Tang 2005), allergic diseases (Chatila 2005) and transplantation (Waldmann, Chen et al. 2006). Both a lack of regulatory $CD4^+CD25^+$ T cells with normal regulatory function (Boyer, Saadoun et al. 2004; Meloni, Vitulo et al. 2004) and a dysfunction of regulatory $CD4^+CD25^+$ T cells with defective suppressive properties have been described (Viglietta, Baecher-Allan et al. 2004).

In our previous study (Louis, Braudeau et al. 2006), we showed that patients with chronic rejection display a decreased number of $CD4^+CD25^{high}$ blood T cells expressing CTLA4, GITR, CCR4 and CD103 markers with a lack of FOXP3 transcripts in $CD4^+$ T cells, a gene associated with immunoregulatory T cell activity (Hori, Nomura et al. 2003; Ziegler 2005). These data suggest that a defect in $CD4^+CD25^{high}FOXP3^+$ T cell number may contribute to chronic rejection. In contrast, patients with good and stable graft function did not display a higher $CD4^+CD25^{high}$ T cell number, suggesting that $CD4^+CD25^{high}$ T cell activity may also be intrinsically different in these two situations. In this paper, we analyzed the $CD4^+CD25^{high}$ T

cell activity profile in patients with chronic rejection compared to patients with long term graft survival (including drug-free recipients) and healthy individuals. We showed that, when activated with an anti-CD3 antibody in the presence of autologous PBMC, $CD4^+CD25^{high}$ blood T cells from patients with chronic rejection displayed impaired suppressive properties on the proliferation of autologous $CD4^+CD25^-$ T cells compared to those from patients with long-term graft survival who displayed $CD4^+CD25^{high}$ T cells with a similar suppressive activity as healthy individuals. These data suggest that graft outcome is not only associated with a change in $CD4^+CD25^{high}$ T cell numbers but also with a modulation of their regulatory properties.

MATERIALS and METHODS

Patients

A total of 39 patients, matched for age, were included in the study. The clinical history of the patients is outlined in Table 1. The study was approved by our institutional University Hospital Ethical Committee and written informed consent was obtained from all patients. Donor cells were either unavailable or, when available, were unable to activate third party cells in mixed leukocyte culture, probably due to the long storage periods (a decade in many cases), confounded by the fact that PBMC harvested from cadaveric donors are known to be fragile. Because of the rarity of patients and because the experiments performed span several years, the different analyses were not performed on all patients.

Patients with long term graft survival (LTS): (n=22; mean age 51.8 ± 11.5 years; range 33-76) This group included 15 patients on maintenance immunosuppression with long term kidney graft survival (LTS-IS) and good renal function (clearance above 40 ml/min and proteinuria below 1g/day) (Table 1) and 7 patients with long term kidney graft survival who had been without any immunosuppressive drugs for 8.9 ± 5.3 years (range 2-17) (LTS-NoIS) (Roussey-Kesler, Giral et al. 2006). These recipients with a stable clearance above 38 ml/min and proteinuria below 1g/24h, are referred to as “operationally tolerant” (LTS-NoIS). Because of normal and stable graft function, no biopsy was taken from the operationally tolerant patients due to recommendations by the University Hospital Ethical Committee and the Committee for the Protection of Patients from Biological Risks.

Patients with chronic rejection (CR): (n=17; mean age: 50.3 ± 12.7 years; range 27-75). The grafts of these patients displayed a histology of chronic allograft nephropathy which has been associated with chronic rejection (Nankivell and Chapman 2006). Chronic rejection was

defined at histology by endarteritis and sclerosing transplant vasculopathy and/or transplant glomerulopathy with double contours of the glomerular basement membrane on light microscopy associated with frequent glomerular depositions of IgM by immunofluorescence. The patients with chronic rejection (CR) included in this study exhibited a glomerulopathy and a progressive degradation of their renal function under classical immunosuppression with a proteinuria above 1g/24h (Table 1). All patients received a maintenance immunosuppressive treatment (Table 1).

Healthy volunteers (HV): (n=10; mean age: 46.9 ± 7.9 years; range 36-61; sex 2M/8F)

Healthy individuals were age-matched volunteers with a normal blood formula and no infectious disease for at least 6 months prior to the study.

Isolation and purification of blood cell subsets from PBMC: Venous blood samples were collected in EDTA test tubes and processed for analysis within 6 hours. Peripheral Blood Mononuclear Cells (PBMC) were isolated by Ficoll hypaque gradient (Eurobio, Les Ulis, France). Cells were then incubated with microbeads and $CD4^+$ cells were purified using magnetic separation columns (Miltenyi Biotec, Germany). The negatively selected cells ($>90\%$ $CD4^+$) were subsequently incubated with $CD25$ microbeads and the $CD25^{high+}$ and $CD25^-$ cells were purified using magnetic cell separation columns (Miltenyi Biotec, Germany). To increase cell purity of the $CD4^+CD25^{high+}$ population, the positive fraction was separated in a second column leading to a purity $> 95\%$.

Reagents and FACS analysis: All patients and healthy individuals were first examined for complete blood count and screened for the presence of $CD4^+CD25^{high}$ T cells by flow cytometry as described elsewhere (Louis, Braudeau et al. 2006). Antibodies were purchased

from Becton Dickinson (San Jose, CA) (CD25-PE and PC5) Beckman Coulter (Marseille, France) (CD4-FITC) and BD Biosciences (Mountain View, CA) (CD3-PC7 and PC5, CD4-APC). Staining for intracellular FOXP3 protein was performed on PBMC with the PE-conjugated FOXP3 (PCH101) Ab according to the manufacturer's instructions (eBioscience, Clinisciences, France). Peripheral CD4⁺CD25^{high} T cells were analyzed by direct whole blood staining (Beckman Coulter Marseille, France). Flow cytometry was performed on a FACSCalibur with Cell Quest Pro software (BD Biosciences, Mountain View, CA).

CD4⁺CD25^{high} T cell proliferation: Experiments were performed on 5 LTS (5 LTS-NoIS and 5 LTS-IS) (mean age: 52.1 ± 11.8 years; range: 38-76; sex: 7M/3F; clearance 67.4 ± 19.5 ml/min; proteinuria: 0.3 ± 0.4 g/24h), 5 CR (mean age: 54.8 ± 12.5 years; range 42-75; sex 5F; clearance 34 ± 22.5 ml/min; proteinuria: 3.4 ± 2.4 g/24h) and 7 HV (mean age: 48.3 ± 9.5 years; range 36-61; sex 2M/5F). To test their proliferative response CD4⁺CD25^{high} T cells were stimulated with autologous irradiated (35Gy) PBMC. Twenty thousand autologous irradiated PBMC were added to 20,000 CD4⁺CD25^{high} T cells in a round bottom 96-well plate previously coated overnight at 4°C with 1 µg/ml CD3 mAb (Orthoclone OKT3®, Janssen-Cilag, Germany). After 72 hours of culture, 100 µl of supernatant was collected from each well to test for cytokine production, and cells were pulsed for 8 hours with 1 µCi per well of [3H] thymidine (Amersham Biosciences, UK). Cells were harvested and counted in a scintillation counter.

CD4⁺CD25^{high} T cell suppressive activity: CD4⁺CD25⁻ T cells responder cells were stimulated in the presence of 20,000 irradiated autologous PBMC cells in a round bottom 96-well plate previously coated overnight at 4°C with 1 µg/ml CD3 mAb (Orthoclone OKT3®, Janssen-Cilag, Germany). CD4⁺CD25⁻ T cells were plated at 20,000 cells/well in a final

volume of 200µl in complete medium. CD4⁺CD25^{high} T cells were added at ratios ranging from 1:1 to 1:4. Each proliferation assay was carried out in duplicate. Recombinant human IL2, anti-IL10 or anti-TGFβ were added to the culture to give a final concentration of 100U/ml, 1µg/ml and 10µg/ml respectively. After 72 hours of coculture, 100µl of supernatant was collected from each well to test for cytokine production, and cells were pulsed for 8 hours with 1µCi per well of [3H] thymidine (Amersham Biosciences,UK). Cells were harvested and counted in a scintillation counter. The effect of the addition of IL2 100UI/ml (Proleukin®, Chiron Corporation, Emeryville, CA), anti-IL10 1µg/ml (R&D Systems, UK) and anti-TGFβ 10µg/ml (clone 2G7) was analyzed. Culture medium consisted of RPMI-1640 supplemented with 2mM L-glutamine, 100U/ml penicillin, 0.1mg/ml streptomycin, 1% non-essential amino acids, 1mM sodium pyruvate and 10% heat inactivated human serum (Sigma, St Louis, USA).

Cytokine production: Cytokines were quantified in supernatants of CD4⁺CD25⁻ mixed lymphocyte reactions before and after addition of CD4⁺CD25^{high} T cells at day 3, using a multiplex fluorescent bead immunoassay, the FlowCytomix Multiplex human Th1/Th2 Kit II (Bender MedSystems, Tebu-bio, France). IFNγ, IL1β, IL2, IL4, IL5, IL6, IL8, IL10 and TNFα protein levels in cell culture supernatants were measured. Samples were analyzed on a BD FACSCalibur flow cytometer, according to the manufacturer's instructions. For each cytokine, the minimum detectable level was between 4.5 and 9.7 pg/ml. Values of $p < 0.001$ were considered as significant.

Statistical analysis: Data were presented as mean ±SD. The comparisons among the three groups of patients were performed using one-way analysis of variance (ANOVA) Kruskal-Wallis test and Dunn's test for multiple comparisons. The Mann-Whitney test, equivalent to

the Kruskal–Wallis test was used when applied to two groups. Values of $p < 0.05$ were considered as significant.

RESULTS

Patients with chronic rejection display fewer CD4⁺CD25⁺FOXP3⁺T cells than patients

with long term graft survival and healthy individuals: We previously showed that the T cell regulation-associated markers CTLA4, GITR, CCR3 and CD103 were expressed on CD4⁺CD25^{high} blood T cells from patients with chronic rejection at levels comparable to those found in age-matched healthy volunteers and patients with long term graft survival. Nevertheless, we also showed that the CD4⁺ T cells from patients with chronic rejection displayed decreased FOXP3 at the transcript level (Louis, Braudeau et al. 2006). In this study, we found a significant decrease in the co-expression of FOXP3⁺ and CD25⁺ protein on the CD4⁺ T cells from patients with chronic rejection ($p<0.01$ and $p<0,007$, patients with long term graft survival *versus* healthy individuals respectively) (Figure 1A, 1B). Within the patients with long-term graft survival, no difference was observed between operationally tolerant patients (free of immunosuppression) and patients under standard immunosuppression (Figure 1B). In contrast, the FOXP3 expression level on CD4⁺CD25^{high} was not different between the 3 groups (Figure 1C). These data show that blood from patients with chronic rejection display less CD4⁺CD25^{high}FOXP3⁺ T cells with a normal level of FOXP3 expression within these cells.

CD4⁺CD25^{high} blood T cells from patients with chronic rejection and long-term graft

survival display different suppressive functions: CD4⁺CD25^{high} T cells were purified from PBMC with microbeads. Because donor cells were no longer available, CD4⁺CD25⁻ T cells from patients were isolated in parallel and used as targets of suppression upon activation in the presence of anti-CD3 mAb and autologous irradiated PBMC. In all groups, a strong and similar CD4⁺CD25⁻ T cell proliferative response was obtained by day 3 of activation whereas CD4⁺CD25^{high} T cells poorly proliferated in the same conditions (Figures 2, 3, 4, 5). There was no difference between the proliferations of CD4⁺CD25^{high} T cells among the three groups (*data*

not shown). These data were confirmed by the absence of IL2 production by the stimulated $CD4^+CD25^{high}$ T cells in the three groups (*data not shown*). The $CD4^+CD25^{high}$ T cell unresponsiveness was reversed by addition of IL2, indicative of a state of anergy (Figures 2, 3, 4, 5). $CD4^+CD25^{high}$ T cells from healthy volunteers suppressed proliferation of autologous $CD4^+CD25^-$ T cells stimulated with anti-CD3 and autologous PBMC by an average of $65.4\% \pm 10.9\%$ (Figures 2, 6A). In comparison, $CD4^+CD25^{high}$ T cells from patients with chronic rejection and patients with long-term graft survival suppressed proliferation of autologous $CD4^+CD25^-$ T cells by an average of $53.1\% \pm 17\%$ and $73.8\% \pm 10.2\%$ respectively (Figures 3, 4, 5, 6A). Thus, the suppressive function of $CD4^+CD25^{high}$ T cells from patients with long-term graft survival was significantly increased compared to that of $CD4^+CD25^{high}$ T cells from patients with chronic rejection ($p < 0.03$) (Figures 6A). Within the patients with long-term graft survival, the suppressive activity of $CD4^+CD25^{high}$ T cells from operationally tolerant patients (free of immunosuppression) ($69.7\% \pm 7.4$) did not differ significantly from that of patients under standard immunosuppression ($77.9\% \pm 11.8$) (Figure 3, 4).

In both groups, $CD4^+CD25^{high}$ T cells were able to inhibit $CD4^+CD25^-$ T cell proliferation in a dose-dependent manner with almost complete suppression at a 1:1 ratio (Figure 6C). In all groups, IL2 reversed the suppressive properties of the $CD4^+CD25^{high}$ T cells (Figures 2, 3, 4, 5), whereas the blockade of IL10 or TGF β production with antibodies did not affect their inhibitory properties (Figure 6D). $CD4^+CD25^{high}$ T cell suppressive activity was stable in time in all patients, as shown in Figure 7.

These results show that $CD4^+CD25^{high}$ T cells from kidney recipients with chronic rejection display a defective suppressive function compared with those from recipients with long-term graft survival. In contrast, patients with long-term graft survival displayed $CD4^+CD25^{high}$ T cells with regulatory properties similar to healthy individuals.

Cytokine production during $CD4^+CD25^{high}/CD4^+CD25^-$ T cell coculture does not differ between recipients with chronic rejection or long-term graft survival.

Since $CD4^+CD25^{high}$ T cells from patients with chronic rejection and long-term graft survival displayed significantly different suppressive activities towards autologous $CD4^+CD25^-$ T cells, we next investigated whether cytokine production in $CD4^+CD25^{high}/CD4^+CD25^-$ T cell coculture was altered in the three groups. No difference was observed in coculture between patients with chronic rejection, patients with long term graft survival or healthy volunteers for any of the cytokines tested ($IFN\gamma$, $TNF\alpha$, $IL1\beta$, $IL10$, $IL4$, $IL5$, $IL6$, $IL8$ and $IL2$) suggesting an absence of modulation of cytokine production by $CD4^+CD25^{high}$ T cells from the different groups of patients (Figure 6B).

DISCUSSION

There is now compelling evidence that, in humans, $CD4^+CD25^{high}$ T cells may be involved in regulating the immune system and particularly in determining transplant outcome (Wood and Sakaguchi 2003; von Boehmer 2005). In the present study, we investigated the function of $CD4^+CD25^{high}$ T cells in blood from different cohorts of kidney recipients with contrasted clinical situations: patients with long-term graft survival with or without immunosuppressive therapy (patients under standard immunosuppression and drug-free operationally tolerant patients), patients with chronic rejection and healthy volunteers. We used a conventional approach to examine the functional regulatory capacity of $CD4^+CD25^{high}$ T cells in these different groups by assessing their ability to suppress the proliferation of autologous $CD4^+CD25^-$ T cells and to modulate their accompanying cytokine production. $CD4^+CD25^{high}$ T cells isolations were performed using immunomagnetic beads designed to preferentially purify the $CD4^+CD25^{high}$ cell subset that has been reported to harbour the most potent regulatory capacity (Baecher-Allan and Hafler 2006).

We have previously shown that, in these three groups of patients, $CD4^+CD25^{high}$ T cells express the surface markers distinctive of regulatory T cells (Louis, Braudeau et al. 2006). We also showed that patients with chronic rejection display a lack of $CD4^+CD25^{high}$ T cells associated with a decrease in FOXP3 transcript in $CD4^+$ T cells, whereas patients with long term graft survival displayed normal $CD4^+CD25^{high}$ blood T cell numbers and FOXP3 transcriptional level compared to healthy volunteers. In this study, we showed that, whereas the level of the FOXP3 protein remains unchanged at the cell surface level of the 3 groups of patients, patients with chronic rejection displayed a defect in $CD4^+CD25^{high}FOXP3^+$ T cell numbers. FOXP3 has been shown to play a major role in the differentiation of $CD4^+CD25^{high}$ regulatory T cells and to be expressed constitutively at high level in these cells (Fontenot, Gavin et al. 2003). Defective FOXP3 expression has been evidenced in patients with IPEX, a

condition associated with a deficiency in $CD4^+CD25^{high}$ regulatory capacity (Bacchetta, Passerini et al. 2006). The aim of this study was thus to investigate whether the lack of $CD4^+CD25^{high}FOXP3^+$ T cells in chronic rejection may also be associated with a decrease or a lack of their suppressive properties *in vitro* and if long-term graft survival may be associated with a variation of their suppressive properties compared to healthy volunteers.

$CD4^+CD25^{high}$ T cells from the 3 different groups were unresponsive to anti-CD3 and autologous PBMC activation whereas their $CD4^+CD25^-$ T cell counterparts proliferated strongly. $CD4^+CD25^{high}$ T cells did not proliferate following TCR-mediated activation unless IL2 was present. In the presence of an anti-CD3 antibody and autologous PBMC, we show that freshly isolated $CD4^+CD25^{high}$ T cells from patients with chronic rejection were not only present in a lower number but also displayed a significant decrease in their ability to suppress the proliferation of their autologous $CD4^+CD25^-$ T cell counterparts, compared to patients with long term graft survival who displayed a similar suppressive activity relative to healthy volunteers. A defect in the suppressive function of $CD4^+CD25^{high}$ T cells has already been reported in a number of autoimmune diseases in humans, such as multiple sclerosis (Viglietta, Baecher-Allan et al. 2004), type 1 autoimmune diabetes (Lindley, Dayan et al. 2005) and psoriasis (Sugiyama, Gyulai et al. 2005). A similar observation was performed in the thymus of patients with myasthenia gravis (Balandina, Lecart et al. 2004). Recently, Bachetta et al. showed a defective regulatory function in patients with IPEX syndrome (Bacchetta, Passerini et al. 2006) and Salama et al showed that the risk of graft loss in renal transplant recipients correlated with a decrease in $CD4^+CD25^+$ T cell regulation (Salama, Najafian et al. 2003).

We show here that the modulation of the $CD4^+CD25^{high+}$ T cell suppressive activity is independent of IL10 and $TGF\beta$, suggesting naturally occurring $CD4^+CD25^+$ T regulatory cells (Battaglia, Gregori et al. 2006). We also show that the cytokine production in coculture is not affected by addition of $CD4^+CD25^{high+}$ T cells. These findings contrast with reports suggesting

that a decrease in CD4⁺CD25^{high} T cell suppressive activity is accompanied by a decrease in IFN γ or IL2 production (Bacchetta, Passerini et al. 2006). Our data suggest that CD4⁺CD25^{high} T cells from patients with chronic rejection are able, as are those from the other two groups, to suppress the production of IFN γ by alloreactive CD4⁺CD25⁻ T cells, despite displaying a defect in suppressive function. The lack of viable donor cells meant that we were not able to directly test a possible specific anti-donor response in our patients, however, a recent study from Fudaba et al. have reported that operationally tolerant patients transplanted with kidney and bone marrow showed no significant anti-donor (or anti-host) mixed lymphocyte culture responses (Fudaba, Spitzer et al. 2006).

Because a single reliable surface marker has not yet been identified for regulatory T cells, it is possible that isolated putative CD4⁺CD25^{high} regulatory T cells from patients with chronic rejection could also contain effector T cells resulting in an apparent lack of *in vitro* regulation. However, this is unlikely, because the CD4⁺CD25^{high} T cells isolated from the PBMC of patients with chronic rejection and patients with long term graft survival displayed all the classical phenotypic and functional properties of regulatory cells: CD4, CD25 and CTLA-4 co-expression (Louis, Braudeau et al. 2006), high expression of FOXP3, low proliferative responses, absence of IL2 production and inhibition of co-cultured T cell proliferation in response to stimulation. In addition, if the regulatory T cell population from patients with chronic rejection had contained more effector CD4⁺CD25⁺ T cells than the other groups, their proliferative response would be expected to have been higher, which was not the case. Thus, the observed impairment of regulation in chronic rejection is very unlikely to be attributable to contamination with proliferative effector CD4⁺CD25⁺ T cells. Finally, some reports demonstrated that immunosuppressive treatment could influence regulatory T cell function (Baan, van der Mast et al. 2005; Coenen, Koenen et al. 2006; Zeiser, Nguyen et al. 2006). However, our data showed that the decrease of regulatory activity of CD4⁺CD25^{high} T

cells from patients with chronic rejection was not influenced by immunosuppression since no difference in suppressive activity was observed in patients with long-term graft survival with or without immunosuppression (*data not shown*).

We previously showed that patients with chronic rejection also display more CD8⁺CD28⁻CD27⁻ T cells with a cytotoxic and apoptotic phenotype (Baeten, Louis et al. 2006). These data, together with those described here, suggest that the low number of CD4⁺CD25^{high} regulatory T cells in patients with chronic rejection may not efficiently balance effector CD8⁺CD28⁻CD27⁻ T cells and thereby favor graft failure (Zheng, Sanchez-Fueyo et al. 2003). Conversely, the normal suppressive activity and numbers of CD4⁺CD25^{high+} T cells in patients with long term graft survival may be sufficient to counteract the cytotoxic activity of these effector cells. This hypothesis does not exclude the possibility that patients who suffer chronic rejection may initially display fewer blood CD4⁺CD25⁺ T cells than patients with long term graft survival before transplantation. This hypothesis is unfortunately impossible to test due to the lack of cells from these recipients pre-transplantation. However, neither immunosuppressive treatment nor renal insufficiency has been shown to influence the number of CD4⁺CD25⁺ T cells in these patients (Louis, Braudeau et al. 2006).

Finally, our findings show that long-term graft survival is not associated with an increased activity of CD4⁺CD25^{high} T cells compared to healthy individuals and suggest that chronic rejection is not only associated with a defect in the number of CD4⁺CD25^{high+}FOXP3⁺ regulatory T cells but also with a decrease in their immuno-regulatory properties.

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LEGENDS

Table 1: Demographic characteristics of patients: Recipients with chronic rejection (CR) and patients with long term graft survival (LTS) were matched for age, sex, and time post transplantation. Clearance and proteinuria were measured the day of blood sampling. Data are presented as median (range). C= Calcineurin inhibitor, M= mycophenolate mofetil, S= steroid, Aza= Azathioprine, Sirol= Sirolimus.

Figure 1: FOXP3 protein expression: FOXP3 protein was measured on CD4⁺CD25⁺ T cells by intracellular staining. A) CD4⁺ T cells have been double stained with anti-CD25 Ab and anti-FOXP3 Ab. Absolute value of CD25⁺FOXP3⁺ T cells have been calculated for healthy individuals (HV) patients with chronic rejection (CR) and with long term graft survival (LTS). B) Absolute value of CD25⁺FOXP3⁺ T cells in patients with chronic rejection (CR), with long term graft survival under immunosuppression (LTS-IS) and operationally tolerant patients (LTS-NoIS). C) Percentage of FOXP3 protein expression on CD4⁺CD25^{high} T cells.

Figure 2: CD4⁺CD25^{high} T cell suppressive activity in healthy volunteers

The ability of CD4⁺CD25^{high} T cells, freshly isolated from HV, to suppress CD4⁺CD25⁻ T cell responders was assessed. Responder cells were activated in the presence of 20,000 irradiated

(35Gy) autologous PBMC plus anti-CD3 mAb (1µg/ml) (see Methods for details). CD4⁺CD25^{high} T cells were added to activate responder cells and [3H] thymidine incorporation was measured after 72 hours. IL2 was added at 100U/ml. Percentages indicate inhibition of proliferation.

Figure 3: CD4⁺CD25^{high} T cell suppressive activity in LTS-NoIS patients

The ability of CD4⁺CD25^{high} T cells, freshly isolated from operationally tolerant patients (LTS-NoIS), to suppress CD4⁺CD25⁻ T cell responders was assessed. [3H] thymidine incorporation was measured after 72 hours. Percentages indicate inhibition of proliferation.

Figure 4: CD4⁺CD25^{high} T cell suppressive activity in LTS-IS patients

The ability of CD4⁺CD25^{high} T cells, freshly isolated from LTS with immunosuppression (LTS-IS), to suppress CD4⁺CD25⁻ T cell responders was assessed. [3H] thymidine incorporation was measured after 72 hours. Percentages indicate inhibition of proliferation.

Figure 5: CD4⁺CD25^{high} T cell suppressive activity in CR recipients

The ability of CD4⁺CD25^{high} T cells, freshly isolated from patients with chronic rejection, to suppress CD4⁺CD25⁻ T cell responders was assessed. [3H] thymidine incorporation was measured after 72 hours. Percentages indicate inhibition of proliferation.

Figure 6: Characterization of CD4⁺CD25^{high} T cell suppressive activity

A) Average inhibition of proliferation in HV, LTS, and CR. B) Table detailing cytokine production in coculture supernatants using a multiplex fluorescent bead immunoassay. IFN γ , IL2, IL10, IL6, IL4, IL5, IL1 β , and TNF α protein levels in cell culture supernatants were detected following cocultures in HV, LTS and CR recipients. C) Dose-dependant suppression.

CD4⁺CD25⁻ cells and CD4⁺CD25^{high} T cells were cultured at various ratios, with anti-CD3 and irradiated autologous PBMC. In the coculture, the number of CD4⁺CD25⁻ responder cells was constant whereas the number of CD4⁺CD25^{high} T cells varied by serial 2-fold dilutions. [3H] thymidine incorporation was measured after 72 hours. D) Effect of the addition of anti-IL10 (1µg/ml) and anti-TGFβ (10µg/ml) antibodies to the coculture.

Figure 7: Stability of the suppressive activity of CD4⁺CD25⁺ T cells over time

The ability of CD4⁺CD25^{high} T cells, freshly isolated from HV, LTS and CR recipients, to suppress CD4⁺CD25⁻ T cells responders was assessed over time, as described in the legend to Figure 2. Graphs show HV2 with a 9 month interval, LTS-NoIS2 with a 6 month interval and CR2 with a 2 month interval. Percentages indicate inhibition of proliferation.

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	CR	LTS
Number	17	22
Age	50.3 ± 12.7 (27-75)	51.8 ± 11.5 (33-76)
Sex	6M/11F	12M/10F
Clearance ml/min	25.3 ± 15.5 (10-69)	77.7 ± 41.3 (38-216)
Proteinuria g/24h	2.4 ± 2.3 (0.12-7.75)	0.2 ± 0.3 (0-1)
Time post-Tpx. (years)	7.9 ± 4.6 (2-17)	11.1 ± 7.1 (4-33)
Immunosuppression		
No	-	7
C	2	1
C/M	10	10
C/M/S	4	1
C/S	1	-
C/Aza	-	2
C/Sirol	-	1

Table1

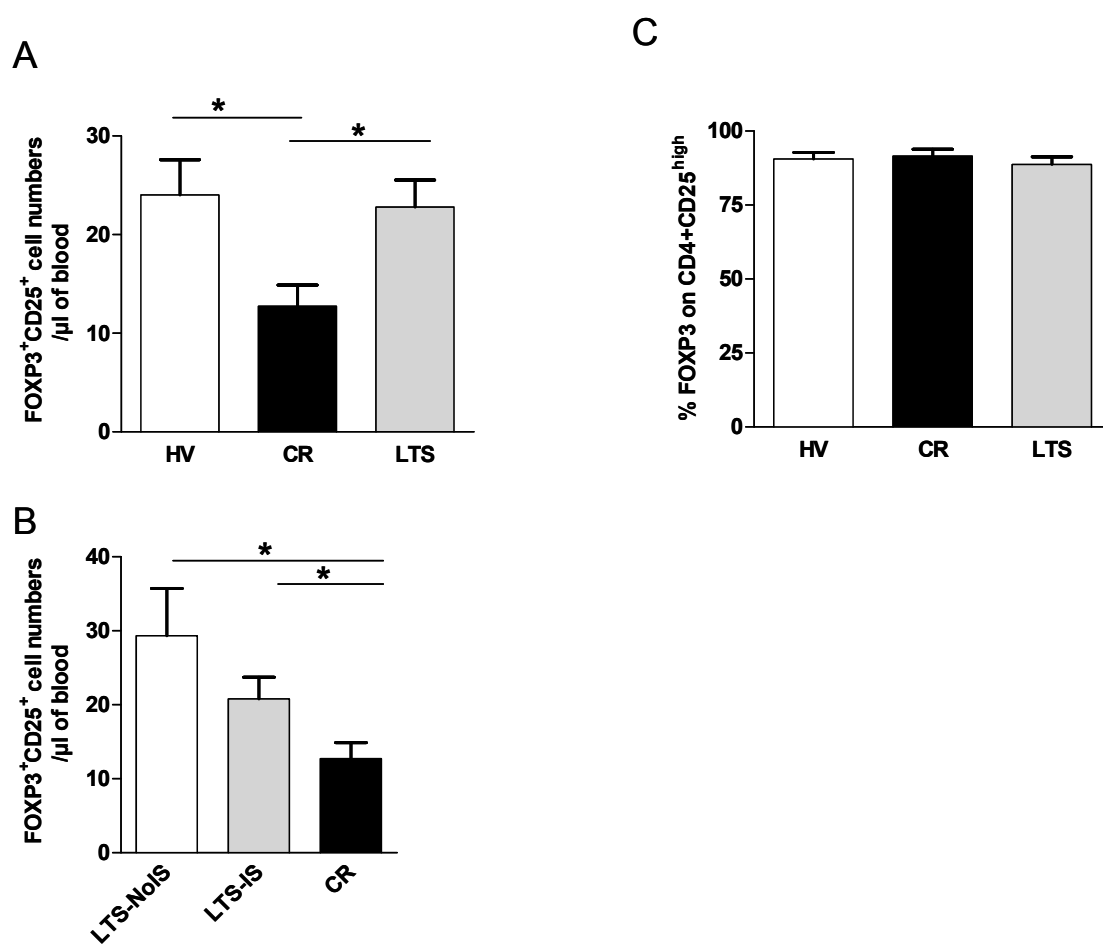


Figure 1

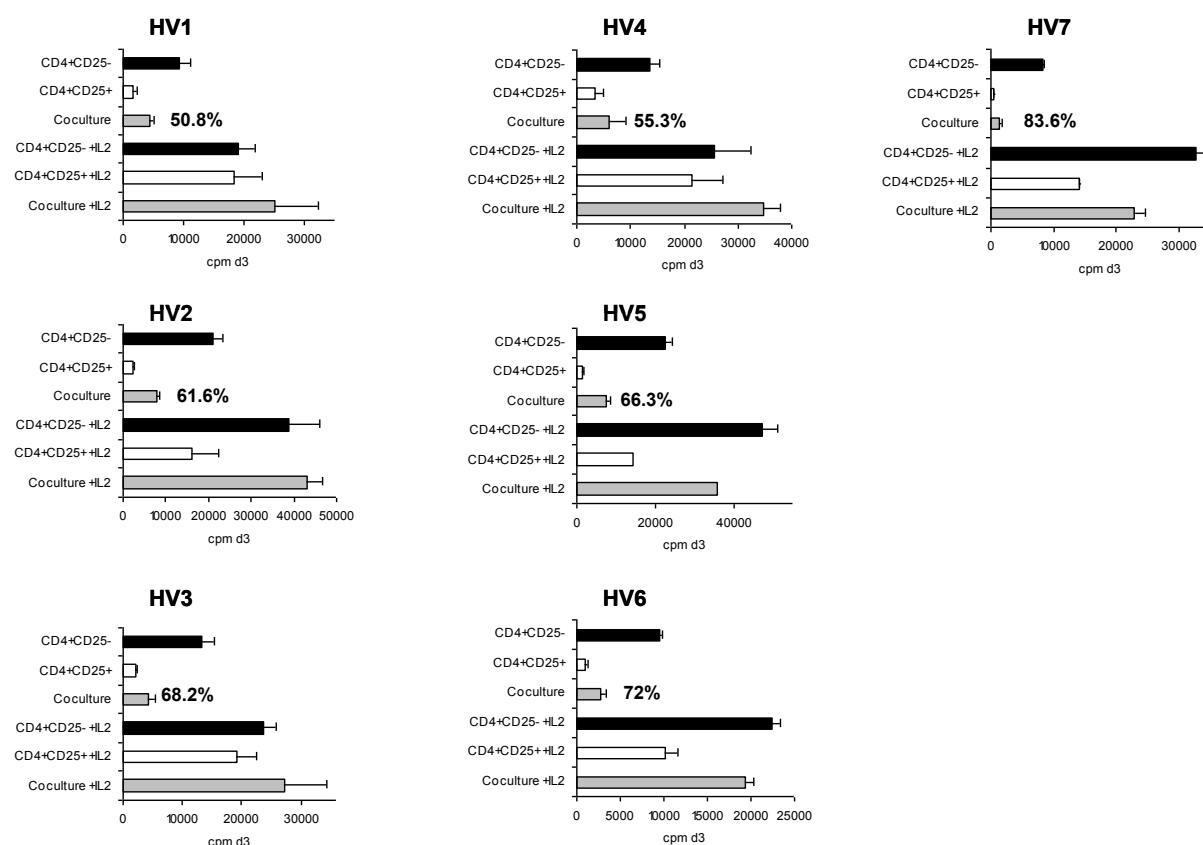


Figure 2

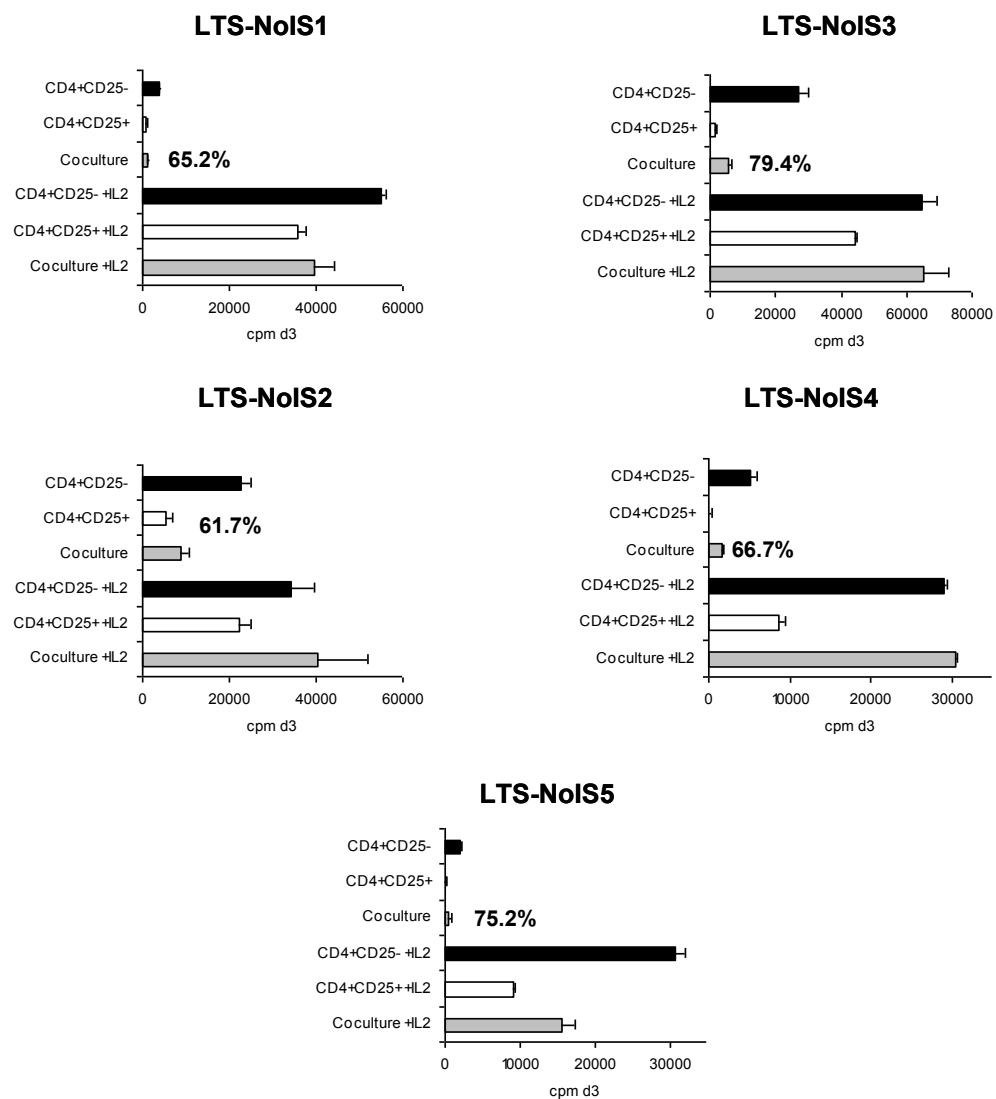


Figure 3

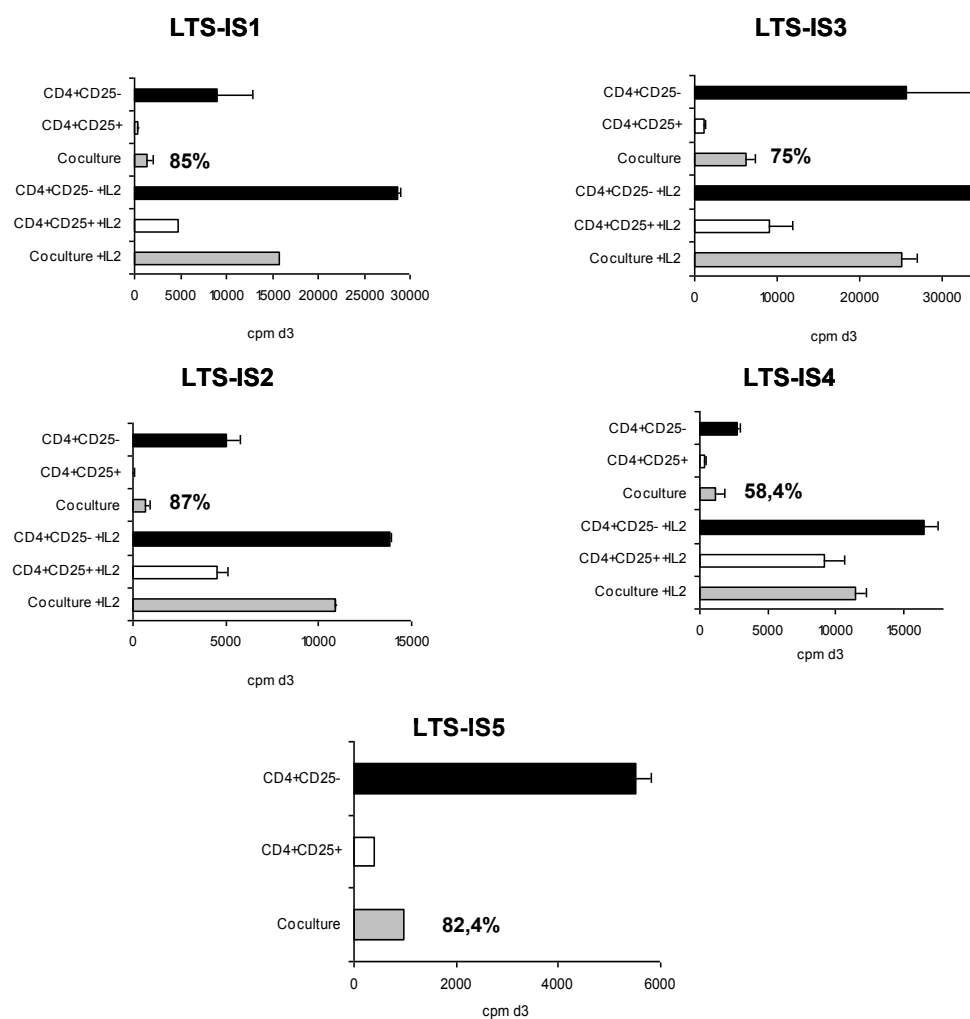


Figure 4

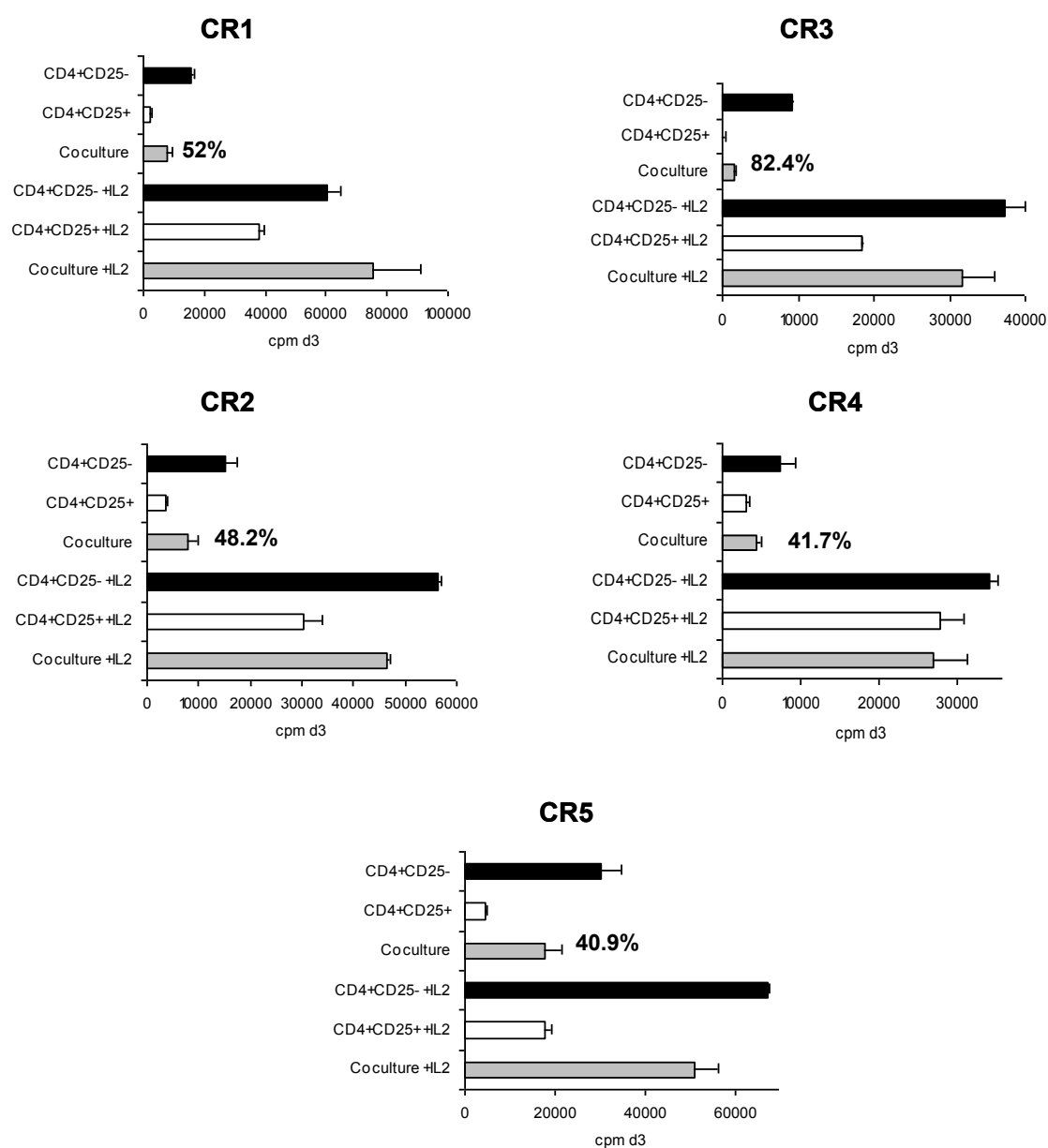
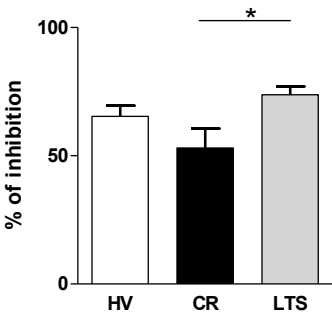


