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Analyse des mécanismes de la tolérance à l'allogreffe de rein chez le rat suite à l'administration de sérum anti-CMH de classe II du donneur

THESE DE DOCTORAT

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Abréviations

TCR: T cell receptors

CMH: complexe majeur d'histocompatibilité

HLA: human leukocyte antigens

APC: antigen presenting cells

DC: dendritic cells

mHAg: complexe mineur d'histocompatibilité

DTH: delayed-type hypersensivity

BM: bone marrow

GVHD: graft-vs-host disease

Treg: T regulatory cells

AICD: active induced cell death

PCD: passive cell death

IL: interleukin

NHP: non-human primates

OWM: Old World Monkeys

NWM: New World Monkeys

LEW: Lewis

PCR: Polymerase chain reaction

IDO: indoleamine-2,3-dioxygenase

iNOS: inducible nitric oxide synthase

CTLA4 : cytotoxic T-lymphocyte antigen 4

BN: Brown Norway

TGF-β: transforming growth factor β

DST: donor specific transfusion

IFN: Interferon

FoxP3: Forkhead box P3

HO-1: heme-oxygenase-1

IS: immunosuppression

GOT: glutamic-oxaloacetic transaminase

SCR: subclinical rejection

CAN: allograft nephropathy

MMP7: Matrix Metalloproteinase 7

WNT: wingless

TNF: tumor necrosis factor

DSG: deoxyspergualine

GIC: graft infiltrating cells

CML: cellules musculaires lisses

Avant propos

Aujourd'hui, l'allotransplantation des cellules et des organes représente un traitement de choix pour les pathologies humaines avec une dysfonction totale de l'organe. La transplantation rénale est la meilleure option thérapeutique pour les patients en phase terminale d'une maladie rénale.

L'histoire de la transplantation a commencé, il y a plus de cent ans, quand E. Ullmann a réalisé la première transplantation rénale expérimentale entre deux chiens. En 1906, M. Jaboulay a tenté la greffe de vaisseaux rénaux provenant soit d'un mouton soit d'un cochon avec les vaisseaux brachiaux de deux patients mourant de dysfonction rénale (Morris, 2004). Les greffons ont été rejetés, et ceux-ci constituent les premiers essais de greffes chez l'homme (Morris, 2004). Entre les deux guerres mondiales, des essais de transplantation ont été réalisés occasionnellement sans aucun succès. Dans l'histoire de la transplantation, un nouveau chapitre commence le 23 décembre 1954 à Boston où J. Murray et son équipe a prélevé le rein du donneur sain et l'a transplanté chez son frère jumeau (Merrill et al., 1956). L'organe a fonctionné immédiatement et a survécu pendant neuf ans. L'amélioration en transplantation est venue avec la découverte du gène codant pour le CMH humain - human leukocyte antigen (HLA) en 1958 (Albert et al., 1978). Dix ans plus tard, la recherche de l'incompatibilité HLA est devenu disponible en transplantation clinique (Mathe et al., 1963). Une nouvelle ère de la transplantation débute avec l'utilisation de protocole immunosuppresseur en clinique. Durant les années soixante et au début des années soixante-dix, la proportion de transplantations réussites entre les donneurs compatibles a

augmenté grâce à l'introduction d'azathioprine et de corticostéroïdes, (Murray et al., 1963). Au début des années quatre-vingt, l'introduction de la cyclosporine, a augmenté le taux de survie du greffon à un an de 70% à plus de 80% (Kahan, 1989). La découverte du tacrolimus et son utilisation en clinique au début des années quatre-vingt dix est une nouvelle avancée dans l'immunosuppression (Goto et al., 1991).

Le traitement avec des immunosuppresseurs entraîne de nombreux effets secondaires, comme des complications dues à des maladies infectieuses (Soulillou and Giral, 2001), des tumeurs malignes (Dantal et al., 1998) ou des désordres métaboliques (Halloran, 2004). Les patients sous thérapie immunosuppressive doivent prendre des médicaments à vie et ce traitement induit le rejet chronique qui est aujourd'hui la première cause d'échec de greffe rénale (Dantal and Soulillou, 2005). Aujourd'hui la transplantation pourrait bénéficier du développement des stratégies immunosuppressions afin d'assurer la tolérance opérationnelle (Waldmann and Cobbold, 2004). Pour être plus précis, l'induction de la tolérance immune spécifique d'un organe transplanté (sans effets délétères sur l'immunocompétence) représenterait le «saint graal de la transplantation» (Auchincloss, 2001).

Introduction

I. La réponse immunitaire

I.1. Les principales caractéristiques de la réponse immunitaire

Le terme immun vient du mot Latin – *immunitas* qui réfère à la protection de la vindicte légal proposer aux sénateurs Romains. Dans le contexte médical, le terme immun signifie la protection contre la maladie. Les cellules et molécules responsables de l'immunité forment le système immunitaire et la réponse contre le pathogène s'appelle la réponse immune. En parlant d'immunité, on peut distinguer l'immunité innée (naturel) et l'immunité adaptative. L'immunité adaptive se développe comme une réponse contre l'infection qui a deux caractéristiques principales – la *spécificité* et l'*habilité de mémoriser* qui permettent une réaction rapide et vigoureuse contre une infection répétée. Aussi, avec l'immunité adaptive on peut distinguer deux types de réponses immunitaires – l'immunité humorale et l'immunité à médiation cellulaire.