Figure 5

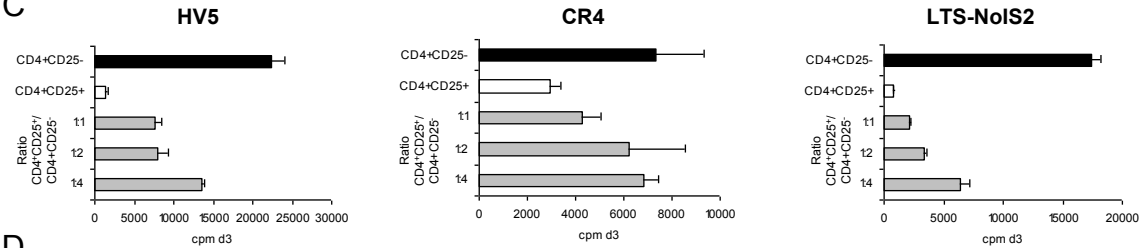
A



B

In Coculture	HV	CAN	LTS	p
Number	4	3	8	
pg/ml				
TNFa	449±216	331±121	124±156	ns
IL1b	8.7±2.6	3.1±2.6	4.7±5.7	ns
IL5	22.2±19	9.7±17	7.8±13.8	ns
IL4	32.7±13	16.7±15	11.1±15.4	ns
IL6	37.6±37	7.9±13	150±232	ns
IL10	8.1±11	0	2.8±7.3	ns
IL2	54.6±26	49.3±8.4	41.5±60.5	ns
IFNg	31.8±22	21.9±25	3.3±7.1	ns
IL8	14871±7225	16150±2636	14879±6037	ns

C



D

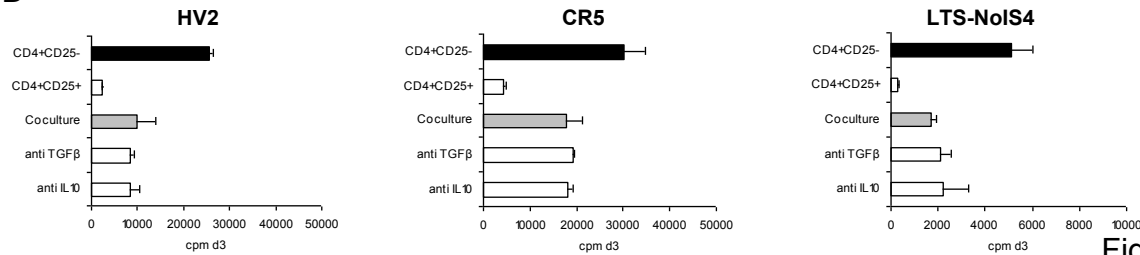


Figure 6

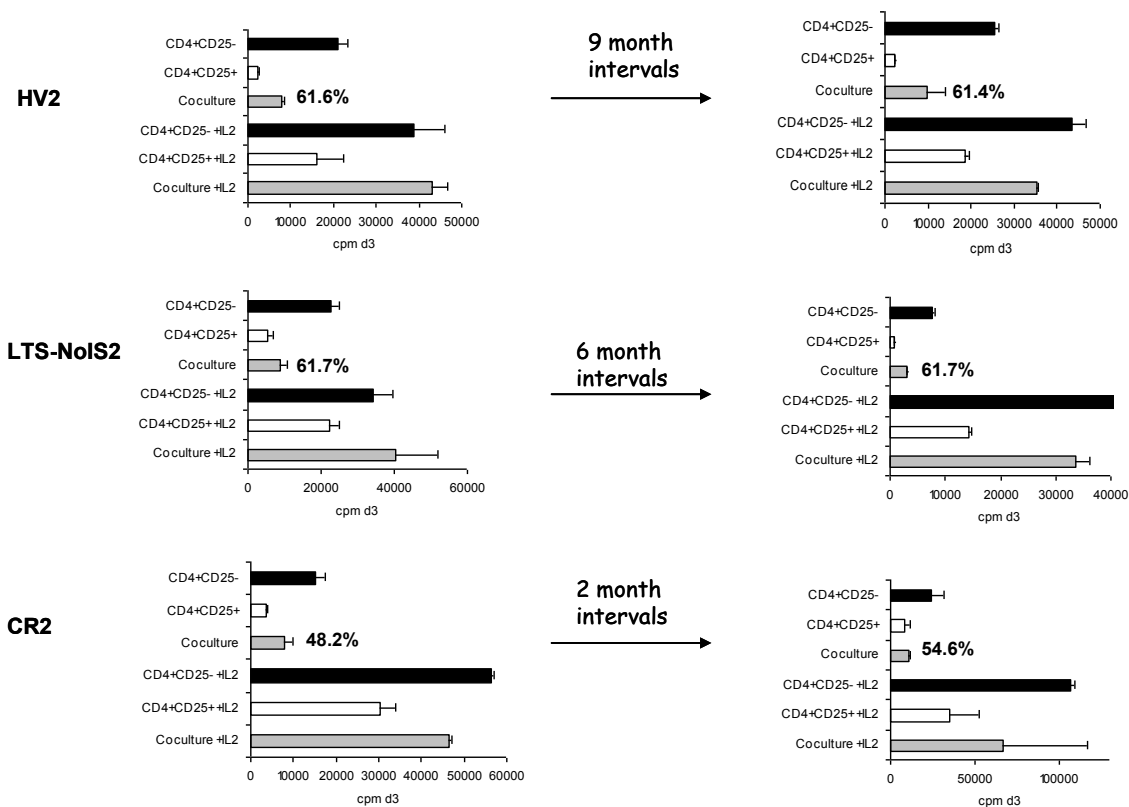


Figure 7

Discussion générale et Perspectives

Notre objectif étant de trouver de biomarqueurs de la tolérance, nous avons choisi d'effectuer nos études à partir du sang des patients, ce qui représente un moyen d'étude non invasif pour le patient. De telles études peuvent permettre l'identification de marqueurs du rejet ou de la tolérance, toutefois, les observations faites à partir du sang peuvent ne pas refléter les événements qui se déroulent dans le greffon.

Nous avons montré que les patients en rejet chronique présentent dans leur sang moins de lymphocytes T régulateurs $CD4^+CD25^{high}$ que les patients opérationnellement tolérants et les sujets sains. En revanche, ces cellules comme celles des 2 autres groupes présentent un profil régulateur caractérisé par la présence des marqueurs FOXP3, CTLA4, GITR, CCR4 et CD103.

Les résultats montrent que cette diminution du nombre de LT $CD4^+CD25^{high}$ chez les patients en rejet chronique n'est liée ni au traitement immunosuppresseur, ni à la dialyse, ni à l'insuffisance rénale. Elle n'est pas non plus liée à des infections virales ou au traitement d'induction post transplantation. Nous ne pouvons pas exclure que les cellules régulatrices de nos patients en rejet chronique se soient accumulées au niveau du greffon, ce qui expliquerait leur diminution dans le sang. Nous réaliserons des études sur les biopsies, en collaboration avec le service d'anatomopathologie du CHU de Nantes, afin de déterminer le pourcentage de cellules régulatrices au niveau de greffon. Nous ne pouvons pas exclure non plus, que les patients aient un niveau de LT $CD4^+CD25^{high}$, avant greffe, faible. Nous ne disposons pas de cellules avant la greffe pour vérifier ou non cette hypothèse chez cette cohorte de patients. C'est pourquoi une des perspectives de ce travail est de corrélérer le nombre et la fonction de cellules régulatrices avant la greffe au devenir du greffon à 1 an (analyse histologique après biopsie systématique) chez 200 patients transplantés rénaux. A 1an post greffe 4 patients de cette cohorte dégradent et les autres maintiennent leur fonction rénale.

Une des hypothèses serait que le rejet chronique pourrait être lié à un défaut du nombre de LT $CD4^+CD25^{high}$. Plusieurs études ont déjà associé une pathologie à un défaut du nombre des cellules T $CD4^+CD25^{high}$ régulatrices (Boyer, Saadoun et al. 2004) (Meloni, Vitulo et al. 2004). Ainsi, Meloni et al. ont montré qu'une diminution des LT $CD4^+CD25^{high}$ régulateurs périphériques était associée au développement d'un rejet chronique chez les patients greffés du poumon et suggèrent leur confinement au niveau du greffon. Nos résultats sont également similaires avec ceux de Salama et al. qui ont montré que le risque de perte de greffon rénal était corrélé à une diminution des lymphocytes T $CD4^+CD25^+$ dans le sang (Salama, Najafian et al. 2003). De même, comme pour nos patients tolérant spontanément leur greffe de rein, Li et al. ont montré, dans le cadre d'une greffe de foie, que les patients opérationnellement tolérants ont

un nombre de lymphocytes T $CD4^+CD25^{high}$ régulateurs dans le sang identique à celui des sujets sains (Li, Koshiba et al. 2004).

En l'absence de cellules du donneur, nous avons mis au point un test de coculture utilisant les cellules T $CD4^+CD25^-$ du patient, activées par un anticorps anti-CD3, en présence de PBMC autologues irradiés. L'effet suppresseur des cellules T $CD4^+CD25^+$ ajoutées au système est «titré» par la mesure de la prolifération et la production en cytokines des cellules T $CD4^+CD25^-$ stimulées. Les cellules $CD4^+CD25^{high}$ des patients en rejet chronique présentent une fonction suppressive sur la prolifération des cellules T $CD4^+CD25^-$ diminuée par rapport à celle des patients opérationnellement tolérants et des patients présentant une fonction stable de leur greffon sous traitement immunosuppresseur.

Nous avons ainsi montré que le rejet chronique était non seulement associé à une diminution du nombre de cellules régulatrices T $CD4^+CD25^{high}FOXP3^+$ mais aussi à une diminution de leur fonction régulatrice. Bachetta et al. ont montré chez les patients atteints d'IPEX déficients en FOXP3, une diminution de l'activité suppressives des cellules T $CD4^+CD25^{high}$ (Bacchetta, Passerini et al. 2006). Un défaut de fonction régulatrice des cellules T $CD4^+CD25^{high}$ a également été décrit dans plusieurs maladies autoimmunes comme la sclérose en plaque (Viglietta, Baecher-Allan et al. 2004), le diabète de type 1 (Lindley, Dayan et al. 2005) le psoriasis (Sugiyama, Gyulai et al. 2005) ou encore la myasthénie (Balandina, Lecart et al. 2004). Afin de différencier les cellules régulatrices induites des cellules régulatrices naturelles, nous avons également testé leur mode d'action. Les premières agissant par la sécrétion de cytokines telles que IL10 et/ou TGF β (Groux, O'Garra et al. 1997) (Battaglia, Gregori et al. 2006), nous avons analysé l'effet d'anticorps neutralisants anti-IL10 et anti-TGF β ajoutés dans la coculture décrite précédemment. Cette suppression est indépendante de l'IL10 et du TGF β .

Une autre étude menée dans notre laboratoire sur ces patients a montré une augmentation très significative du nombre de lymphocytes T $CD8^+CD28^-$ chez les patients en rejet chronique (Baeten, Louis et al. 2006) (Annexe 2). Ces cellules sont $CD27^-CD57^+$ et expriment fortement perforine et granzyme A. L'ensemble de ces résultats suggère donc une diminution des cellules régulatrices T $CD4^+CD25^{high}$ et une augmentation des cellules effectrices cytotoxiques chez les patients en rejet chronique. Ainsi, comme l'ont proposé Zheng et al. (Zheng, Sanchez-Fueyo et al. 2003), de l'équilibre entre cellules alloréactives ($CD4^+CD25^-$ ou $CD8^+CD28^-$?) et cellules régulatrices dépendrait l'état de tolérance ou le rejet. Chez les patients en rejet chronique, non seulement les cellules T $CD4^+CD25^{high}$ seraient en nombre insuffisant pour contrôler les cellules alloréactives, mais elles présenteraient aussi un défaut de régulation sur ces dernières.

Par ailleurs, les patients opérationnellement tolérants présentent un nombre et une activité suppressive des lymphocytes T $CD4^+CD25^{high}$ similaires aux sujets sains. Les études phénotypiques ont également montré que les patients opérationnellement tolérants exprimaient les mêmes marqueurs que les sujets sains. Le groupe de M. Sykes a récemment montré que des patients greffés simultanément avec un rein et des cellules hématopoïétiques de la moelle osseuse du donneur, et devenus opérationnellement tolérants, ne présentaient pas de réponse spécifique anti-donneur en culture lymphocytaire mixte et ont une réponse suppressive comparable à celle de sujets sains (Fudaba, Spitzer et al. 2006). Ces résultats suggèrent que l'état de tolérance opérationnelle pourrait se rapprocher d'un phénomène de tolérance au soi, rendant ainsi complexe la détermination de marqueurs biologiques de la tolérance.

Conclusions et perspectives

Les perspectives de ce travail sont nombreuses mais néanmoins limitées par le faible nombre de patients et par le volume de sang disponible à chaque prélèvement. Pour pallier à ce problème, nous souhaitons mettre en place un protocole d'expansion des cellules T $CD4^+CD25^{high}$ afin d'en étudier les mécanismes d'action, notamment leur rôle et les modes d'interaction avec les cellules $CD4^+CD25^{high}$ et les cellules alloréactives $CD8^+CD28^-$ dans le rejet chronique (**Figure 15**). Kreijveld et al. ont récemment décrit un protocole d'expansion des cellules T $CD4^+CD25^{high}$ à partir de faible quantité de sang de patients greffés rénaux, avec le l'IL2 et de l'IL15 (Kreijveld, Koenen et al. 2006). Aucune modification de leur phénotype, de leur activité suppressive et de leur répertoire T n'a été observée (Kreijveld, Koenen et al. 2006).

D'autre part, nous essayons de déterminer si d'autres mécanismes seraient impliqués dans le rejet chronique et la tolérance. Deux hypothèses de travail sont actuellement étudiées.

1^{ère} hypothèse:

Les Toll-like récepteurs (TLR) seraient impliqués dans le rejet chronique.

Nous cherchons à savoir s'il existe un lien entre le défaut en lymphocytes T régulateurs $CD4^+CD25^+$ et certains TLR. En effet, plusieurs études ont récemment suggéré que les TLR peuvent moduler la réponse immunitaire adaptative en stimulant ou en inhibant l'activité des cellules T régulatrices $CD4^+CD25^+$ (Pasare and Medzhitov 2003; Kubo, Hatton et al. 2004; Netea, Suttmüller et al. 2004; Peng, Guo et al. 2005; Zhai, Meng et al. 2006) (Chen, Wang et al.

2006). Nous avons montré par PCR quantitative une surexpression des TLR 2, 4, 5 et 8 dans les PBMC des patients en rejet chronique comparativement aux patients opérationnellement tolérants (**Figure 8**).

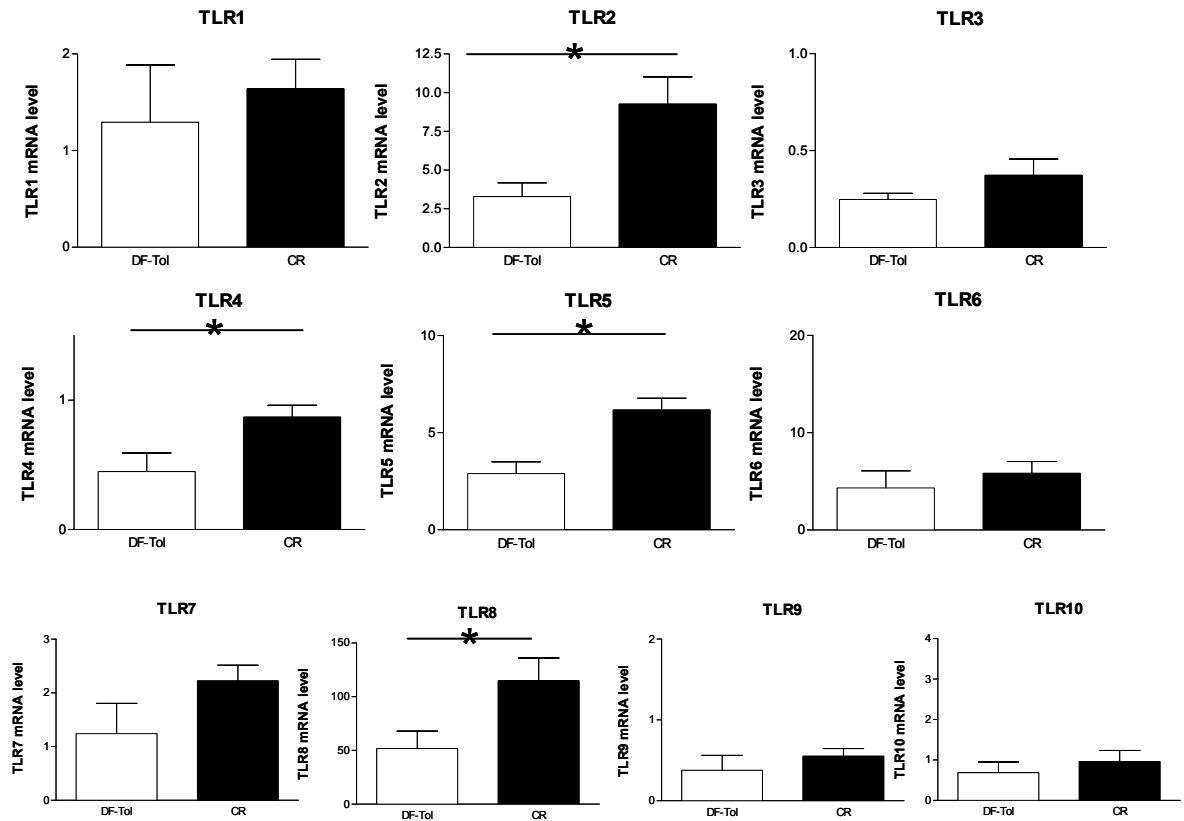


Figure 8: Expression des ARNm des TLR dans les PBMC des patients tolérants (DF-Tol) et en rejet chronique (CR)

Nous avons également montré, une augmentation de l'ARNm de la protéine adaptatrice MyD88 chez les patients en rejet chronique, comparativement aux patients opérationnellement tolérants (**Figure 9**). Ce résultat est donc concordant avec l'augmentation des ARNm de certains TLR (2, 4, 5, 8), suggérant une activation de la voie MyD88 et donc un rôle de la voie des TLR dans le rejet chronique.

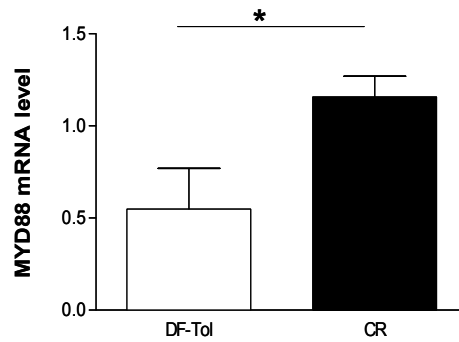


Figure 9: Expression de l'ARNm de MyD88 dans les PBMC des patients tolérants (DF-Tol) en rejet chronique (CR) ou avec une fonction stable sous immunosuppresseurs (Sta)

Nous nous sommes ensuite intéressés à l'expression des TLR au niveau protéique. Les TLR sont exprimés par une grande variété de types cellulaires (Iwasaki and Medzhitov 2004) (Hornung, Rothenfusser et al. 2002). Ils sont différenciellement exprimés selon les populations de DC et selon leur état de maturation, par les neutrophiles et les éosinophiles (Iwasaki and Medzhitov 2004), les macrophages, les monocytes (Muzio, Bosisio et al. 2000), les mastocytes (McCurdy, Lin et al. 2001; Supajatura, Ushio et al. 2001) les lymphocytes B, T et cellules NK (Hornung, Rothenfusser et al. 2002). Les cellules T régulatrices $CD4^+CD25^+$ exprimeraient plus particulièrement les TLR4, 5, 7, 8 (Caramalho, Lopes-Carvalho et al. 2003) (Crellin, Garcia et al. 2005; Peng, Guo et al. 2005). Les TLR1, 2, 4, 5, 6 sont exprimés à la surface cellulaire alors que les TLR3, 7, 8 et 9 sont exprimés dans les compartiments intracellulaires comme les endosomes (Heil, Ahmad-Nejad et al. 2003; Matsumoto, Funami et al. 2003; Latz, Schoenemeyer et al. 2004).

Nous avons analysé, par cytométrie en flux, l'expression des 4 TLR dont les ARNm étaient surexprimés dans les PBMC des patients en rejet chronique comparativement aux patients tolérants dans les lymphocytes T ($CD3^+$), les lymphocytes B ($CD19^+$) et les monocytes ($CD14^+$). La surexpression du TLR4 a été confirmée au niveau protéique dans les monocytes ($CD14^+$) des patients en rejet chronique comparativement aux patients tolérants (**Figure 10**).

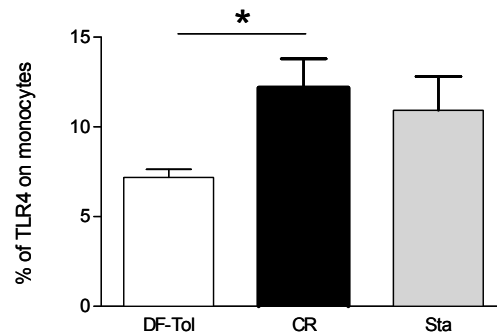


Figure 10: Pourcentage de TLR4 sur les monocytes de patients tolérants (DF-Tol), en rejet chronique (CR) et sous immunosuppresseurs (Sta)

Les Toll-like récepteurs (TLR), représentent des sentinelles du système immunitaire inné en reconnaissant des séquences spécifiques des composants microbiens (PAMPs pour Pathogen-Associated Molecular patterns) (Medzhitov, Preston-Hurlburt et al. 1997). Des travaux ont montré qu'ils intervenaient également dans le système immunitaire acquis en participant à des réactions inflammatoires générées par des ligands endogènes (Janeway and Medzhitov 1998; Ohashi, Burkart et al. 2000) (Akira, Takeda et al. 2001) (Johnson, Brunn et al. 2002) (Termeer, Benedix et al. 2002). Les TLR appartiennent à une superfamille incluant le récepteur à l'IL-1. Actuellement 10 TLR ont été identifiés (TLR1 à 10) chez l'homme et un pseudogène (TLR11) (Takeda and Akira 2005) (**Figure 11**). Les TLR sont caractérisés par un domaine cytoplasmique TIR (Toll/IL1 receptor) appelé ainsi par homologie avec le récepteur à l'IL1. La voie de signalisation du Toll présente de grande similarité avec celle de l'interleukine 1 (IL1) qui conduit à l'activation de NF- κ B, un facteur de transcription impliqué dans les réponses immunitaires.

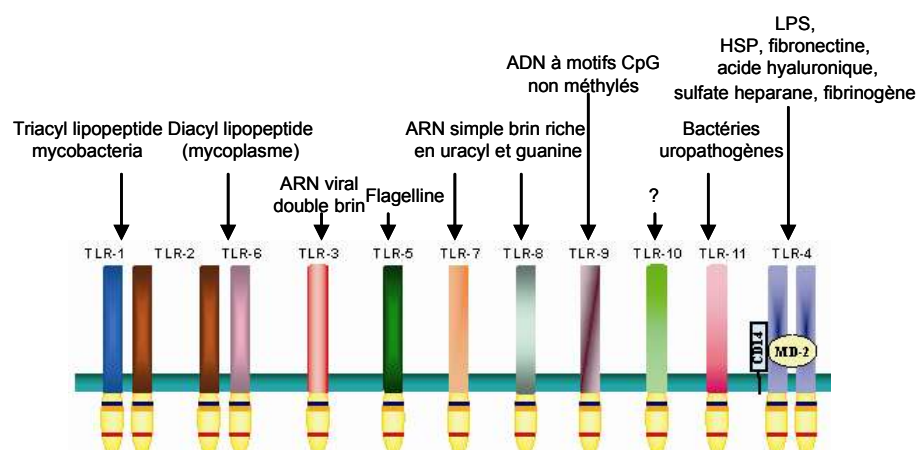


Figure 11: Les TLR et leurs ligands
D'après (Akira and Takeda 2004)

TLR 2:

Le TLR2 peut être associé au TLR1 ou au TLR6 formant ainsi un hétérodimère permettant respectivement la reconnaissance de lipopeptides triacyl ou diacyl (Takeuchi, Sato et al. 2002). Le TLR2 reconnaît les lipoprotéines des pathogènes tels que les bactéries Gram-négatives, les mycoplasmes, les composés protéoglycanes (zymosan) (Takeuchi, Hoshino et al. 1999) (Takeuchi, Hoshino et al. 1999; Takeda, Kaisho et al. 2003). De plus, le TLR2 reconnaît le LPS des non entérobactéries. La structure de ce LPS différerait du LPS typique des bactéries Gram-négatives reconnu par le TLR4 (Hirschfeld, Weis et al. 2001) mais cela reste encore controversé.

TLR4 :

Le TLR4 est un récepteur essentiel dans la reconnaissance du LPS (mais aussi du taxol, un antimitotique présentant une activité anti-tumorale chez l'homme) (Poltorak, He et al. 1998; Kawasaki, Akashi et al. 2000). Ce TLR est aussi impliqué dans la reconnaissance de ligands endogènes comme les protéines de choc thermique (HSP60, HSP70), l'extradomaine A de la fibronectine, les oligosaccharides d'acide hyaluronique (Termeer, Benedix et al. 2002), de sulfate heparane (Johnson, Brunn et al. 2002) et le fibrinogène (Smiley, King et al. 2001). Cette reconnaissance n'implique pas uniquement le TLR4. Plusieurs protéines sont impliquées dans la reconnaissance du LPS et le récepteur fonctionnel du LPS forme un large complexe TLR4/CD14/MD2 (da Silva Correia and Ulevitch 2002).

TLR5 :

Le TLR5 reconnaît des domaines conservés de la flagelline, composé protéique du flagelle (Hayashi, Smith et al. 2001). Il est exprimé à la surface basolatérale des cellules épithéliales et des cellules endothéliales intestinales (Maaser, Heidemann et al. 2004).

TLR 8 :

Les TLR7 et 8 sont des protéines très conservées qui peuvent dans certains cas reconnaître les mêmes ligands. Ils sont associés aux réponses anti-virales et à la production d'IFN α (Hemmi, Kaisho et al. 2002). Ils ont été étudiés à l'aide de composés synthétiques comme l'Imidazoquinoline, qui est un de leurs ligands. Une étude récente a décrit le ligand de l'Imidazoquinoline comme correspondant à des séquences spécifiques de motifs d'ARN riches en guanine et uracile (Diebold, Kaisho et al. 2004). Les TLR7 et 8 humains reconnaissent des ARN de virus riches en guanosine ou uridine (single-stranded RNA, ssRNA).

Nous avons donc commencé une étude des voies de signalisation des TLR. La stimulation des TLR par leurs ligands conduit à l'expression de plusieurs gènes impliqués dans la réponse immune. MyD88 joue un rôle crucial dans la signalisation des TLR car des souris invalidées pour le gène MyD88 ont une absence de production de cytokines inflammatoires comme l'IL12p40, l'IL6 et le TNF α en réponse à tous les ligands de TLR (Hacker, Vabulas et al. 2000; Schnare, Holt et al. 2000). Les mécanismes moléculaires impliqués dans l'induction de ces gènes sont largement étudiés et deux types de voies de signalisation des TLR ont été identifiés: la voie MyD88 dépendante et la voie MyD88 indépendante (**Figure 12**)

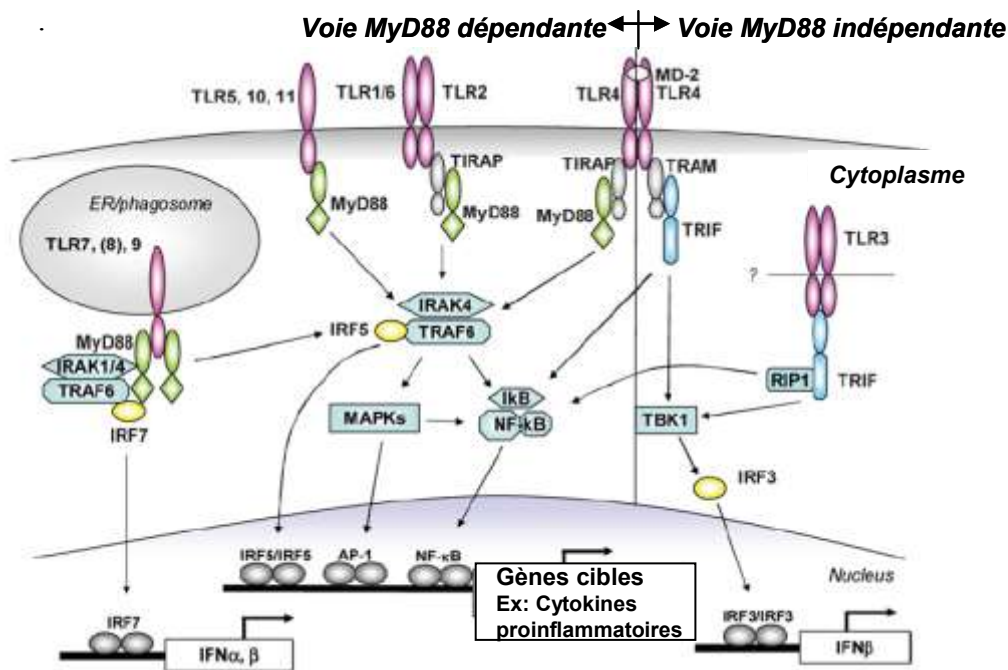


Figure 12: Représentation schématique des voies de signalisation des TLR
D'après (Ishii, Coban et al. 2005)

Comme nous l'avons vu, les cellules régulatrices semblent jouer un grand rôle en transplantation (Waldmann, Chen et al. 2006) et nos résultats suggèrent un défaut du nombre et de la fonction des lymphocytes T régulateurs CD4⁺CD25⁺ chez nos patients en rejet chronique. Nous cherchons à savoir si un lien est possible entre ces défauts des lymphocytes T régulateurs CD4⁺CD25⁺ et la diminution d'expression des TLR2, 4, 5, 8. Plusieurs études ont récemment suggéré que les TLR peuvent moduler la réponse immune adaptative en stimulant ou en inhibant l'activité des cellules T régulatrices CD4⁺CD25⁺ (Pasare and Medzhitov 2003; Kubo, Hatton et al. 2004; Netea, Suttmuller et al. 2004; Peng, Guo et al. 2005; Zhai, Meng et al. 2006) (Chen, Wang et al. 2006). Les études réalisées jusqu'à ce jour restent encore contradictoires. Bien que la production de cytokines proinflammatoires (IL1 β , TNF α) soit normale, celle de

l'IL10 est diminuée fortement chez les souris TLR2^{-/-} (Netea, Suttmuller et al. 2004) (Liu, Wu et al. 2006). Ceci est accompagné par une diminution de 50% des cellules T régulatrices CD4⁺CD25⁺ chez ces souris (Netea, Suttmuller et al. 2004). D'autre part, des études *in vitro* ont montré que la survie des T régulateurs était augmentée par des agonistes du TLR2 (Netea, Suttmuller et al. 2004) (Yang, Huang et al. 2004) (Andrade, Waddell et al. 2005). Cependant Liu et al. ont montré récemment qu'un agoniste (BLP) du TLR2 aurait l'effet inverse et diminuerait l'effet suppresseur des Treg sur les effecteurs (Liu, Komai-Koma et al. 2006).

Par ailleurs, Peng et al. ont établi que le TLR8 était exprimé par les lymphocytes T régulateurs humains et murins. L'activation de la voie TLR8-MyD88-IRAK4 entraîne une inhibition de l'activité suppressive des lymphocytes T CD4⁺CD25⁺. *In vivo*, dans un modèle de tumeur, l'injection de T régulateurs activés par un ligand de TLR8, inhibe la croissance de la tumeur chez la souris (Peng, Guo et al. 2005). Cependant, Caramalho et al. ont montré chez la souris que les T régulateurs exprimaient principalement les TLR4, 5, 7, 8 et que le LPS augmente l'activité suppressive des T régulateurs. *In vivo*, l'injection de T suppresseurs traités au LPS empêche le développement d'une maladie inflammatoire (Caramalho, Lopes-Carvalho et al. 2003). Dans un modèle murin de transplantation cardiaque, Zhai et al. ont récemment étudié l'effet de l'activation du TLR4 sur l'induction et la maintenance d'une tolérance induite par des LT CD4⁺ régulateurs. Ils ont montré qu'un défaut de TLR4 n'interfère pas avec l'établissement d'une tolérance. L'activation du TLR4 par son ligand, le LPS, n'entraîne pas de rupture de la tolérance, malgré une augmentation du TNF α et une diminution du ratio FOXP3/Tbet dans le greffon. Ainsi, l'activation du TLR4 ne semblerait pas nécessaire pour l'induction et la fonction des T régulateurs dans ce modèle de tolérance (Zhai, Meng et al. 2006).

De façon contradictoire, Chen et al. ont montré récemment qu'un engagement d'un seul TLR est suffisant pour bloquer la tolérance induite par l'anti-CD154 dans un modèle d'allogreffe cardiaque chez la souris. Le rejet est alors corrélé à une inhibition du recrutement de cellules régulatrices FOXP3⁺ au niveau du greffon. Enfin, une absence de MyD88 chez le donneur ou le receveur entraîne l'acceptation d'une greffe de peau chez les souris traitées avec l'anti-CD154. Les auteurs ont ainsi montré que l'activation des TLR pouvait empêcher l'établissement d'une tolérance (Chen, Wang et al. 2006).

Avec la découverte des TLR et de leurs ligands endogènes (HSP), le rôle des TLR commence, depuis quelques années, à être analysé dans le rejet aigu et chronique après une greffe d'organe. Le TLR4 reste le TLR le plus étudié en transplantation. Methe et al. ont analysé le rôle de la réponse innée et des TLR dans le rejet d'allogreffe cardiaque chez la souris, en comparant le niveau d'ARNm du TLR4 dans le rejet aigu et chronique (Methe, Zimmer et al.

2004). Les souris qui développent un rejet chronique présentent un taux élevé de TLR4 comparativement à une greffe syngénique, suggérant une contribution du TLR4 dans ce rejet. Cependant, Goldstein et al. ont montré que les souris TLR2^{-/-} avaient une prolongation de survie, petite mais significative, de leur greffe de peau HY incompatible, tandis que les souris TLR4^{-/-} ne présentaient aucune différence avec les souris contrôles (Goldstein, Tesar et al. 2003). Samstein et al. ont également utilisé un modèle de greffe de peau pour étudier le rôle du TLR4 en transplantation (Samstein, Johnson et al. 2004). Ils ont établi grâce à 2 souches de souris déficientes en TLR4, qu'un défaut de TLR4 n'affectait pas le rejet de peau dans des modèles de CMH incompatibles majeurs ou mineurs. D'autre part, Goldstein et al. ont montré que le rejet de greffe de peau ne pouvait pas se développer en l'absence de MyD88 (Goldstein, Tesar et al. 2003).

Les premières études chez l'homme ont été réalisées en transplantation pulmonaire, le poumon étant perpétuellement exposé aux pathogènes et toxines de l'environnement. Elles ont suggéré un rôle important du TLR4 dans le rejet aigu d'une greffe de poumon (Belperio 2003) (Palmer 2003) (Palmer, Burch et al. 2005). Le groupe de Palmer a mené des études sur le polymorphisme de l'ADN de 147 receveurs et de leurs donneurs de greffe de poumon. Ils ont établi qu'un polymorphisme du TLR4, Asp299Gly et Thr399Ile, chez le receveur, était associé à une réduction de l'incidence du rejet aigu et une tendance à une diminution de celle du rejet chronique chez des patients ayant reçu une greffe de poumon (Palmer 2003) (Palmer, Burch et al. 2005). Ducloux et al. ont récemment étudié le polymorphisme du TLR4 sur 238 patients greffés rénaux. L'impact de ce polymorphisme sur le devenir du greffon est une diminution du rejet aigu chez les patients portant une mutation du gène TLR4 ainsi qu'une diminution des complications athérosclérotiques post-transplantation (Ducloux, Deschamps et al. 2005). Cependant, une augmentation des infections a été observée chez ces patients. Les auteurs suggèrent que ces patients pourraient bénéficier d'une diminution de leur traitement immunosuppresseur avec une augmentation des mesures préventives contre les agents infectieux. Methe et al. ont également décrit une augmentation du TLR4 sur les monocytes de patients ayant subi une greffe cardiaque et présentant un rejet chronique. Cette augmentation du TLR4 est accompagnée d'une élévation de la production de l'IL12 et du TNF α par les monocytes activés avec du LPS (Methe, Zimmer et al. 2004).

Toutes ces données, bien qu'encore contradictoires, suggèrent que les TLR pourraient être directement impliqués dans la réponse immune en transplantation. Nous allons donc poursuivre cette étude en analysant précisément par cytométrie en flux, les types cellulaires

exprimant les TLR2, 4, 5, 8. Suite aux différents résultats indiquant que les TLR peuvent moduler l'activité des cellules T régulatrices (Pasare and Medzhitov 2003; Kubo, Hatton et al. 2004; Netea, Suttmuller et al. 2004; Peng, Guo et al. 2005; Zhai, Meng et al. 2006) (Chen, Wang et al. 2006), il paraît important de déterminer, chez nos patients, si les lymphocytes T $CD4^+CD25^+$ expriment certains TLR et si cela peut avoir une incidence sur leur capacité suppressive. Des expériences de blocage de TLR lors de coculture pourront être envisagées afin de répondre à cette question.

Les 2 voies d'activation des TLR (MyD88 dépendante et indépendante) seront également étudiées par Western blot (IRF3, TRAF6, NF- κ B, AP-1). Nous allons stimuler des monocytes issus des différents groupes de patients avec le ligand du TLR4, le LPS, afin de quantifier la production de cytokines telles que le TNF α , et nous réaliserons un phénotype de ces cellules activées. Aux vues de nos premiers résultats et de la littérature, nous pensons que les monocytes des patients en rejet chronique, stimulés avec le LPS, pourraient présenter une augmentation de leur production de cytokines proinflammatoires, contrairement aux patients tolérants. Les cellules exprimant les TLR2, 5, 8, une fois identifiées, pourront également être stimulées avec les ligands des TLR, et nous doserons la production de cytokines de la même façon. En fonction de tous ces résultats, nous étudierons si le niveau de TLR peut être en relation avec les lymphocytes $CD4^+CD25^+$ (**Figure 15**).

2^{ème} hypothèse :

La molécule CD30, membre de la famille du TNFR, pourrait être impliquée dans le devenir du greffon

Nous avons dosé le CD30 soluble (CD30s) dans le sérum des patients greffés (**Figure 13**).

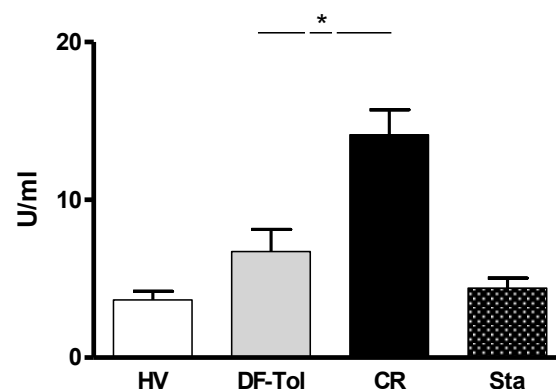


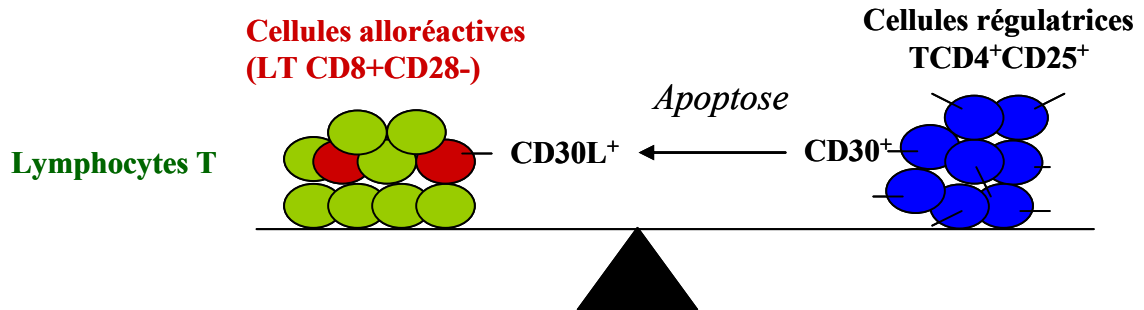
Figure 13: Dosage du CD30s dans le sérum de patients transplantés rénaux (DF-Tol, CR, Sta) et de sujets sains (HV)

Nous avons montré que les patients opérationnellement tolérants ont peu de CD30s dans leur sérum comparativement aux patients en rejet chronique, qui comme décrits dans la littérature présentent de forte concentration de CD30s dans leur sérum. En effet, plusieurs travaux ont montré une augmentation du CD30 soluble dans le rejet aigu (Pelzl, Opelz et al. 2003) et chronique (Susal, Pelzl et al. 2002) (Rajakariar, Jivanji et al. 2005; Weimer, Susal et al. 2006). Le CD30 est une glycoprotéine de la famille du TNF récepteur, exprimée par les lymphocytes B et T activés et les cellules CD8 mémoires (Horie and Watanabe 1998). Le CD30 ligand est porté par les LT activés, les macrophages et les lymphocytes B au repos (Horie and Watanabe 1998). Une fois activés, les lymphocytes T relarguent par clivage protéolytique du CD30 soluble (Hansen, Kisseleva et al. 1995). La liaison CD30-CD30L induit l'activation de TRAF2 et TRAF5 et finalement NF- κ B (Duckett, Gedrich et al. 1997) (Lee, Lee et al. 1996). Il a été démontré *in vitro* que des lymphocytes de lymphome (lignée YT) exprimant le marqueur CD30 présentaient une diminution de leur fonction cytotoxique avec une baisse de perforine, granzyme et FasL, mais une augmentation de la molécule de homing CCR7 (Muta, Boise et al. 2000). L'expression de CCR7 entraînerait le départ des cellules du site d'inflammation. Par ailleurs, Plusieurs travaux ont suggéré l'implication de la voie CD30/CD30L dans l'induction d'une apoptose (Telford, Nam et al. 1997) (Dai, Li et al. 2004). Dai et al. ont montré chez la souris que les T régulateurs $CD4^+CD25^+$ induites par les antigènes du donneur, et exprimant le CD30, bloquaient de manière allospécifique, le rejet de greffe de peau et de cœur induit par les TCD8 mémoires, en induisant leur apoptose (Dai, Li et al. 2004). De plus, Zeiser et al. ont récemment montré que les lymphocytes T régulateurs $CD4^+CD25^+$ issus de souris déficientes en CD30, étaient incapables de protéger les souris de la GVH après une greffe de cellules hématopoïétiques de moelle osseuse, contrairement aux lymphocytes T régulateurs issus de souris normale (Zeiser, Nguyen et al. 2006). Comparativement à l'étude de Dai et al., ils ont également montré que les LT régulateurs $CD4^+CD25^+$ induisaient l'apoptose de LT $CD4^+$ et $CD8^+$ par l'intermédiaire de CD30/CD30L (Zeiser, Nguyen et al. 2006).

Notre hypothèse est donc la suivante : le faible nombre de $CD4^+CD25^+$ (exprimant le $CD30^+$?) chez les patients en rejet chronique ne suffirait pas à moduler l'activité des $CD8^+CD28^-$ cytotoxiques (absence d'apoptose) (Baeten, Louis et al. 2006) (**Figure 14, 15**). Les LT $CD4^+CD25^+$ portant le $CD30^+$ interagiraient avec les LT $CD8^+CD28^-$ alloréactifs qui exprimeraient le ligand du CD30 ($CD30L^+$ ou CD153). Mais les LT $CD4^+CD25^+$ ($CD30^+$?) seraient en nombre insuffisant pour induire une apoptose totale des cellules alloréactives LT $CD8^+CD28^-$ ($CD30L^+$?) cytotoxiques chez les patients en rejet chronique. L'accumulation de cellules LT $CD8^+CD28^-$ ($CD30L^+$?) cytotoxiques pourrait ainsi expliquer le rejet chronique.

Dans l'état de tolérance opérationnelle, les LT $CD4^+CD25^+$, en nombre normal, induiraient via le $CD30^+$ l'apoptose des cellules alloreactives $TC8^+CD28^-$ favorisant ainsi le maintien de la balance cellules alloreactives/cellules régulatrices favorisant ainsi le processus de tolérance.

Tolérance



Rejet Chronique

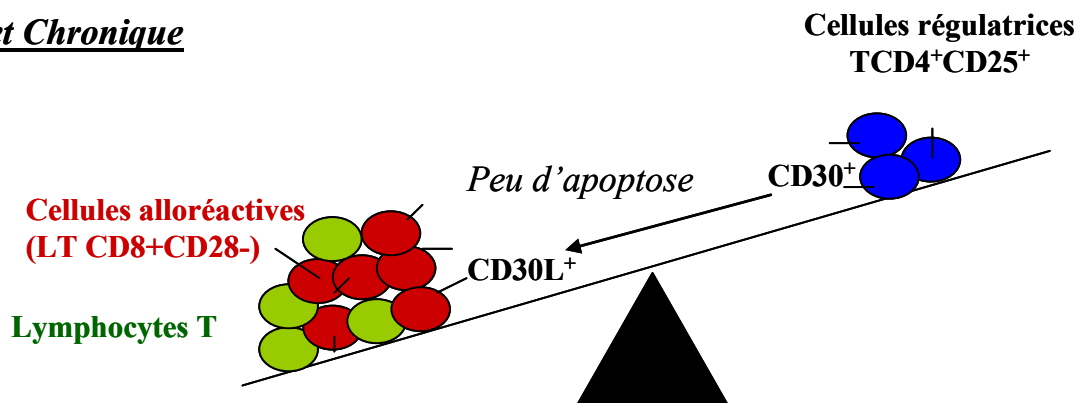


Figure 14: Hypothèse sur le rôle de la voie $CD30/CD30L$ dans l'apoptose des cellules $CD8^+CD28^-$

Nous allons donc étudier le phénotype ($CD30/CD30L$) des cellules $CD4^+CD25^+$ et $CD8^+CD28^-$. Nous réaliserons, chez les patients en rejet chronique, des cultures avec des LT $CD4^+CD25^+$ mis en présence de LT $CD8^+CD28^-$ et nous déterminerons les effets du blocage de la molécule $CD30$ et/ou $CD30L$ sur l'apoptose de ces LT $CD8$.

Collaborations

Différentes études sont menées en collaboration sur la caractérisation des cellules sanguines de ces patients opérationnellement tolérants.

Analyse des lymphocytes B, collaboration S. Hillion, CHU de Brest.

Nos résultats préliminaires indiquent que, si les patients tolérants présentent un nombre normal de lymphocytes B par rapport aux sujet non greffés, les patients présentant un rejet

chronique ont significativement moins de lymphocytes B circulants. Une étude a par ailleurs rapporté une augmentation du nombre de lymphocytes B (LB) circulants chez les patients tolérants une greffe de foie par rapport aux volontaires sains et aux patients stables sous immunosuppression (Li, Koshiba et al. 2004) .

Bien que les LT puissent être considérés comme des acteurs clefs de la reconnaissance allogénique aboutissant à la destruction du greffon, les LB pourraient jouer un rôle sous estimé et mal connu dans la réponse allogénique. Il semble désormais acquis que les LB jouent un rôle central dans le développement et le contrôle de l'immunité. Les LB peuvent agir par exemple dans l'ontogenèse des cellules dendritiques folliculaires (Cerny, Zinkernagel et al. 1998) mais également comme cellules présentatrices d'antigène dans l'activation des lymphocytes T CD4 et T CD8. Il a même été proposé récemment l'existence d'une catégorie de cellules B régulatrices (Serra and Santamaria 2006) productrices d'IL10 qui pourraient, à l'instar des cellules T régulatrices CD4⁺CD25⁺, réguler les différents phénomènes immuns et auto immuns. Enfin, des arguments récents suggèrent un rôle crucial des LB dans l'induction des phénomènes de tolérance au niveau des LT périphériques ouvrant ainsi une nouvelle voie de recherche dans l'étude de la réponse allogénique (Raimondi, Zanoni et al. 2006) (Raimondi, Zanoni et al. 2006).

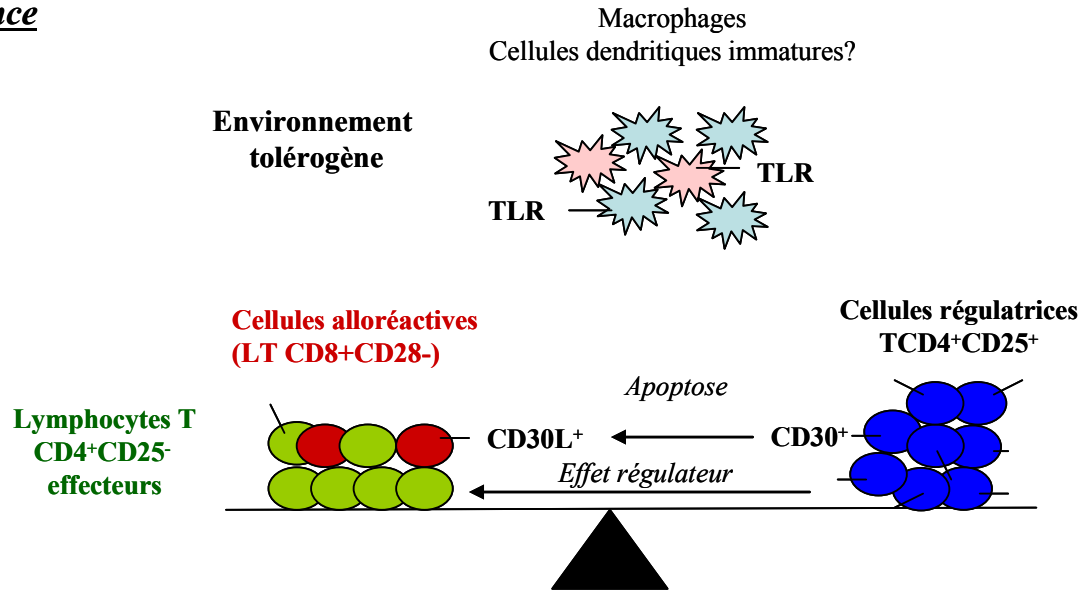
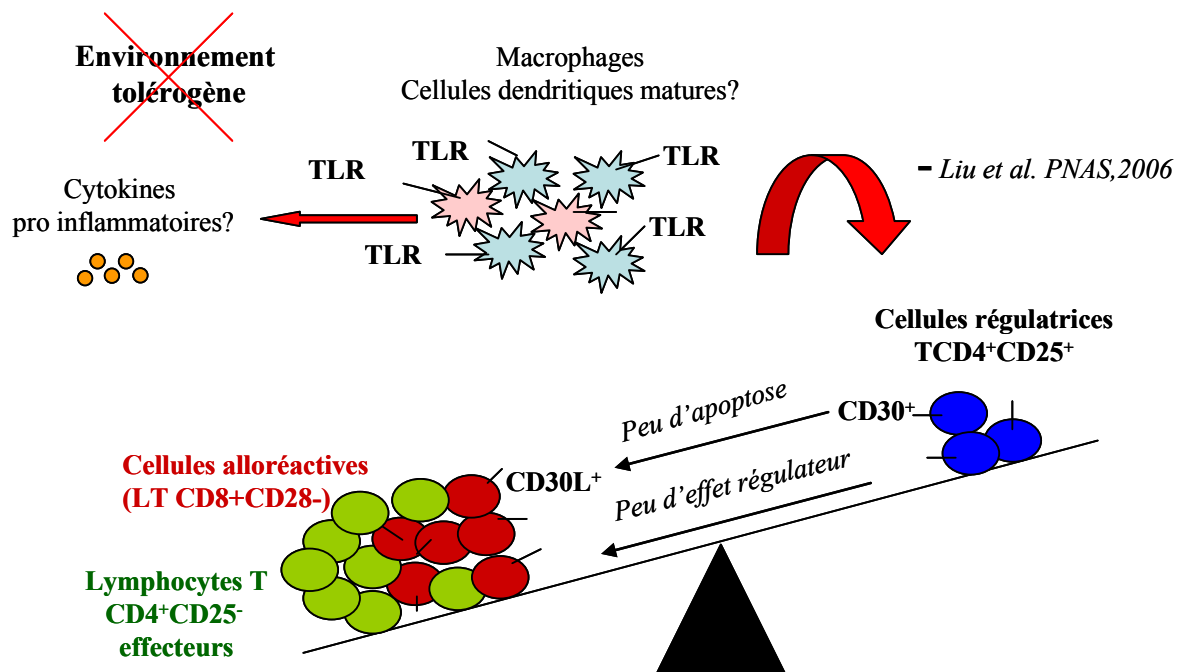
L'analyse des différentes sous populations de LB n'a pour l'instant jamais été réalisée dans le cadre de la transplantation. Cette analyse phénotypique originale des LB sera accompagnée d'une analyse fonctionnelle cherchant à caractériser l'action des LB sur les autres cellules immunitaires ainsi qu'une étude plus particulière des LB régulateurs, ouvrant alors une voie de recherche plus fondamentale. Nous chercherons à caractériser les populations B circulantes par le phénotypage précis de chacune des populations de B matures (LB naïfs Bm1 IgD⁺/CD38⁻, les LB activés Bm2 IgD⁺/CD38⁺, les cellules fondatrices du centre germinatif Bm2' IgD⁺/CD38^{high}, les cellules du centre germinatif (centroblastes et centrocytes Bm3 et Bm4) IgD⁻/CD38^{high}, les cellules B mémoires précoces EBm5 (IgD⁻/CD38⁺), les cellules B mémoires Bm5 IgD⁻/CD38⁻, les plasmocytes IgD⁻/CD38^{high} CD138⁺) (Hillion, Saraux et al. 2005). Une étude sur coupe sera également menée afin d'identifier la présence d'un infiltrat B ou plasmocytaires sur des coupes de biopsie rénale des patients en rejet chronique. Le rôle de ces populations B sur d'autres populations lymphocytaires (LT) sera ensuite évalué en cultures mixtes. Nous espérons ainsi améliorer la compréhension des mécanismes immunologiques du rejet chronique et démontrer l'implication ou non des cellules B dans le maintien d'une fonction stable du greffon voir de l'établissement d'un état de tolérance.

Analyse des cellules dendritiques, collaboration R Josien, INSERM U64, Nantes

Nous avons également débuté, en parallèle, une étude de la répartition et du phénotype des cellules dendritiques (DC) chez ces patients. Chez l'homme, Thomson et al ont montré une augmentation du nombre des DC plasmacytoides dans le sang périphérique chez les patients tolérant une greffe de foie sans ou sous faible dose d'immunosuppresseurs comparativement à ceux qui développent un rejet (Mazariegos, Zahorchak et al. 2003) (Mazariegos, Zahorchak et al. 2005). Les cellules dendritiques jouent également un rôle critique dans l'induction et la régulation de la réponse immune. De nombreuses études suggèrent une tolérogénicité des DC dans le contexte de la reconnaissance du soi et de la réponse allogénique (Banchereau and Steinman 1998) (Steinman, Turley et al. 2000). Deux sous populations de DC on été décrites chez l'homme, les DC myéloïdes (DC1) et plasmacytoides (DC2) (Kohrgruber, Halanek et al. 1999) (Robinson, Patterson et al. 1999). Ces 2 populations cellulaires circulent dans le sang et se différencient par l'expression de marqueurs spécifiques (HLADR+ lineage- CD11c+ pour les DC1, CD123+ pour les DC2). Les DC1 induiraient une différenciation des LT vers un profil Th1 et sont responsables d'une réponse immune tandis que les DC2 favoriseraient une différenciation des LT vers un profil Th2 (Rissoan, Soumelis et al. 1999) et seraient favorables à l'induction d'une tolérance (Liu, Kanzler et al. 2001). Récemment il a été montré que des DC immatures sont incapables de fournir des signaux d'activation efficaces pour l'activation de lymphocytes T et ainsi peuvent induire un état de tolérance (Fu, Li et al. 1996) (Lutz, Suri et al. 2000). Ces données laissent penser que le fort niveau de TLR observé chez nos patients en rejet chronique pourrait être corrélé à une augmentation de la maturation des DC et donc contribuer au rejet, tandis que le faible taux de TLR chez les patients tolérants contribuerait à leur état de tolérance opérationnelle (**Figure 15**).

L'induction d'une tolérance spécifique à une allogreffe serait une avancée considérable dans le domaine de la transplantation. Dans cette étude, nous avons analysé le phénotype et la fonction des cellules sanguines, chez des patients greffés rénaux opérationnellement tolérants ou en rejet chronique. Nous avons comparé l'effet suppresseur des LT CD4⁺CD25⁺ des volontaires sains, des patients traités par des immunosuppresseurs, des patients transplantés en rejet chronique et des patients opérationnellement tolérants. Les résultats de cette étude suggèrent que les cellules régulatrices des patients opérationnellement tolérants ne sont pas significativement différentes de celles des volontaires sains, puisque ces patients présentent des LT CD4⁺CD25⁺FOXP3⁺ avec un phénotype et des fonctions régulatrices comparables. Au contraire, les patients qui

développent un rejet chronique, présentent un déficit en nombre et en fonction de leurs cellules suppressives T CD4⁺CD25⁺FOXP3⁺ (**Figure 15**). Notre objectif est maintenant d'en étudier plus précisément les mécanismes d'action.

Tolérance**Rejet Chronique****Figure 15: Schéma général**

Hypothèses : *Dans l'état de tolérance opérationnelle*, les LT CD4⁺CD25⁺ en nombre normal exercent un effet régulateur vis-à-vis de la prolifération des cellules effectrices LT CD4⁺CD25⁻. Ces LT CD4⁺CD25⁺ induiraient via le CD30⁺ l'apoptose des cellules alloréactives CD8⁺CD28⁻. Peu d'expression des TLR et la présence de cellules dendritiques immatures favoriseraient l'établissement d'un environnement tolérogène.

Dans le rejet chronique, les LT CD4⁺CD25⁺ seraient en nombre insuffisant pour induire une apoptose totale des LT CD8⁺CD28⁻ cytotoxiques et pour contrôler la prolifération de cellules effectrices LT CD4⁺CD25⁻. La surexpression des TLR et la présence de cellules dendritiques matures empêcheraient l'établissement d'un environnement tolérogène.

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Annexes

Annexe 1

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

Par: Degauque N. *, Lair D. *, **Braudeau C.**, Haspot F., Sebille F., Dupont A. Merriau E., Brouard S. * and Souillou JP*.

** Les auteurs ont contribué de manière égale à ce travail*

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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. The *in vitro* suppressive activity of CD25⁻ T cells required both cell contact and soluble factors (IL-10 and IFN- γ). 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 T_{REG} 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 and cytokine mediated. 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; $p < 0.05$). 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 (1×10^5 naive CD25⁻ T responder cell cultured with 1×10^5 CD25⁻ T cell from tolerant recipient), the mean percentage of proliferation was $44\% \pm 9\%$ 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 $166\% \pm 18\%$ 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 tolerant 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 $\pm$ 1 days (excepted for 2 out of 17 animals). Adoptive transfer of 50x10⁶ 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 50x10⁶ T cells from naive LEW.1A rat (n=2; Fig.3) did not prolong graft survival. Finally, 50x10⁶ 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 2x10⁶ CD4⁺CD25⁺ T cells from desoxyspergualine derivate-treated animals was efficient in transferring tolerance in the same strain combinaison [29]. Adoptive transfer of 4x10⁶ CD25⁺ T cells from tolerant animals did not prolong significantly heart survival to LEW.1A hosts (15.5 days; n=4; Fig.3).

***In vitro* suppressive activity of CD25⁺ T cells from tolerant animals is both cell-contact dependent and IL-10 / IFN- γ dependent.**

The mechanisms involved in the hyporesponse of CD25⁺ T cells to allostimulation were then investigated. The inhibition of IDO, iNOS (data not shown) and specific blocking antibodies against IL-4, IL-10, or TGF- β (data not shown) did not restore the alloreactivity of CD25⁺ T cells of tolerant rats. However, neutralization of IFN- γ as well as addition of exogenous IL-2 significantly restored CD25⁺ T cell responses of tolerant rats to donor APCs (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.

To further study the suppressive mechanisms of CD25⁺ T cells from tolerant animals, different inhibitors were added to the coculture system. Again, the *in vitro* suppressive activity of CD25⁺ T cells from tolerant animals were not modified when IDO or iNOS were inhibited (data not shown) or when blocking antibodies against IL-4, or TGF- β were used (data not shown). However, suppressive activity of CD25⁺ T cells from tolerant animals could be abolish by neutralizing IL-10 or IFN- γ or by the addition of exogenous IL-2 (Fig.4.C).

Taken together, we have shown that CD25⁺ T cells from tolerant animals are able *in vitro* to inhibit the proliferation of naïve CD25⁺ T cells through a composite mechanism, that required (i) direct cell-contact and (ii) soluble factors (IL-10 and IFN- γ) (Figure 4.D).

We then tested the effect on the regulatory functions of separating CD4⁺ and CD8⁺ CD25⁺ T cells of tolerant animals. Neither CD4⁺ CD25⁺ T cells nor CD8⁺ CD25⁺ T cells from tolerant animal inhibited the naïve CD25⁺ T cells allogeneic response (data not shown). Taken together, our data suggested that both CD4⁺ CD25⁺ and CD8⁺ CD25⁺ T cells of tolerant animals have to be present to mediate a full regulation of alloreactivity.

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 naïve 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 naïve 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 (5-fold) in CD25⁺ T cells of tolerant animals vs. CD25⁺ T cells of naïve animals ($p < 0.001$; Fig.5.B). To assess the extend of Foxp3 expression detected in CD25⁺ T cells from tolerant recipients, we analyze the Foxp3 expression in the CD25⁺ T cells fraction from naïve and tolerant animals. Naïve CD25⁺ T cells expressed 500 fold higher levels of Foxp3 transcripts as compared to naïve CD25⁺ T cells and 100 fold as compared to tolerant CD25⁺ T cells (Fig.5.B). CD25⁺ T cells from tolerant recipients accumulate Foxp3 transcripts without reaching the statistical significance (3 fold increase as compared to naïve CD25⁺ T cells; 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 mediated effect [36-39], the reversal of the inhibition by exogenous IL-2 [40] and the absence of CDR3 length distribution biases (our unpublished data) [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, 4x10⁶ of 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 T_{REG} 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 (50×10^6) 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- β 1 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- β 1 antibody abrogated allograft tolerance [13]. The role of TGF- β 1 has also been shown in another strain combination using another protocol to induce tolerance [50]. Fantini *et al.* have demonstrated that TGF- β 1 *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- β 1 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.

Our transcriptional profiling of CD25⁺ T regulatory cells show an increase in level of expression of IL-10 and IFN- γ transcripts. We also demonstrate that these two cytokines were mandatory *in vitro* for the tolerant CD25⁺ T cells to inhibit the proliferation of naïve CD25⁺ T cells. These findings are in agreement with several reports in the literature, using similar DST protocol. Koshiba *et al.* have reported that tolerated grafts but not rejected ones exhibit high level of IL-10 and IL-4 expression in DST-treated long term recipients of heart allografts [52]. 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 administered early following grafting inhibited rather than potentialized the effect of DST on a subsequent heart transplant [55]. Implications of these two cytokines remained to be determined on the mechanism of long term maintenance of heart allografts.

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 naïve 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 congenic LEW.1A (RT1^a), LEW.1W (RT1^u) and BN (RT1^a) rats were purchased from Janvier (Savigny/Orge, France). LEW.1W or BN heart grafts were implanted heterotopically 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. CD25⁻ T cells were negatively selected using Ox39 (anti-CD25) mAb, followed by incubation with Dynabeads[®] Pan Mouse IgG according to the manufacture's recommendations (purity >95%). To purify CD25⁺ T cells, spleen cells were incubated with anti-rat CD25 mAb (Ox39 mAb) followed by incubation with anti-mouse IgG1 microbeads (Miltenvi Biotec). Magnetic separation was then performed with LS-positive selection column according to manufacture's recommendations.

Mixed lymphocyte reaction (MLR) and transwell experiments.

According to experiments, 1×10^5 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 2×10^4 of APC, obtained as described elsewhere [57], from either LEW.1W or BN rats for 5 days at 37°C/7% CO₂. 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. 6×10^5 CD25⁻ T cells of naive LEW.1A rats were stimulated with 3×10^5 of APC from LEW.1W rats. Additionally, 6×10^5 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- β 1 (clones 2G7) [58], anti-rat IL-10, anti-rat IFN- γ 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-rat IL-4 was used at 10 μ g/mL, anti-rat IL-10, anti-rat IFN- γ and anti-rat TGF- β 1 antibodies at 100 μ g/mL, 5 μ L of L-N Methyl Arginine (Sigma) or 1mg/mL of 1-methyltryptophane (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 μ g RNA was reverse-transcribed as previously described [59]. A constant amount of cDNA was amplified in 25 μ L SYBR® Green PCR Core Reagent (Applied Biosystems, Foster city, CA) with 1mg/mL of BSA, 3 mM MgCl₂, 200 μ M 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 one out of five 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 1×10^5 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 1×10^5 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 = 16 days, n=17); ..▲.., sub irradiated LEW.1A recipients transferred with 50×10^6 T cells from naive animals (MST = 14 days, n=2); —■—, sub irradiated LEW.1A recipients transferred with 50×10^6 CD25⁻ T cells from tolerant animals (MST > 100 days, n=9; p<0.05); —●—, sub-lethally irradiated LEW.1A recipients transferred with 4×10^6 CD25⁺ T from tolerant animals

(MST = 15.5 days, n=4). **■**, sub irradiated LEW.1A recipients transferred with 50×10^6 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. CD25⁻ T cells were obtained from tolerant recipients (black bar) or naive (white bar) LEW.1A rats and were stimulated with LEW.1W rat APCs. Isotype control or anti-IL-10 or anti IFN- γ antibodies or IL-2 were added to the culture. Bars represent mean $\pm$ SEM of one out of at least 3 experiments. 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 $\pm$ SEM obtained in the lower chamber. C. CD25⁻ T cells obtained from tolerant recipients (black bar) or naive (white bar) LEW.1A rats, were added at a ratio 1:1 to naive CD25⁻ T cells in LEW.1W MLRs. Isotype control or anti-IL-10 or anti-IFN- γ antibodies or IL-2 were added to the coculture system. Bars represent mean $\pm$ SEM of one of out at least 3 experiments.

Figure 5. CD25⁻ T cells from long term tolerant rats accumulate IFN- γ , IL-10 and FoxP3 transcripts. (A). Real-time RT-PCR analysis of cytokines (IL-2, TNF- α , IFN- γ , IL-10, IL-13, TGF- β 1) expression in CD25⁻ T cells from either tolerant (n=6) or naive (n=6) LEW.1A rats. (B). FoxP3 expression in CD25⁺T cells and CD25⁻T cells from naïve and tolerant LEW.1A rats (n=3 at least). Bars represent mean $\pm$ SEM and statistical significance was tested using the Mann-Whitney test.

Fig.1

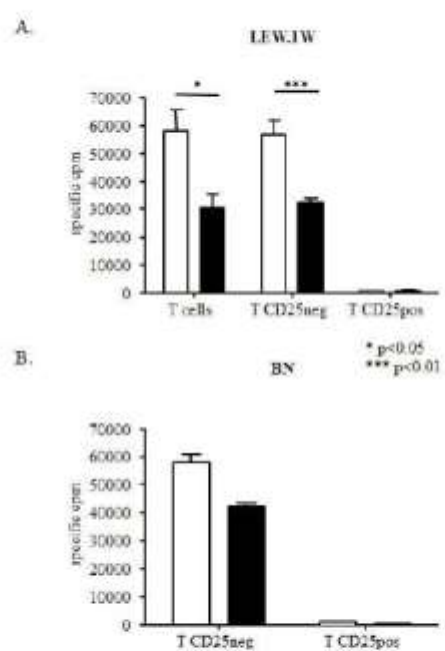


Fig.2

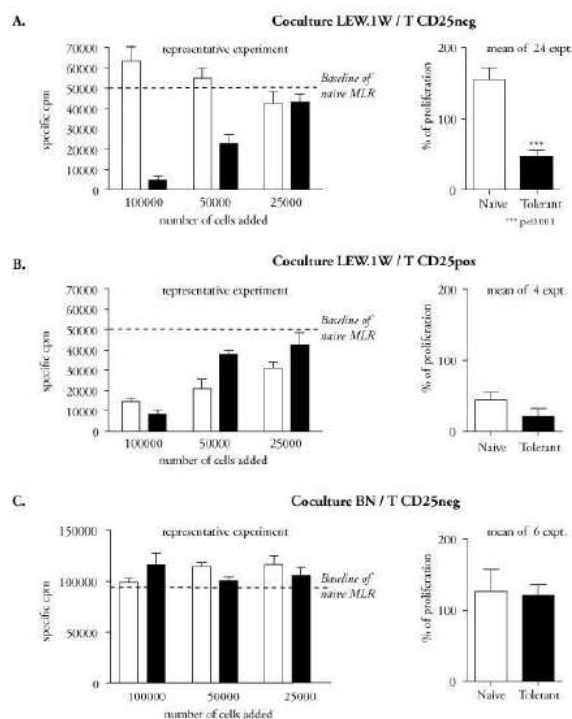


Fig.3

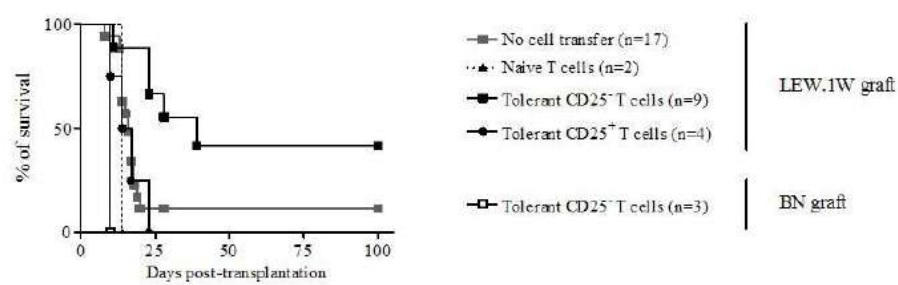


Fig.4

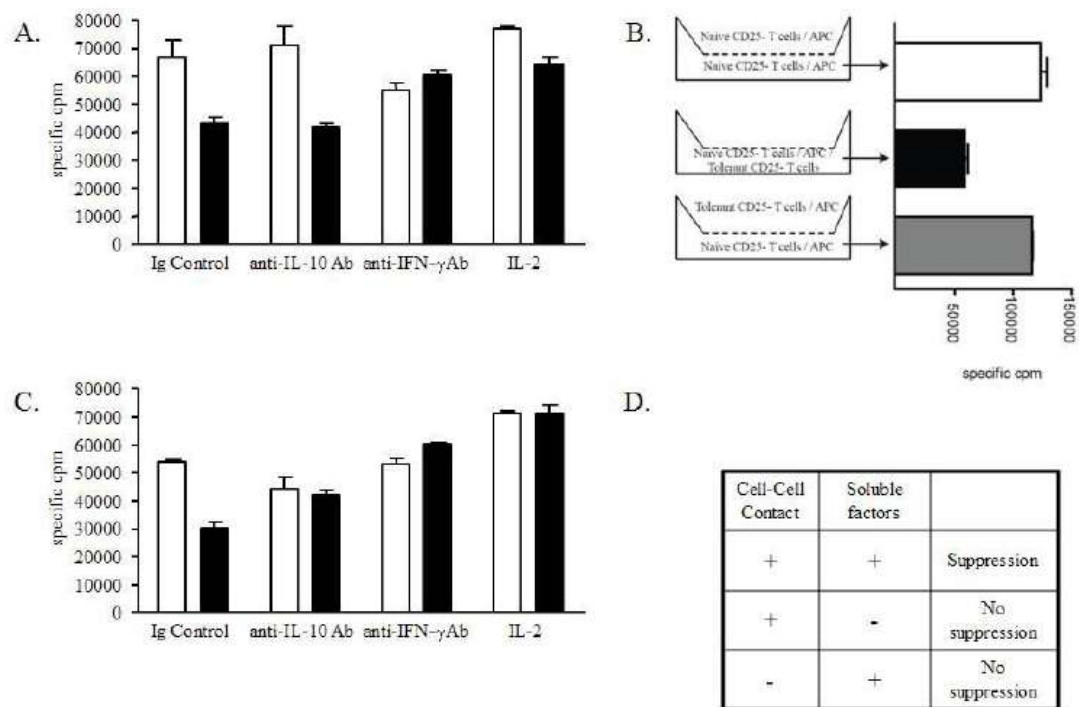
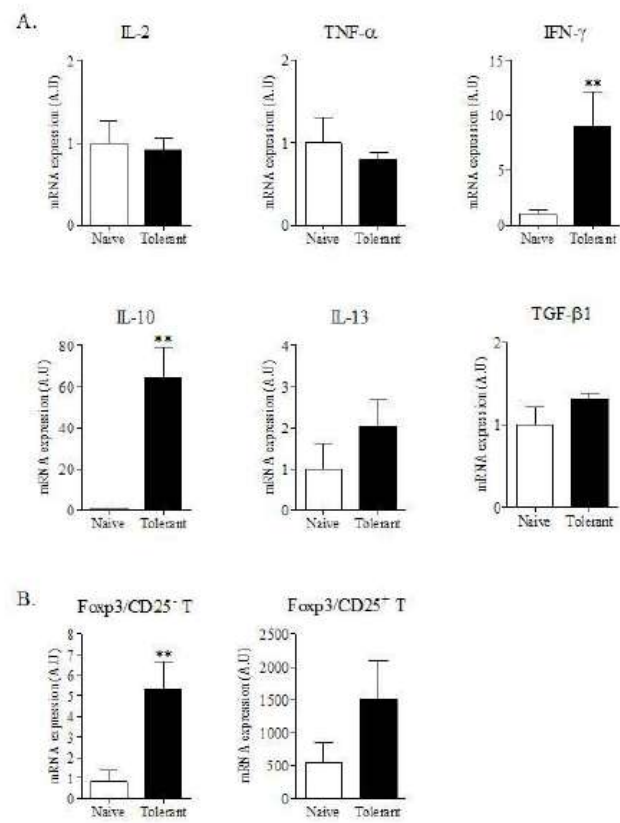


Fig.5



Annexe 2

*Phenotypically and functionally distinct CD8⁺
lymphocyte populations in long term drug-free tolerance
and chronic rejection in human kidney graft recipients*

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Phenotypically and Functionally Distinct CD8⁺ Lymphocyte Populations in Long-Term Drug-Free Tolerance and Chronic Rejection in Human Kidney Graft Recipients

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A substantial proportion of long-term kidney graft recipients, including those with a stable renal function in the absence of immunosuppressive therapy, present a skewed T cell receptor (TCR) V β chain usage, essentially in the CD8⁺ subset. This study analyzed in more detail phenotypical and functional alterations of CD8⁺ lymphocytes in drug-free tolerant patients (DF-Tol) compared with recipients with chronic rejection (CR). Phenotyping revealed a significant increase in central memory and a decrease in effector CD8⁺ lymphocytes in DF-Tol versus CR. The expression of CD28⁺ and CD27⁺ on these effector cells was significantly decreased in CR. These profiles were stable over time and independent of treatment. Functionally, the CD8⁺CD28⁺ lymphocytes were less sensitive to apoptosis than their CD8⁺CD28⁺ counterparts, without differences in polyclonal proliferation. The CD8⁺CD28⁺ cells did not express GITR and FoxP3 but were characterized by high levels of preformed perforin and granzyme A, pointing toward a cytotoxic rather than a suppressor function. CD8⁺CD28⁺ lymphocytes did not show antigen-specific degranulation when co-cultured with targets that bear donor HLA class I antigens, suggesting that the cytotoxicity is directed either to other determinants of the graft or to nongraft epitopes. Of interest, CD8⁺ cells from DF-Tol displayed the same profile as healthy individuals, indicating an increase in CD8⁺CD28⁺ effector lymphocytes in CR rather than a decrease in DF-Tol. CD8⁺ lymphocytes from stable kidney recipients under conventional maintenance immunosuppression displayed a mixed profile, independent of treatment and time of sampling. Taken collectively, these data show a strong cytotoxicity-associated CD8⁺CD28⁺ signature in CR and suggest a suppression of pathologic cytotoxicity in DF-Tol. Further prospective studies should assess whether serial CD8⁺ phenotyping may help to identify patients who are at risk for CR when immunosuppression is tapered.

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Drug-free tolerance, defined as long-term maintenance of graft integrity and function without immunosuppression, is a rare event in human kidney transplantation because interruption of immunosuppressive treatment usually leads to acute or chronic graft rejection. Nevertheless, this phenomenon is of unique interest to study the physiologic basis of graft tolerance in humans. On the one hand, long-term drug-free tolerant patients (DF-Tol) represent a unique model to study the extent to which mechanisms of tolerance defined experimentally, such as active suppression by regulatory lymphocytes, ignorance of alloantigens, chimerism, homeostatic

regulation or clonal deletion, are relevant to this human situation (1–4). Most studies in rodents analyzed the induction rather than the maintenance phase of tolerance, and discrepancies with the human situation may exist, as exemplified by the role of alloreactive CD8⁺ central memory cells in rejection and tolerance induction (5,6). On the other hand, the characterization of peculiar immunologic profiles in DF-Tol may be clinically important to identify biologic signatures that are associated with graft tolerance. Considering the major medical and economic burden of chronic immunosuppression and that operational tolerance may be more common than expected but could be masked in long-term immunosuppressed patients, the identification of specific biologic signatures of tolerance could open new perspectives for rational rather than empiric minimizing of immunosuppressive drugs in well-selected patients (6–10).

As a proof of concept of the relevance of the DF-Tol model to study human tolerance, we described recently a number of specific immunologic features in these patients. First, DF-Tol

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were characterized by a maintenance of CD25^{high}CD4⁺ lymphocytes that express regulation-associated molecules such as FoxP3, CTLA4, GITR, CCR4, and CD103, in comparison with patients with chronic rejection of their allograft (CR) (Louis *et al.*, unpublished observations). Second, peripheral blood T cells of a substantial proportion of DF-Tol and CR displayed a skewed T cell receptor (TCR) V β chain usage, which was observed mainly in the CD8⁺ subset (11). The T cells with skewed V β profiles from DF-Tol were characterized by a decrease in cytokine transcripts (IL-2, IL-13, and IFN- γ) compared with CR, suggesting a state of hyporesponsiveness or anergy (11).

On the basis of the data of this latter study, we performed here a systematic analysis of CD8 phenotypes in DF-Tol *versus* CR and compared this with healthy individuals (HI). Our data show that a population of CD8⁺CD28⁻ lymphocytes was dramatically increased in CR. This subpopulation was characterized further with regard to apoptosis and proliferation, regulatory markers such as GITR and FoxP3, and general as well as donor-specific cytotoxicity. Finally, we evaluated the presence of this CD8⁺ population in kidney graft recipients with stable graft function under standard maintenance immunosuppressive therapy (Sta).

Materials and Methods

Patients

A total of 38 individuals were included in the study. The protocol was approved by the University Hospital Ethical Committee and the Committee for the Protection of Patients from Biologic Risks. All patients signed a written informed consent before inclusion.

The study included six DF-Tol. Operational tolerance was clinically defined as stable graft function and absence of clinical or biologic signs of chronic rejection (blood creatinemia <150 mmol/L, proteinuria <1.5 g/24 h) for at least 2 yr (median 8 yr; range 2 to 12 yr) after complete interruption of all immunosuppressive therapy. Immunosuppressive treatment was stopped because of lymphoma in two patients and noncompliance in the four others (11). These normally functioning kidneys were not biopsied for ethical reasons. The CR group included 14 kidney graft recipients with a degradation of the renal function (blood creatinemia >150 mmol/L) and histologically proven chronic rejection lesions. As control groups, we included six HI and 12 kidney

graft recipients with stable renal function under Sta. Demographic and clinical data are shown in Table 1.