I.2. Les récepteurs aux antigènes

I.2.1. Origine de la reconnaissance moléculaire par les cellules T

Le système immunitaire adaptif chez les vertébrés a évolué et a acquis la capacité à produire une grande quantité de lymphocytes B et T. Les anticorps et les récepteurs de cellules T pour l'antigène (TCR) peuvent reconnaître une grande quantité d'antigènes différents comme les protéines, les carbohydrates,

les petits composés organiques, et les acides nucléiques. L'analyse des molécules de surface du lymphocyte a aidé à comprendre les mécanismes de reconnaissance d'antigène par TCR et par les immunoglobulines membranaires des cellules B. L'identification de ses molécules a commencé avec la caractérisation des différences allotypiques entre différentes souches de souris et avec la découverte des anticorps monoclonaux (Williams et al., 1977). En 1974 Zinkernagel et Doherty ont montré que l'effet cytotoxique de cellules T dépend de la présentation d'antigène par le CMH (Zinkernagel and Doherty, 1974). Aujourd'hui, sur la surface des cellules T, on a identifié trois groupes de molécules qui sont responsables de l'initiation ou de la modulation de la «réponse T» les récepteurs de cellules T pour l'antigène (TCR), les molécules «co-récepteurs» CD4 et CD8 et les protéines responsables pour la co-stimulation (CD28 et CTLA-4).

I.2.2. Différentiation et sélection des cellules T

La différentiation et la sélection des cellules T prend place dans le thymus (Miller, 1961), (Bevan, 1977), (Zinkernagel et al., 1978). Le développement des cellules T se déroule en plusieurs étapes: l'entrée des cellules précurseurs dans le thymus puis développement des cellules double positive (DP) $CD4^+CD8^+$ thymocytes dans le cortex thymique extérieur. Au niveau du cortex thymique, les thymocytes subissent une sélection positive et négative. La sélection positive terminera son développement par interaction avec les cellules épithéliales dans la zone médullaire thymique. Cette étape du développement va assurer la

tolérance centrale. A la fin, les cellules T vont être exporter du thymus (pour la revue cf (Takahama, 2006)).

Les thymocytes $CD4^-CD8^-$ migrent de la zone cortico-médullaire à la zone cortex thymiques (la région sub-capsulaire) et pour ce déplacement plusieurs récepteurs de cytokine sont important (CXCR4, CCR7, CCR9) (Plotkin et al., 2003) (Misslitz et al., 2004).

Les thymocytes double négatifs $CD4^-CD8^-$ subissent un re-arrangement des chaînes TCRβ et pre-Tα polypeptides. La signalisation qui passe par le complexe pre-TCR va résulter d'une expression simultanée de $CD4^+$ et $CD8^+$ co-récepteurs double positive (DP) - thymocytes $CD4^+CD8^+$.

Les thymocytes $CD4^+CD8^+$ contiennent les TCR non- sélectionnés et ils subissent une sélection très rigoureuse (sélection positive puis négative) (Jameson et al., 1995). Les TCR sont testé avec le complexe CMH-peptide dans le cortex et les interactions avec une avidité faible vont induire un signal de survie et pour la différentiation en thymocytes simple positif - lymphocytes T CD4+ ou lymphocytes T CD8+. Cette étape est une sélection positive ou les cellules T, potentiellement réactifs contre les antigènes étrangers, sont préservées. Le processus de la sélection négative va résulter en l'élimination des cellules T qui reconnaissent les antigènes du Soi présentés par les molécules du CMH à la surface des cellules présentatrices d'antigènes (CPA). Les cellules T dont les TCR ne reconnaissent aucun CMH dans le thymus vont mourir par apoptose. Le résultat cette sélection très forte est que 1 à 3% des

thymocytes sont exportés hors du thymus (Scollay et al., 1980) (Goldrath and Bevan, 1999).

I.3. Complexe majeur d'histocompatibilité (CMH)

Le rôle du complexe majeur d'histocompatibilité (CMH) est la présentation des antigènes aux cellules T. Le CMH a été découvert comme un locus génétique responsable pour la rejection rapide de la greffe de peau entre les différentes souches des souris. En 1940, Snell et son équipe utilisaient des techniques génétiques pour analyser le rejet de greffe de peau. Les résultats montraient que les greffes autologues étaient acceptées, ce qui n'était pas le cas pour les greffes alloqeniques (McDevitt, 2000). Les gènes déterminant si le greffon est similaire ou différent de son propre tissu sont appelés gènes d'histocompatibilité.

I.3.1. Les gènes du CMH de classe I et de classe II

Il y a deux type de gènes du CMH – classe I et classe II. Les gènes du CMH sont les gènes les plus polymorphiques dans le génome et il sont exprimé simultanément dans chaque individu. La version humaine des molécules du CMH est connue sous le nom de *human leukocyte antigenes* (HLA). HLA-A, HLA-B, HLA-C regroupent les gènes du CMH de classe I et HLA-DR, HLA-DP, HLA-DQ ceux du CMH de classe II. A notre connaissance le système HLA est impliquée dans les maladies infectieuses, les maladies auto-immunes, le cancer et la transplantation (Klein and Sato, 2000).

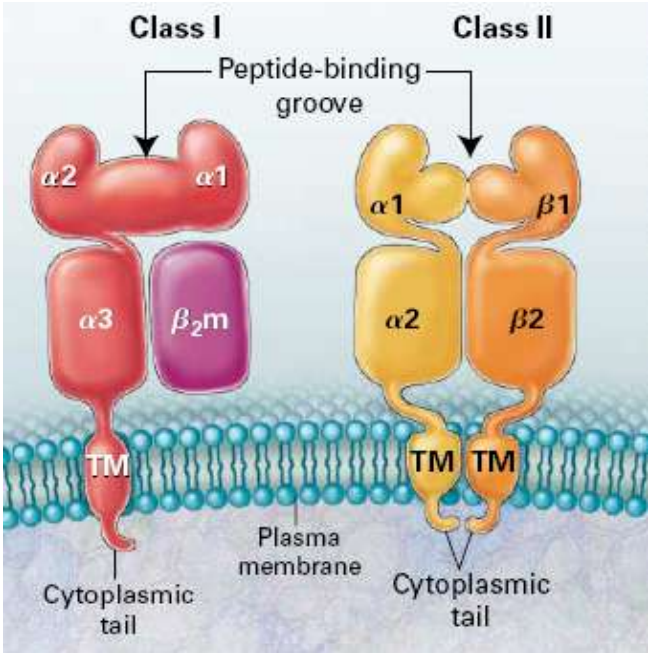

Figure 1: La structure du HLA de Classe I et de Classes II (Klein and Sato, 2000)

I.3.1.1 Le CMH de classe I

Les molécules du CMH de classe I sont exprimées virtuellement sur toutes les cellules qui contiennent un noyau et le niveau d'expression du CMH de classe I varie et dépend du type de tissu (Klein and Sato, 2000). La molécule du CMH de classe I consiste en deux chaînes polypeptidiques connectées de façon non covalente. La chaîne α a cinq domaines: deux sites de liaisons de peptides (α1 et α2), un domaine immunoglobuline (α3), la région transmembranaire et la queue cytoplasmique (Klein and Sato, 2000) (Bjorkman et al., 1987). Le domaine immunoglobuline α3 et microglobuline β2 forment le site de liaison pour le CD8, donc les lymphocytes T $CD8^+$ cytotoxique sont CMH de classe I restreint. Les protéines antigènes sont proteolysées dans les CPA pour generer des peptides. Ces peptides sont liés et présentés par le CMH. La nature de la liaison entre le

CMH et le peptide est non covalente. Chaque CMH de classe I présente des peptides de 7 à 10 acides aminés et l'origine de ces peptides est endogène (Ackerman and Cresswell, 2004; Ackerman et al., 2005) (Hammer et al., 2006) (Shastri et al., 2005).

I.3.1.2 Le CMH de classe II

Les gènes du CMH de classe II sont exprimés normalement sur les cellules B, les cellules T activées, les macrophages, les cellules dendritiques et les cellules épithéliales du thymus. En présence de l'interféron γ, d'autres types de cellules peuvent exprimer le CMH de classe II (Klein and Sato, 2000). Les chaînes α et β du CMH de classe II ont quatre domaines: les sites de liaison peptidiques (α1 ou β1), les domaines immunoglobulines (α2 ou β2) la région transmembranaire et la queue cytoplasmique.

Les segments α1 et β1 du CMH de classe II forment le site de liaison du peptide. Le segment β2 est reconnu par CD4 donc le CMH classe II est restreint pour les cellules T helper $CD4^+$. Il faut évoquer le peptide non-polymorphique connu sous le nom de «chaîne invariante». Cette chaîne est associée avec le CMH de classe II récemment synthétisé. La chaîne invariante joue un rôle importante dans transfert du CMH de classe II et empêche le chargement d'antigènes (Ackerman and Cresswell, 2004). Les antigènes présentés par le CMH de classe II sont d'origine exogène et la taille est de 13 à 26 amino acides (Maffei and Harris, 1998).

I.4. Les voies de présentation d'antigènes

Les cellules présentatrices d'antigènes (CPA) utilisent les mécanismes distincts d'endocytose pour introduire des antigènes solubles dans les différents compartiments intracellulaires. Ce processus est suivi par la présentation d'antigènes aux lymphocytes T $CD8^+$ ou $CD4^+$. Dans le chapitre précèdent, il a été décrit que les molécules du CMH de classe II sont constituées de deux chaînes α et β et d'une chaîne invariante. La chaîne invariante a deux fonctions: lier les ligands dans le réticulum endoplasmique et transférer le CMH de classe II dans la voie endosomale (Cresswell, 1994).

I.4.1. La voie du CMH de classe I

Les molécules du CMH de classe I présentent les peptides dérivés de protéines dégradés dans le cytosol. Dans la plupart des cellules dendritiques l'origine de ces protéines est endogène (les protéines sont synthétisées par la cellule elle même) (Villadangos and Schnorrer, 2007).

I.4.2. La voie du CMH de classe II

Les molécules du CMH de classe II acquièrent des peptides générés par la dégradation protéolytique dans les compartiments endosomaux. Les précurseurs de ces peptides viennent des matériaux exogènes (matériaux endocytosés). Les complexes peptides-CMH sont présentés à la surface de cellules pour qu'ils puissent interagir avec les $CD4^+$ (Li et al., 2005). En plus du rôle de présentation

d'antigène, les molécules du CMH de classe II peuvent être des récepteurs qui régulent leurs activités (Al-Daccak et al., 2004) (Truman et al., 1994).