Phenotypic Characterization by Flow Cytometry

Peripheral blood mononuclear cells (PBMC) were isolated using a Ficoll gradient (Eurobio, Les Ulis, France) and incubated for 30 min with the following fluochrome-labeled mAb for phenotypic characterization: Anti-CD45RA-FITC, anti-CD57-FITC, anti-KIR-NKAT2-FITC, anti-CD94-FITC, anti-CD28-PE or anti-CD28-APC, anti-CD27-PE, anti-CD69-PE, anti-GITR-PE, anti-CCR7-PC7, anti-CD3-Cy-Chrome or CD3-PC7, anti-CD8-PE-Cy5.5 or anti-CD8-APC, anti-CD95-APC, and anti-NKG2D-APC (all mAb from BD Biosciences Pharmingen, San Diego, CA). For the assessment of intracellular proteins, PBMC were permeabilized with saponin 1% for 15 min, and intracellular perforin and granzyme A were stained with PE-labeled mAb (BD Biosciences Pharmingen). The labeled PBMC were washed, fixed in PBS/formaldehyde 1%, and analyzed by four-color flow cytometry (FACSCalibur, Becton Dickinson, San Diego, CA) using Cellquest Pro software (Becton Dickinson). T lymphocytes were identified using a forward and side scatter gate for lymphocytes in combination with a gate on CD3⁺ cells. Nonspecific staining and autofluorescence were determined by isotype-matched control mAb. Results are expressed as mean percentage of positive cells or as mean fluorescence intensity.

Detection of Apoptosis

For *in vitro* induction of apoptosis, PBMC were cultured for 18 h at 37°C in serum-free RPMI 1640 medium (Sigma, St. Louis, MO). As negative controls, PBMC were cultured in RPMI with 10% heat-inactivated FCS. After 18 h, PBMC were labeled with phenotypic markers as described and with annexin V-APC (BD Biosciences Pharmingen). The percentage of annexin V-positive cells was measured by flow cytometry after exclusion of dead cells by propidium iodide labeling.

CFSE Proliferation Assay

PBMC were stained with 1 μ M of carboxyfluorescein diacetate succinimidyl ester (CFSE) for 3 min, washed extensively, and adjusted to 5×10^5 cells/ml in RPMI 1640 with 10% FCS. CFSE-labeled PBMC were stimulated with 1 μ g/ml plate-bound anti-CD3 antibody (Orthoclone OKT3; Janssen-Cilag, Germany) with or without 100 U/ml IL-2 (Proleukin; Chiron Corp., Emeryville, CA). After 72 h, cells were stained for 15 min with anti-CD8-PE-Cy5.5 and anti-CD28-APC antibodies. Pro-

Table 1. Demographic and clinical data of the patient cohorts^a

	DF-Tol (n = 6)	CR (n = 14)	HI (n = 6)	Sta (n = 12)
Gender, male/female	5/1	7/7	4/2	8/4
Median age, yr (range)	57 (37 to 73)	53 (35 to 72)	53 (33 to 62)	53 (30 to 76)
Median yr after transplantation (range)	14 (5 to 30)	7 (2 to 6)	NA	12 (4 to 24)
Corticosteroids	0/6	6/14	0/6	6/12
Azathioprine	0/6	1/14	0/6	6/12
Calcineurin inhibitor	0/6	11/14	0/6	5/12
Mycophenolate mofetil	0/6	6/14	0/6	5/12

^aDF-Tol, kidney graft recipients who were operationally tolerant after interruption of immunosuppressive therapy (drug-free tolerant); CR, patients with chronic graft rejection despite immunosuppressive treatment; HI, healthy individuals; Sta, kidney recipients with stable renal function while treated with standard immunosuppressive maintenance regimen; NA, not applicable. Calcineurin inhibitors comprise cyclosporin A and tacrolimus.

liferation was analyzed by measuring the CFSE signal on gated CD8⁺CD28⁺ and CD8⁺CD28⁻ cells by flow cytometry.

Real-Time PCR

CD8⁺ T cells from six CR were negatively isolated by magnetic bead sorting (Milteny Biotec, Bergisch Gladbach, Germany). Then, CD8⁺CD27⁺ and CD8⁺CD27⁻ subsets were separated by a positive magnetic selection (Milteny Biotec). Because CD27 and CD28 expression correlated perfectly on CD8⁺ T lymphocytes, anti-CD27 rather than anti-CD28 beads were used to avoid cell activation. The sorted CD8⁺CD27⁺ and CD8⁺CD27⁻ populations contained >90% CD8⁺CD28⁺ and CD8⁺CD28⁻ lymphocytes, respectively, as assessed by flow cytometry. Purified cells were frozen in Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) for RNA extraction according to the manufacturer's instructions. Total mRNA was reverse-transcribed using a cDNA synthesis kit (Boehringer Mannheim, Indianapolis, IN). Real-time quantitative PCR was performed using labeled TaqMan probes specific of FoxP3 and normalized against the hypoxanthine phosphoribosyl transferase-1 (HPRT) transcript level, as described previously (11).

Cytotoxicity-Associated Degranulation Assay

CD8⁺ lymphocytes of seven CR were assessed for antigen-specific degranulation by the CD107 mobilization assay, as described previously (12,13). Because PBMC of the kidney graft donors were not available several years after transplantation, we collected from healthy blood donors surrogate PBMC that were matched for the HLA class I molecules of the kidney graft donors. Recipient PBMC from CR were incubated for 5 h either with irradiated surrogate PBMC at a 1:1 ratio or with a pool of common viral peptides at 10 µg/ml (gift of J.-G. Guillet, Institut Pasteur, Paris, France) in RPMI medium with 10% human serum and 2 µM monensin (Sigma-Aldrich). Unstimulated recipient PBMC were used as negative control, whereas plate-bound anti-CD3 antibody or phytohemagglutinin stimulation was used as positive control. Degranulation of CD8⁺CD28⁻ lymphocytes was assessed by flow cytometric analysis with a mix of anti-CD107a-FITC and anti-CD107b-FITC antibodies (BD Biosciences Pharmingen).

Statistical Analyses

The Mann-Whitney *U* test (for unpaired samples) and the Wilcoxon test (for paired samples) were used to assess differences between groups. Correlations were calculated with the Spearman's ρ rank correlation test. $P < 0.05$ was considered as statistically significant. Classification of Sta according to their CD8⁺ phenotype was performed by Predictive Analysis of Microarray data software (14).

Results

Increase of CD8⁺CD28⁻ Effector Lymphocytes in CR

Peripheral blood CD8⁺ T lymphocytes from DF-Tol and age-matched CR were analyzed for the surface expression of CD45RA and CCR7 to distinguish naive (CD45RA⁺CCR7⁺), effector (CD45RA⁺CCR7⁻), central memory (CD45RA⁻CCR7⁺), and effector memory (CD45RA⁻CCR7⁻) CD8⁺ lymphocytes (15,16). As shown in Table 2, there was a significant increase in central memory ($P = 0.032$) and decrease in effector ($P = 0.048$) CD8⁺ lymphocytes in DF-Tol versus CR. Of interest, the percentage of CD45RA⁺CCR7⁻ effector CD8⁺ lymphocytes in age-matched HI was similar to DF-Tol but significantly lower than in CR ($P = 0.048$), indicating that these differences correspond to an increase in CD8⁺ effectors in CR rather than a decrease in DF-Tol.

Effector as well as effector memory CD8⁺ cells can be subdivided further according to the loss of surface expression of CD28 and CD27 during terminal differentiation (15). As shown in Table 2, the percentage of CD28⁺ ($P = 0.040$) and CD27⁺ ($P = 0.018$) CD8⁺ effector lymphocytes was significantly higher in DF-Tol than in CR. A similar difference in CD28 ($P = 0.002$) and CD27 ($P = 0.002$) expression was observed in the effector memory subset. This was also reflected by a significantly higher expression of both CD28 ($P = 0.001$) and CD27 ($P = 0.006$) on the global CD8⁺ population, with a high correlation between both markers ($r = 0.91$, $P < 0.001$; Figure 1). Analysis of age-matched HI revealed that the expression of CD28 and CD27 on the different CD8⁺ lymphocyte subsets was similar in

Table 2. Phenotypic analysis of CD8⁺ T lymphocytes in DF-Tol, CR, and age-matched HI^a

	DF-Tol (n = 6; %)	CR (n = 14; %)	HI (n = 6; %)
CD8 ⁺			
CD45RA ⁺ CCR7 ⁺	33.9 ± 19.5	24.4 ± 11.7	26.0 ± 14.1
CD45RA ⁺ CCR7 ⁻	19.6 ± 7.6 ^b	11.7 ± 5.7 ^b	13.6 ± 6.4
CD45RA ⁻ CCR7 ⁺	25.1 ± 13.9	31.3 ± 11.4	35.9 ± 13.8
CD45RA ⁻ CCR7 ⁻	21.4 ± 9.6 ^b	32.7 ± 11.3 ^{b,c}	24.8 ± 3.8 ^c
CD8 ⁺ CD45RA ⁻ CCR7 ⁻			
CD27	77.1 ± 14.7 ^b	31.8 ± 16.9 ^{b,c}	82.5 ± 14.1 ^c
CD28	78.3 ± 3.4 ^b	23.6 ± 15.7 ^{b,c}	77.1 ± 14.4 ^c
CD8 ⁺ CD45RA ⁺ CCR7 ⁻			
CD27	44.0 ± 15.0 ^b	17.6 ± 14.1 ^{b,c}	45.7 ± 3.8 ^c
CD28	30.8 ± 3.7 ^b	10.9 ± 12.6 ^{b,c}	29.3 ± 4.5 ^c

^aThe percentage of naive (CD45RA⁺CCR7⁺), central memory (CD45RA⁻CCR7⁺), effector memory (CD45RA⁻CCR7⁻), and effector (CD45RA⁺CCR7⁻) cells was analyzed, as well as the expression of CD28 and CD27 on the effector and effector memory subsets. Results are expressed as mean ± SD percentage of positive cells. There were no significant differences between DF-Tol and HI.

^b $P < 0.05$ between DF-Tol and CR.

^c $P < 0.05$ between CR and HI.

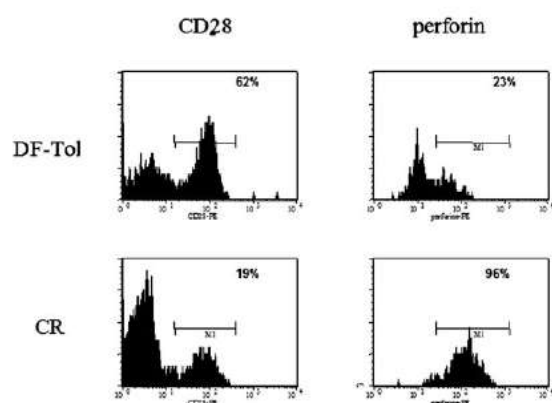


Figure 1. Analysis of the expression of CD28 on the cell surface and of intracellular perforin in peripheral blood CD8⁺ lymphocytes in drug-free tolerant kidney graft recipients (DF-Tol) and in patients with chronic rejection of the graft (CR). Representative histograms show the increased expression of CD28 and the decrease of intracellular perforin in DF-Tol *versus* CR.

DF-Tol and HI but significantly decreased in CR (Table 2). Taken together, these data indicate an increase in CD8⁺CD28⁺ effector lymphocytes in CR, whereas DF-Tol exhibited a pattern close to that of HI.

Increase of Effector CD8⁺CD28⁺ Lymphocytes in CR Is Stable over Time and Independent of Treatment

To investigate whether the increase of CD8⁺CD28⁺ lymphocytes was a stable phenomenon related directly to CR, paired PBMC that were obtained in five patients at two different time points with an interval of 7.4 (2 to 16) months were analyzed. There was no significant variation of the percentage of CD45RA⁺CCR7⁺CD8⁺ effector cells (35.5 ± 8.5 *versus* $29.4 \pm 7.3\%$) or of the expression of CD28 (19.2 ± 10.7 *versus* $17.5 \pm 10.7\%$) and CD27 (32.3 ± 13.8 *versus* $34.4 \pm 19.7\%$), indicating that the observed CD8 profile was stable over time (Figure 2A).

Considering the heterogeneity of treatment in CR (Table 1), we next analyzed the potential effect of treatment on these profiles. Comparison of CR who were treated or not with corticosteroids, calcineurin inhibitors (CNI), or mycophenolate mofetil did not show a significant difference for the percentage of CD45RA⁺CCR7⁺CD8⁺ effector cells (37.4 ± 12.6 *versus* $29.2 \pm 9.4\%$, 30.4 ± 11.3 *versus* $41.1 \pm 6.8\%$, and 37.1 ± 10.9 *versus* $29.4 \pm 11.0\%$, respectively), CD28 expression (15.1 ± 9.7 *versus* $24.0 \pm 14.3\%$, 21.5 ± 14.0 *versus* $15.4 \pm 13.1\%$, and 18.4 ± 10.9 *versus* $21.5 \pm 15.9\%$, respectively), or CD27 expression (26.8 ± 13.1 *versus* $36.2 \pm 18.4\%$, 32.6 ± 16.8 *versus* $26.7 \pm 14.4\%$, and 27.5 ± 12.9 *versus* $33.1 \pm 17.7\%$, respectively; Figure 2, B through D). Accordingly, after exclusion of corticosteroid-, CNI-, or mycophenolate mofetil-treated CR, there was still a significant decrease of CD28 expression ($P = 0.001$, $P < 0.001$,

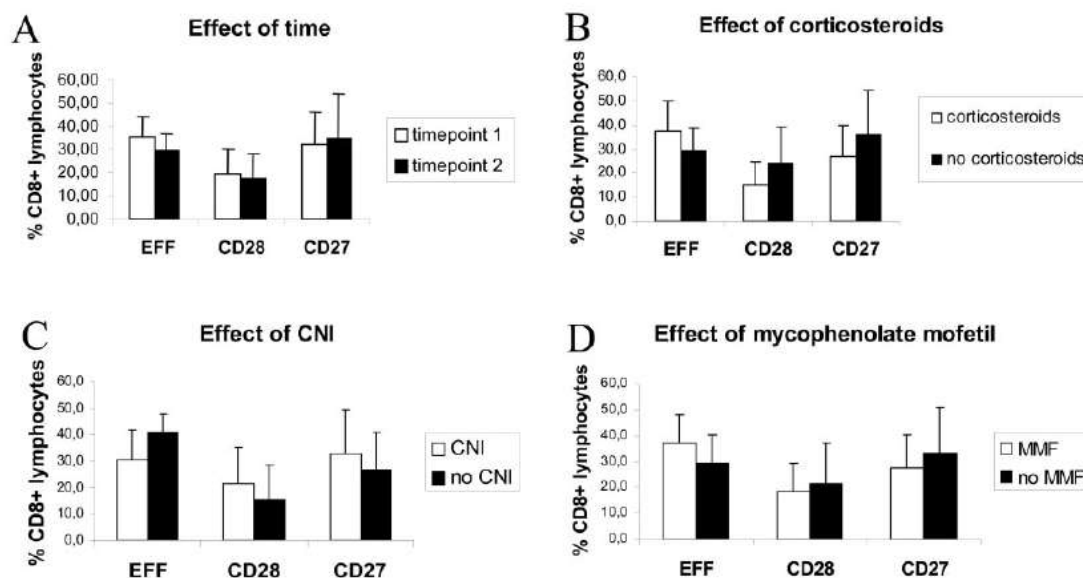


Figure 2. Stability of the effector CD8⁺CD28⁺CD27⁺ lymphocyte population in CR. Peripheral blood CD8⁺ lymphocytes were analyzed by flow cytometry for the percentage of CD45RA⁺CCR7⁺ effector (EFF) cells, CD28⁺ cells, and CD27⁺ cells. Comparisons were performed between two different time points in five patients (A). In addition, patients with ($n = 6$) and without ($n = 8$) corticosteroid treatment (B), patients with ($n = 11$) and without ($n = 3$) calcineurin inhibitor (CNI) treatment (C), and patients with ($n = 6$) and without ($n = 8$) mycophenolate mofetil treatment (D) were compared. Data are represented as mean $\pm$ SD. None of the comparisons was statistically significant.

and $P = 0.001$, respectively) and of CD27 expression ($P = 0.032$, $P < 0.001$, and $P = 0.012$, respectively) in CR *versus* DF-Tol with a similar trend toward increase of CD8⁺ effector lymphocytes.

Alterations of Apoptosis but not Proliferation of CD8⁺CD28[−] Lymphocytes

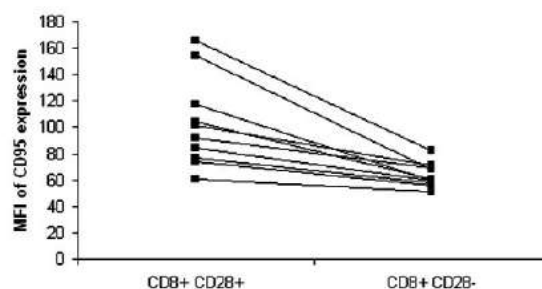
To characterize further the increase of CD8⁺CD28[−] effector lymphocytes in CR, we studied the sensitivity to apoptosis and the proliferative capacities of these cells in comparison with their CD8⁺CD28⁺ counterparts. There was no significant difference in Fas expression on the CD8⁺CD28[−] lymphocytes in DF-Tol *versus* CR (data not shown). However, although nearly all CD8⁺CD28[−] cells expressed Fas (percentage of positive cells), the expression levels (mean fluorescence intensity) were significantly lower than on their paired CD8⁺CD28⁺ counterparts (63.3 ± 9.0 *versus* 103.3 ± 34.2 ; $P = 0.002$; Figure 3A). Accordingly, the CD8⁺CD28[−] subset was less sensitive to apoptosis induced by serum deprivation than the paired CD8⁺CD28⁺ cells (15.5 ± 5.2 *versus* $31.1 \pm 10.0\%$; $P = 0.002$; Figure 3B).

As to the proliferative capacity, there was no significant difference between the CD8⁺CD28[−] and CD8⁺CD28⁺ T subsets with regard to the percentage of cells that proliferated upon anti-CD3 stimulation (46.1 ± 17.8 and $52.1 \pm 23.5\%$, respectively; Figure 3C). Addition of IL-2 did not further increase the proliferation of either subset. Also, the degree of proliferation assessed by the number of divisions per cell was not different between both subsets (Figure 3C). Finally, no significant difference was observed in proliferative capacity between DF-Tol and CR for these two populations (data not shown).

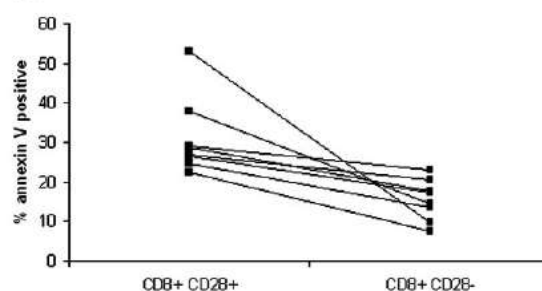
CD8⁺CD28[−] Lymphocytes in CR Have No Characteristics of Suppressor T Cells

To explore the functional features of the CD8⁺CD28[−] lymphocytes in CR, we first compared these cells with the recently described CD8⁺CD28[−] suppressor lymphocytes (17–22). As indicated previously, the CD8⁺CD28[−] cells that were detected in CR were CD45RA⁺CCR7[−]CD27[−], which contrasted with the described phenotype of the suppressor lymphocytes (CD45RO⁺CD62L⁺CD27⁺) (21,22). Flow cytometric analysis of GITR, which is expressed on suppressor cells (19,21), revealed low expression on CD8⁺ cells of CR ($2.8 \pm 2.4\%$) compared with DF-Tol ($12.1 \pm 6.2\%$; $P = 0.011$) and HI (16.0 ± 15.5 ; $P = 0.033$; Figure 4A). Accordingly, real-time PCR showed low levels of FoxP3 transcripts in CD8⁺ lymphocytes from CR compared with DF-Tol ($P = 0.006$) and HI (NS; Figure 4B). Moreover, real-time PCR on purified lymphocyte subsets in CR showed that FoxP3 expression was very low in both the CD8⁺CD28⁺CD27⁺ and CD8⁺CD28[−]CD27[−] cells (Figure 4C). Taken together, these data clearly distinguish the CD8⁺CD28[−] population that was detected *in vivo* in CR from the previously described CD8⁺CD28[−] suppressor lymphocytes that were obtained after several rounds of *in vitro* stimulation.

A Fas expression of CD8⁺ lymphocyte subsets



B Apoptosis of CD8⁺ lymphocyte subsets



C Proliferation of CD8⁺ lymphocyte subsets

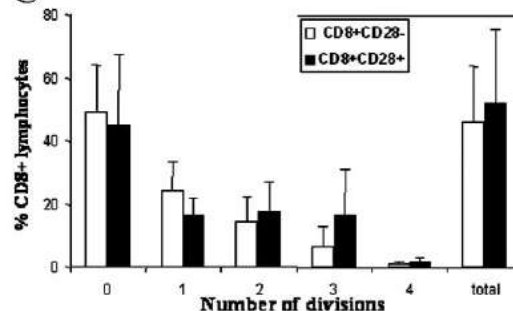


Figure 3. Analysis of apoptosis and proliferation of CD8⁺CD28[−] and CD8⁺CD28⁺ peripheral blood lymphocytes. Expression of Fas (CD95), assessed as mean fluorescence intensity (MFI) by flow cytometry, was significantly lower in CD8⁺CD28[−] lymphocytes than in the paired CD8⁺CD28⁺ counterparts ($P = 0.002$; A). Accordingly, these cells were less sensitive to apoptosis upon serum deprivation, as assessed by annexin V staining ($P = 0.002$; B). In contrast, there were no significant differences for the total number of cells that proliferated or for the number of divisions per cell upon anti-CD3 stimulation of CD8⁺CD28⁺ and CD8⁺CD28[−] lymphocytes, as assessed by CFSE-based flow cytometry (C).

CD8⁺CD28[−] Lymphocytes Exhibit Markers of Differentiated Cytotoxic Cells

Considering that the CD8⁺CD28[−] lymphocytes in CR were different from suppressor cells and belonged to the effector

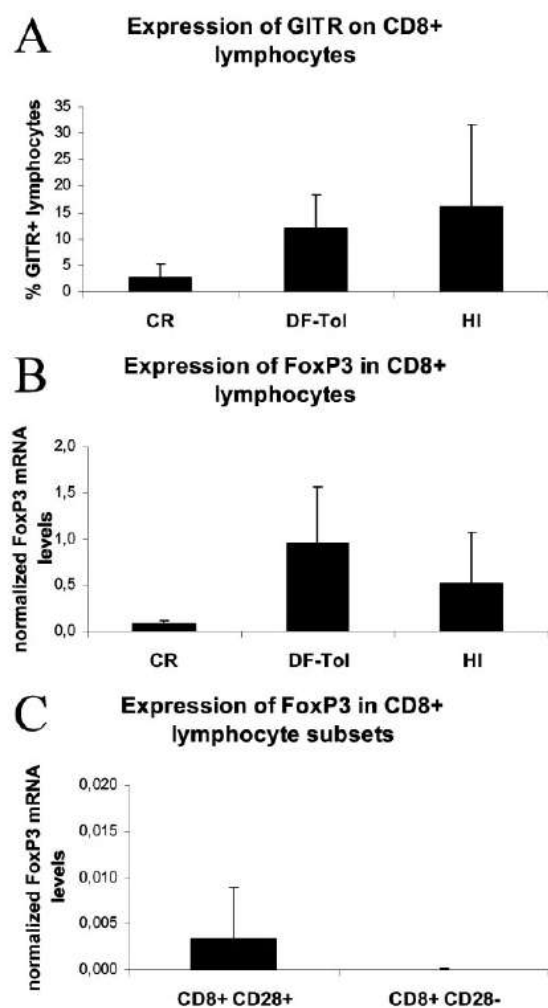


Figure 4. Analysis of the expression of the regulatory markers GITR and FoxP3 on CD8⁺ peripheral blood lymphocytes in CR in comparison with DF-Tol and healthy individuals (HI). Expression of GITR as assessed by flow cytometry was significantly lower on CD8⁺ lymphocytes of CR compared with DF-Tol ($P = 0.011$) and HI ($P = 0.003$; A). Similarly, real-time PCR indicated that the FoxP3 mRNA levels were decreased in CD8⁺ cells from CR compared with DF-Tol ($P = 0.006$) and HI (NS; B). Moreover, real-time PCR analysis of purified lymphocyte subsets of CR showed that FoxP3 expression was very low in both the CD8⁺CD28⁺(CD27⁺) and CD8⁺CD28⁻(CD27⁻) cells (C). Data are represented as mean $\pm$ SD.

subset, we next analyzed the presence of markers of cytotoxicity. There was a highly significant increase of the number of CD8⁺ lymphocytes that were positive for intracellular perforin (85.6 ± 16.8 versus $30.1 \pm 5.3\%$; $P < 0.001$) and for granzyme A (32.7 ± 15.7 versus $6.8 \pm 8.4\%$; $P = 0.010$) in CR versus DF-Tol

(Figure 1). However, there was no difference for perforin or granzyme A between HI (30.1 ± 10.1 and $5.3 \pm 7.2\%$, respectively) and DF-Tol. Perforin and granzyme A correlated strongly ($r = 0.723$, $P = 0.009$) and both markers correlated inversely with CD28 expression ($r = -0.947$, $P < 0.001$; and $r = -0.745$, $P = 0.007$, respectively). Also the surface expression of CD57 was significantly increased on the CD28⁻ subset ($67.0 \pm 20.4\%$) compared with the CD28⁺ counterpart ($22.2 \pm 10.6\%$; $P = 0.031$; Figure 5). Whereas a similar difference in CD57 expression was found between both subsets in DF-Tol (70.9 ± 12.7 versus $26.2 \pm 16.5\%$; $P = 0.046$), there was no difference for the expression of these cytotoxicity-associated markers on the CD8⁺CD28⁻ lymphocyte subset between CR and DF-Tol, indicating that the number of these cells rather than their cytotoxicity-associated profile differentiated both situations (Figure 5). Finally, the analysis of the expression NK-cell receptors (KIR-NKAT2, CD94, and NKG2D) on CD8⁺ lymphocytes showed no difference between DF-Tol and CR or between the CD8⁺CD28⁻ and CD8⁺CD28⁺ subsets in both situations (Figure 5).

To identify the cytotoxic targets of the CD8⁺CD28⁻ lymphocytes in CR, we next performed a CD107 mobilization assay to assess donor antigen-specific degranulation. Unstimulated CD8⁺CD28⁻ cells showed low levels of degranulation ($6.0 \pm 2.0\%$), which were significantly increased upon nonspecific stimulation with anti-CD3 antibody ($14.5 \pm 3.9\%$) or phytohemagglutinin ($37.9 \pm 21.4\%$). Incubation with surrogate donor cells, which

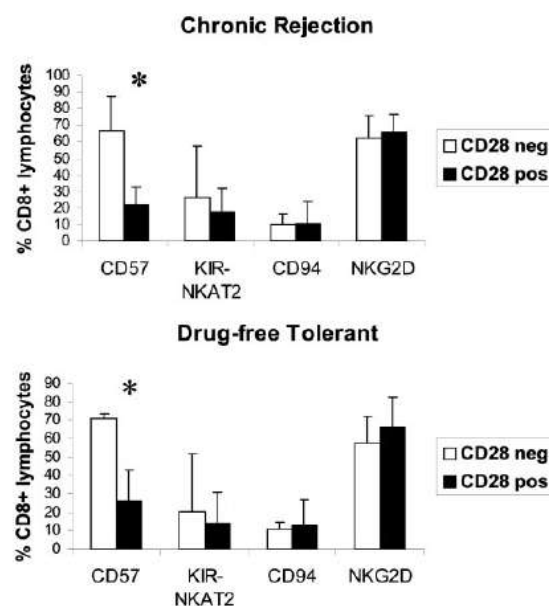


Figure 5. Flow cytometric assessment of the expression of markers associated with cytotoxicity and NK-cell receptors on the cell surface of peripheral blood CD8⁺ lymphocytes in CR and in DF-Tol. The CD8⁺CD28⁻ subset was compared with the CD8⁺CD28⁺ subset. Data are represented as mean $\pm$ SD. * $P < 0.05$.

were matched for HLA class I with the original kidney graft donor, did not increase degranulation ($4.9 \pm 2.5\%$). Similarly, stimulation with a pool of common viral peptides did not significantly increase the degranulation of CD8⁺CD28⁻ T lymphocytes ($8.2 \pm 3.9\%$). Taken together, these data do not provide functional evidence that the targets of this cell population are donor-specific HLA class I molecules or common viral antigens.

Sta Recipients Display a Mixed CD8⁺ Lymphocyte Profile

Finally, we analyzed whether the described CD8⁺ lymphocyte profile that was associated with the chronic rejection process could be observed also in immunosuppressed kidney graft recipients without clinical or biologic signs of rejection. The CD8⁺ lymphocytes of five patients showed a similar CD28, CD27, and granzyme A expression as DF-Tol, whereas two had an intermediate profile and five had similar expression levels as CR (Figure 6, A through D). As to the percentage of effector CD45RA⁺CR7⁻CD8⁺ cells, seven Sta recipients had similar numbers as CR, whereas five had low numbers such as in DF-Tol. Similar to our observations in CR, these profiles were not dependent on the treatment with CNI (Figure 6E) or other immunosuppressive drugs (data not shown), and a kinetic analysis in five patients at a median of an 18-mo interval (16 to 24 mo) showed that these profiles were stable over time (Figure 6F). To analyze the Sta profiles in more detail, we set up a model using Predictive Analysis of Microarray data software based on the CD8⁺ lymphocyte phenotypes in DF-Tol *versus* CR. Cross-validation of this model using the DF-Tol and CR profiles classified correctly 13 of the 14 CR samples and five of the six DF-Tol samples (Figure 6G). Applying this model to the Sta patient cohort, the CD8⁺ lymphocyte phenotype of seven patients resembled CR, whereas the others had a profile more closely related to DF-Tol (Figure 6H).

Discussion

Considering the major burden of immunosuppression in organ transplantation and the discrepancies between tolerance in rodents and humans, spontaneous drug-free tolerance in humans is a rare but unique model to study clinically relevant immune phenomena related to graft integrity and survival. In this context, we analyzed alterations of the CD8⁺ lymphocytes in kidney graft recipients who tolerated their graft for several years without any immunosuppressive or corticosteroid treatment. The main finding is the reduced numbers of circulating CD8⁺CD28⁻ effector lymphocytes compared with patients with chronic graft rejection. However, the DF-Tol had a similar number of CD8⁺CD28⁻ effector lymphocytes as HI, indicating an abnormal increase of this cell population in CR rather than a primary alteration in DF-Tol. Because the expression of CD28 on CD8⁺ cells decreases with age (23), it is important to notice that the study groups were age matched. A bias caused by immunosuppressive therapy is also unlikely because we found no effect of the different treatment regimens on the CD8⁺ lymphocyte profiles in CR. Finally, the increase in CD8⁺CD28⁻ effector cells was stable over time in CR.

Several mechanisms can lead to an increase of CD8⁺CD28⁻

lymphocytes. During aging, effector CD8⁺ lymphocytes can lose the surface expression of CD28 by replicative senescence as a result of extensive homeostatic proliferation (16,23). A distinct mechanism is observed after TCR-mediated activation, which is associated mostly with a decreased susceptibility to undergo apoptosis (24). In this respect, CD8⁺CD28⁻ cell populations can appear as a consequence of extensive rounds of antigen-induced division such as in chronic infections or malignancies (25–27) and, in contrast with senescent cells, are characterized by increased effector functions rather than functional anergy (13,28,29). Investigation of the sensitivity to apoptosis and the proliferative capacity of the CD8⁺CD28⁻ lymphocytes in CR indicated that the loss of CD28 was associated with a decreased susceptibility to apoptosis, which related both to the Fas-mediated pathway and to the sensitivity to growth factor withdrawal (30–32). However, in contrast with replicative senescence, which is characterized by an irreversible nondividing state (30,33,34), the CD8⁺CD28⁻ lymphocytes in CR proliferated at a similar level as their CD28⁺ counterparts upon CD3 stimulation. Because CD28 is required for IL-2 production and subsequent sustained proliferation, it is likely that both in our assay and *in vivo* IL-2 could be provided in a paracrine manner by CD28⁺ lymphocytes rather than in an autocrine manner by the CD8⁺CD28⁻ lymphocytes themselves (35). The impaired sensitivity to apoptosis and the maintained proliferative potential of the CD8⁺CD28⁻ lymphocytes may influence the balance between clonal expansion and contraction and thereby contribute to the increase of this cell population in CR.

A subset of CD8⁺CD28⁻ cells have been described recently as suppressor lymphocytes, which, in contrast to our study, were obtained by multiple rounds of stimulation *in vitro* (17,18). These cells were described to express GITR and FoxP3 and to suppress CD4 responses by tolerization of antigen-presenting cells through an upregulation of the inhibitory molecules ILT3 and ILT4 (19,20). Whereas animal models support an *in vivo* function for these suppressor lymphocytes (36,37), their natural presence, exact phenotype, and suppressive function in humans still are a matter of debate (22). The suppressor CD8⁺CD28⁻ lymphocytes seem to be central memory cells (CD62L⁺CD45RO⁺) that co-express CD27 (21,22), in contrast with the cell population in CR, which are CD27⁻ effector cells (CCR7⁻CD45RA⁺). Moreover, the regulatory-associated markers GITR and FoxP3, which were described on the suppressor cells (19,21), were not expressed on CD8⁺ lymphocytes in CR, thereby clearly indicating that the CD8⁺CD28⁻ cells that we described here are different from suppressor lymphocytes.

In contrast to a suppressor function and in agreement with the association between loss of CD28 and marked cytotoxicity of CD8⁺ effector cells (13,28,29), the CD8⁺CD28⁻ effector population in CR was characterized by high levels of perforin, granzyme A, and CD57. Of interest, however, we found no differences for these markers in CD8⁺CD28⁻ effector lymphocytes between DF-Tol and CR, indicating a quantitative rather than a qualitative difference between both situations. An important question raised by this observation is the target of these cells and the potential functional consequences for the graft. On the basis of reports in animal models (38–41), it would be

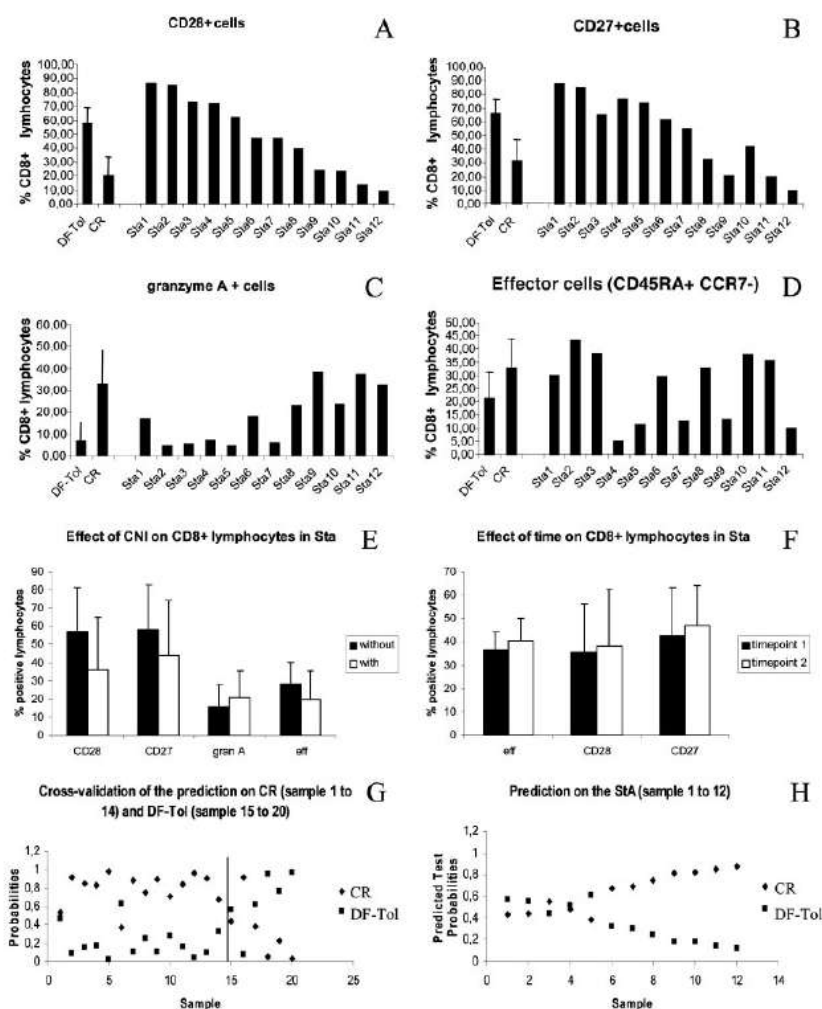


Figure 6. CD8⁺ lymphocyte profiles as assessed by flow cytometry on peripheral blood lymphocytes of 12 kidney graft recipients with stable renal function under immunosuppressive therapy (Sta). The percentage of CD8⁺ lymphocytes that expressed CD28 (A), CD27 (B), and intracellular granzyme A (C) and the number of effector CD45RA⁺CCR7⁻CD8⁺ lymphocytes (D) are shown. For comparison, the mean $\pm$ SD is depicted for DF-Tol ($n = 6$) and CR ($n = 14$). Similar to the data in CR, there was no influence of treatment with CNI on the CD8⁺ T lymphocyte profiles in Sta (E), and kinetic analysis of five patients at an interval of 18 mo showed that these profiles were stable over time (F; data are represented as mean $\pm$ SD). Using the CD8⁺ lymphocyte phenotypes from CR and DF-Tol, a model was set up for classification of individual samples. Cross-validation indicated that 13 of 14 CR and five of six DF-Tol were classified correctly (G). When applied to samples of Sta patients, the model classified the CD8⁺ lymphocyte profiles of seven of 12 as CR, whereas five showed an intermediate profile (H).

tempting to speculate that these cells are induced by donor antigens by either a direct or an indirect pathway of allorecognition. Using donor HLA class I-matched cells as surrogate targets, however, our attempt to provide functional evidence that the described CD8⁺CD28⁻ lymphocyte subset is indeed committed to donor determinants was unsuccessful. Whereas our study is hampered by the lack of original donor cells, future prospective and/or experimental research is needed to address

this in more detail and to evaluate the eventual contribution of other donor determinants. An alternative hypothesis would be that pre-existing cytotoxic CD8⁺ lymphocytes directed against viruses and pathogens cross-react with the graft, as suggested by the fact that heterologous immune memory has been described as a potential to transplantation tolerance (5,6,42,43). Also here, however, we were unable to demonstrate antigen-specific degranulation of CD8⁺CD28⁻ lymphocytes against a

pool of common viral peptides. A third and most interesting possibility that should be explored further is reactivity against self-determinants as suggested by recent experimental evidence (44,45).

Independent of the precise primary target of the CD8⁺CD28[−] effector cells in CR, the normal expression level of the activating cytotoxic receptor NKG2D, which is not counterbalanced by an increase of MHC class I-binding inhibitory receptors such as CD94/NKG2A and KIR-NKAT2, is compatible with functional cytotoxicity of these cells (46–49). In this context, it is interesting to note that cell-mediated alloimmunity has been demonstrated to contribute to chronic allograft nephropathy in renal transplant recipients (50). Moreover, Li *et al.* (51) indicated recently that operational tolerance in liver transplantation was associated with a decrease of NK cells, a distinct cytotoxic lymphocyte population that was not investigated in this study.

A final important issue is raised by the fact that some of the patients who had stable graft function and were analyzed in our study display a similar CD8⁺ lymphocyte phenotype as CR, whereas others had an intermediate profile. This indicates that the described CD8 profile is strongly but not exclusively associated with active chronic rejection. Because an increase of granzymes and perforin was also reported to precede allograft rejection in rodents as well as humans (52,53), it would be interesting to analyze whether such a CR-like signature in seemingly stable patients may help to identify a poorer prognosis and eventually subsequent rejection. All stable patients who were analyzed in our study, however, maintained a normal graft function for now almost 3 yr of follow-up.

These data also emphasize the complexity of such studies in human subjects. First, the large interindividual variability precludes conclusive statements in cross-sectional studies and requires validation by serial, prospective analyses in which each individual with changes in clinical status can serve as his own control over time. In this context, a gradual increase of a cytotoxic CD8⁺CD28[−] population over time may be more relevant than an absolute value as such at the individual level. Second, because most of the patients with kidney transplantations remain stable for years under conventional immunosuppression, only large studies over a longer period will be able to relate the described phenotype to poor clinical outcome. An alternative would be a prospective tapering of the immunosuppression in patients with a profile close to DF-Tol, but this is still medically and ethically unacceptable without a clear, coherent, and reliable signature based on a broader panel of immunologic parameters (11). Finally, studies on spontaneous tolerance in kidney transplantation are severely hampered by the extreme rarity of these patients. Our studies have analyzed the largest number of these patients in the current literature, making it difficult to validate these findings in an independent cohort. Performing similar analyses in other types of solid organ transplantation, where tolerance is more frequently observed, such as for liver, therefore may be an interesting alternative (51).