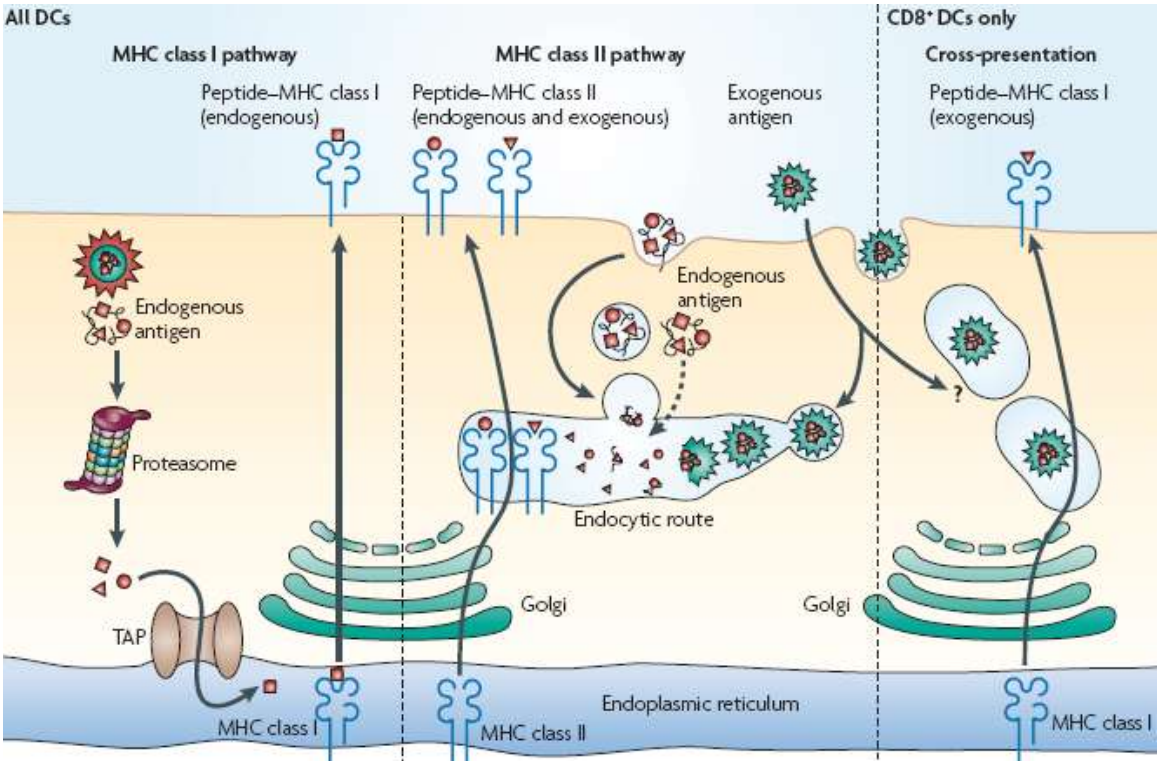

Figure 2: Les voies de présentation des antigènes dans les cellules dendritiques (Villadangos and Schnorrer, 2007)

I.4.3. La «présentation croisée»

La présentation des antigènes exogènes n'est pas limitée à la voie du CMH de classe II. Certaine cellules ont la capacité de présenter les antigènes d'origine exogènes par les molécules de CMH de classe I. Ce phénomène est connu sous le nom de « présentation croisée » et il a été proposé par Bevan (Bevan, 1976). Dans ces expériences, avec des animaux immunisés avec des cellules

alloqeniques (la différence vient des CMH et des antigènes mineurs), certaines cellules T cytotoxiques générées sont devenues spécifiques des antigènes mineurs du greffon présentés par les CMH de classe I du receveur. Cette découverte indique que pendant le traitement des antigènes, les antigènes mineurs ont été transférés des cellules du greffon et présentés par les cellules du receveur (Bevan, 1976).

Aujourd'hui les cellules dendritiques paraissent être le principale type cellulaires pouvant effectuer la présentation croisée d'antigène in vivo (Jung et al., 2002). Pour être plus précis, les cellules dendritiques (DC) $CD8^+$ sont les plus efficaces en présentation croisée, probablement grâce à leur capacité de phagocytose (den Haan et al., 2000). Les mécanismes impliqués dans cette voie ne sont pas complètement connus. Il y a des évidences qui montrent que chaque forme d'endocytose ne délivre pas des antigènes à la présentation croisée. Par exemple, un antigène capté par le récepteur au mannose sera présenté par la présentation croisée ce qui n'est pas le cas s'il est intériorisé par pinocytose (Burgdorf et al., 2007). Certaines études ont montré que la voie d'endocytose et la présentation des antigènes chez les DC $CD8^+$ sont «ramifiées» (contrairement à la voie «linéaire» observée chez les autres types de CPA). Il subsiste toujours une interrogation quant aux conditions et aux critères requis pour la présentation croisée. Il pourrait s'agir de l'activité de protéases spécifiques, de différences de pH entre les compartiments cellulaires ou de la présence de mécanismes particuliers de transfert direct des antigènes vers le cytosol (pour la revue cf (Villadangos and Schnorrer, 2007)).

II. Immunologie de la transplantation allogénique

II.1. Alloreconnaissance

La limite la plus importante pour le succès de la transplantation est la réponse immunitaire du receveur contre le tissu du donneur. Le développement et la sélection des cellules T chez un individu sain conduit à l'élimination de toutes les cellules autoréactives et va ainsi fournir une discrimination efficace du Soi et du non-Soi (Wraith, 2006). Dans une situation où le greffon est syngénique (c'est-à-dire génétiquement identique – par exemple une transplantation entre des jumeaux identiques) ou autologue (par exemple une autotransfusion) l'histocompatibilité est parfaite, la réponse immune n'est pas provoquée et le greffon est toléré (Murray, 1992).

Les allogreffes constituent des cellules ou des organes transplantés entre deux individus de la même espèce, génétiquement différents. A cause de la non-*histocompatibilité*, les molécules d'une allogreffe sont reconnues comme étrangères (alloantigènes) et une réponse immunitaire est induite (Starzl and Zinkernagel, 2001). Les cellules T répondent aux molécules de CMH étrangères (CMH alloqéniques) de la même façon qu'à n'importe quelle protéine étrangère: elles vont sécréter des cytokines, se diviser et se différencier (Walsh et al., 2004). Les alloantigènes peuvent être séparés en deux groupes: les alloantigènes du complexe majeur d'histocompatibilité (CMH) et du complexe

mineur d'histocompatibilité (mHAg). Les molécules alloqéniques de CMH sont présentées et reconnues (alloreconnaissance) de trois façons.

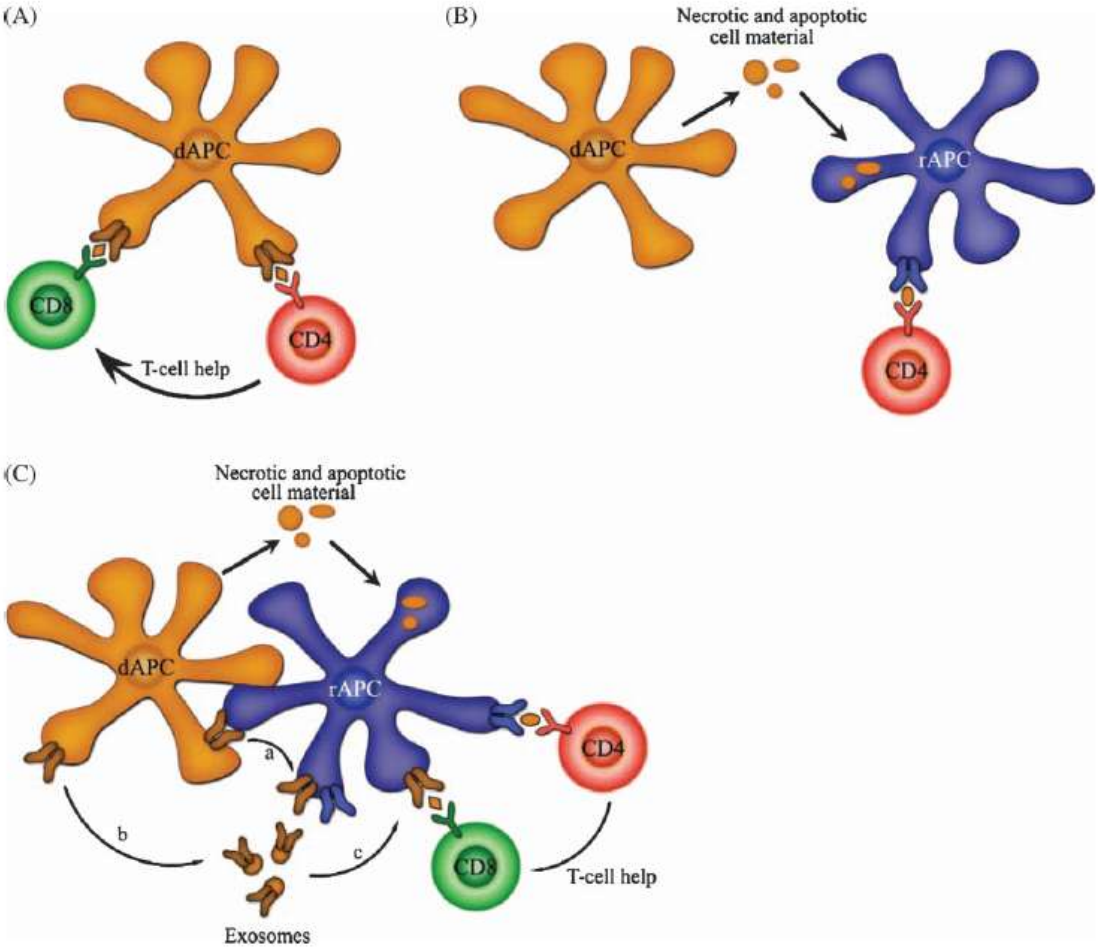

Figure 3 : Les voies de l'alloreconnaissance directe, indirecte et semi directe (Afzali et al., 2007)

II.1.1. L'alloreconnaissance directe

Au cours de l'alloreconnaissance directe, les molécules de CMH du donneur présentes à la surface des CPA du donneur sont reconnues directement par les

cellules T du receveur. Ce mécanisme est si rapide qu'il semble être impliqué dans le rejet aigu (Warrens et al., 1994). Le caractère vigoureux d'une telle réponse est le résultat de la reconnaissance directe des molécules de CMH intactes par les cellules T, sans nécessité d'un « processing » du CMH du donneur ou d'une présentation des antigènes par le CMH du receveur, ce qui montre la grande fréquence des cellules participant à l'alloreconnaissance directe (Baker et al., 2001). Cette hypothèse semble confirmée par le fait que le blocage du TCR par des peptides synthétique (Schneck et al., 1989) ou les mutations «site spécifique» vont inhiber les alloréponses spécifiques (Villadangos et al., 1994). Une explication présume l'inhibition du contact entre le TCR et le CMH (Lombardi et al., 1991).

II.1.2. L' alloreconnaissance indirecte

Au cours de l'alloreconnaissance indirecte, les molécules de CMH du donneur sont internalisées, partiellement digérées et présentées sous forme de peptides portés par les molécules de CMH du receveur à ses cellules T (d'une façon similaire à la celle de la présentation des antigènes classiques) (Lechler and Batchelor, 1982). Puisque les CPA du donneur sont déplétées au cours du temps, la voie indirecte semble prédominer de plus en plus au cours de la progression de l'alloréponse (Chiffoleau et al., 2003).

II.1.3. L' alloreconnaissance semi directe

Au cours de l'alloreconnaissance semi directe, les molécules intactes de la surface des cellules (dont le CMH) peuvent être échangées entre les différentes cellules du système immunitaire. Les mécanismes d'échange peuvent impliquer un contact direct entre cellules («cell-to-cell») (Game et al., 2005) ou l'intervention de petites vésicules («exosomes) (Morelli et al., 2004). La voie semi direct de l'alloreconnaissance peut connecter les voies directe et indirecte dans une seule cellule CPA et faire une communication croisée entre les cellules T CD4 et CD8 (Afzali et al., 2007).