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Please see the related Controversies in Nephrology article by Wilkinson in this month's *CJASN* (pages 130–137), which reviews the pros and cons of serial biopsies in renal transplant patients.

Annexe 3

Serial evolution of TCR β chain transcript mobilization in HIV-1 infected patients following vaccine immune stimulation and HAART

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Serial Evolution of TCR β Chain Transcript Mobilization in HIV Type-1-Infected Patients Following Vaccine Immune Stimulation and HAART Interruption

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ABSTRACT

In this article, we studied the T cell receptor (TCR) β chain transcript mobilization in peripheral blood lymphocytes harvested from HIV-1-infected patients before and after vaccination with a mixture of six lipopeptides and at the moment and serially after highly active antiretroviral therapy (HAART) interruption. This study was performed by using a combined qualitative and quantitative assessment of V β mRNA alterations at the level of complementary determining region 3 length distribution (CDR3-LD) of the TCR. Whereas healthy individuals displayed both stable CDR3-LD profiles and V β transcript accumulations over time, the four HIV-1-infected patients in a quiescent disease phase under HAART have a highly significantly biased CDR3-LD. In addition, they displayed a significant further increase of alterations of their β CDR3-LD profile after vaccination and both a more altered CDR3-LD ($p < 0.05$) and an increased transcript accumulation of some V β families after HAART interruption. These modifications mostly concerned the CD8⁺ T cells. Such a global approach of TCR alterations may help to follow the immune response of these patients and allow targeting of more complex *in vivo* studies by identifying the T cells with a selected repertoire.

INTRODUCTION

LYMPHOCYTES PLAY A CRUCIAL ROLE in immune responses against viral diseases in which processed determinants are recognized through the T cell receptors (TCR) that carry the specificity for antigen. Analysis of TCR usage therefore indirectly reflects the host–virus relationship at various stages of the disease. The genes encoding for the heterodimeric $\alpha\beta$ chains of the TCR are composed of a rearrangement of V (variable), D (diversity), and J (junction) genes.^{1,2} The random association of these V(D)J segments leads to the formation of a hyper-variable region, referred to as complementary determining region 3 (CDR3) involved in the recognition of the foreign pep-

tides presented by major histocompatibility complex (MHC) molecules on infected targeted cells.³ T cells from human blood can display about 10⁶ different β chains.⁴ Identifying and then following T cells specifically involved during a complex immune response *in vivo* such as during HIV infection is thus difficult. The Immunoscope/Spectratype approaches have made it possible to identify V β chains selected on the basis of bias in their CDR3 length distribution (CDR3-LD), altered after a T cell selection.⁵ This approach recently provided evidence of a public V β 13.2-specific usage in some HIV-1-infected patients.⁶ However, estimating the magnitude of the pool size of T cells using a given CDR3-LD alteration and, in the presence of a gaussian CDR3-LD, discriminating between resting and poly-

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clonally activated T cells, requires a quantitative assessment of the transcripts involved.^{7,8} By combining information on V β mRNA usage at the CDR3-LD level with assessment of the amount of all possible CDR3 length restricted mRNA in each V β family, patterns of resting and activated T cells can be better discriminated and set in a hierarchy.^{7,8}

Highly active antiretroviral therapy (HAART) has profoundly changed the outcome of HIV infection. In particular, it is associated with a strong decrease of viral load and subsequent repletion of CD4⁺ T cells.⁹ However, this treatment also induces a strong decrease of CD8⁺ T cell responses.^{10,11} Because of this, structured therapeutic interruption protocols and the associated relapse of viral load are being proposed to boost CD8⁺ anti-HIV immune responses.¹² Other strategies may consist of selectively stimulating T cell immune responses through therapeutic vaccination.¹³ Following the immune responses in HIV-1-infected patients in different therapeutic protocols as well as under HAART and after HAART interruption is therefore important to make further progress in the management of this disease.

We study four chronically HIV-1-infected patients who are on HAART and are enrolled in a therapeutic vaccine study consisting of three injections with a mixture of lipopeptides, followed by HAART interruption. In particular, T cell mobilization in peripheral blood lymphocytes (PBL) serially harvested from these chronically HIV-1-infected patients has been stud-

ied through a combined qualitative and quantitative assessment of V β mRNA alterations. The study was performed on the whole T cell population, in purified CD4⁺ and CD8⁺ T cells from patients in a quiescent phase of the disease during HAART, before and after vaccination while on HAART, and serially after HAART cessation. We showed that HIV-1 infection is characterized by an altered V β transcriptome pattern even during the quiescent stage of the disease on HAART. Vaccination as well as removal of HAART in these patients were associated with a further serial increase in alterations in their V β transcriptional patterns, mostly involving the CD8⁺ T cell compartment. Implications of these findings for a better understanding of the disease are discussed.

MATERIALS AND METHODS

HIV-infected patients and vaccination protocols

The study was performed on PBL serially harvested from four HIV-1-infected patients, to whom a phase II clinical trial of vaccination and a therapeutic interruption were proposed (LIPATHERA study). The patients were immunized with a mixture of six lipopeptides (3 mg) injected intramuscularly at 0, 21, and 42 days (Fig. 1A). Three lipopeptides were derived from HIV-LAI Nef proteins: L-N1 (aa 66–97), L-N2 (aa 117–141),

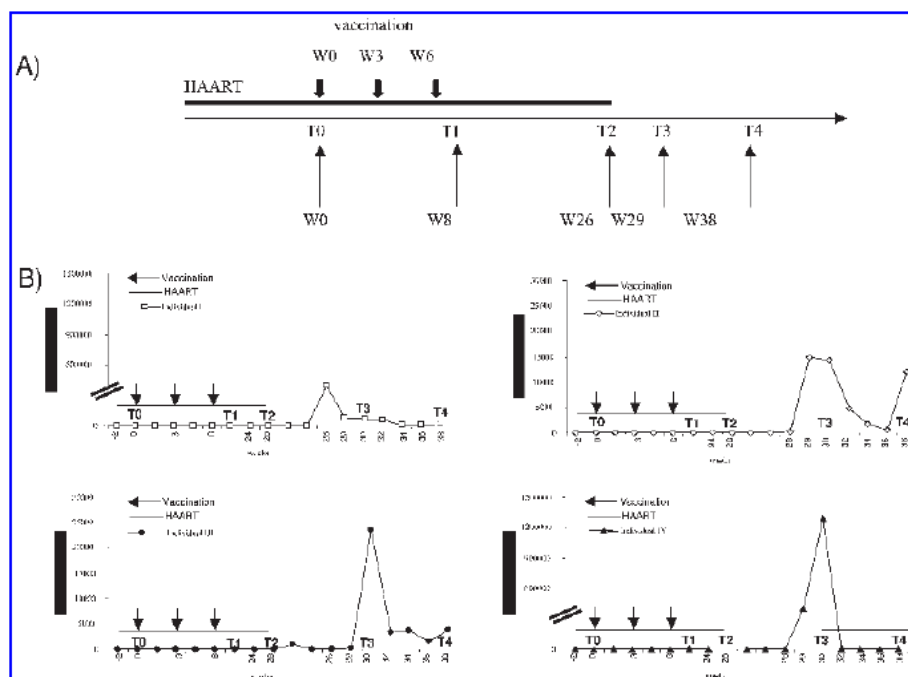


FIG. 1. Timing of the therapeutic protocols and evolution of the viral load in the four HIV-1-infected patients. (A) Timing of vaccination and HAART interruption as well as the time period of blood harvesting. (B) Evolution of the viral load in the four HIV-1-infected patients according to the harvesting time.

TABLE 1. PATIENTS CHARACTERISTICS AT THE TIME OF INCLUSION^a

		HIV-1 Years seropositivity old (months)	LT cells			Plasma HIV-1 RNA load			Kind of HAART 2 N + (NN or PI) ^c
			CD4 ⁺ nadir (cells/mm ³)	CD4 ⁺ current (cells/mm ³)	CD8 ⁺ current (cells/mm ³)	Pre-HAART viral load (copies/ml)	Time below 500 ^b copies/ml (months)	HAART length (months)	
C12J	37	94	432	608	593	8100	41	43	PI
C11K	35	71	481	783	639	102,000	42	43	NN
R13K	57	103	338	1046	1923	161,900	31	33	NN
R04A	44	102	220	558	1090	2750	54	63	NN

^aAll patients were infected for at least 6 years and received HAART treatment for more than 2 years.

^bDetection threshold in 1996, but the viral load was <50 copies/ml at the time of screening.

^cN, nucleoside reverse transcriptase inhibitor; NN, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitor.

and L-N3 (aa 182–205); two lipopeptides were derived from the HIV-LAI Gag proteins: L-G1 (aa 183–214) and L-G2 (aa 253–284); and one lipopeptide was derived from a V3-ENV gp120 consensus sequence, also found in the HIV-BX08 strain: L-E (aa 303–335).¹⁴ Inclusion criteria were HIV-1 chronic infection >18 months, HAART >12 months, plasma HIV-1 RNA <50 copies/ml >6 months, and CD4⁺ T cells count >350/mm³ >6 months. The clinical status of the four HIV-1 chronically infected patients is summarized in Table 1. Eighteen weeks after the last vaccination, HAART treatment was interrupted. To avoid transient monotherapy, nonnucleoside reverse transcriptase inhibitors (NNRTI) were stopped 1 week before the other drugs. HAART was reintroduced 2 months later if the viral load was above 30,000 copies/ml on two measurements and/or until CD4⁺ T cell counts decreased by half or below 250 cells/mm³. This study was approved by the ethics committee of the Cochin Hospital and all participants gave their written informed consent.

Healthy individuals

Healthy individuals ($n = 15$), 25–60 years of age, with normal blood results and no known infectious pathology for at least 6 months prior to the study were also included in this study as references for CDR3-LD and V β /HPRT values.¹⁵ In addition, the TCR distribution of three healthy individuals was analyzed over time at 0, 1, and 2 months.

Blood harvesting procedure and conditioning procedure

PBL were harvested before vaccination (T0) and 14 days after the last lipopeptide injection (T1 = T0+8 weeks), at HAART cessation (T2 = T0+26 weeks), and then at two later time points (T3 = T0+29 weeks and T4 = T0+38 weeks) (Fig. 1A). For comparison, PBL from three healthy individuals were serially harvested at month 0, 1, and 2 to check the stability over time of the V β transcriptome patterns. A volume of phosphate-buffered saline (PBS) or NaCl solution was added to whole blood (vol/vol) harvested from the three healthy individuals and from the four HIV-1-infected patients. This mixture was slowly laid on Ficoll and spun for 30 min at 2200 rpm, without brakes. Cells were then collected at the interface and washed three times. An equal volume of freezing media [60%

RPMI + 20% fetal bovine serum (FBS)] was added to the cells resuspended in RPMI at 10⁷/ml. Then, 20% dimethyl sulfoxide (DMSO) was added drop by drop. Freezing medium was slowly added to the cells before storage at –70°C in freezing tubes. After a few days, cells were transferred to a liquid nitrogen tank.

Purification of CD4⁺ and CD8⁺ T cells

Magnetic cell separation of CD4⁺ and CD8⁺ T lymphocytes was realized using magnetic cell sorting (MACS) columns A1 (Miltenyi Biotec, Bergish Gladbach, Germany) according to the manufacturer's procedures. Briefly, 10⁷ peripheral blood mononuclear cells (PBMC) were incubated in 100 μ l of buffer [PBS, 0.5% bovine serum albumin (BSA), 2 mM ethylenediaminetetraacetic acid (EDTA)] with microbeads directly conjugated with either mouse antihuman CD4 or antihuman CD8 monoclonal antibodies (20 μ l of magnetic microbeads per 10⁷ PBMC, Miltenyi Biotec) at 4°C for 20 min. After washing with 3 ml buffer and centrifugation, the pellet was resuspended in 0.5 ml of buffer and applied onto an MS+ column placed in a MiniMACS magnet (Miltenyi Biotec). The column was rinsed with buffer and then the elution of the positively selected cells was achieved by removing the column from the magnet and flushing with PBS.

TCR repertoire analysis

RNA extraction and cDNA synthesis. Cells were thawed slowly and total RNA from PBL was extracted using the technique of Chomczynski Sacchi;¹⁶ 2 μ g RNA was reverse transcribed, using the Boehringer cDNA synthesis kit (Boehringer Mannheim, Indianapolis, IN), and diluted to a final volume of 100 μ l.

PCR amplification and elongation reactions for analysis of CDR3 length. cDNA was amplified by PCR using a C β primer and one of the 26 V β -specific primers. The amplifications were performed in a 9600 Perkin-Elmer Automate (Applied Biosystems, Foster City, CA) as previously described.⁵ Each amplification product was used for an elongation reaction using a dye-labeled C β primer,⁵ then heat denatured, loaded onto a 6% acrylamide–8 M urea gel, and electrophoresed for 9 h using an

Applied Biosystems 373A DNA Sequencer (Perkin-Elmer). TCR repertoire alterations were studied as previously detailed elsewhere.⁵ Briefly, Immunoscope software was used to display the distribution profiles of CDR3 lengths, in amino acids, of the amplified and elongated products.⁵ The percentage of alteration for each V β family and global percentages of alteration for all V β families in each individual were obtained as previously described.¹⁷ The ABI PRISM 7700 Sequence Detection Application program (Applied Biosystems) was used to detect and measure fluorescence emitted during PCR amplification of a given target sequence in a 96-well reaction plate. Data were collected during each PCR cycle, which was conducted in real time as previously described.¹⁸ Each specific V β family transcript amount was divided by the number of hydroxyphosphoribosyltransferase (HPRT) transcripts to correct for variability in the initial concentration and quality of total RNA and in the conversion efficiency of the reverse transcription reaction. Moreover, all experiments were run in duplicate. The MatLab software was used to compute and display the data.^{7,8} In the integrated landscapes, referred to as TcLandscape, which gives a direct assessment of the contribution that each Vb chain makes to the total T cell repertoire, the *x*-axis displays the 26 V β families analyzed. The *z*-axis shows the ratio between the number of V β transcripts and the number of HPRT transcripts. The *y*-axis gives the various possible CDR3 lengths. Colors represent the percentage of alterations. The color code ranges from deep blue to dark red and is indicated on the right hand side of the figure.

Statistical analysis

A randomization test was used to analyze the "people" and "time" effect in the serial study of healthy individuals. In this test, for each V β peak coordinate, the variance at this coordinate across values obtained over time of the same individual was calculated. Each person was thus assigned the average of the variances, giving a "variability index" for the different harvesting times within one individual. The averages from the three healthy individuals were then averaged to give a "global variability index" within normal people and the statistic test was based on comparison with this average. An ANOVA and a *t*-test were used to analyze the data. This parametric test makes it possible to compare two samples. This test enabled us to compare the patterns obtained at different time points from the same individual. For analysis of the CDR3-LD in HIV-1-infected patients over time, a linear regression on the CDR3-LD alteration square values was also performed. Using time as the variable, the hypothesis was the linearity of the evolution of the CDR3-LD over time. A conventional *p*-value of <0.05 was used to indicate significance.

RESULTS

TCR V β transcriptome in normal individuals

The study of TCR V β chain mRNA regulation at the CDR3-LD and V β /HPRT transcript ratio levels in a given infectious process, mainly during the quiescent stage of a disease or during a clinical therapeutic protocol, first requires comparison with patterns obtained from healthy individuals. Taken together,

healthy individuals exhibited only slight global CDR3-LD alterations ($10 \pm 3\%$ on average) and a low V β /HPRT transcript ratio (<2). As this study of HIV-1-infected patients was longitudinal and spanned over 12 weeks, we also analyzed the V β transcriptome obtained from three healthy individuals serially harvested every month for 3 months (data not shown). TcLandscape from each healthy individual showed a roughly stable pattern over time, both at the CDR3-LD alteration and V β /HPRT transcript ratio levels. However, healthy individuals exhibited some isolated V β families with altered CDR3-LD profiles and elevated V β /HPRT transcript ratios. For instance, individual I displayed a low CDR3-LD alteration level but high V β /HPRT transcript ratios for the V β 21 and V β 22 families. Individual II showed only a slight accumulation of V β /HPRT transcript ratios but a strong and stable CDR3-LD alteration level for the V β 7 family (48%, 59%, and 56% of CDR3-LD alterations, respectively, for months 0, 1, and 2). The same observations were done for individuals III and IV.

A randomization test was then performed on the panels of these individuals to investigate the "people" and "time" effect on the TcLandscape topologies. The "global variability index" (see Material and Methods), which mixed the CDR3-LD alteration and V β /HPRT transcript ratio values obtained from the three healthy individuals tested over time, was 0.16. In contrast, the same index obtained from the three healthy individuals, one compared to the others, was 0.03, indicating that the patterns from single individuals harvested serially were much less variable than the patterns obtained from various individuals. TcLandscape patterns from healthy individuals may thus be considered stable over time.

TcLandscape patterns obtained from chronically HIV-1-infected patients in a quiescent phase under HAART (T0) showed numerous V β families with a strongly altered CDR3-LD

Figure 2 shows the patterns obtained from the PBL harvested from four HIV-1 chronically infected patients before (T0) and after vaccination (T1) under HAART and after HAART cessation (T2 to T4). The inset displays the "top views" of the TcLandscapes, which visualized the CDR3-LD alterations for each individual. TcLandscape patterns obtained from chronically HIV-1-infected patients in a quiescent phase under HAART (T0) are displayed on the left hand side of Fig. 2. The V β transcriptional patterns of these four HIV-1 chronically infected patients were significantly different ($p < 0.01$) from healthy individuals. Indeed, these patients displayed strongly altered CDR3-LD in some V β families ($\geq 50\%$ of alterations for the V β 8 family of individual I, V β 8 and V β 13.1 families of individual II, V β 24 family of individual III, and V β 15 family of individual IV; Fig. 2). Interestingly, individuals I and III also exhibited some V β families (V β 15 for individual I and V β 3 and V β 11 for individual III) with an unusually long CDR3 length (circled in the Immunoscope profiles, Fig. 3). These unusual patterns were reproducibly found over time and were not related to artifactual migratory events (see Discussion).

The corresponding V β /HPRT transcript ratios in these individuals, represented by the height of the peaks in the TcLandscapes, were also analyzed. Two V β families (V β 3 and V β 11) presented high V β /HPRT ratios in all four individuals compared

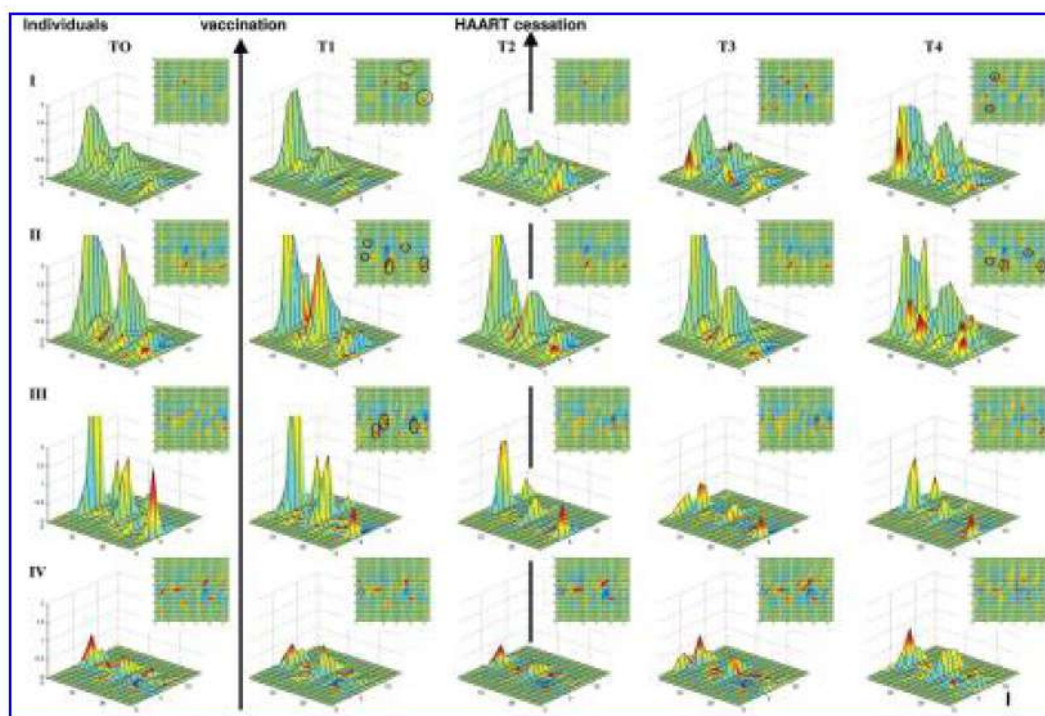


FIG. 2. TcLandscape topologies of HIV-1-infected patients at each studied time point. Each TcLandscape topology was associated with an inset representing only the qualitative analysis. The TcLandscape analysis was performed in each individual before (T0) and after (T1) vaccination, when the patients were under HAART, as well as after HAART interruption (T2, T3, T4). Some modifications at both the V β /HPRT transcript ratio and CDR3-LD levels were observed over time in each patient. For the sake of clarity, not all changes of CDR3-LD were circled on the top view of the landscape, only alterations discussed in the Results section are circled.

with the other V β family transcripts. Although these high ratios could be observed in some of the healthy individuals studied, they were less frequent and not as high as in HIV-infected patients.

Effect of vaccination on the CDR3-LD profile and V β transcript accumulation

Figure 2 shows the TCR patterns obtained before (T0) and 8 weeks after (T1) vaccination in the four HIV-1-infected patients. The vaccination was associated with some new biases of the CDR3-LD in several V β families. As illustrated in the top views, in individual I, the V β 13.5, V β 15, and V β 23 families (only new alterations are circled in black) displayed a more pronounced percentage of CDR3-LD alteration following vaccination as compared with the pattern observed before vaccination. In individual II, the V β 3, V β 4, V β 9, V β 14, and V β 24 families also exhibited a more altered pattern after vaccination, as was the case for the V β 6.1, V β 7, and V β 17 families in individual III. Globally, CDR3-LD profiles were significantly more altered after (T1) than before (T0) vaccination ($p < 0.0001$ to 0.018).

At a quantitative (V β /HPRT ratio) level, the V β 6.4, V β 14, and V β 22 transcript levels decreased in individual I (2-fold

less). This was also observed for the V β 4 and V β 5.2 family transcripts in individual II as well as for the V β 15 and V β 22 transcript families in individual III. In contrast, in these same individuals, transcripts of some V β families were also found to be accumulated more (2-fold more) following vaccination (V β 18 in individual I, V β 9 in individual II, and V β 2 in individual III). Finally, in individual IV, the V β 1 and V β 7 family transcripts were accumulated at least 2-fold more following vaccination. Nevertheless, it is not possible to know if these variations could be related to vaccination.

Effect of HAART interruption on CDR3-LD alterations and V β /HPRT transcript ratios in the whole blood T cell population

Taken individually, the four patients displayed different CDR3-LD profiles, affecting specific V β families, following treatment interruption. After HAART interruption, the global CDR3-LD alteration levels were significantly more altered with time (pattern at T4 more altered than at T3 and pattern at T3 more altered than at T2— $p < 0.0001$ to 0.018). In individuals I and II, the increased CDR3-LD levels were associated with a

strong accumulation of many $V\beta$ transcript families at T4 (for instance, $V\beta 5.2$ and $V\beta 6.4$ families in individual I and $V\beta 5.2$, $V\beta 8$, $V\beta 16$, and $V\beta 23$ families in individual II). However, these quantitative changes were not a common feature shared by all patients and did not reach statistical significance.

We then compared the $V\beta$ transcriptome patterns with the viral load in each patient obtained at T2 and serially after HAART interruption (T3 and T4) (Fig. 1B). The statistically significant increase in the level of CDR3-LD alterations in the four patients reported above occurred whatever the viral load evolution. For example, in individual I, the viral load increased between T2 and T4 and was associated with an increase of some $V\beta$ /HPRT transcript ratios and the appearance of new CDR3-LD alterations (Fig. 2). However, in patients II and III, the viral load remained roughly stable over the 12 weeks of analysis and only slight modifications of the $V\beta$ transcriptome were observed at a distance from HAART interruption (T4) in patient III. No analysis of individual IV was possible because of an unscheduled prescription of HAART between T3 and T4.

Different $V\beta$ transcriptional patterns in purified $CD4^{+ve}$ and $CD8^{+ve}$ T cells

We then compared the TCR profiles in patterns obtained from purified $CD4^{+ve}$ and $CD8^{+ve}$ T cells before and after vaccination in two out of the four patients (Fig. 4, left panel) as

well as at a distance from HAART interruption (patients I, III, and IV; Fig. 4, right panel). As shown in Fig. 4 (left panel), after vaccination, modifications of the TCR repertoire were observed mostly in the CD8 T cell compartment in one of the two patients (patient II, Fig. 4, left panel). The trend for more pronounced alterations in CD8 T cells was more evident following HAART interruption, particularly in individual I (Fig. 4, right panel). The patterns from the $CD4^{+ve}$ T cell compartment were similar at T2 and T4, for both individuals, at the $V\beta$ /HPRT transcript ratio and CDR3-LD levels. Taken together, our data suggest that most of the $V\beta$ transcriptome modifications (CDR3-LD alteration and $V\beta$ /HPRT transcript ratios) involve the $CD8^{+ve}$ T cells in HIV-1-infected patients.

DISCUSSION

In this report, we studied TCR $V\beta$ regulation through a global appraisal of the $V\beta$ transcriptome (CDR3-LD alterations and $V\beta$ /HPRT transcript ratios)^{8,15,19,20} in patients with HIV-1 infection displaying a quiescent disease following vaccination and HAART interruption. For comparison, three healthy individuals were also serially studied over time. We showed that HIV-1 infection profoundly affects the $V\beta$ transcriptome and that additional changes mostly involved the $CD8^{+ve}$ T cell

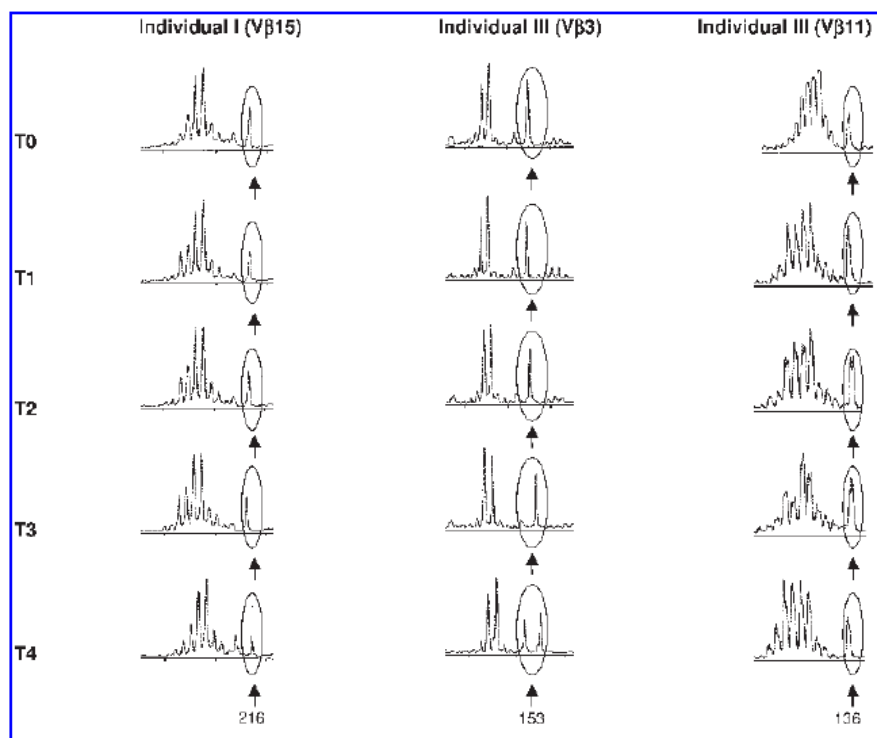


FIG. 3. Reproducibility over time of the unusual CDR3 length observed in some CDR3-LD profiles. Individual I exhibited a $V\beta$ family ($V\beta 15$) with an atypical CDR3-LD. In this family, some transcripts (circled in the Immunoscope profiles) used unusually large CDR3 lengths. A similar observation was also noted in the $V\beta 3$ and $V\beta 11$ families in individual III.

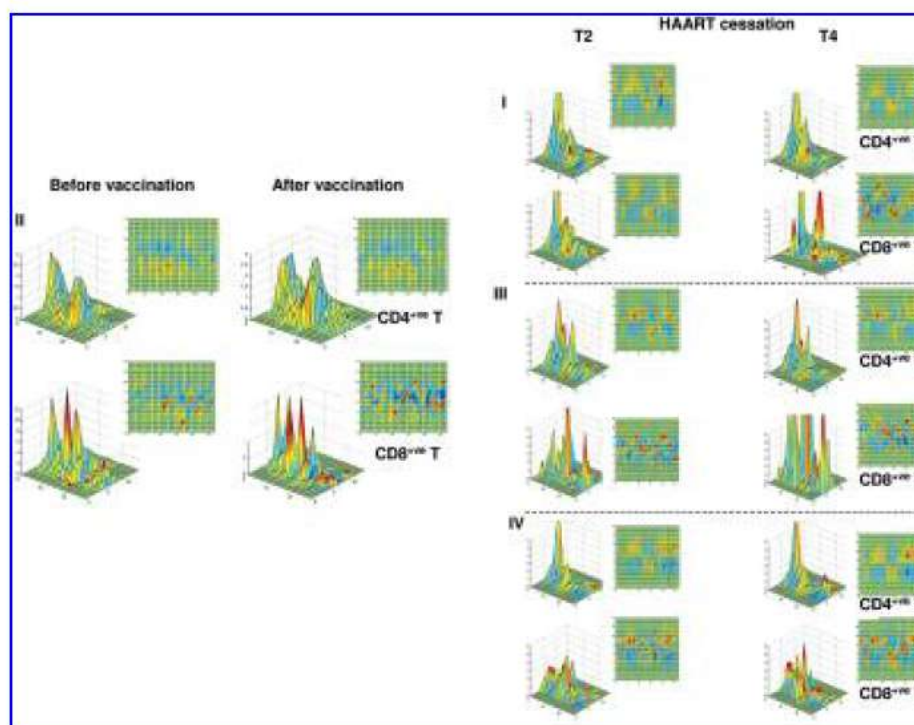


FIG. 4. Analysis of the TCR β chain transcripts in purified CD4⁺ve and CD8⁺ve T cells before (T0) and after (T1) vaccination. In patient I, modifications of the CDR3-LD and V β /HPRT transcript ratio were observed in some V β families of both compartments. In contrast, the modifications observed in individual II mostly concern the CD8⁺ve T cells. Analysis of the TCR β chain transcript in purified CD4⁺ve and CD8⁺ve T cells after HAART interruption (T2 and T4). In each of the three patients analyzed, no qualitative or quantitative modifications of the V β chain transcripts were observed in the CD4⁺ve T cells between T2 and T4. In contrast, in the CD8⁺ve T cells, the CDR3-LD and V β /HPRT transcript ratios of some V β families were found modified at T4 compared with T2, mostly in patients I and III.

compartment. The relationship between the viral load and serial TcLandscape patterns was studied.

First, healthy individuals used for serial comparison exhibited roughly stable TCR profiles characterized by only some V β families with altered CDR3-LD. Such "private" alterations have already been reported in healthy individuals.^{21–23} Altered V β families, persisting over time, were observed in CD8⁺ve T cells during influenza virus responses²⁴ and from EBV-infected individuals.²⁵ Human cytomegalovirus (CMV)-specific CD4⁺ve memory T cells were also characterized by strongly altered V β families.²⁶ These dominant clonotypes involved 0.3–4% of peripheral blood memory CD4⁺ve T cells in CMV-infected individuals (>60% of adults).²⁶ Thus, the occasional oligoclonal patterns that we observed in our healthy individuals, reproducibly found over time, are likely linked to the "immunological history" of each individual. Our observations thus extend the concept that T cells from healthy individuals do not have a gaussian CDR3-LD in each TCR V β family as usually observed in laboratory strains of rats or mice.^{8,27} The presence of persisting CDR3-LD alterations in healthy individuals indicates that the study

of the TCR repertoire must be done using samples serially harvested over time to be relevant for understanding complex *in vivo* immune responses.

In this report, we combined the CDR3-LD analysis in each V β family with a quantitative assessment of all V β transcripts. Such an approach^{8,15,19,20} makes it possible to better establish a hierarchy of the amount of mRNA corresponding to each V β family displaying altered as well as gaussian CDR3-LD. Statistical analyses, performed on both the CDR3-LD and V β /HPRT transcript ratio values of healthy volunteers, showed that the TcLandscape topologies obtained serially in time in each individual were more stable and comparable than in different individuals studied independently. This suggests that the TcLandscape analysis can be useful in following longitudinally the V β TCR mRNA regulation in patients and in contributing to the study of the global evolution of T cell responses over time in patients with autoimmune diseases¹⁹ or cancer²⁰ as well as following an allograft.^{8,15}

In this paper, such an analysis was performed in HIV-1 chronically infected patients to better describe the T cell modifications associated with a particular clinical context. Overall,

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the V β alterations observed in chronically infected asymptomatic patients were much more pronounced and systematic than in healthy individuals, even if these patients who had received HAART treatment for a long time. Indeed, Soudeyns *et al.*¹¹ showed a reduction in the global oligoclonality following at least 7 months of HAART compared with the HIV-specific CD8⁺ T cell clones with a predominant V β usage involved in the primary immune response to HIV.^{27,28} These CDR3-LD alterations are likely related to memory cells resulting from the sustained antigenic stimulation due to chronic HIV-1 infection.^{17,29,30}

However, our data did not allow us to determine if such T cells with skewed TCR V β usage were only committed against HIV-1 determinants or if clones committed to other types of viral infection (such as CMV), which are frequently encountered in these patients, contribute to these patterns. Only sorting such CDR3-LD altered V β families using anti-V β antibodies or MHC class I peptide tetramers would make it possible to test these T cells against relevant viral determinants. Interestingly, as recently described by Dong *et al.* in long-term survivor HLA-B8-restricted humans infected with HIV-1,⁶ we also observed atypical CDR3-LD profiles in two out of the four patients analyzed. Such an unusual CDR3 length in V β transcripts has also been described in some overstimulated alloreactive T cell lines in mice.³¹ However, we never observed such long CDR3 lengths among the 15 healthy individuals and more than 80 patients with autoimmune disease,¹⁹ cancer,²⁰ or an allograft¹⁵ so far studied in our laboratory and who also presented chronic stimulations of their immune system. These unusual CDR3 lengths were not artifactual since they were serially observed in independent experiments and remained stable over time. In addition, they were not dependent on the age of the patient (individual I was 38 years old and patient III was 58 years old). It can be hypothesized that long-term active chronic viral stimulation and/or specific peptide/MHC conformations³¹ can result in the selection of T cells with such TCR usage biases.

The TCR β chain regulation of these HIV-1-infected patients was serially analysed in a phase II vaccination protocol and after HAART cessation. Our data suggest that both vaccination and HAART cessation are associated with a reshaping of the T cell repertoire toward more clonal selection. When assessed over time, the global TCR repertoire exhibited more pronounced CDR3-LD alterations when the patients progressed through the two protocols. We also found that most of the selected clones belong to the CD8⁺ T cell compartment. Nevertheless, it is difficult to precisely draw conclusions from these experiments on the relative effects of vaccination and HAART withdrawal on TCR changes, and larger cohorts of patients studied with this technology are needed. No clear correlation between viral load and TCR repertoire alteration was observed. Nevertheless, interestingly, HAART interruption in patient I was associated with both an increase in viral load and an increased clonal selection. The accumulation of transcripts of the altered V β families observed at T4 suggests that these T cell clones, mostly concerning the CD8⁺ T cells, could contribute to the stabilization of the viral load observed between T3 and T4. In patients II and III, the relative stability of the viral load over time, possibly resulting in a lower antigenic stimulation, could explain the low level of modifications of the V β transcriptome after HAART interruption.

Taken together, our study showed that HIV-1 chronically infected patients exhibited identifiable patterns of their V β transcriptome topologies. In addition, with other monitoring tests, such an approach could help to better envision global T cell V β transcriptome regulation in HIV-1-infected patients. The combination of the analysis of the CDR3-LD profile and the transcript accumulation may also help to classify the various populations exhibiting CDR3-LD alterations and may provide a guide for sorting specific V β ⁺ altered clones for further analysis.

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Annexe 4

*Operationally Tolerant and minimally
immunosuppressed kidney recipients display strongly
altered blood T cell clonal regulation*

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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) V β 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: NI, 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, phycoerythrin; 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 ($n = 6$) (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 Star group ($n = 6$) (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 (Stal) 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 (IDF-CRI) 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 (IN⁻ 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 separated 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 V β 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 V β family individually. The alteration per V β 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 V β families in one sample gives the global percentage of alteration for each individual. To assess the magnitude of the V β transcript accumulation, the level of V β RNA was measured by real-time quantitative PCR. The data are displayed as a tridimensional 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 V β families in humans. Correspondence between numbers indicated on the X-axis and the different V β families is given in Figure 2. The Y-axis gives the 13 CDR3 lengths and the Z-axis gives the amount of each V β 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	66	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	1990	1993	1974	1991	1997	1990	1998	1983	1980
Induction therapy (duration)	Anti-IL2 mAb (10 days)	No	No	No	ATG (6 days)	ATG (11 days)	ATG (17 days)	ATG (11 days)	ATG (19 days)	No
HLA-A-B-D-R inc.*	3 ^a	3 ^a	3 ^a	ND	3	3 ^{mb}	0 ^a	gmb	4 ^a	2 ^a
Anti-T FRA prior to transplantation (%)	0	20	0	ND	0	0	72	0	60	20
Anti-T FRA 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)	No	No
Reason for IS interruption	PTLD	Non-compliance	CMV 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 mAb (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 3) received a second graft. The mean HLA-A, -B and -DR incompatibility was 2.8 ± 1.9 . The mean pre-graft anti-T FRA was $30 \pm 33\%$. Four patients received ATG induction therapy for 1.4 ± 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 serious 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.