II.2. L'alloréponse

Le rejet d'un allogreffe est le résultat des mécanismes qui associent les réponses d'immunité innée (naturel) et d'immunité adaptive. En parlant du type de rejet d'une allogreffe, on peut différencier quatre situations. Le rejet hyper aigu, le rejet aigu, le rejet chronique et la réaction d'hyper sensibilité retardée.

II.2.1. Le rejet hyper aigu

Le rejet hyper aigu concerne une perte du greffon précoce, généralement pendant les premières 48 heures. Les anticorps, spécifiques pour les antigènes du donneur, préformés et présents dans le sérum du receveur, peuvent détruire les cellules vasculaires du greffon. Au niveau histologique, le rejet hyper aigu est caractérisé par l'hémorragie ou la thrombose (Busch et al., 1975). La fixation des anticorps IgM et IgG sur ces cibles activent la coagulation, les voies de

signalisation du complément et du kinine, et ainsi provoquer la thrombose intra vasculaire, l'ischémie et la nécrose (Afzali et al., 2007).

En clinique, le rejet hyper aigu a été observé chez les patients pré-sensibilisés par la transfusion du sang, la grossesse ou les greffons rejetés. Avec les derniers protocoles cliniques le problème du rejet hyper aigu est presque éliminé (Magee, 2006), mais chez 1% de tous les patients cette situation apparaît pour des raisons inconnus (Scornik et al., 1988).

Aujourd'hui, dans le but d'éliminer le rejet hyper aigu, l'évaluation immunologique s'applique avant la transplantation. Cette évaluation comprend la détermination du group sanguin, le typage HLA, dépistage pour les anticorps anti-HLA et «cross-matching» (Gallon et al., 2002).

Le problème du rejet hyper aigu présente aujourd'hui la plus grande barrière pour la xeno transplantation. Dans la xeno transplantation la situation de rejet aigu implique les aspects du rejet humoral (Schuurman et al., 2003) et du rejet cellulaire (Davila et al., 2006), (Dorling et al., 1996) (Cardozo et al., 2004).

II.2.2. Le rejet aigu

Au cours du rejet aigu, les lésions vasculaires et parenchymales sont provoquées par les cellules T et les anticorps. En clinique, cette forme de rejet se produisent entre 5 jours et trois mois après la transplantation (Neuberger, 1999). Néanmoins, le problème du rejet aigu est résolu avec le traitement des immunosuppresseurs (Meier-Kriesche et al., 2004). Au niveau histologique, les signes du rejet aigu sont caractérisés par les infiltrats de cellules T CD4+ et

CD8+. Parmi les CD8+, les majoritaires sont les cellules de phénotype CD45RO+ (apoptose résistant) (Ibrahim et al., 1995).

II.2.2. Le rejet chronique

La perde tardive du greffon, provoqué par la réponse immunitaire du receveur contre le greffon, est connu sous le nom de rejet chronique (Colvin, 2003). Les protocoles cliniques appliqués aujourd'hui à court terme prolongent la survie des greffes de cœur et de rein de 88 à 95% (la période à court terme est définie par un traitement lors des 12 premiers mois). Probablement, le meilleur critère de signes histologiques de rejet chronique est défini pour le greffe du rein - le system de Banff. La conférence Banff '05 a inclus plusieurs caractéristiques de rejet chronique comme les modifications au niveau des artères et des capillaires, les dépôts de C4d et la présence de anticorps anti-donneurs dans le sang (Solez et al., 2007). Pour illustrer la survie de la greffe rénale aujourd'hui, on peut prendre les résultats de l'Agence de la biomédecine pour 2006. Les patients greffés entre le 1995 et 1999, en première année ont eu une survie du greffon à 90.1%. Cinq ans plus tard, pour le même groupe la survie a été 79.8% et après dix ans de 62.3%. Tous ces résultas indiquent l'importance du rejet chronique en transplantation.

II.2.1. La réaction d'hyper sensibilité retardée

La réaction d'hyper sensibilité retardée (delayed-type hypersensivity DTH) est caractérisée par le gonflement de tissu, l'augmentation de la perméabilité vasculaire et par la présence d'infiltrat inflammatoire (les cellules T, les

macrophages et les neutrophiles). La réaction d'hyper sensibilité immédiate est provoquée par les anticorps préformés ce qui n'est pas le cas avec la DTH. Plusieurs jours sont nécessaires pour l'activation des cellules Th1 et pour la production d'IFN-γ et de TNF-α. Ensuite, les macrophages sont activés pour produire les molécules toxiques comme l'oxyde-nitrique (NO), TNF-α et d'autres molécules activées. Plusieurs indices confortent l'hypothèse que la réaction d'hyper sensibilité retardée est responsable du rejet du greffon (Grimm et al., 1999) (Worrall et al., 1995).

III. La tolérance de l'allogreffe

Comme Zheng a évoqué, la tolérance immunologique est définie comme l'état où le système immunitaire, en absence d'immunosuppresseur exogène, ne provoque pas de réponse pathologique contre les antigènes du donneur (Zheng et al., 2003).

En transplantation, accomplir un état de tolérance est un procès actif et comprend de multiples étapes. Comme lors de la tolérance naturelle, en transplantation on peut distinguer deux types de mécanismes qui contrôlent la réactivité des cellules T – la tolérance centrale et la tolérance périphérique.

III.1. La tolérance de l'allogreffe centrale

La tolérance centrale ou délétion central thymique correspond à l'élimination de cellules dirigées contre les antigènes du donneur alors que la sélection positive permet le maintien de cellules qui ne réagissent pas contre le greffon (Cosimi and Sachs, 2004). Le mécanisme de la tolérance centrale est basé sur l'établissement d'un système immun chimérique. Dans le thymus, les cellules T du receveur apprêtent des antigènes du donneur conduisant à l'élimination de toutes les cellules potentiellement réactives contre le greffon (Chiffoleau et al., 2003). Dans les conditions expérimentales, le système immun chimérique peut être établi par injections intra-thymiques des allopeptides (peptides du donneur). Medawar a montré que chez les souris nouveau-nés, en absence de tous les moyens d'immunosuppression, la transfusion de cellules allogénique du donneur

conduit à une tolérance de la greffe de la peau du même donneur (Billingham et al., 1953). L'induction d'un état chimérique hématopoïétique a été étudié dans plusieurs modèles chez le rongeur (Ildstad and Sachs, 1984) (Wekerle et al., 2000). En clinique, plusieurs études ont montré l'importance du chimérisme du donneur dans la tolérance. La transplantation de la moelle osseuse chez des patients souffrants de leucémie aigue peut induire le chimérisme du donneur. Quelques années plus tard le même groupe de patients, à cause de dysfonction rénale, a été greffé avec les reins des donneurs qui avaient déjà donné leur moelle osseuse (Sayegh et al., 1991) (Spitzer et al., 1999) (Buhler et al., 2002). Les greffes rénales ont été acceptées sans aucune thérapie immunosuppressive grâce à un chimérisme établi. De plus, chez des patients ayant reçu une greffe de foie (Fogt et al., 2002) (Gao et al., 2001), de cœur (Quaini et al., 2002) ou de poumon (Kleeberger et al., 2003), plusieurs cas de chimérisme ont été rapportés. Le phénomène du chimérisme peut être expliqué par le mécanisme de la tolérance centrale ou les cellules dirigées contre les antigènes du donneur sont éliminées. Cependant le phénomène du chimérisme doit être traité avec prudence car les cellules chimériques qui ne proviennent pas du donneur peuvent être présentes avant la transplantation (Koopmans et al., 2005). Certaines études ont montrés que chez 30% des femmes après l'accouchement d'un fils, les cellules mâles (d'origine fœtale) sont présentent dans le corps pendant 30 ans (Evans et al., 1999). De plus chez la femme, après la transfusion, les cellules mâles peuvent être identifiées dans la circulation et

créent une situation chimérique (Lee et al., 1999) qui n'est pas corrélée avec la tolérance à l'allogreffe.

III.2. La tolérance de l'allogreffe périphérique

La tolérance à l'allogreffe périphérique comprend plusieurs mécanismes qui maintiennent les cellules T alloréactives en périphérie.

III.2.1. Les cellules T régulatrices

La suppression par les cellules T régulatrices (Tregs) est essentielle pour l'induction et le maintien de la tolérance aux antigènes du Soi et il a été montré que ces cellules ont une activité régulatrice après la transplantation. Les premières expériences indiquent que les Tregs ont un rôle important dans la tolérance chez la souris (absence de réponse immunitaire contre une greffe de peau) (Kilshaw et al., 1975). Les études effectuées chez le rat (Hall et al., 1998) (Kitade et al., 2005) ont montré que les $CD4^+$ ont une activité régulatrice spécifique pour les antigènes du donneur. Les cellules Treg $CD4^+$ sont aussi implique dans la tolérance d'allogreffe chez l'homme et chez le souris. Pour être plus précis le phénotype $CD4^+CD25^+$ a été étudié plus en détail, chez l'homme (les exemples sont discutés plus tard) et chez la souris (Adeegbe et al., 2006; Bushell and Wood, 2007; Xia et al., 2006) et les résultats pour cette phénotype montrent une rôle importante dans la tolérance du greffon. A part les T $CD4^+$, la capacité régulatrices a été aussi confirmé pour les $CD8^+$ chez l'homme (Gilliet and Liu, 2002) et chez le rat (Zhou et al., 2001). Les expériences de transplantation faites chez le rat ont montrées que les deux, $CD4^+$ et $CD8^+$ ont

une capacité régulatrice, même si les $CD4^+$ jouent un rôle dominant (Kataoka et al., 2003). Chez la souris, les autres sous types cellulaires ayant un rôle suppressive dans la tolérance en transplantation sont: les $CD8^+CD28^-$ (Ciubotariu et al., 1998), les cellules T $CD4^-CD8^-$ (double négative) (Zhang et al., 2000), les Natural Killer (NK) (Seino et al., 2001).

Les cellules régulatrices les plus caractérisées sont les cellules T $CD4^+CD25^+$. Hall et al. furent les premiers a montrer la fonction des $CD4^+CD25^+$ dans la tolérance d'allogreffe cardiaque a long terme chez le rat. Chez la souris, des expériences in vivo montrent que les cellules régulatrices $CD4^+CD25^+$ sont importantes dans l'induction et le maintien de la tolérance (Hara et al., 2001) (Kingsley et al., 2002). Les cellules $CD4^+CD25^+$ ont aussi la capacité d'inhiber la «graft-vs-host-disease» (GVHD) (Trenado et al., 2006).

A propos des Tregs, il est important d'évoquer le facteur de transcription «forkhead box P3» (FoxP3). Aujourd'hui FoxP3 est reconnu comme le «gène référence» des Treg. En effet son expression est spécifique des Treg chez la souris et prédominantes chez l'homme (Long and Wood, 2007). Chez le rat les Treg ayant une capacité de transfert de la tolérance affichent une accumulation de FoxP3 (Guillonneau et al., 2007) (Degauque et al., 2007) (Degauque et al., 2006).