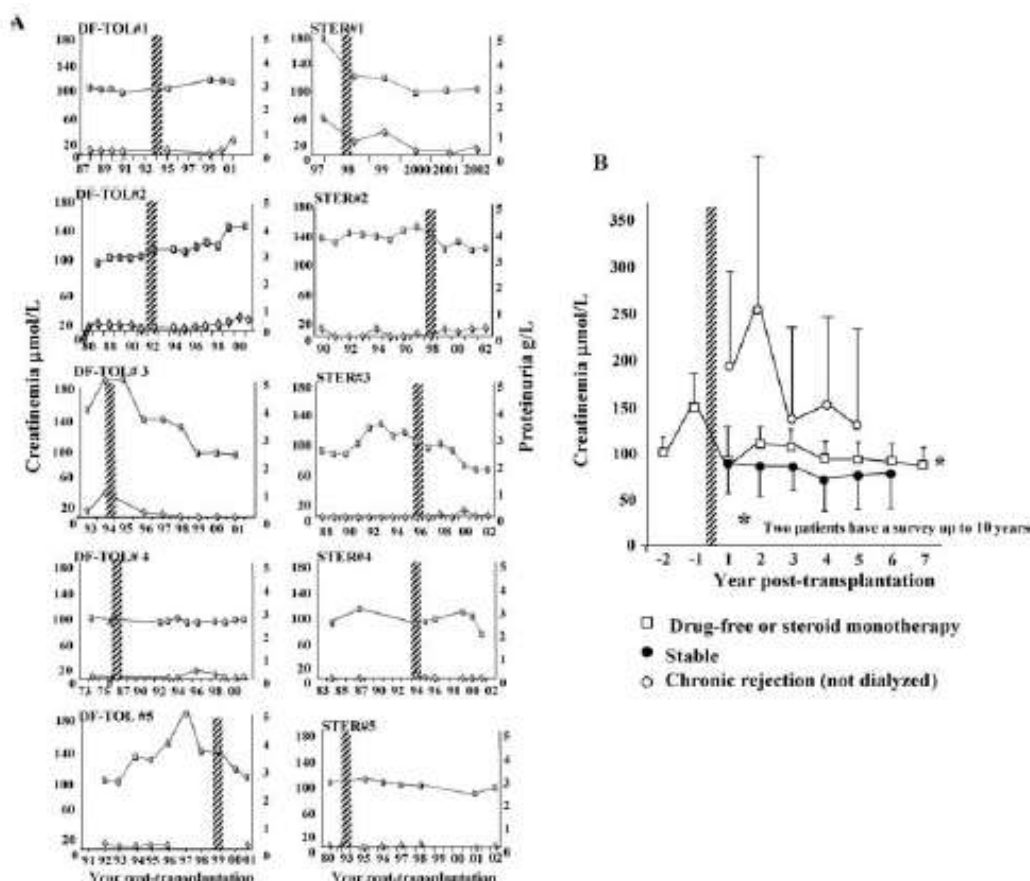


Figure 1: Graft function in the various kidney recipient groups. (A) The left-hand panel shows the creatinemia (■) and proteinuria levels (●) for each drug-free patient (DF-Tol group) before and after withdrawal of immunosuppressive treatment until the time of analysis. The corresponding data for patients 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 (ΔHPRT 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 V β families positively selected using MACS[®] anti-PE micro-beads and a MS⁺ type separation column (Mil-

tarry 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 V β surface staining

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

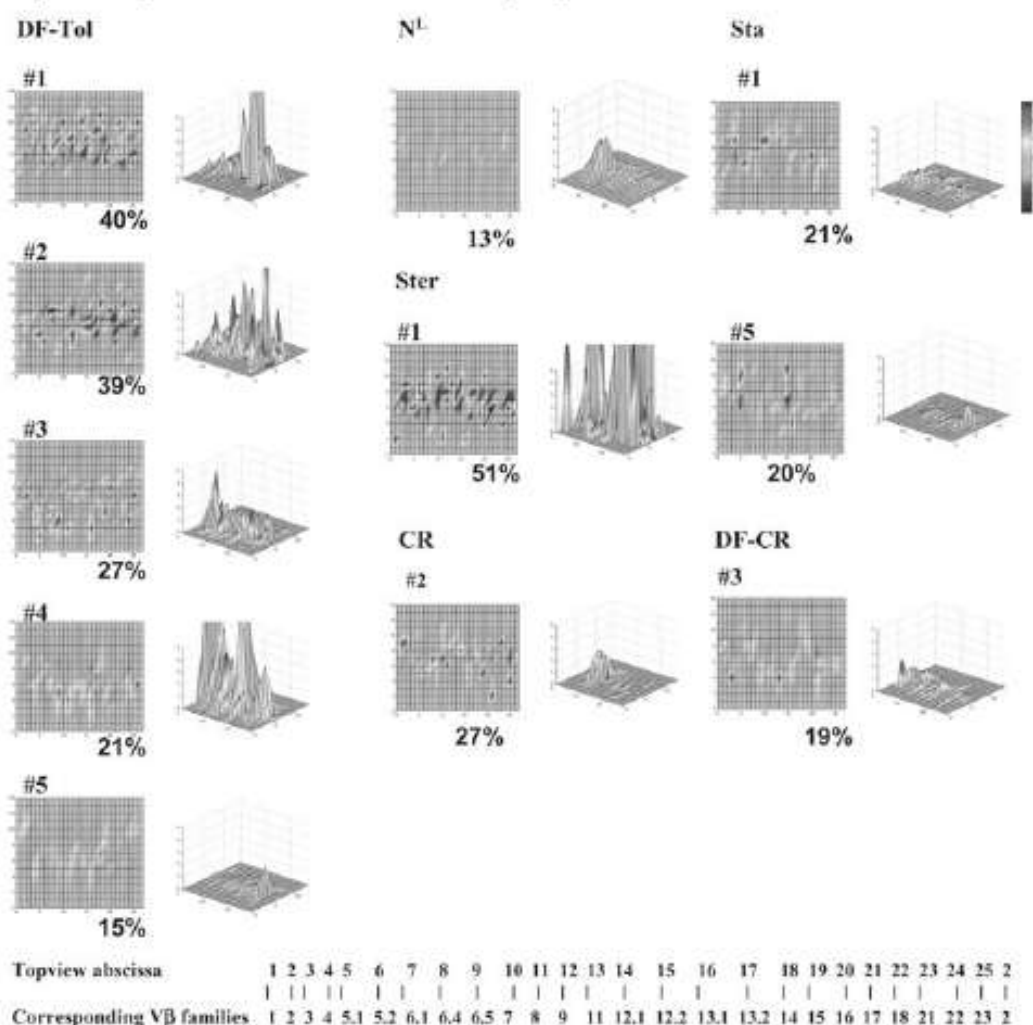
V β transcriptome alterations in blood of kidney recipients

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

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

Statistical analysis

The different groups were compared on the basis of CDR3-LD and V β /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 V β /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 (Stal), 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 V β /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

V β 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 V β /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 V β families displaying <30% CDR3-LD alteration and about 1% of V β 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 V β families for each individual) remained within $10.9 \pm 2.9\%$. In addition, in normal individuals, 83% of V β families had a V β /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 V β /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 V β families showed more than 50% CDR3-LD alteration (Table 2) whereas the global CDR3-LD alteration was $28.3 \pm 11\%$. In addition, these recipients showed high V β /HPRT transcript ratios with approximately 8% more than 15 (Table 2). These ratios corresponded to a strong accumulation of V β transcripts since HPRT levels were unchanged in operationally tolerant

Table 2: CDR3-LD alteration and V β /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
V β /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 V β families in the alteration interval.

Note: (a) the distribution of V β families according to the percentage of CDR3-LD alteration, global CDR3-LD alterations and (b) the V β /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-matched individuals.

patients compared to the other groups (data not shown). This phenomenon was not associated with deletion of the remaining V β 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-V β antibodies. For example, the transcripts of the V β 1, 4 and 11 families represented 29, 6.5 and 12%, respectively, of the whole V β transcriptome in one representative patient (DF-Tol 4 patient) whereas the percentages of T-cells staining for the corresponding V β protein were only 6.9, 0.2 and 0.5%, respectively, (Figure 3C) and did not differ from the values of normal 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 V β 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 V β families showed more than 50% CDR3-LD alteration and the global CDR3-LD alteration was extremely high ($42 \pm 15\%$) (Table 2). Furthermore, these patients had high V β /HPRT transcript ratios with 8% of V β families having a V β /HPRT ratio >15 (Table 2). Thus, drug-free patients and those under low-dose steroid

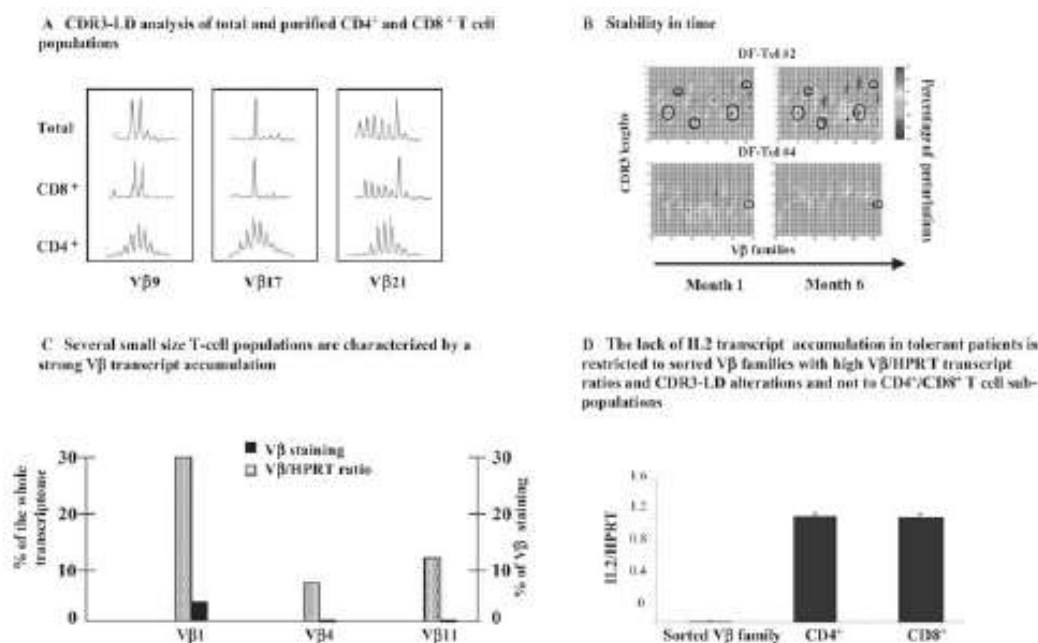


Figure 3: (A) CDR3-LD analysis was first performed on purified CD4⁺ and CD8⁺ T-cell populations in 4 DF-Tol patients. Figure 3A gives a representative example of a CDR3-LD analysis performed on three families sorted from patient DF-Tol 2. The alterations were mainly found to correspond to a CD8⁺ T-cell 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 fam-

ilies, 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 V β families showed CDR3-LD alterations below 30 % whereas only 1 % of families displayed alterations >50 % (Table 2) and their global CDR3-LD alteration was $20.9 \pm 4.8\%$. These patients also displayed low V β /HPRT transcript ratios with 98 % of V β 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 V β /HPRT transcript ratios, statistical analysis (Manova) showed that both of these groups displayed significantly stronger CDR3-LD alteration and higher V β /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 V β /HPRT ratio. Finally, the listed clinical parameters were also analyzed for their possible influence on CDR3-LD and V β /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 tran-

scriptional 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 V β 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

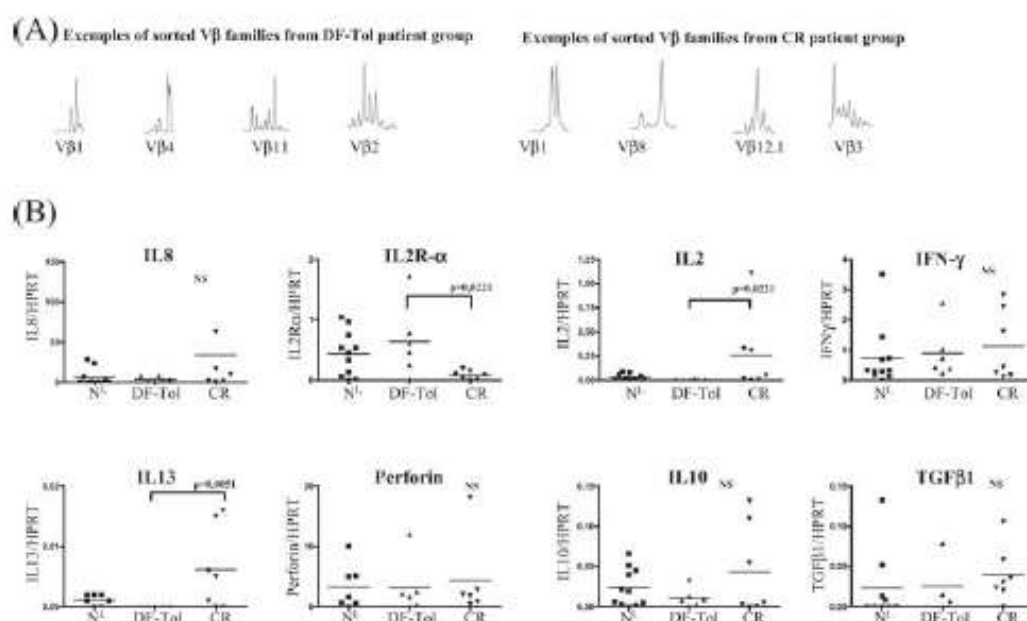


Figure 4: Cytokine transcript accumulation in sorted Vβ families from drug-free operationally tolerant patients and recipients with chronic rejection. (A) CDR3-LD of T cells in sorted Vβ families analyzed for cytokine transcript accumulation. (B) Cytokine transcript measurements were performed on sorted 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/ μ L 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 V β /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 V β /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 V β /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 5

Clinical operational tolerance after kidney transplantation

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Clinical Operational Tolerance after Kidney Transplantation

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Induction of allograft-specific tolerance and the detection of a "tolerance" state in recipients under immunosuppression with long-term stable graft function are major challenges in transplantation. Clinical "operational tolerance," defined as stable and acceptable graft function without immunosuppression for years, is a rare event. There is no report on the clinical history of such patients. In this article, we report on the medical history of 10 kidney recipients who display an immunosuppressive drug-free "operational tolerance" for 9.4 ± 5.2 years. Clinical factors that may favor such a tolerant state are underlined. Firstly, most of the patients interrupted immunosuppression over a long time period (until 4 years), which mimics the procedure of intentional immunosuppression weaning following liver transplantation. Secondly, donor age was younger (median 25 years) than the one of the general transplanted population, suggesting that graft quality is one of the conditions favoring "operational tolerance." Moreover, the "operationally tolerant" recipients may be 'low responders' to blood transfusions (PRA $6 \pm 5.4\%$, six blood transfusions). We also show that "operational tolerance" occurs in the presence of anti-donor class II antibodies, as assessed in two patients. Finally, two patients degraded their renal function 9 to 13 years after treatment withdrawal, however only one presented histological lesions of chronic rejection.

Key words: Operational tolerance, kidney transplantation, immunosuppression withdrawal

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Introduction

The advent of renal transplantation has notably reduced morbidity and mortality in patients with end-stage renal disease. New immunosuppressive molecules have dramatically reduced the risk of acute rejection (1) and prolonged graft survival (2). However, immunosuppressive treatments have numerous side effects, including infectious complications (3), malignancies (4,5) and metabolic disorders (6). Calcineurin inhibitor-related-nephrotoxicity also greatly contributes to the development of chronic allograft nephropathy in long-term functioning grafts (7). Moreover, immunosuppression poorly influences chronic rejection, the main cause of graft loss in renal transplantation (8,9). Understanding the mechanisms of immune tolerance in humans is therefore crucial if induction of graft-specific tolerance is to become achievable.

Tolerance has rarely been established in large animals models and is rare in humans. "Operational tolerance" is a clinical situation defined as a stable graft function without clinical features of chronic rejection and in the absence of any immunosuppressive drugs, usually for more than 1 year. This is observed more frequently in liver transplant patients than in other organ recipients (10,11). Albeit rare, "spontaneous" operational tolerance has also been reported in kidney recipients (12–21), although unidentified non-compliance to treatment may lead to underestimation of the phenomenon.

Such patients offer a unique opportunity to study the clinical and biological characteristics potentially specific to and associated with tolerance. Despite an active research on their biological characteristics, no detailed clinical history of such patients has been reported to date. In this article, we report on the clinical description of a cohort of 10 kidney recipients displaying clinical "operational tolerance" to their graft years after interruption of their immunosuppressive treatment.

"Operationally Tolerant" Kidney Recipients

Patients, Materials and Methods

Clinical "operational tolerance" was defined as a stable kidney graft function, without clinical markers of chronic rejection, and without any immunosuppressive drugs for more than 1 year. A creatininemia and proteinuria below 150 $\mu\text{mol/l}$ and 1g/24h, respectively were defined as acceptable thresholds. Graft biopsies were not performed on these normally functioning kidneys for ethical reasons. However, biopsies were performed in patients who, over time, failed to meet the criteria of "operational tolerance" mentioned above. Anti-HLA class I and II antibodies were screened by microlymphocytotoxicity on a selected panel (PRA) and, more recently, by flow cytometry (Luminex technology). Specificity was determined with a cytotoxicity assay. The characteristics of the 10 patients meeting these clinical criteria are detailed in the case reports and are summarized in Tables 1 and 2. Creatininemia and proteinuria evolution are depicted for each patient in Figure 1.

Case reports

Case 1: A.S., born in 1931, developed a membrano-proliferative glomerulopathy. He received a first living-related HLA-identical donor kidney, which was immediately lost because of an arterial thrombosis. This patient was not HLA-immunized (previous blood transfusions not documented). In 1973, he received a second renal transplant, from a 25-year-old deceased donor with one HLA class I incompatibility (donor class II undetermined). His immunosuppressive treatment consisted of an initial cyclophosphamide perfusion relayed with corticosteroids (CS) and azathioprine (AZA). Graft function was delayed until day 15. During this period an acute rejection was suspected but not biopsy-proven, and treated with Cyclophosphamide and CS boluses. A Hepatitis C Virus (HCV) infection was clinically quiescent and he spontaneously recovered from a Hepatitis B Virus (HBV). Five years after transplantation, a systematic graft biopsy showed a discrete interstitial mononuclear infiltrate, and C3 and IgM deposits on arteriolar walls, interpreted as a border-line rejection, but he was not treated at this time. Twelve years after transplantation, the patient progressively stopped taking his immunosuppressive medication (incompliance) over an estimated period of 4 years. Twenty-six years later, his renal function is stable (creatininemia 113 $\mu\text{mol/L}$) and he is only treated for arterial hypertension.

Case 2: F.J., born in 1966, had an obstructive uropathy. He was 15 years old when hemodialysis was started. Despite six blood transfusions, a weak anti-HLA immunization was detected only once (PRA 7%). A few months later, in 1981, he received a deceased, 3 HLA class I incompatible renal transplant from a 7-year-old donor (donor class II undetermined). His immunosuppressive treatment was composed of CS and AZA. Seven days after transplantation he was treated with CS boluses for a clinically suspected acute rejection (not biopsy-proven). He was found to be positive for HCV and this disease is still clinically quiescent. Because of non-compliance, he stopped his immunosuppressive treatment 16 years ago, over an estimated time period of 2 years. Since this withdrawal, his renal function has remained stable (creatininemia 139 $\mu\text{mol/L}$).

Case 3: M.R., born in 1945, presented an end-stage renal disease related to an undetermined glomerulopathy. He was transfused four times before grafting; no HLA immunization was detected. In 1987, he received a renal transplant from a 16-year-old deceased donor, with 4 HLA incompatibilities. He received ATG and his maintenance immunosuppressive regimen consisted of Cyclosporine (CsA), CS and AZA. The graft began functioning after 8 days. CS were stopped at 3 months. After 13 years, and following an estimated period of 2 years of inconsistent drug intake, the patient finally stopped taking his immunosuppressive medication (incompliance). Five years following total interruption, his creatininemia is at 89 $\mu\text{mol/L}$ and he is without proteinuria. He is only treated for hypertension.

Case 4: Y.M.L., born in 1968, had an obstructive nephropathy. In 1987, he received a deceased 4 HLA incompatible renal transplant. His therapy consisted of an induction with ATG and a maintenance regimen of CsA, AZA and CS. An acute rejection was suspected on day 6 post-surgery and treated with CS boluses; no graft biopsy was performed. Nine years after transplantation, he started to space out his immunosuppressive drug intake and stopped it definitively 1 year later. Eight years after immunosuppression withdrawal, his renal function remains stable (creatininemia 150 $\mu\text{mol/L}$) and he is only treated for hypertension.

Case 5: S.P., born in 1978, had an obstructive uropathy. He presented a weak HLA immunization secondary to two blood transfusions. In 1991, 1 year after dialysis, he received a kidney transplant from a 21-year-old deceased donor with 3 HLA incompatibilities. He was given ATG and his maintenance treatment was an association of CsA, AZA and CS. He displayed no delayed graft function (DGF). He had six graft biopsies during the first 5 years post-transplantation, all because of moderate episodes of renal dysfunction. All, but one, showed only mild interstitial fibrosis. One biopsy showed moderate focal lymphoid infiltration, with mild tubulitis, suggesting borderline changes, which was treated with steroid boluses. He presented a primo-infection with Epstein-Barr Virus (EBV) the year following transplantation. CS were progressively withdrawn 7 years after transplantation. In 1999, he developed a multifocal post-transplantation lymphoproliferative disorder (PTLD) relative to EBV, and CsA and AZA were abruptly interrupted. PTLD treatment consisted of chemotherapy and radiotherapy. Currently, 6 years after PTLD treatment, he is being treated with valacyclovir and considered to be cured of his disease. His renal function remains stable (creatininemia 123 $\mu\text{mol/L}$).

Case 6: H.L., born in 1929, whose initial disease was an interstitial nephropathy, received a deceased kidney transplant in 1993 from a 39-year-old donor with 3 HLA incompatibilities. Despite having previously transfused 24 times and being pregnant twice, she presented low levels of PRA (9%) before transplantation. Her immunosuppressive treatment consisted of CsA, CS and mycophenolate mofetil (MMF), without induction. She displayed a DGF, which was interlinked with an acute rejection episode (Banff grade Ib) treated with CS boluses. CS were stopped 5 months after transplantation, and MMF 1 month later (cytopenia). A second acute rejection episode (Banff Ia) occurred 7 months later and 3 CS boluses were administered. During the first year of transplantation, she presented numerous bacteriological infections and a basocellular carcinoma. Because of poor graft function, hemodialysis was started 12 months after the transplantation. CS and CsA were subsequently stopped. At this time, anti-donor class II antibodies were detected. Surprisingly, after months of dialysis, her renal function progressively recovered and she no longer required dialysis. Renal-DMSA scintigraphy performed in 2002 showed no residual native kidney function, but a functional kidney graft. Eleven years after immunosuppression withdrawal, her creatininemia is 96 $\mu\text{mol/L}$ and her proteinuria is below 0.5g/d.

Case 7: F.C.E., born in 1967, presented a renal failure related to renal hypoplasia. A weak pre-graft immunization was noted secondary to five blood transfusions. In 1994, she received a deceased kidney from a 30-year-old donor with 4 HLA incompatibilities. She received an induction therapy with ATG. Her maintenance regimen consisted of CsA, CS and AZA. She displayed no DGF. She progressively stopped her immunosuppressive treatment due to incompliance over a 4-year period. She displayed a donor-class II immunization during CsA monotherapy. She has now been without treatment for 1 year and her renal function remains stable (creatininemia 128 $\mu\text{mol/L}$).

Case 8: I.K., born in 1966, suffered from a testicular cancer treated with chemotherapy and bone marrow auto-transplantation. He then developed renal failure attributed to drug toxicity and resulted in hemodialysis. He was transfused twice and did not develop PRA. In 1998, he

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Table 1: Main characteristics of "operationally tolerant" patients

Case (year of transplantation)	Initial renal disease	Recipient age	Donor age	Pre-graft blood transfusions	PRA	HLA incompatibility number	HLA DR incompatibility number	Cold ischemia (hours)	DGF Yes (d/5)	Induction therapy	Acute rejection Yes	Cancer	Calcineurin inhibitor exposure (years)	IS withdrewal time to transplantation	Reason for IS withdrewal	Tolerance duration (y)	antiHLA antibody since IS withdrewal
1 (1973)	Membrano- proliferative glomerulopa- thy	42	25	ND	No	1*	ND	14	Yes (d/5)	Cyclo- phosphamide	No	None	12	Non-compliance	20	None	None
2 (1981)	Obstructive uropathy	15	7	6	7%	3*	ND	37	No	No	Yes	None	8	Non-compliance	16	None	None
3 (1987)	Undetermined glomerulopa- thy	41	16	4	No	4	1	36	Yes (d/5)	ATG	No	None	13	Non-compliance	5	None	None
4 (1987)	Obstructive uropathy	19	ND	ND	ND	4	2	ND	ND	ATG	Yes	None	10	Non-compliance	8	ND	ND
5 (1991)	Obstructive uropathy	13	21	2	12%	3	2	29	No	ATG	Yes	PTLD	8	PTLD	6	None	None
6 (1993)	Interstitial nephropathy	64	39	24	9%	3	1	40	Yes (d/9)	No	Yes	Basocellular carcinoma	1	Initial renal dysregulation	11	anti-donor anti-class II (DR11)**	anti-donor anti-class II (DR4)**
7 (1994)	Renal hypoplasia	27	30	5	15%	4	1	24	No	ATG	No	None	10	Non-compliance	1	None	None
8 (1998)	Interstitial nephropathy	27	28	2	No	0	0	3	No	No	No	None	1.5	Non-compliance	5	None	None
9 (1999)	Hypocomple- mentemic glomerulopa- thy	43	17	2	7%	3	1	18	Yes (d/5)	No	No	None	9	Non-compliance	13	None	None
10 (1987)	Undetermined glomerulopa- thy	52	30	4	4%	3	0	38	Yes (d/2)	anti-IL2R	No	PTLD	7	PTLD	9	suspected for class II	

*donor class II undetermined; **anti-class II determined with PRA.

ND: Not determined.

"Operationally Tolerant" Kidney Recipients

Table 2: Current biological parameters of 'operationally tolerant' patients

Case	Creatininemia ($\mu\text{mol/L}$)	Proteinuria (g/d)	WBC (/mm ³)	Lymphocytes (/mm ³)	Hb (g/dl)	γ globulin (g/L)	Treatment
1	113	0.22*	4100	980	12.3	11.5	calcic inhibitor, β -blocker
2	139	0.93	8050	3120	14.6	10.5	None
3	89	0.07	5800	2480	16.3	12.2	ACE inhibitor
4	150	0.11	6850	2898	14.4	ND	β blocker and central anti-hypertensor
5	123	0.33	7130	2180	16.7	ND	valaciclovir
6	96	0.50	7150	2480	13.5	ND	
7	128	0.11	7190	720	11.6	ND	None
8	115	0.05	6490	ND	12.5	10.2	β blocker
Mean $\pm$ SD	119 $\pm$ 20	0.20 $\pm$ 0.18	6595 $\pm$ 1195	2123 $\pm$ 924	14 $\pm$ 1.8	11 $\pm$ 1	

*proteinuria in g/L.

ND: Not determined.

received a renal transplant from his HLA-identical 28-year-old brother. He was treated with CsA, MMF and CS without induction therapy. CS were stopped 1 month later. Less than 1 year after transplantation, he spaced out his immunosuppressive drug administration due to in compliance and definitively interrupted his immunosuppressive medication 2 years after transplantation. He is now being treated only for arterial hypertension and, 5 years after immunosuppression withdrawal, continues to display a normal renal function (creatininemia 115 $\mu\text{mol/L}$).

Case 9: P.P., born in 1937, suffered from a hypocomplementemic glomerulopathy leading to renal failure. He received two blood transfusions and had low PRA levels. He had a chronic VHB assessed with the presence of AgHBs. In 1980, he received a kidney transplant from a 3 HLA incompatible 17-year-old deceased donor. He was treated with AZA and CS. He progressively stopped his immunosuppressive medication between 9 and 11 years after transplantation due to in compliance. After 13 years of "operational tolerance," his renal function started to degrade. A subsequent graft biopsy revealed grade I chronic allograft nephropathy with mild nephroangiosclerosis without significant lymphoid infiltration or specific changes suggestive of chronic rejection (Figure 2). C4d staining was negative. No anti-HLA antibodies have been detected.

Case 10: M.C., born in 1935, presented an undetermined glomerulopathy. He was transfused four times and developed low PRA levels. In 1987, he received a kidney transplant from a 3 HLA incompatible 30-year-old deceased donor. He received an anti-IL2 receptor antibody together with CsA, AZA and CS. CS were stopped 2 months later. Seven years later he developed a PTLD that was treated with chemotherapy and his immunosuppression was abruptly withdrawn. His renal function remained stable for 7 years without immunosuppression, with an apparently cured PTLD, until a significant proteinuria appeared. His renal function progressively degraded, requiring dialysis in 2004. At this time, the presence of anti-HLA class II antibodies was suspected, but could not be confirmed on additional analysis. A graft biopsy, performed in 2002, showed grade Ib chronic allograft nephropathy with allograft glomerulopathy, but without C4d staining (Figure 2).

Cross-sectional analysis

Donor and recipient ages, DGF and cold ischemia: The dates of transplantation for these patients are spread over a long period of time (1973 to 1998). Their median age at the time of transplantation was 34 years (range 13 to 64). Their donors (all but one deceased) were unusually young with a median age of 25 years (range 7 to 39). We compared the age of the donor for each recipient studied with the confident interval of the mean donor age in each corresponding year of graft. This test suggests a trend for younger donor in tolerant patients (i.e. outside of the confidence interval). However we realize that this approach is only informative as statistical significance

was not reached. It was a first kidney transplantation for 9 out of 10 patients. Cold ischemia was on average 26.4 ± 12.8 hours. Four patients had a delayed graft function, defined as the requirement of at least one dialysis during the first week post-transplantation.

Blood transfusions, pre-graft immunization and HLA incompatibilities:

Eight patients were multi-transfused before transplantation (6 ± 7.3 blood transfusions). Possible blood transfusions before transplantation could not be documented for the two other patients. Among the eight transfused patients, six presented a low anti-HLA immunization, with mean PRA levels of $9 \pm 3.9\%$ (range 4 to 15%). Taking into account the nine patients whose PRA had been tested before transplantation, the mean PRA was $6 \pm 5.4\%$. Most of these blood transfusions were performed before 1990 and without leukocyte depletion. Mean HLA incompatibilities were 3 ± 1.5 . The quality of matching varied between 1973 and 1998, and donor MHC class II was not determined for two patients transplanted in 1973 and in 1981 (Table 3).

Induction and maintenance treatment: Six patients received an induction therapy: four with ATG, one with an anti-IL2 receptor antibody and one with cyclophosphamide. Three patients underwent transplantation before the ciclosporine era, and received a maintenance regimen of AZA and CS. All of the others received ciclosporine for a mean period of 7.2 ± 4.9 years before interruption.

Rejection and post-transplant immunization: Five patients were clinically diagnosed with acute rejection during the follow-up before immunosuppression withdrawal, but this was biopsy-proven for only two patients (one borderline). Histological findings of transplant glomerulopathy were present for only one of the two patients whose renal function finally degraded years after immunosuppression withdrawal. Lack of C4d staining suggested no humoral participation. Neither sample showed staining for anti-C4d, suggesting no significant involvement of antibodies, particularly in the patient with peripheral anti-class II antibodies. Interestingly, two other patients developed anti-donor class II antibodies: once for patient 6 (and this was not checked thereafter), and for four consecutive years for patient 7. No concurrent impairment of graft function was observed.

Infectious complications before and after treatment withdrawal

(Table 4): Before immunosuppression withdrawal, three patients presented a symptomatic herpetic manifestation, one of them (patient 5) also suffered from an EBV primo-infection 1 year after transplantation and a zoster infection the following year. Four patients suffered from recurrent bacteriological infections, essentially urinary, pulmonary and ORL infections. Three patients presented tumoral disorders after transplantation: a skin basocellular carcinoma in one case (patient 6) and PTLD in two cases (patients 5 and 10).

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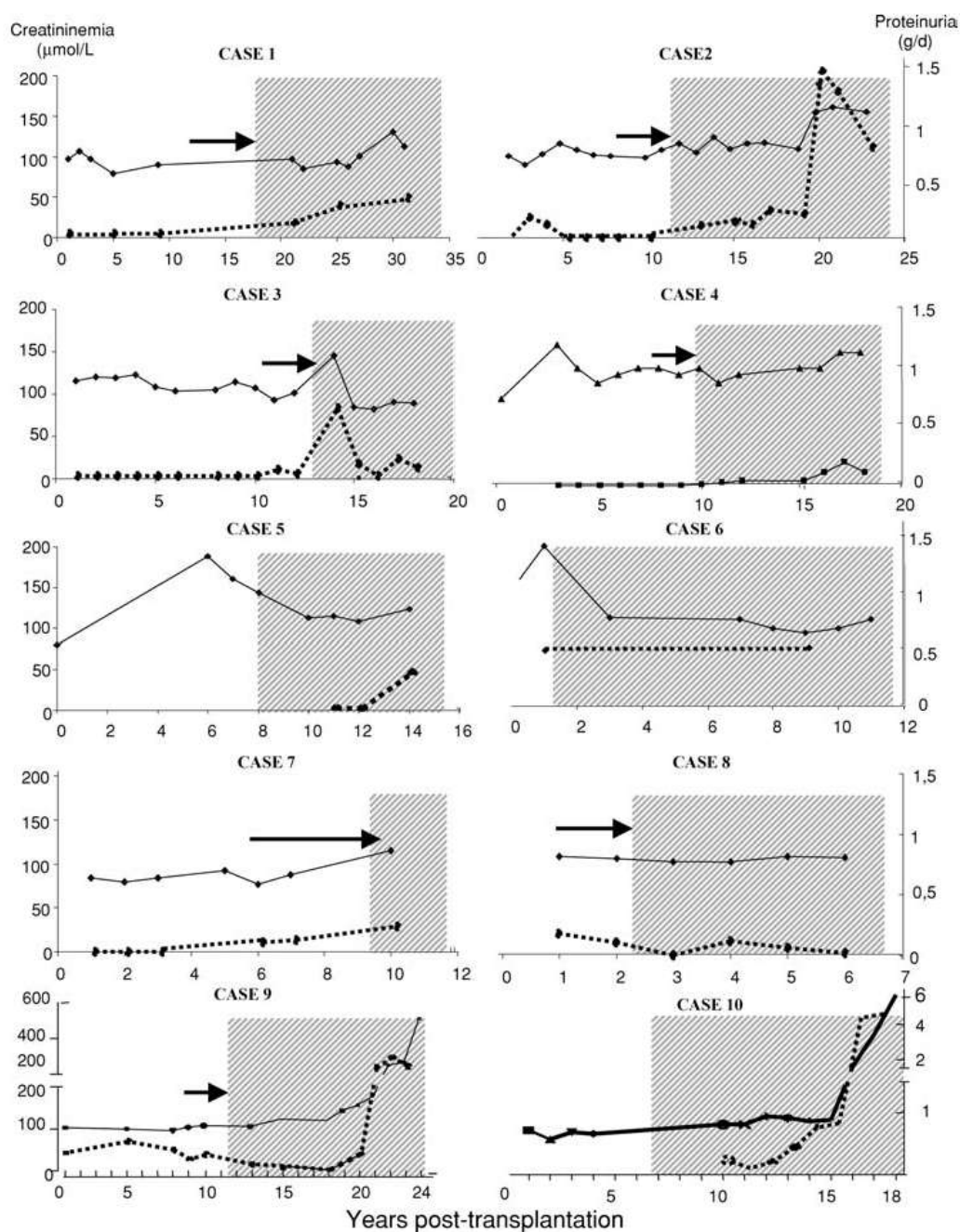


Figure 1: Creatininemia and proteinuria evolution since transplantation. Each graph indicates post-transplantation evolution of creatininemia ($\mu\text{mol/L}$) and proteinuria (g/d) for each patient. The horizontal arrow corresponds to the period of progressive immunosuppressive medication withdrawal. The grey part indicates the period off-immunosuppression. Creatininemia and proteinuria remain stable over time, except for cases 9 and 10 whose renal function degraded 14 and 9 years, respectively after immunosuppression cessation. In these two last cases the scale of the graph was adjusted to the level of creatininemia and proteinuria.

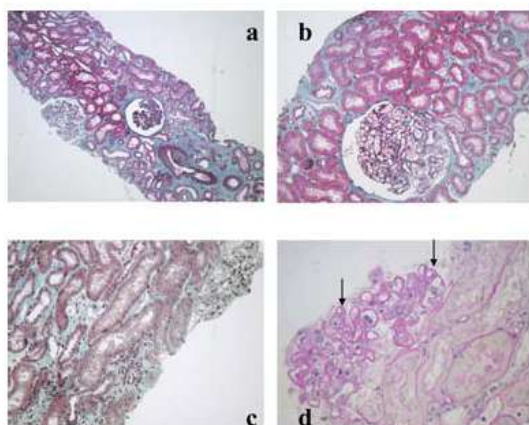


Figure 2: Graft biopsy performed following degradation of renal function in cases 9 and 10. Panels 2A and 2B correspond to the graft biopsy for patient 9 (Masson's trichrome coloration). It only revealed mild focal interstitial fibrosis and tubular atrophy (Figure 2A). Minimal arterial fibrous intimal thickness was detected and one glomeruli presented with a retracted flocculus. The other ones showed no glomerulitis or double contours (Figure 2B). Lesions suggested nephroangiosclerosis rather than chronic allograft rejection. No acute component was present in this biopsy. Panels 2C and 2D correspond to graft biopsy of patient 10 (Masson's trichrome coloration for Figure 2C and PAS coloration for Figure 2D). It showed focal fibro-edema associated with mild mononuclear infiltration. Glomeruli showed double contours in 26 to 50% of peripheral capillary loops, which, together with the presence of parietal IgM and C1q deposits (not shown), were in favor of allograft glomerulopathy. There was no acute rejection lesion but moderate arteriolar hyalinosis (not shown) was detected.

Table 3: Immunological characteristics of "operationally tolerant" patients

Blood transfusions	8 patients/8 (undetermined for 2)
Pre-graft immunization	6 patients/9 (undetermined for 1)
Mean PRA before transplantation	$6 \pm 5.4^*$
HLA incompatibilities	3 ± 1.5
Induction therapy	6 patients/10
Calcineurin inhibitor	7 patients/10
Acute rejection episode**	5 patients/10
Post-graft immunization	3 patients/9 (anti-donor class II for 2)
IS withdrawal (years from transplantation)	7.8 ± 4.3
Withdrawal through non-compliance	7 patients/10
Tolerance duration (years)	9.4 ± 5.7

*Mean PRA in 9 out of 10 tested "operationally tolerant" patients.

**Biopsy-proven or clinically suspected.

After treatment withdrawal, two patients (patients 1 and 5) continued to present recurrent bacteriological infections. EBV reactivation was checked for five patients several years after treatment withdrawal and was negative for three of them. One patient had a weak EBV reactivation and the other

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(patient 5 who had an EBV-induced PTLD) is still presenting significant EBV replication and is being treated with valacyclovir.

Concerning VHB, two patients presented a VHB infection from which they spontaneously recovered. One patient had a chronic VHB infection at the time of transplantation, but without significant cytolysis after transplantation. Six other patients retained protective anti-HBs titres after treatment withdrawal, but one of them (patient 10) lost his protective antibodies (10 years after immunosuppression withdrawal).

Overall, infections before and after the immunosuppressive drug-free phase were uncommon.

Causes and modalities of immunosuppression disruption, stability of operational tolerance: Immunosuppression was stopped at a mean of 7.8 ± 4.3 years post-transplantation. The reason for drug withdrawal was PTLD in two patients and non-compliance in seven. The non-compliant patients did not interrupt their drugs abruptly, but over a long time interval. Although no precise time period can be reported, the mean period of progressive interruption has been estimated to 1.8 ± 1.5 years. For the last patient (patient 6), immunosuppressive medication was withdrawn because of drug toxicity and a very poor clinical condition. The mean period of stable "operational tolerance" was 9.4 ± 5.7 years (range 1 to 20). Two out of 10 patients (patients 9 and 10) developed a renal failure after 9 to 13 years of stable "operational tolerance." A biopsy on one patient (patient 10) showed transplant glomerulopathy (Figure 2). The eight other patients still display a stable renal function, with a creatininemia at $119 \pm 20 \mu\text{mol/L}$ (range 89 to 150) and a proteinuria at $0.20 \pm 0.18 \text{ g/d}$ (range 0.07 to 0.93) (cf. Table 2).

Discussion

In this article, we report on a cohort of 10 kidney recipients with clinical immunosuppression drug-free "operational tolerance." For the first time, we present a detailed account of their medical history and their long-term clinical evolution. The median period of "operational tolerance" observed at the time of this study is 9.4 ± 5.7 years, with 8 out of 10 patients still "tolerant." Anecdotal cases of long periods of "operational tolerance" previously reported in the literature (see Table 5 for review) (14–20). All but one recipient in our cohort received a kidney from a deceased mismatched donor (3 ± 1.5 HLA incompatibilities), contrasting with the previously described "operationally tolerant" kidney recipients, all but two of whom received grafts from living-related donors (14–20).

"Operational tolerance" in allotransplantation remains a clinically defined concept, with a stable graft function at a good or acceptable level for several months or years in the absence of immunosuppressive treatment. Clinical "operational tolerance" should be distinguished from "almost tolerance," which corresponds to a stable graft function in minimally immunosuppressed recipients (low dose monotherapy) (22, 23). Graft biopsies were not proposed to these patients since they displayed normal renal function, without significant proteinuria. Despite this restriction, their acceptance of a mismatched deceased kidney, without any immunosuppressive drugs for years fulfils the most optimistic definition of tolerance and offers

Table 4: Infections and neoplasia during immunosuppressive treatment and after withdrawal

During immunosuppression			After IS withdrawal				
Viral infections	Bacteriological infections	Neoplasia	Viral infections	Bacteriological infections	Neoplasia	EBV reactivation after IS withdrawal	HBV status
1 Quiescent HCV	Abces	None	Quiescent HCV	Recurrent abces, infectious diarrhea, pneumopathy, sinusitis	None	ND	HBV infection spontaneously recovered
2 Quiescent HCV		None	Quiescent HCV		None	ND	Protective anti-HbS Ab (last control: 192 UI/ml)
3 Herpetic manifestation	Pneumopathy	None		Pneumopathy	None	negative	Protective anti-HbS Ab (last control: 106 UI/ml)
4		None		Abces	None	ND	ND
5 EBV primoinfection, herpetic manifestation, zoster,	Urinary and pulmonary infections	None	EBV-induced PTL	Recurrent ORL infections,	None	positive (290/10 ⁵ PBMC)	Protective anti-HbS Ab (last control: 215 UI/ml)
6 Herpetic manifestation	Pulmonary and urinary infections, infectious colitis	Basocellular carcinoma			None	ND	ND
7 Recurrent verruca	Recurrent sinusitis	None			None	negative	Protective anti-HbS Ab (last control: 95 UI/ml)
8	ORL infections	None			None	negative	HBV infection spontaneously recovered
9		None		Arthritis	None	positive (low level)	HBV (AgHbS positive)
10		PTLD			None	ND	Protective anti-HbS Ab lost 10 years after IS withdrawal

ND: Not determined.

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Table 5: Reported cases of "operationally tolerant" patients

Patients	Donor	Numbers of HLA incompatibilities	Immunosuppressive treatment	Reason for IS withdrawal	Tolerance duration	Histology	Mechanisms	References
1	Living related	?	ND	Non-compliance	?			(13)
6	Living related	0	ND	Non-compliance	3 to 8 years			(14)
7	Living related	?	AZA (+CS for acute rejection) DST and CsA+AZA+CS		3 to 38 years	"normal"		(15)
1	Living related	3 (haplo-identical)		Non-compliance	5 years	focal lymphocyte infiltrate and interstitial fibrosis, arteriolar intimal hyperplasia	Microchimerism, regulation, anergy	(16)
1	Living related	3 (haplo-identical)	ND	PTLD	> 3 years		anti-donor hyporesponsiveness (anergy? Clonal deletion?)	(17)
1	Deceased	6	Total lymphoid irradiation and ATG+ CS	Protocol	12 years		no microchimerism, anti-donor reactivity restored <i>in vitro</i> regulation	(19)
2	Living related (1) and deceased (1)	0 for one patient, 2 for the other patient	Induction therapy (OKT3) for one patient, no induction for the other	Non-compliance	5 and 27 years	Focal infiltrate confined to interstitium		(20)
1	ND	1 HLA class I mismatch (class II ND)	AZA+CS	Non-compliance	9 years			(18)

ND: Not determined.

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a unique opportunity to understand its mechanisms and to find a clinical and biological signature associated with tolerance in humans.

Before immunosuppression withdrawal, the clinical histories of these "operationally tolerant" patients do not seem to differ greatly from kidney recipients in general. However, although the relatively low number of such patients precludes relevant statistical analysis, the detailed clinical review of the patients described here revealed some interesting findings. Firstly, no evidence for a strongly biased proportion of original disease was observed, for example, glomerulopathy was the initial disease in 4 of the 10 patients, two undetermined) compared to 47% of the last 522 patients of our databank. Secondly, the main cause of immunosuppressive treatment cessation was non-compliance (7 out of 10 patients) and the patients usually discontinued their drugs progressively. They usually withdrew one drug and spaced out the administration of the remaining immunosuppressive drug, with several phases of 'stop and start' intake. The exact period of time during which treatment was spaced out is difficult to determine but in most cases probably lasted several years (1 to 4 years). This progressive "non-compliance procedure," which mimics in some respects what is sometimes applied to liver recipients considered as being at low risk of rejection (10,11,24) may select these patients and favor a transition to a state of "operational tolerance." The occurrence of such "operational tolerance" might be under-estimated because non-compliance is more frequent than actually confessed (25,26), varying from 18% (27) to 52% (28). These patients are at a higher risk of late acute rejection and of increased serum-creatinine (29). However, non-compliance also reveals states of clinical tolerance, as described in this article.

The third notable point concerns donor status. All the donors in our study were younger than the mean donor age of the corresponding year, and were also younger than the recipients themselves. Renal graft survival has been shown to be significantly reduced when the donors are older than 50 to 55 years, attributed in part to a reduced functional reserve (30–32). Kidneys from aging donors are usually more susceptible to ischemia-reperfusion lesions and to delayed graft function, and so may display higher antigenicity (32–34). One can hypothesize that good "quality" of the graft, with few age-related lesions (glomerulosclerosis, interstitial fibrosis), may provide better condition for the establishment of tolerance.

Finally, these "operationally tolerant" patients did not seem to be non-specifically immunosuppressed because they did not present significant opportunistic, frequent or severe viral infections or neoplasia following immunosuppression withdrawal. Preliminary data on the response of some of these "operationally tolerant" patients to influenza vaccination suggest that they present humoral and cel-

lular responses not significantly different from those of healthy volunteers (35). Furthermore, their protective anti-HBs titres remain stable, also indirectly reflecting immunocompetence. Six patients out of nine had presented anti-HLA immunization after blood transfusions. Most of the transfusions were performed with blood non-depleted in leukocytes. This proportion of sensitized patients is comparable of what was usually observed in the literature, with range of immunized patients from 4.4% (36) to 90% (37) after five or more blood transfusions. However, the levels of PRA of "operationally tolerant" patients before the transplantation were low ($6 \pm 5.4\%$), nearly the half of the mean PRA observed by Fehrman et al. (mean PRA 11% after five blood transfusions) (38). Moreover, although three of these patients were treated for suspected or biopsy-proven ($n = 2$, with one borderline) acute rejection, all episodes were easily reversed by steroid pulses. Altogether, this could suggest that the "operationally tolerant" recipients described here, despite not being clinically immunoincompetent, may be low alloreactive responders. A larger cohort is needed to confirm this trend.

Although 8 of the 10 patients continue to display stable renal function, the renal function of two patients degraded during the study period despite no indication of poor graft quality (donor age below 30 years, no prolonged delayed graft function). These two patients did not present acute rejection. Graft biopsies performed when renal function started to degrade showed no signs of acute or chronic rejection for one patient (patient 9) but rather lesions suggestive of nephroangiosclerosis. In contrast, the biopsy from the other patient (patient 10) who presented one positive PRA test showed allograft glomerulopathy (Figure 2). Nevertheless, there was no active or humoral component, as suggested by an absence of C4d staining. Three previous cases of graft biopsies performed during the phase of "operational tolerance" have been reported in the literature. One of them presented a moderate and focal infiltrate with an arteriolar intimal hyperplasia, suggesting the beginnings of chronic allograft nephropathy lesions (16), another one only displayed a focal interstitial infiltrate (20) and the last one was described as normal (15). In our study, a biopsy was proposed only for patients with creatinine or proteinuria evolving outside of the defined values. Recommendation to not biopsy such patients presenting a stable function years following withdrawal of immunosuppression may appear questionable since the operational tolerant state is not necessarily permanent (Figure 1). Minimal lesions could be detected by histology, justifying immunosuppressive drug reintroduction. However, such a strategy would require serial biopsies (every year?) and such multiple procedures would certainly carry substantial risks (44). In addition, the reintroduction of immunosuppression based on the presence of minimal lesions is also questionable, particularly in view of the decade(s) of stable function enjoyed by these patients; alteration of active regulation could be also imagined.

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Interestingly, two patients had anti-donor class II antibodies without showing degradation of renal function. We and others have shown that the late appearance of anti-donor antibodies is statistically associated with the secondary apparition of chronic rejection (39,40). However, the two patients who had anti-donor class II antibodies, still exhibit a stable renal function more than 4 and 10 years on.

The main mechanisms associated with experimental tolerance in allotransplantation are clonal deletion, sometimes associated with mixed chimerism, anergy and regulation (41). Regulation, alone or associated with anergy, has been suggested to play a role in previous reported cases of "operational tolerance" (16,19,42). VanBuskirk et al. have demonstrated an immune regulation implicating regulatory cell activity in some "operationally tolerant" kidney recipients using a "trans-vivo" delayed-type hypersensitivity assay (20). Clonal deletion does not seem to be crucial in "spontaneous tolerance," as observed by Strober (19), as also suggested by the presence of anti-donor reactivity (anti-donor antibodies) in two patients of our cohort. We also showed that the T cells of "operationally tolerant" patients are characterized by low levels of cytokine transcripts, contrasting with profiles observed in recipients with chronic rejection (21). Blood cell phenotype studies suggest that the profile of "operationally tolerant" kidney recipients does not differ from those of healthy volunteers (43) whereas recipients with chronic rejection present significantly less CD4⁺ CD25^{hi} cells (associated with regulatory cells) (Louis S. et al., submitted). In liver transplantation, "operationally tolerant" patients present a higher number of CD4⁺ CD25^{hi} cells than healthy volunteers (45). Finally, although these patients presented a heterogeneous clinical history, they offer the opportunity to search for a common tolerance signature that would help to identify the state of "operational tolerance" in other recipients under classical immunosuppression (46). However, these observations are clearly not substantial enough for an immunosuppression weaning procedure to be undertaken in these patients. For this to be feasible safer, parameters of tolerance prediction will need to be available. What recommendations could be made for the follow-up of such "operationally tolerant" patients? One could try to monitor the renal function of these in-compliant patients more closely (every month) and screen for panel reactive antibodies, which have been associated with an increased risk of developing chronic rejection (39). One could also recommend these patients to consult in the case of intercurrent infection episodes (47). Graft biopsies could be performed in the case of a proteinuria exceeding 1g/d, a creatinemia increase of more than 10% or the apparition of an anti-donor immunization.

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Annexe 6

Induction of long-term cardiac allograft survival by heme oxygenase-1 gene transfer

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RESEARCH ARTICLE

Induction of long-term cardiac allograft survival by heme oxygenase-1 gene transfer

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Elevated expression of heme oxygenase-1 (HO-1), an intracellular enzyme that degrades heme into carbon monoxide (CO), biliverdin and free iron, has anti-inflammatory and antiapoptotic effects in diverse models. Here, we analyzed the effects of specific overexpression of HO-1 following adenovirus-mediated (AdHO-1) gene transfer in an acute cardiac allograft rejection model. The intra-graft (i.g.) injection of AdHO-1 into cardiac allografts, as well as intramuscular (i.m.) or intravenous (i.v.) administration, prolonged allograft survival with, respectively, 13.3, 62.5 and 80% of the grafts surviving long term (>100 days), whereas control grafts were rejected with acute kinetics. HO-1 overexpression was associated with inhibited allogeneic responses in MLRs using graft-infiltrating leukocytes and

splenocytes, but not with lymph node cells. The inhibition of splenocyte proliferation was mediated by soluble factors and was dependent on the presence of APCs, since purified T cells proliferated normally. i.v. but not i.g. AdHO-1 administration decreased the number of graft-infiltrating leukocytes, cytokine mRNA accumulation and apoptosis in transplanted hearts, whereas i.v. and i.g. AdHO-1 did not modify normal immune responses against cognate antigens, indicating that there was no general immunosuppression. These results indicate that HO-1 overexpression prolongs the survival of vascularized allografts by promoting tolerogenic mechanisms acting on allogeneic cellular immune responses. Gene Therapy (2004) 11, 701–710. doi:10.1038/sj.gt.3302208
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Introduction

Heme oxygenase (HO) is the rate-limiting intracellular enzyme that degrades heme to biliverdin, free iron and CO.^{1,2} Biliverdin is subsequently reduced to bilirubin by biliverdin reductase. Free iron is involved in intracellular metabolism and ferritin production.^{1,2} Three HO isoforms (HO-1, -2 and -3), products of three distinct genes, have been identified so far.² The HO-2 and -3 isoforms are constitutively expressed, whereas HO-1 expression is induced by free heme and a large variety of cellular insults.^{1,2} This suggests that HO-1 is involved not only in heme degradation and iron reutilization but also in tissue protection against a variety of cell injuries.^{1,2}

Recently, the overexpression of HO-1 has been associated with prolongation of graft survival following the administration of metalloporphyrins^{3–5} or immunomodulatory peptides,^{6,7} which modulate HO-1 activity. Unfortunately, metalloporphyrins can also modulate the action of other heme-containing molecules such as NO synthases (NOS) or soluble guanylate cyclases^{8,9} and immunomodulatory peptides may have effects on other molecules independent of HO-1.⁷ Subsequently, the

capacity of HO-1 to prolong graft survival has been demonstrated by specific overexpression of HO-1 by gene transfer in allograft models^{10–12} and by the use of HO-1-deficient mice in xenograft models.²

At the present time, little is known about the effect of selective upregulation of HO-1 on acute allograft rejection. In addition, it is unclear whether elevated HO-1 expression prolongs graft survival through protection of the graft from injury or inhibition of antigraft immune responses.

The aim of this study was to analyze the effects of gene transfer-induced HO-1 overexpression in a model of acute cardiac allograft rejection. Our results show that HO-1 gene transfer into recipients gave rise to long-term cardiac allograft survival associated with an inhibition of allogeneic cellular immune responses.

Results

Adenovirus-mediated gene transfer of HO-1 prolongs cardiac allograft survival

To evaluate the effect of HO-1 overexpression on allograft survival, AdHO-1 was injected at various different sites: either i.g. into the graft myocardium, i.m. into the hind limb or i.v. into the tail vein.

Survival (MST ± s.e. days) of cardiac allografts injected with the noncoding adenovirus Add1324 (7.4 ± 0.7, *n* = 8) was not statistically different from that of control untreated hearts (10.1 ± 0.8, *n* = 19) (Figure 1). AdHO-1

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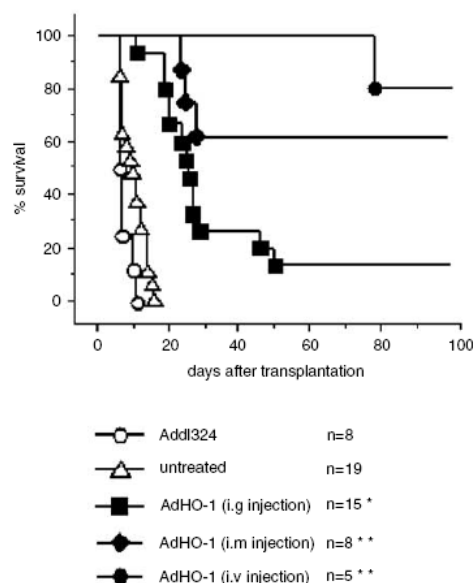


Figure 1 Prolonged survival of cardiac allografts after local or systemic treatment with AdHO-1. LEW.1W cardiac allografts transplanted into LEW.1A recipients were untreated ($n=19$) or underwent i.g. injection (10^{10} IP) with the noncoding adenovirus Add324 ($n=8$) or AdHO-1 ($n=15$). AdHO-1 was also injected i.m. into the hind limb ($n=8$) or i.v. ($n=5$). * $P<0.05$ versus Add324 or untreated groups, ** $P<0.05$ versus Add324, untreated or AdHO-1 i.g.-treated groups as determined by Kaplan-Meier log-rank statistical analysis.

administration i.g. resulted in a significant increase in graft survival ($>38.2 \pm 8.4$, $n=15$) compared to both control groups and 13.3% of the allografts showed long-term survival (Figure 1). i.m. administration of AdHO-1 significantly increased allograft survival ($>72.2 \pm 13.6$, $n=8$) compared to both control groups, and 62.5% of the cardiac grafts showed long-term survival (Figure 1). Lastly, AdHO-1 administration i.v., which results in the majority of viruses being trapped and expressed within the liver, lungs and spleen,¹³ also significantly delayed heart allograft rejection ($>95.6 \pm 4.4$, $n=5$) compared to both control groups, and 80% of the allografts displayed long-term survival (Figure 1). Allograft survival in the groups injected with AdHO-1 i.m. or i.v. was significantly prolonged compared to the group injected i.g.

These results show that adenovirus-mediated HO-1 gene transfer resulted in abrogation of acute rejection and long-term allograft survival, but that gene transfer into a distant site was more efficient than into the transplanted heart.

Detection of adenovirus-derived HO-1 expression following gene transfer

At 5 days after AdHO-1 injection i.g., the expression of AdHO-1-encoded HO-1 was analyzed by immunohistology and Western blot analysis. Immunohistology showed that AdHO-1-encoded HO-1 was strongly expressed, mainly in cardiomyocytes, as assessed using anti-HO-1 and anti-Flag antibodies (Figure 2b and d).

Control noncoding adenovirus Add324-injected cardiac allografts showed the expression of endogenous HO-1 in scattered infiltrating leukocytes and the anti-Flag antibody was negative (Figure 2a and c). Western blot analysis 5 days after gene transfer showed high expression of adenovirus-derived HO-1 in AdHO-1-transduced hearts but not in control noncoding adenovirus Add324-treated tissue. Endogenous HO-1 has a molecular weight of 32 kDa, while AdHO-1-encoded HO-1 migrates as a 33 kDa band (Figure 2e). At 10 days after transplantation and gene transfer, the expression of adenovirus-derived HO-1 was weak or not detectable in AdHO-1-transduced cardiac allografts, when analyzed by immunohistology and Western blot (data not shown). At 5 days after the i.m. or i.v. injection of AdHO-1, graft expression of AdHO-1-encoded HO-1 was absent when analyzed by immunohistology (data not shown).

Adenovirus-derived HO-1 expression was also detected in the spleen. At 2 days after transplantation and treatment of the recipients with the i.g., i.m. or i.v. injection of AdHO-1, immunohistological analysis revealed HO-1 expression mostly in the red pulp and to a lesser degree in the white pulp of spleens (Figure 3a, c, and e). Immunohistological analysis showed AdHO-1-derived HO-1 expression in the spleen of only one of the three AdHO-1 i.m.-treated recipients, but reverse-transcriptase polymerase chain reaction (RT-PCR) analysis showed HO-1 expression in the three tested recipients (Figure 3g). Adenovirus-derived-HO-1 expression was undetectable 5 days after gene transfer by both immunohistological (Figure 3b, d, and f) and RT-PCR analysis (data not shown).

Immunohistology of liver sections from animals treated with AdHO-1 i.v., i.g. or i.m. and analyzed at day 5 after gene transfer showed high, moderate and no hepatocyte expression of adenovirus-derived HO-1, respectively. Analysis at day 10 after gene transfer showed lower levels of HO-1 expression as compared to day 5 with comparable levels for i.v. and i.g. and no expression for the i.m. route (data not shown).

These observations indicate that prolongation of allograft survival after HO-1 adenovirus-mediated gene transfer was correlated with HO-1 overexpression in the spleen and not in the liver or the graft, regardless of the route of administration.

Analysis of leukocytes and levels of apoptosis in the grafts

Graft-infiltrating leukocytes and activation markers were analyzed by histology 5 days after transplantation and gene transfer. Cardiac grafts from recipients treated with AdHO-1 i.v. showed a marked decrease in infiltration by CD45+ total leukocytes, monocytes/macrophages (ED1+) and $\alpha\beta$ T (R.73+) cells, as compared to noncoding adenovirus-treated or -untreated recipients (Figure 4 a-f). Grafts from recipients treated with AdHO-1 i.g. showed similar infiltration by total leukocytes (CD45+ cells) monocytes/macrophages, $\alpha\beta$ T, CD4+, CD8+, iNOS, MHC class II molecules, CD54 and α IL-2R-positive cells (data not shown) as compared to control noncoding adenovirus-treated or -untreated grafts.

Apoptosis was analyzed by TdT-mediated dUTP nick-end labeling (TUNEL) assays 5 days after trans-

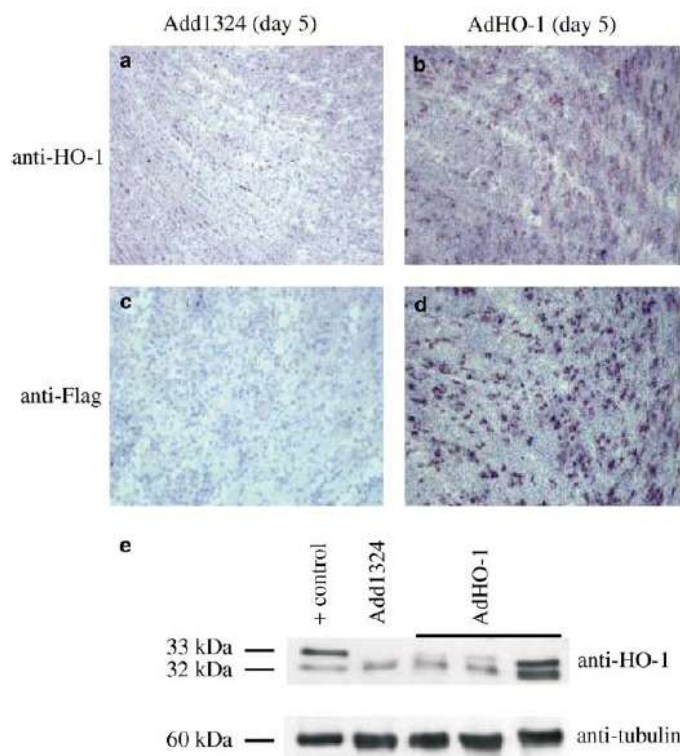


Figure 2 Expression of HO-1 in heart transplants after AdHO-1-mediated gene transfer. Rats were transplanted and received the noncoding adenovirus Add1324 or the AdHO-1 i.g. Grafts were harvested on day 5 after gene transfer and analyzed by immunohistology with anti-HO-1 (a and b) and anti-Flag antibodies (c and d) (objective $\times 10$). Results are representative of three samples for the control noncoding adenovirus Add1324-transduced heart group and of six samples for the AdHO-1-transduced group. (e) Western blot analysis of HO-1 expression in hearts transduced with the noncoding adenovirus Add1324 or with AdHO-1 and harvested 5 days after transplantation using an anti-HO-1 antibody. NR8383 cells expressing HO-1-Flag were used as a positive control. Anti- β -tubulin was used as a loading control.

plantation and gene transfer. The apoptotic cells were observed mostly among graft-infiltrating lymphocytes (GICs) and apoptotic cardiomyocytes were seldom detected as previously described in this model.¹⁴ Cardiac grafts from recipients treated with AdHO-1 i.v. showed a significant decrease in apoptotic cells as compared to noncoding adenovirus-treated grafts (mean $\pm$ s.e., 20.5 ± 3.54 cells/mm², $n=2$ versus 3.54 ± 0.74 cells/mm², $n=2$, respectively, $P=0.03$) (Figure 4g-h). Grafts from recipients treated i.g. displayed comparable numbers of apoptotic cells in noncoding adenovirus Add1324- and AdHO-1-treated heart grafts (mean $\pm$ s.e., 29.84 ± 2.26 cells/mm², $n=3$ versus 31.13 ± 1.46 cells/mm², $n=6$, respectively) (data not shown).

These results indicate that treatment of the recipients with AdHO-1 i.v. injection decreased graft infiltration and apoptosis, whereas grafts from recipients treated with AdHO-1 i.g. displayed graft infiltration and apoptosis comparable to that observed with the control noncoding adenovirus.

Analysis of cytokine mRNA levels in the grafts

The analysis of cytokine mRNA levels 5 days in the grafts after i.v. gene transfer and transplantation using

quantitative real-time RT-PCR revealed a significant decrease in TGF β 1, TNF α , IFN γ , IL-10 and iNOS transcript levels. AdHO-1-treated rats as compared to noncoding adenovirus Add1324-treated rats (Figure 5). The analysis of cytokine mRNA levels in the grafts 5 days after gene transfer i.g. and transplantation showed no significant differences for IFN γ , TNF α and IL-10 expression in AdHO-1- and noncoding adenovirus Add1324-treated grafts (data not shown). However, TGF β 1 transcript levels were slightly but significantly reduced in grafts of AdHO-1-treated animals (mean $\pm$ s.d., 1.14 ± 0.31 versus 0.65 ± 0.09 , $P=0.003$, respectively).

These observations indicate that treatment of the recipients with AdHO-1 i.v. decreased the expression of cytokines within the graft.

Lack of proliferation in MLRs using splenocytes after HO-1 gene transfer into the recipient

To further explore the immune mechanisms underlying the inhibition of acute allograft rejection following HO-1 gene transfer, we evaluated MLRs with spleen cells, T cells or lymph node cells, harvested 5 days after

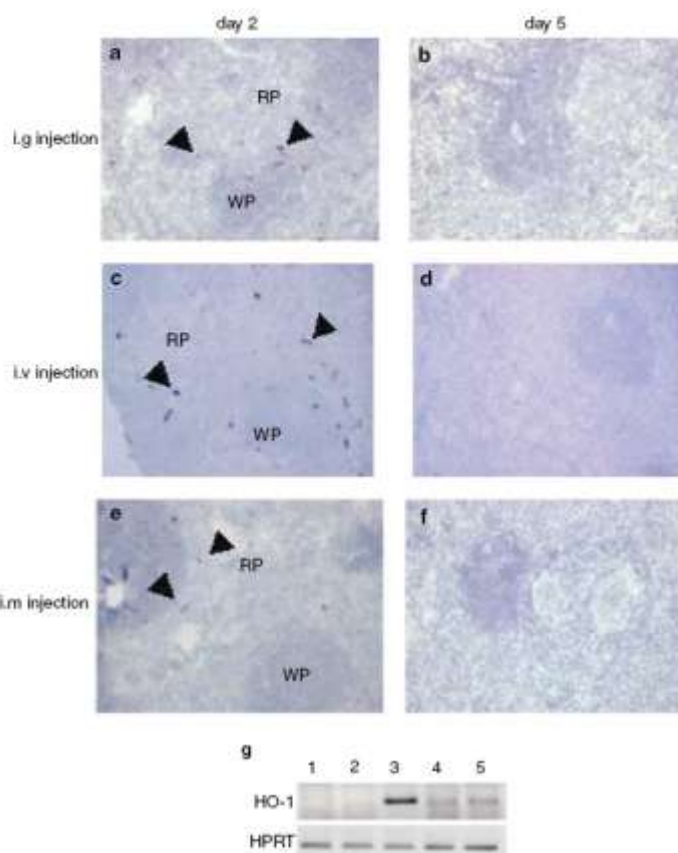


Figure 3 Expression of HO-1 in the spleen after AdHO-1-mediated gene transfer. Rats were transplanted and received AdHO-1 i.g. (a and b), i.v. into the tail vein (c and d) or i.m. into the hind limb (e and f). Spleens were harvested on days 2 (a, c and e) and 5 (b, d and f) after gene transfer and analyzed by immunohistology with anti-Flag antibodies (objective $\times 20$). WP: white pulp, RP: red pulp. (g) At 2 days after gene transfer, RT-PCR analyses of spleens from recipients that received control noncoding adenovirus Ad324 (two animals, lines 1 and 2) or AdHO-1 (three animals, lines 3–5) were performed. HPRT was used as a loading control.

transplantation and cultured with donor APCs for 3 or 5 days. Since the MLR kinetics were the same for cells from animals with AdHO-1- or noncoding adenovirus Ad324-treated grafts, results are given for the peak of cell proliferation (day 3 for T cells and lymph nodes cells and day 5 for splenocytes). A profound decrease in proliferation in response to donor APCs was observed with cell populations from recipients treated with i.v. AdHO-1 at day 5 for three of the four animals tested (mean % of inhibition $\pm$ s.e.: $93.8 \pm 6.3\%$, $n=3$) (Figure 6) and at day 3 (data not shown). Interestingly, T cells purified from splenocytes from AdHO-1 i.v.-treated recipients proliferated normally (Figure 6). In contrast to splenocytes, mesenteric lymph node cells from animals bearing grafts from recipients treated with i.v. AdHO-1 showed proliferative responses to donor APCs comparable to those of animals treated with noncoding adenoviruses (Figure 6). Proliferation of splenocytes against concanavalin A was comparable to

that of control cells (Figure 6). A similar inhibition of MLRs using total splenocytes ($80.2 \pm 5.3\%$, $n=20$) and GICs ($97.9 \pm 4.8\%$, $n=4$), but not purified T cells or mesenteric lymph nodes, was observed after the administration of AdHO-1 i.g., whereas proliferation against concanavalin A was normal in all cases (data not shown).

These results suggest that HO-1 overexpression resulted in allogeneic unresponsiveness in some (spleen) but not all (mesenteric lymph nodes) lymphoid compartments. The lack of proliferation in MLRs was due to inhibition by non-T cells, since isolated T cells proliferated normally, suggesting the existence of suppressive interactions between T and non-T cells within the spleen.

Lack of proliferation in MLRs is due to soluble factor(s)
To investigate the mechanism(s) underlying the inhibition of MLRs, we initially evaluated whether the

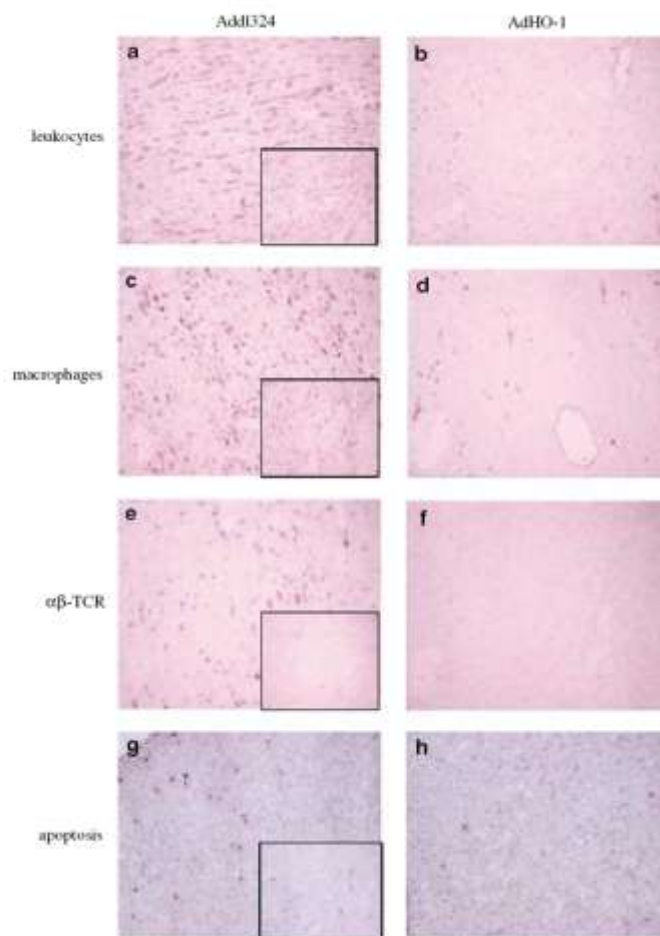


Figure 4 Analysis of heart leukocyte infiltration and apoptosis at day 5 after transplantation and gene transfer. Rats were transplanted and received control noncoding adenovirus Add324 (a, c, e, and g) or AdHO-1 (b, d, f, and h) i.v. Hearts were harvested on day 5 after gene transfer and analyzed by immunohistology with OX1/OX30 anti-CD45 mAbs (a and b), anti-macrophage ED1 mAbs (c and d) and anti- $\alpha\beta$ TCR K.7.3 mAbs (e and f). Apoptosis was evaluated by the TUNEL technique (g and h). Inserts correspond to untreated grafts (objective $\times 20$).

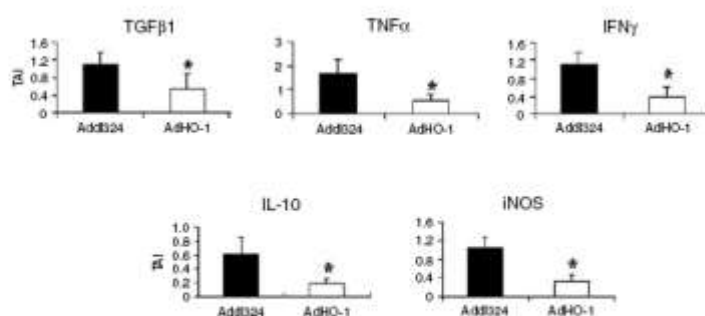


Figure 5 Analysis of cytokine mRNA accumulation at day 5 after transplantation and gene transfer. Total RNA was extracted from cardiac grafts 5 days after i.v. gene transfer, retrotranscribed and analyzed by quantitative real-time PCR. Data represent the mean $\pm$ s.e. of five rats for the noncoding adenovirus Add324-treated group and four rats for the AdHO-1-treated group. * $P < 0.05$ as compared with the noncoding adenovirus Add324-treated group.

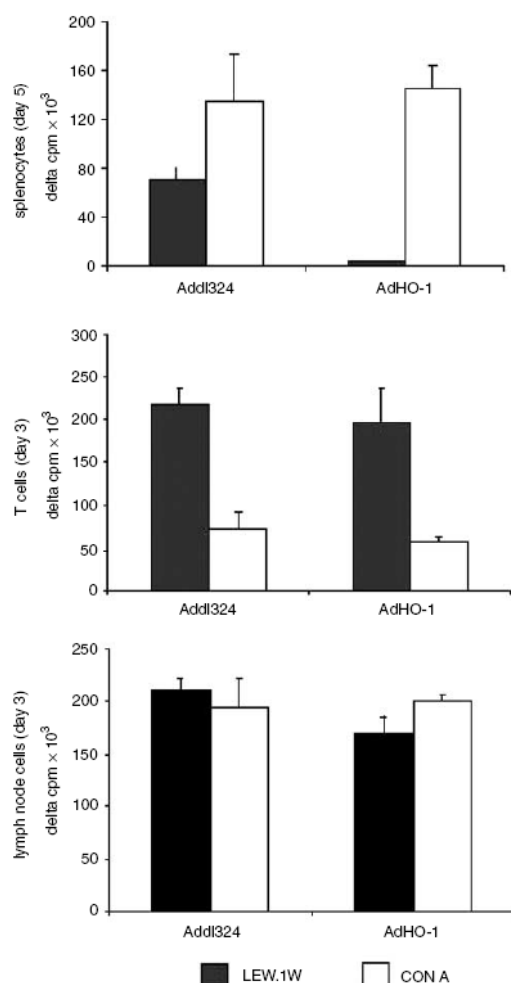


Figure 6 Inhibition of one-way MLRs using splenocytes from recipients grafted and treated with AdHO-1. Recipients were transplanted and received control noncoding adenovirus Addl324 or AdHO-1 i.v. MLRs of splenocytes, T cells purified from the same splenocytes or lymph node cells were performed 5 days after transplantation. Cellular allogeneic responses against donor-irradiated APCs were analyzed after 3 and 5 days of culture. Results correspond to the peak of proliferation of cells from a single animal for each treatment and are representative of three samples for splenocytes of AdHO-1 i.v.-treated animals in which AdHO-1 was injected into the tail vein and two to five animals for the other groups. Results are expressed as delta cpm (allogeneic - spontaneous) $\pm$ s.d. of triplicate wells.

lack of proliferation to alloantigens was dependent on the production of soluble products or required direct cell contact. Proliferation of naïve LEW.1A splenocytes in response to irradiated LEW.1W APCs was assessed in the lower compartments of transwell plates in the presence, in the upper compartments, of splenocytes from recipients treated with i.v. AdHO-1 or noncoding adenoviruses, and cultured with irra-

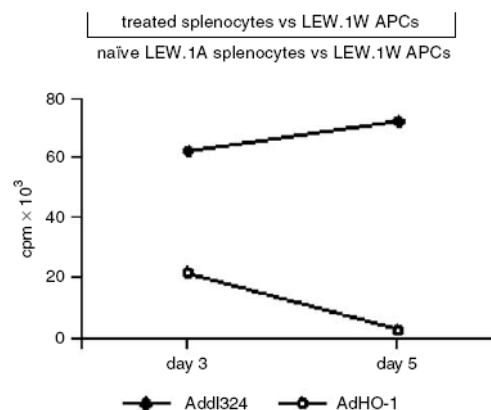


Figure 7 Inhibition of MLRs using transwell cultures. Splenocytes from naïve LEW.1A animals were cultured in the lower compartment of transwell plates with irradiated LEW.1W APCs and proliferation was evaluated at days 3 and 5 of culture. The upper compartment contained splenocytes from LEW.1A rats transplanted with LEW.1W hearts and injected i.v. with AdHO-1 or control noncoding adenovirus Addl324, harvested on day 5 after transplantation and cultured with irradiated LEW.1W APCs. Results correspond to cell proliferation in the lower compartment and are representative of two identical experiments with a total of five noncoding adenovirus Addl324- and three AdHO-1-treated animals. Each curve represents cpm proliferation of cells.

diated LEW.1W APCs. These experiments showed that proliferation of naïve splenocytes was strongly reduced in the presence of splenocytes from AdHO-1-treated rats for three of the four tested animals (mean $\pm$ s.d., $n=3$) after 3 ($78 \pm 10.8\%$) or 5 ($93 \pm 1.3\%$) days of culture as compared to that observed in the presence of splenocytes from control Addl324-treated animals (Figure 7). A similar inhibition of naïve MLRs after 3 ($53 \pm 8.7\%$, $n=3$) or 5 ($77.3 \pm 6\%$, $n=3$) days of culture was observed in the presence of splenocytes from recipients treated with AdHO-1 i.g. (data not shown).

These results indicate that the unresponsiveness of splenocytes from allograft recipients overexpressing HO-1 involves the presence of soluble factor(s).

Normal anticognate immune responses after AdHO-1 treatment

To evaluate whether the expression of HO-1 resulted in the inhibition of immune responses against environmental antigens, rats injected i.v. with AdHO-1 were immunized with Keyhole limpet hemocyanin (KLH) and humoral anti-KLH immune responses were evaluated. Compared to recipients treated with control noncoding adenoviruses, recipients treated with AdHO-1 i.v. showed comparable levels of anti-KLH Abs at all dilutions tested (Figure 8). Similarly, recipients treated with AdHO-1 i.g. at the moment of KLH immunization also showed levels of anti-KLH Abs comparable to those of noncoding adenovirus Addl324-treated controls (data not shown). These results suggest that HO-1 overexpression did not result in a general inhibition of immune responses.

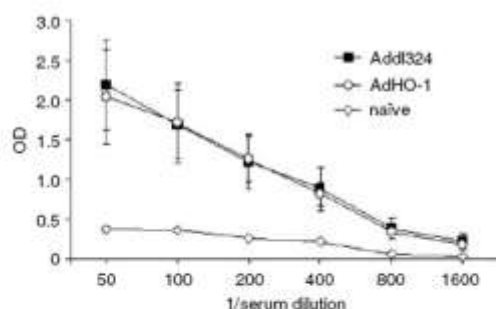


Figure 8 Conserved anti-KLH immune responses after HO-1 gene transfer. Rats treated i.v. with AdHO-1 ($n=3$) or Add324 noncoding adenovirus ($n=3$) were immunized with KLH on the day of gene transfer. A nonimmunized rat (naïve) was used as a negative control. Levels of anti-KLH Abs were quantified in serially diluted serum by ELISA at day 11 after gene transfer.

Discussion

Our results demonstrate that specific HO-1 overexpression following gene transfer results in long-term cardiac allograft survival. Furthermore, our studies demonstrate an inhibition of cellular allogeneic immune responses following HO-1 overexpression.

Similar to our findings, increased HO-1 expression following the administration of immunoregulatory peptides or metalloporphyrins has been shown to prolong allograft survival moderately.⁵⁻⁷ While these results suggest a potential role for HO-1 in prolonging allograft survival, immunomodulatory peptides and metalloporphyrins exert other functions.⁷⁻⁹ More recently, gene transfer of HO-1 resulted in a decrease in graft lesions in aortic and heart chronic rejection and liver transplantation models.¹⁰⁻¹²

Our results extend and confirm a role for HO-1 in prolonging allograft survival in a strong cardiac rejection model. Prolongation of allograft survival following HO-1 gene transfer was not only observed after i.g. gene transfer but also after i.v. as well as i.m. administration. A common mechanism for these three gene transfer protocols could be the transduction of donor and/or recipient APCs by AdHO-1, either in the graft, in the spleen or in peripheral tissues (such as the skeletal muscle of the hind limb), followed by migration into the spleen and inhibition of allogeneic immune responses. *In vivo* transduction of dendritic cells and macrophages by recombinant adenovirus has been previously described after i.v.^{13,15} or i.m. injection.^{16,17} This hypothesis is strengthened by the detection of transduced cells in the spleen after the i.g., i.m. and i.v. injection of AdHO-1. HO-1 expression in the spleen after i.g. gene transfer could be due to the well-known migration of donor DCs from the heart into the spleen and not mesenteric lymph nodes^{18,19} after AdHO-1 transduction or due to the migration of adenoviral particles from the heart to the spleen where they could transduce recipient DCs or macrophages.¹³ Finally, the inhibition of MLRs by non-T cells suggests that spleen APCs could mediate this inhibition.

Despite the fact that elevated HO-1 expression reduces graft lesions in allogeneic¹⁰⁻¹² and xenogeneic² models, the mechanisms involved are not completely understood. HO-1 expression could prolong allograft survival by its cellular protective functions,^{1,2} such as anti-apoptotic^{11,20} or anti-inflammatory² activities, which result in reduced graft inflammation and leukocyte recruitment in certain models.^{10,12} Alternatively, HO-1 activity could inhibit the initiation, amplification or effector phases of allogeneic immune responses. The fact that i.g., i.m. and i.v. AdHO-1 gene transfer resulted in prolongation of allograft survival in the presence of adenovirus-derived HO-1 expression in the spleen for the three gene transfer routes, whereas expression in the cardiac graft was only observed after i.g. administration, argues in favor of a modification of allogeneic immune response in the spleen. The modification of effector immune responses, decreased leukocyte infiltration and cytokine mRNA in the graft and the decreased frequency of apoptotic graft-infiltrating cells may only be secondary to the effect of priming of allogeneic immune responses in the spleen. Prolongation of graft survival after i.g. gene transfer was less pronounced compared to i.m. or i.v. administration and this could be due to local inflammation and immune responses against the adenoviruses. Our results are in agreement with those of Araujo *et al*'s²¹ very recently published paper describing longer allograft survival in transgenic mice expressing HO-1 systemically rather than in the graft.

Lymphocytes infiltrating HO-1-overexpressing grafts displayed a greatly reduced proliferative response to alloantigens, indicating a reduced capacity to amplify an allogeneic immune response locally. The possibility that HO-1 in this model is acting more prominently in reducing the immune response, rather than protecting graft cells, is strongly supported by the inhibition of the proliferative responses of spleen cells and GICs against alloantigens. Cells expressing adenoviral-derived HO-1 were detected in the spleen of treated animals on day 2 after transplantation but were no longer present on day 5, the time point at which MLRs were performed. This suggests that the inhibition of MLRs was not due to the expression of adenovirus-derived HO-1 during the MLR but rather before, in the spleen. This is supported by the absence of Ad-derived HO-1 in the same MLRs (data not shown). The inhibition of MLRs using spleen cells or GICs, but not mesenteric lymph node cells, could be dependent on the presence of donor APCs in the graft and in the spleen, but not in lymph nodes.^{18,19} In keeping with this anatomical distribution, AdHO-1 probably concentrated in the spleen and not in mesenteric lymph nodes.^{13,15} Alternatively, activated tolerogenic T cells may circulate through the spleen but may not be found in lymph nodes, since activated T cells lose their expression of CD62L and gain the expression of VLA4,²² resulting in a diminished capacity to home to lymph nodes and an increased capacity to home to the graft.

The inhibition of the MLRs of AdHO-1-treated grafts involved the secretion of unidentified soluble factor(s). The inhibition of MLRs by secreted products has been described for Tr1 cells and Th3 cells (producers of IL10 and TGF β , respectively), and by cell-contact-dependent mechanisms for CD4+CD25+ regulatory T cells.²³

Suppressive APCs acting through the production of extracellular suppressive products have also been described.²⁴⁻²⁶ Further studies, which are beyond the scope of this paper, are necessary to define the cellular and molecular mechanisms implicated in MLR inhibition and the presence of different populations of suppressive cells.

After i.v. HO-1 gene transfer, cellular and humoral immune responses to cognate antigens introduced into peripheral tissues were normal. Such immune responses were initiated in the lymph nodes. These results, in combination with the observation of normal MLR responses using lymph node cells, may be explained by three nonmutually exclusive alternatives: (a) i.v. gene transfer of HO-1 may exert less inhibitory effects in lymph nodes as a result of the cell migratory mechanisms described above; (b) the direct stimulation of DCs by the adjuvant used in immunization may bypass the inhibitory actions of HO-1 acting at upstream steps of DC activation; or (c) HO-1 gene transfer and Ag presentation need to occur in the same lymphoid compartment for inhibition to occur.

The mechanism by which HO-1 prolonged allograft survival likely relies on the generation of end products of heme degradation acting on both immune and non-immune components and still need to be analyzed in this model.^{1,2} For example, the induction of ferritin by free iron has been shown to inhibit leukocyte migration,²⁷ proliferation of T cells²⁸ and to promote the development of DCs capable of inhibiting MLRs.²⁹ Bilirubin and biliverdin have been demonstrated to inhibit complement activation³⁰ and bilirubin has been described as a potent antioxidant,³¹ as well as been shown to inhibit lymphocyte proliferation, IL-2 production and cytotoxicity.^{32,33} Recently, CO has been shown to have anti-inflammatory and protective roles in xenograft³⁴ and allograft rejection.^{10,11} Delivery of CO through CO inhalation or methylene chloride administration markedly prolonged cardiac allograft survival in the cardiac transplantation model described in this work (manuscript in preparation).

Our data are consistent with a novel activity of HO-1: the inhibition of cellular allogeneic responses *in vivo* through the modulation of APC-T cell interactions in the spleen, resulting in the inhibition of allogeneic immune responses.

Materials and methods

Animals and heart transplantation

The rats used in this study were 250 g inbred male Lewis.1W (LEW.1W, haplotype RT1ⁿ), LEW.1A (haplotype RT1^a) and Brown Norway (BN, haplotype RT1ⁿ) (Centre d'Élevage René Janvier, Le Genest St Isle, France). LEW.1W and LEW.1A are congenic strains mismatched for the entire MHC region. Heterotopic heart grafts were performed using the Ono and Lindsey technique.³⁵ Graft function was evaluated by daily palpation through the abdominal wall. Rejection was defined as total cessation of cardiac beating and was confirmed by direct examination following laparotomy. The results of graft survival are expressed as mean survival times (MST ± s.e. days) and by arbitrarily defining 100 days survival as long-term graft survival.

Recombinant adenovirus coding for HO-1 and gene transfer into the heart

An adenovirus coding for HO-1 (AdHO-1) was constructed using the pAdEasy and pAdTrack-CMV system³⁶ in 293 cells.³⁷ The human HO-1 cDNA (kindly provided by Pr S Shibahara, Tohoku University School of Medicine, Sendai, Japan) fused to a Flag sequence at its 3'-end was placed under the control of the human CMV promoter. The noncoding adenoviral vector Add1324 has been described previously.³⁸ Recombinant adenoviruses were purified and titered (infectious particles, IP) as described previously.³⁷ Adenovirus stocks were tested for the absence of replication-competent adenoviruses by PCR amplification of the E1 adenoviral region (the detection limit was one adenoviral particle in 10⁹ IP of recombinant adenoviruses).

For gene transfer into the grafted heart, 10¹⁰ IP of recombinant adenoviruses in a total volume of 100 µl were slowly injected into the apex and ventricular walls of the heart at four different points as previously described in detail.³⁷ Gene transfer into the hind limb muscle was performed by the injection of 10¹⁰ IP of recombinant adenovirus in a total volume of 100 µl at two injection points into the tibialis. Gene transfer in recipient animals was also performed by the injection of 10¹⁰ IP of recombinant adenovirus in a total volume of 200 µl into the tail vein.

Western blot analysis

Heart samples were snap-frozen in liquid nitrogen and stored at -80°C until use. Heart samples were homogenized in buffer containing 0.1% SDS, 1% NP40, 0.5% deoxycholic acid, 240 µg/ml AEBBSF (Sigma) and 0.71 TIU/ml aprotinin. Samples containing 20 µg of proteins were boiled and loaded onto 12% SDS-polyacrylamide gels followed by electrophoresis and blotting onto nitrocellulose membranes. Membranes were then blocked (overnight at 4°C) with PBS containing 0.1% Tween-20 and 5% nonfat dry milk, incubated (2 h at room temperature) with a rabbit anti-HO-1 that reacts with HO-1 of both human and rat origin (Stressgen, Victoria, British Columbia, Canada), a mouse MAb anti-Flag (clone M2) (Sigma) or a mouse MAb anti-β-tubulin (Calbiochem, San Diego, CA, USA). They were then washed and incubated (2 h at room temperature) with an HRP-labeled anti-rabbit or anti-mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA, USA) and detected by enhanced chemoluminescence (Amersham, Arlington Heights, IL, USA) using X-ray films.

Immunohistological analysis

Immunohistology was performed on cryostat sections as previously described in detail.¹⁰ Immunohistological analysis of infiltrating leukocytes was performed on day 5 post-transplantation using the following mouse MAbs: a mixture of two anti-leukocyte CD45 MAbs (OX1 and OX30), anti-monocyte/macrophage (ED1), anti-αβ TCR (R.7.3), anti-CD4 (W3/25), anti-CD8 α chain (OX8), anti-monomorphic class II MHC antigens (OX6), anti-CD25 (OX39) (all from ECACC, Wiltshire, UK), anti-CD54 (ICAM-1) (Seikagaku America Inc., Rockville, MA, USA), anti-CD86 (B7.2) (Pharmingen, Franklin Lakes, NJ, USA) and an irrelevant mouse MAb

(3G8, anti-human CD16) (ATCC, Bethesda, MD, USA). Slides were then incubated with a biotin-conjugated anti-mouse immunoglobulin antibody (Vector Laboratories, Burlingame, CA, USA), followed by HRP-conjugated streptavidin (Vector Laboratories) and VIP substrate. Rabbit antibodies were used to detect iNOS (Transduction Laboratories, Lexington, KY, USA) and HO-1 (Stressgen). Biotin-conjugated anti-rabbit antibodies were from Jackson ImmunoResearch. Binding of these antibodies was detected by incubation with HRP-conjugated streptavidin and VIP substrate. Tissue sections were counterstained with hematoxylin and lithium carbonate.

The expression of HO-1 after adenovirus-mediated gene transfer was confirmed on cryostat sections of cardiac allografts, liver and spleen. Cryostat sections were fixed with 2% paraformaldehyde (20 min at room temperature), permeabilized with 0.1% Triton X-100 and incubated (18 h at 4°C) with 200 µl of biotin-conjugated anti-Flag or rabbit anti-HO-1 antibodies (10 µg/ml diluted in PBS with 1% BSA, 1% rat serum and 0.1% Triton X-100). Binding of these antibodies was detected as described previously. All immunohistology experiments included the 3G8 irrelevant MAb or non-immunized control sera from rabbit origin as negative controls.

Detection of apoptosis in the grafts

Apoptosis was detected on cryostat sections using the TUNEL technique with the ApopTag kit (Oncor, Gaithersburg, MD, USA) according to the manufacturer's instructions. The number of apoptotic cells was quantified using the image analysis software Mercator v1.78 h (ExploraNova, La Rochelle, France).

Quantitative RT-PCR

At 5 days after transplantation, heart samples were snap-frozen in liquid nitrogen and stored at -80°C until use. Total RNA was isolated using the TRIzol (Invitrogen, Cergy Pontoise, France) method and 10 µg of mRNA was reverse transcribed using the M-MLV reverse-transcriptase kit (Invitrogen). Transcript levels for cytokines and hypoxanthine phosphoribosyltransferase (HPRT) were quantified using real-time quantitative PCR and the SYBR green DNA dye (ABI Prism 7700, Perkin-Elmer Applied Biosystems, Foster City, CA, USA).³⁷ Specificity of PCRs was confirmed by electrophoresis analysis of the samples on 1.2% agarose gels. The rat primer sequences target for IFN γ , TGF β 1, IL-10, TNF α and HPRT were those described previously,³⁷ and for iNOS were as follows: 5'-GACCAAACTGTGTGCTGGA-3' (sense) and 5'-TACTCTGAGGGTCGACACAAGG-3' (anti-sense). HPRT was used as an endogenous control to normalize RNA amounts. The transcript accumulation index (TAI) was expressed as the fold change in mRNA between a given sample (transplanted heart from AdHO-1-treated rats) (Q) and a calibrator (control noncoding adenovirus-treated transplanted heart) (CB), where the calibrator represents the one-fold expression of each gene. The TAI was calculated as: $TAI = 2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = (C_{tTarget} - C_{tHPRT})_Q - (C_{tTarget} - C_{tHPRT})_{CB}$. A prerequisite for the use of the $2^{-\Delta\Delta C_t}$ method was that the efficiency of each gene-specific PCR was greater than 96% as determined by the slope of the curve $C_t = f(\log[-\log(\text{target DNA})])$.³⁸

Immunizations

KLH (50 µg) (Sigma), emulsified in 200 µl of complete Freund's adjuvant, was injected into the footpad on the day of transplantation.

Detection of anti-KLH antibodies

Anti-KLH antibodies in the sera were analyzed by ELISA as described previously,³⁷ 11 days after immunization. The results were expressed as the mean OD $\pm$ s.d.

Proliferative responses against alloantigens

Spleen and mesenteric lymph node cells were isolated using density gradient centrifugation on Ficoll-Hypaque. T cells were purified from total splenocytes by negative selection using a T-cell purification kit (R&D Systems, Abingdon, UK). GICs were isolated by incubating finely minced heart allografts in 4 ml of collagenase D (2 mg/ml; Boehringer-Mannheim, Indianapolis, IN, USA) for 30 min at 37°C, followed by passage through a stainless-steel mesh and density gradient centrifugation on Ficoll-Hypaque. Cells were resuspended in a culture medium consisting of RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, 1% nonessential aminoacids and 5×10^{-5} M β -2-mercaptoethanol (all from Sigma). Dendritic cells were enriched from LEW.1W spleen fragments that had been digested with collagenase D (2 mg/ml) for 30 min at 37°C and in the presence of 10 µM EDTA for the last 5 min. The cell suspension was washed twice and resuspended in 5 µM EDTA-PBS containing 2% heat-inactivated FCS at 4°C, at $1-2 \times 10^6$ cells/ml. Of this suspension, 4 ml was layered onto 4 ml of 14.5% (wt/vol) Nicodenz (Axis-Shield PoC AS, Oslo, Norway) and centrifuged for 13 min at 2800 g at 4°C. Low-density cells were recovered, resuspended at 10^7 cells/ml and cultured overnight. Nonadherent cells were gently harvested, γ -irradiated (35 Gy) and used as APCs in MLRs. For MLRs in 96-well plates, total splenocytes, purified T cells or GICs were seeded (10^5 cells/well) in triplicate cultures and evaluated for their proliferative response against irradiated APCs (5×10^4 cells/well) or concanavalin A (12.5 mg/kg). For transwell MLRs, 24-well plates with lower and upper chambers separated by a 0.4 µm pore size membrane (Becton Dickinson, Le Pont de Claix, France) were used. The lower compartment contained naïve splenocytes from LEW.1A rats (6×10^5 cells) and irradiated LEW.1W APCs (3×10^5 cells). The upper compartment contained splenocytes from LEW.1A cardiac allograft recipients treated with control adenovirus or AdHO-1 (6×10^5 cells) and irradiated LEW.1W APCs (3×10^5 cells). Cells were cultured for 3 and 5 days, and for the final 8 h of culture, 1 µCi [3 H]thymidine deoxyribose was added to each well. Thymidine incorporation was quantified using a scintillation counter.

Statistical analysis

Statistical significance was evaluated using a one-way analysis of variance test for group comparisons and a Kaplan-Meier analysis for graft survival; *P*-values ≤ 0.05 were considered significant.

Acknowledgements

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Annexe 7

Generation of heme oxygenase-1 transgenic rats.

Par: **Braudeau C**, Bouchet D, Toquet C, Tesson L, Menoret S, Iyer S, Laboisie C, Willis D, Jarry A, Buelow R, Anegon I and Chauveau C.

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Generation of Heme Oxygenase-1-Transgenic Rats

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Heme oxygenase-1 (HO-1) expression protects cells from a variety of cellular insults and inhibits inflammation. However, its role in the regulation of immune responses has not yet been clearly established. We generated HO-1 transgenic rats to directly test the impact of HO-1 on the different immune mechanisms. To temporally control the expression of HO-1, we used a one-plasmid tetracycline (tet)-inducible system. This plasmid contains the H-2K^b promoter, which transcribes the tet transactivator (tTA) and expression of a human HO-1 cDNA is obtained in the absence of tetracycline. The DNA construct was microinjected into one-cell rat embryos and mothers and pups were maintained with tetracycline. Eight transgenic founders were obtained. Analysis of transgene expression in the absence of tet showed that 2 lines (12.4 and 12.6) expressed HO-1 mRNA in several organs (as detected by reverse transcription polymerase chain reaction) and at the protein level only in the thymus. Expression levels of transgene-derived HO-1 increased after withdrawal of tet compared with transgenic rats maintained with tet, as detected by analysis of mRNA levels by quantitative real-time reverse transcription polymerase chain reaction. Gross examination and histopathological analysis of several organs in both lines showed no anomalies. Thymocytes and splenocytes of both lines showed normal cell subpopulations and allogeneic proliferation compared with controls. Systemic immune responses against cognate antigens were normal in both lines, as evaluated by the proliferation of lymph node cells and the production of antibodies against keyhole limpet hemocyanin after immunization. Animals from line 12.6 rejected transplanted allogeneic hearts with the same kinetics as controls. In conclusion, short-term induction of HO-1 overexpression did not modify immune responses compared to

those of control non-transgenic animals. *Exp Biol Med* 228:466–471, 2003

Key words: HO-1; transgenic rats; immune response; transplantation

Heme oxygenase-1 (HO-1) is the rate-limiting enzyme in the oxidative degradation of heme into biliverdin, free iron, and carbon monoxide. This 32-kDa stress-inducible protein provides protection against a variety of cellular injuries, such as oxidative stress, proinflammatory cytokines, and proapoptotic inducers (1–3). HO-1 has been shown to have anti-inflammatory actions (2), to suppress macrophage activation (4), to mediate the anti-inflammatory effects of interleukin-10 (5), and to inhibit allograft and xenograft rejection (2, 6, 7). However, whether HO-1 modulates antigen-specific immune responses or other lymphocyte functions has not yet been established. Previous publications describing transgenic mice for HO-1 have used promoters that are specific for neurons (8), vascular smooth muscle cells (9), lung cells (10), or cardiomyocytes (11, 12).

The aim of this study was to generate HO-1-transgenic rats to directly analyze the impact of HO-1 on various immune functions. We used the ubiquitous H-2K^b promoter to target HO-1 expression primarily to endothelial cells and leukocytes (13) and the tet-off inducible system (expression in the absence of tet) to control transgene expression. We obtained two lines of transgenic rats that, after 10 days of tet withdrawal, overexpressed human HO-1 mRNA in all organs. Transgene-derived HO-1 was detectable at the protein level only in the thymus. Thymocytes from transgenic rats displayed cell subsets and allogeneic proliferative responses *in vitro* comparable to those of nontransgenic animals. HO-1 transgenic rats used as recipients rejected cardiac allografts with the same kinetics as control rats. After immunization, transgenic rats showed normal antibody and proliferative responses against cognate antigens.

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Materials and Methods

DNA Construct. The backbone of the construct (pCombi) used to generate transgenic rats with tet controllable expression was kindly provided by Dr. U. Certa (Hoffmann-La Roche, Basel, Switzerland) and has been used previously to generate transgenic mice (14). In the construct used to generate HO-1 transgenic rats (pCombiHO-1; Fig. 1), the mouse ubiquitous H-2K^b promoter linked to an enhancer from the murine T cell receptor α chain gene (13) and a hybrid intron were inserted 5' of the coding sequence for the tTA and SV40 polyadenylation (polyA) sequences of pCombi. In the absence of tet, the tTA binds to the tet operator (tetOP) and, in conjunction with a CMV minimal promoter (pCMVmin), activates transcription of an expression cassette containing (from the 5' end to the 3' end) a β globin intron, the human HO-1 cDNA (kindly provided by S. Shibahara, Tohoku University School of Medicine, Sendai, Japan) in which a FLAG sequence was inserted in its 3' end, and SV40 polyA sequences.

Generation of HO-1 Transgenic Rats. Transgenic rats were generated by pronuclear microinjection of Sprague-Dawley eggs (IFFA-CREDO, L'Arbresle, France) with the *SalI-BamHI* fragment of pCombiHO-1 (9 kb) using procedures previously described (15, 16). To inhibit transgene expression, tet (1 mg/ml) and sucrose (36 mg/ml) were included in the drinking water (protected from light) of surrogate mothers and litters derived from microinjection. Analysis of transgene expression was performed at least 10 days after tet withdrawal. Transgenic rats were first identified by analysis of tail DNA by polymerase chain reaction (PCR) using human HO-1 primers: sense; 5'-GTC TTC GCC CCT GTC TAC TT-3' and antisense; 5'-CTC TTC TAT CAC CCT CTG CCT-3'. PCR was also performed in parallel for the rat reference gene hypoxanthine phosphoribosyltransferase using the following primers: sense; 5'-GCG AAA GTG GAA AAG CCA AGT-3'; antisense; 5'-GCC ACA TCA ACA GGA CTC TTG TAG-3'. Both PCR reactions consisted of 5 min at 94°C, 5 min at 62°C and 30 cycles of 72°C for 30 sec, 60°C for 30 sec and 94°C for 30 sec. Positive animals were then confirmed by Southern blot analysis of DNA digested with *EcoRV* using a ³²P-labeled dCTP fragment of pCombiHO-1. Determination of zygosity

in HO-1 transgenic animals was performed using a new real-time PCR-based method (17) using an ABI Prism 7700 Sequence Detector System (PE Applied Biosystems, Foster City, CA).

RNA Extraction and Real-Time Quantitative PCR. Total RNA was isolated using Trizol (Life Technologies, Paris, France), treated with DNase (Roche, Indianapolis, IN) and reversed transcribed (Life Technologies, Paris, France) into cDNA. Real-time quantitative PCR was performed using primers for human HO-1: forward; CTC AAC ATC CAG CTC TTT GAG GAG TTG CAG G-3' and reverse; 5'-TGG GAG CGG GTG TTG AGT-3' and a labeled TaqMan[®] probe 5'-FAM-CTC AAC ATC CAG CTC TTT GAG GAG TTG CAG G-TAMRA3'. Rat hypoxanthine phosphoribosyltransferase was amplified using the same primers described above and a labeled TaqMan[®] probe 5'-VIC-CAA AGC CTA AAA GAC AGC GGC AAG TTG AAT-TAMRA3'. Transcript levels were calculated according to the $2^{-\Delta\Delta C_t}$ method (18).

Analysis of HO-1 Expression. Immunohistological analysis was performed on cryostat sections using a rabbit anti-HO-1 antibody (reacting with both human and rat HO-1) (Stressgen, Victoria, British Columbia, Canada) using techniques previously described (7). HO-1 enzymatic activity was analyzed in the microsomal fraction of the thymus as previously described (19).

Keyhole Limpet Hemocyanin (KLH) Immunization. KLH (Sigma, St Louis, MO) was injected in the footpad as previously described (20). Anti-KLH antibodies were detected in sera using an enzyme-linked immunosorbent assay and proliferative responses against KLH were analyzed using popliteal lymph node cells 10 days after immunization as previously described (20).

Mixed Leukocyte Reaction (MLR). Splenic or thymic cells from non-transgenic or HO-1 transgenic rats (MHC haplotype RT1^b) were cultured with γ -irradiated allogeneic antigen presenting cells (APCs) from LEW.1A rats (MHC haplotype RT1^a) with or without recombinant IL-2 (100 U/ml) as previously described (20).

Cytofluorimetry. Cells were incubated with mAbs for 20 min at 4°C, washed twice, and analyzed using a

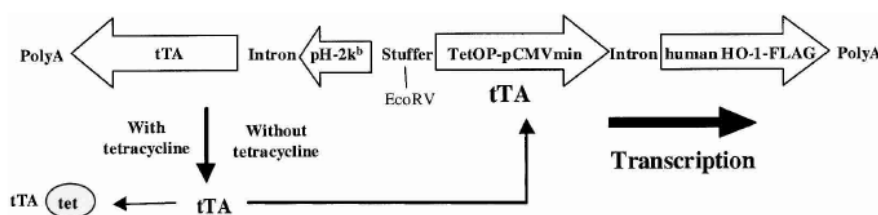


Figure 1. pCombiHO-1 DNA construct used for the generation of HO-1 transgenic rats. The ubiquitous H-2K^b promoter drives the expression of the tet transactivator (tTA). In the absence of tetracycline (tet), tTA binds to the tet operator (tetOP) and with a CMV minimal promoter (pCMVmin), activates transcription of an expression cassette containing a β globin intron, the human HO-1 cDNA with a FLAG sequence in its 3' end and SV40 polyA sequences.

FACScalibur (Becton Dickinson, Mountain View, CA). The following mouse anti-rat mAbs obtained from the European Collection of Cell Culture (Salisbury, UK) were used after coupling to FITC, biotin, or PE (Bioatlantic, Nantes, France): OX6 (MHC class II, B cells, macrophages and activated T cells), W3/25 (CD4; mainly CD4+ T cells), OX7 (Thy-1.1; CD90, thymocytes), OX33 (CD45RB; B cells), OX42 (CD11b and CD11c; macrophages and dendritic cells), OX8 (CD8; CD8 α + T cells), OX39 (CD25; activated T cells), OX22 (CD45RC), and OX85 (CD62L; naive T cells). The JJ319 MAb (CD28; T cells) was kindly provided by Dr. T. Hüning (University of Würzburg, Germany). The FITC-conjugated anti-CD3 (clone G4.18; T cells), anti-B7-2 (CD86; macrophages and dendritic cells), and PE-conjugated anti-B7.1 (CD80; macrophages and dendritic cells) mAbs were purchased from PharMingen (San Diego, CA). The FITC anti-CD161a (NKR-P1A, NK cells) was purchased from Serotec (Oxford, UK).

Heart Transplantation. Cardiac allografts from LEW.1A donors were placed into the abdomen of HO-1-transgenic rats and graft survival was monitored daily by palpation through the abdominal wall. Rejection was defined as cessation of cardiac beating.

Histopathological Analyses. Hematoxylin and eosin-stained tissue sections of paraffin-embedded samples of the indicated organs were analyzed by an experienced pathologist (C.T.).

Results

Generation of HO-1 Transgenic Rats with the Tet-Off System. After transient transfection of COS cells

with pCombiHO-1, expression of HO-1 was undetectable in the presence of tet (2 μ g/ml) and was induced in its absence, as analyzed by Western blot and enzymatic assays in cellular lysates (data not shown).

Immediately after microinjection of the 9-kb fragment of pCombiHO-1, 702 one-cell embryos were transferred into foster mothers and 111 pups were obtained. Eight founders (7.2% of newborns) were identified by PCR analysis and confirmed by Southern blot analysis as carriers of the entire HO-1 transgene integrated in a single-site (data not shown). The frequency of transgenic rats obtained with pCombiHO-1 was comparable to that obtained previously for other transgenes (16, 21, 22). Four founders transmitted the transgene to their descendants and transgenic lines were derived from each of them.

Analysis of Transgene-Derived HO-1 mRNA. Expression of transgene-derived HO-1 mRNA was analyzed using real-time reverse transcription PCR in organs from rats continuously kept with tet or withdrawn from tet for 10 days. Two lines among 4, 12.4 (Fig. 2A) and 12.6 (Fig. 2B), expressed transgene-derived HO-1 mRNA, mainly in lymphoid organs and the highest levels being observed in the thymus, as previously described in transgenic mice generated with the H-2K^b promoter (13). HO-1 mRNA increased in both lines in the absence of tet. Line 12.4 expressed higher levels in the absence and lower levels in the presence of tet than line 12.6. Low levels of HO-1 mRNA were observed in the heart and kidney in line 12.4 but not in line 12.6. The liver did not show HO-1 mRNA accumulation in neither line.

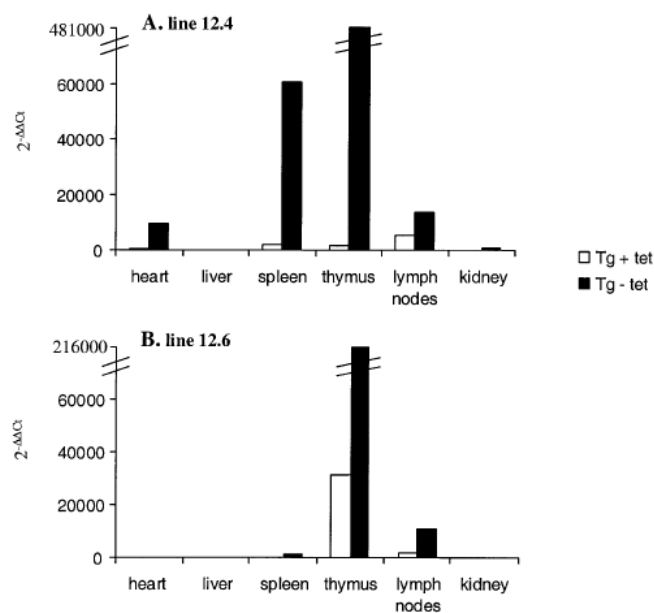


Figure 2. Quantification of hHO-1 mRNA. Real-time quantitative RT-PCR was used to analyze hHO-1 in the indicated organs from (A) line 12.4 and (B) line 12.6 kept with or without tetracycline (tet) for 10 days. Results from one animal in each condition are representative of two to three animals analyzed in each line.

Analysis of Transgene-Derived HO-1 Protein Expression. Homozygous HO-1 transgenic rats from lines 12.4 and 12.6 were continuously kept with tet or withdrawn from tet for 10 days and their organs were analyzed for HO-1 protein expression. As observed by immunohistological analysis (Fig. 3), low levels of endogenous HO-1 were detected in the thymus of control rats and lines 12.4 and 12.6 expressed higher levels. Cells expressing HO-1 in transgenic rats were scattered in the cortex and medulla. Higher expression of HO-1 was confirmed by Western blot analysis in the thymuses of HO-1 transgenic rats versus controls (data not shown).

Analysis of HO-1 enzymatic activity showed increased levels in the thymus in lines 12.6 and 12.4 versus controls (2 and 1.49, respectively vs 0.3 nmol/mg/h). Transgene-derived HO-1 was undetectable in the lymph nodes, spleens, livers, kidneys, and hearts of these animals, as analyzed by immunohistology and Western blot (data not shown).

Thymic Cell Subsets and Immune Responses Were Unmodified in HO-1-Transgenic Rats. Histopathological analysis of the thymus, lymph nodes, spleen, liver, kidney, intestine, lung, and heart in lines 12.4 and 12.6 after tet withdrawal revealed no anomalies (data not shown). Analysis by cytofluorimetry of the major leukocyte subsets (CD4+, CD8+, B cells, macrophages, CD90+ thymocytes, and NK cells) of lines 12.6 (Fig. 4A) and 12.4 (data not shown) revealed no differences compared with controls of the same age. Other markers, such as CD25, CD45RC, CD62L, CD86, and CD80, did not reveal any differences in the thymic cells of transgenic versus nontransgenic rats. The same analysis performed on spleen cells from transgenic lines 12.4 and 12.6 did not show any phenotypic differences compared with controls (data not shown).

Analysis of allogeneic proliferation of thymic cells from transgenic rats (haplotype RT1^a) against APCs from LEW.1A (haplotype RT1^u) rats showed strong proliferation for lines 12.6 (Fig. 4B) and 12.4 (data not shown), which were comparable to those of control nontransgenic rats. The same MLRs performed with spleen cells from transgenic lines 12.4 and 12.6 did not show any differences compared with controls (data not shown).

To evaluate the immune responses of transgenic rats against cognate antigens, the animals were continuously

kept with tet or withdrawn from tet for 10 days. KLH was subsequently injected into the footpad and 10 days later the analyses of proliferative responses of draining lymph nodes cells (Fig. 4C) and sera anti-KLH antibody levels (Fig. 4D) in HO-1-transgenic rats showed responses similar to those of controls.

Allograft Survival Was Not Prolonged in HO-1-Transgenic Rats. We used transgenic rats of both lines as recipients of cardiac allografts from LEW.1A donors. Rejection of LEW.1A heart by non-transgenic controls (6.2 ± 0.8 , $n = 3$) was indistinguishable from those of transgenic recipients (line 12.6, 6 days, $n = 3$; line 12.4, 7 days, $n = 3$).

Discussion

We generated rats transgenic for HO-1, which overexpressed transgene-derived HO-1 protein in the thymus. The H-2K^b promoter was used because of the fact that in transgenic mice, it has been shown to drive expression of transgenes primarily in leukocytes and endothelial cells (13). Overall, the expression levels of HO-1 obtained in the transgenic rats was low because its expression was only detectable in two of four lines and transgene-derived HO-1 protein was only detectable in the thymus. Although transgenic pigs have also been generated using the same promoter (23), we cannot formally exclude the possibility that species restrictions may exist in the rat for high expression of transgenes placed under its transcriptional control. Alternatively, as is the case for every promoter lacking insulating sequences, its activity may be largely dependent on the chromatin configuration at the point of insertion of the transgene into the genome.

The tet-off system integrated into a single DNA construct used in this study has been previously applied to generate lines of transgenic mice, from which a proportion showed tet-controlled expression of the transgene (14). Both rat transgenic lines showed higher HO-1 expression in the thymus in the absence than in the presence of tet at the mRNA level suggesting regulation of HO-1 expression.

Among other observable protective effects, mice transgenic for HO-1 have shown decreased inflammation after oxidative injury (8, 10–12), but the effect of HO-1 in other inflammatory models has not been analyzed in transgenic animals. In our study, rats transgenic for HO-1 displayed thymic and splenic cell subsets as well as cellular and humoral anti-KLH responses comparable with those of non-transgenic rats. In MLRs, thymocytes and splenocytes from HO-1 transgenic rats showed proliferative responses of the same magnitude as control rats. Moreover, in a cardiac allograft model, HO-1 transgenic animals rejected allogeneic hearts with the same kinetics as controls. These results suggest that rats transgenic for HO-1 show normal differentiation of immune cells and immune responses after a relative short-term (10 d) induction of HO-1 expression. Neverthe-

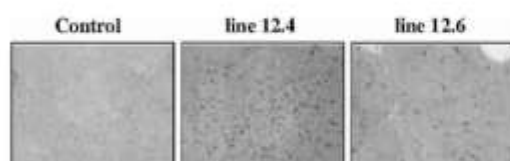


Figure 3. Thymic HO-1 expression. Homozygous HO-1 transgenic rats were maintained without tet for 10 days and HO-1 expression was analyzed on thymus cryostat section by immunohistology using an anti-HO-1 antibody (objective $\times 20$). Results from one animal for each condition are representative of two to three animals analyzed in each line.

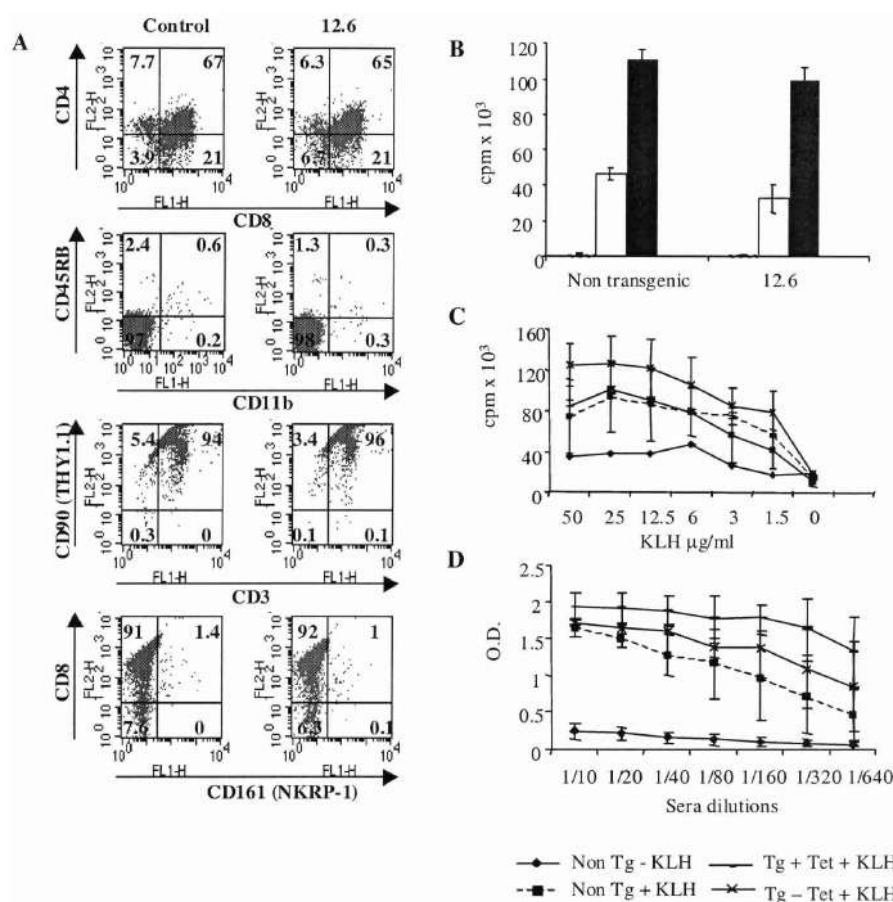


Figure 4. Thymic subsets and immune responses. Nontransgenic litter mates or homozygous HO-1 transgenic rats from line 12.6 maintained with or without tet for 10 days were analyzed for phenotype and function of their immune cells. (A) Thymic cells analyzed by cytofluorimetry. Numbers within each window represent the percentage of positive cells. (B) Proliferation of thymic cells cultured with medium alone (stippled histogram) or with allogeneic APCs (LEW.1A) in the absence (white histogram) or presence (black histogram) of IL-2 (100 U/ml). [³H] thymidine incorporation (mean $\pm$ SD of triplicate cultures) was evaluated after 5 days of culture. Nontransgenic (Non Tg) or transgenic (Tg) rats of line 12.6 were kept with or without tet (+ Tet or - Tet, respectively) for 10 days, immunized or not with KLH in the footpad and analyzed 10 days later. (C) Draining lymph node cells were removed and proliferation against different concentrations of KLH was tested *in vitro*. [³H] thymidine incorporation (mean $\pm$ SD of triplicate cultures) was evaluated after 3 days of culture. Each curve represents the proliferation of cells from a single animal. (D) Anti-KLH antibodies were detected in sera of rats of line 12.6 using an ELISA. The results for the KLH experiments were an average of two animals from each condition. Results are expressed as optical density (OD).

less, the low HO-1 expression observed in secondary lymphoid organs do not allow us to conclude that HO-1 overexpression does not affect immune responses. New transgenic animals with high HO-1 expression in peripheral lymphoid organs are currently being generated. Thymic cells displayed clear HO-1 protein overexpression, and some important cell processes (apoptosis, cytokine production) have not yet been analyzed. Furthermore, overexpression during the whole life of these transgenic rats may modify thymocyte differentiation. The generation of transgenic rodents with expression of HO-1 by cells of the im-

mune system will represent an important tool for the analysis of HO-1 function.

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Annexe 8

Formation Universitaire

Publications personnelles

Communications orales

Posters

Prix

Formation Universitaire

<u>Depuis 2004:</u>	Doctorat de sciences. INSERM U643	Nantes.
<u>2001:</u>	Ecole Pratique des Hautes Etudes (EPHE)	Paris V.
<u>1998:</u>	DEA “ Relation Hôte Greffon ”	Université de Bourgogne.
<u>1997:</u>	Maîtrise de biologie cellulaire et physiologie,	Nantes

Publications

Braudeau C., Louis S., Giral M., Racapé M., Moreau A., Souillou JP. and Brouard S. A distinct pattern of CD4⁺CD25⁺ T cell regulatory function in patients with long term kidney graft survival and chronic rejection. **Soumis.**

Braudeau C., Louis S., Giral M., Dupont A., Moizant F., Robillard N., Souillou JP. and Brouard S. Contrasting CD25^{hi}CD4⁺T cells/FOXP3 patterns in chronic rejection and operational drug-free tolerance **Transplantation**, **2006**, 81:398-407.

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Guillot C, Menoret S, Guilloneau C, **Braudeau C**, and Anegon I. Active suppression of allogeneic proliferative responses by dendritic cells after induction of long-term allograft survival by CTLA4Ig. **Blood**, 2003, 101:3325-33.

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Communications orales

CD4⁺CD25⁺ T cells from drug-free tolerant kidney recipients and chronic rejection patients display the same suppressive activity in vitro as healthy individuals. **7th international conference on new trends in immunosuppression and immunotherapy. Berlin, 2006.**

Low levels of CD25⁺CD4⁺T cells and FOXP3 transcripts together with increased effector memory CD45RA⁺CD62L⁻CD4⁺T cells and CD25⁻CD4⁺T cells with a cytotoxic-associated phenotype in human kidney recipients with chronic rejection. **American Congress of Transplantation. Seattle, 2005.**

Les patients rejetant une greffe rénale présentent moins de cellules T CD4⁺CD25⁺ circulantes et un déficit en FOXP3. **IV Congrès de la société Francophone de transplantation. Bruxelles, 2004.**

Blood CD25⁺CD4⁺ T cells phenotype and regulation associated molecules in “operationally tolerant” versus chronic rejection human kidney recipients. **XX International congress of Transplantation Society. Vienne, 2004.**

Posters

Significantly lower number of CD4⁺CD25⁺ FOXP3⁺ T cells in chronic rejection patients. **Word Transplant Congress, Boston, juillet 2006.**

Patients with chronic rejection display a quantitative deficiency in anergic and hyporesponsive CD4⁺CD25⁺ T cells without defect in suppressive activity. **FOCIS, San Francisco, juin 2006.**

Low accumulation of FOXP3 transcripts in blood CD4⁺ and CD8⁺ T cells of patients with chronic rejection. **FOCIS, Montréal, juillet 2004.**

Low accumulation of FOXP3 transcripts in blood CD4⁺ T cells of patients with chronic rejection. **American Congress of Transplantation, Boston, mai 2004.**

Generation of HO-1 transgenic rats. **2nd Symposium international sur HO-1, Sicile. juin 2002.**

Heme oxygenase-1 (HO-1) gene transfer and CO administration delayed acute allograft rejection in a rat model. **Société Française d'Immunologie, Paris. décembre 2001**

Le transfert du gène de l'hème oxygénase-1 (HO-1) retarde le rejet aigu d'allogreffes cardiaques chez le rat. **Société Française d'Immunologie, Paris. décembre 2000**

Etude des cellules dendritiques chez le rat : manipulations à des fins d'induction de tolérance périphérique. **Société Française d'Immunologie, Paris. Novembre 1998**

Prix[☆]

Young Investigator award. **Vienne, septembre 2004**

Etude du phénotype et de la fonction des cellules régulatrices T CD4⁺CD25⁺ dans le sang de patients tolérant spontanément une greffe de rein

Un des objectifs en transplantation est d'induire un état de tolérance opérationnelle. Cet état, rare en transplantation rénale, se définit comme l'acceptation à long terme d'un greffon à fonction stable sans immunosuppresseur chez un hôte immunocompétent. Ce travail vise à comprendre les mécanismes impliqués dans le processus de tolérance opérationnelle chez les transplantés rénaux. L'étude d'une petite cohorte de patients tolérants a permis d'identifier un phénotype particulier de leurs lymphocytes T sanguins CD4⁺CD25⁺, différent de celui des patients en rejet chronique. Nous avons montré que les patients tolérants présentent un nombre plus important de cellules CD4⁺CD25⁺FOXP3⁺ que les patients en rejet chronique et semblable à celui des individus sains. Ces cellules présentent également une fonction régulatrice semblable à celle d'individus normaux et significativement supérieure à celle de patient en rejet chronique.

Mots-clés : Rejet chronique, tolérance, transplantation rénale

Analysis of the phenotype and function of CD4⁺CD25⁺ regulatory T cells in the blood of spontaneously tolerant kidney allograft recipients.

Despite being efficient in preventing acute rejection, immunosuppressors have little effect on chronic rejection. A major goal in transplantation is therefore to induce a state of operational tolerance. This state, which is rare in kidney transplant recipients, is defined as long-term graft acceptance with a stable function, without immunosuppression in an immunocompetent recipient. Here we tried to understand the mechanisms implicated in operational tolerance to kidney transplants. The study of a small cohort of tolerant patients allowed us to identify a particular phenotypic profile of CD4⁺CD25⁺ blood lymphocytes that was different to that of patients with chronic rejection. Tolerant patients displayed an increased number of CD4⁺CD25⁺FOXP3⁺ T cells compared to patients with chronic rejection. These cells also displayed a regulatory function similar to that of healthy individuals and significantly higher than that of patients with chronic rejection.

Keywords: Chronic rejection, tolerance, kidney transplantation