Le mécanisme de suppression des Treg reste à élucider, mais les derniers résultats indiquent que l'interaction cellule-à-cellule et/ou la production de facteurs solubles (par exemple IL-10) sont nécessaires (Hara et al., 2001) (Dieckmann et al., 2002) (Degauque et al., 2006) (Sanchez-Fueyo et al., 2002).

Une caractéristique très importante des Treg est leur capacité à éduquer et convertir les cellules T effectrices en cellules régulatrices (Jonuleit et al., 2002). La tolérance d'allogreffe peut donc être transférée et ce phénomène est discuté en détail dans l'Annexe 1 – «Transfer of tolerance to heart and kidney allograft in the rat modèle » (Jovanovic et al. *in press*).

III.2.2. La délétion périphérique

La tolérance périphérique peut être établie par le mécanisme d'apoptose. Chez la souris des traitements qui ne tuent pas directement les T activés (agents blocant des voies de co-stimulation), la mort cellulaire induite (activation induced cell death – AICD) et la mort cellulaire passive (passive cell death – PCD) (Wells et al., 1999) (Li et al., 1999) peuvent mener a la délétion. Dans certaines situations, une apoptose massive peut provoquer une forte prolifération de cellules T, ce qui prouve la nécessiter d'améliorer les stratégies existantes. Le fait que les cellules T au repos exprimant des récepteurs de haute affinités pour les facteurs de croissance et pour l'interleukine-2 (IL-2) déclenche l'AICD (là où l'IL15 protége les cellules de la PCD) est utilisé dans le développement de nouvelles stratégies pour la délétion périphérique (Zheng et al., 2003). La découverte que la rapamycine bloque la prolifération mais n'empêche pas les effets pro-apoptotiques de l'IL-2 présente une contribution supplémentaire dans l'amélioration des stratégies existantes (Li et al., 2001) (Ku et al., 2000).

III.2.3.L'Anergie

L'anergie est une situation où les cellules alloreactives existent dans la périphérie mais sont non-réactives contre les antigènes du donneur. Ce phénomène est une conséquence d'activation aberrante du TCR. Les mécanismes moléculaire implicites de l'anergie incluent les voies de signalisation immunologiques et le système ubiquitine-protéasome (pour la revue cf (Fathman and Lineberry, 2007)). La tolérance périphérique est aussi associée avec la suppression des anticorps dirigés contre le Soi produits par les cellules B. L'anergie des cellules B est un mécanisme physiologique clé pour «éteindre» les cellules B alloréactives et donc prévenir l'auto-immunité et aider dans la tolérance de greffe (Ferry et al., 2006). Néanmoins, l'hétérogénéité des mécanismes et des différents modèles complexifie notre compréhension du phénomène d'anergie des cellules B et laisse beaucoup de questions sans réponse (Cambier et al., 2007).

IV. Les protocoles d'induction de tolérance expérimentaux

Medawar a défini la tolérance d'une allogreffe comme : «l'acceptation d'un organe transplanté sans immunosuppression permanente» (Billingham et al., 1953). Aujourd'hui chez les rongeurs, il existe plusieurs protocoles d'induction de la tolérance et parmi eux, certains nombres de protocoles correspondent à la définition de Medawar. Le phénomène de tolérance est classiquement caractérisé par la spécificité aux antigènes du donneurs (Hall et al., 1998) (Degauque et al., 2006), l'absence de signe de rejet chronique (Degauque et al., 2006) (Chiffoleau et al., 2002) et la capacité à transférer la tolérance – la tolérance infectieuse (Qin et al., 1993).

IV.1. Le modèle primate non-humain (NHP) versus le modèle rongeur

Les protocoles d'induction de la tolérance existent chez les grands animaux ainsi que chez les primates non-humains (non-human primates NHP). Chez les rongeurs, l'induction de la tolérance est relativement simple et stable. Les receveurs NHP montrent une acceptation des allogreffes au long terme, mais ce type de tolérance peut simplement être considéré comme la «métastabilité» (Knechtle and Burlingham, 2004).

A ce niveau, on peut se demander pourquoi les stratégies d'induction de la tolérance ont réussi chez les rongeurs mais pourquoi on n'arrive pas à traduire et à appliquer ces protocoles chez les NHP. Et de plus, on peut se demander pourquoi on est loin d'appliquer ces protocoles en clinique. Le modèle NHP est

important parce que il doit permettre de faire le pont entre les protocoles efficaces chez les rongeurs et leur application en clinique.

La question suivante vise à savoir quelles caractéristiques de la réponse immunitaire chez les NHP sont assez importantes pour jouer un rôle dans le transfert des protocoles entre les différentes espèces. Au niveau des séquences d'ADN et des protéines, le système immunitaire des primates a beaucoup plus d'homologies avec celui de l'homme que celui des rongeurs. Il existe entre le singe du vieux monde (Old World Monkeys – OWM) et l'homme une forte similarité au niveau du CMH et d'autres gènes importants pour la réponse immunitaire (Geluk et al., 1993) (Slierendregt et al., 1992) (Jaeger et al., 1994). Ces similarités au niveau du CMH sont particulièrement importantes pour le développement du répertoire du TCR et pour une réponse immunitaire contre les infections virales similaire entre le singe et l'homme. Il existe aussi une grande homologie entre ces deux espèces au niveau des mécanismes des maladies auto-immunes (Watkins, 1995). Chez le singe, la fonction et le phénotype des lymphocytes impliqués dans la réponse immunitaire contre l'allogreffe reflètent de près ce qui est observé chez l'homme (Linley et al., 1998) (Nocera et al., 1989).

Au niveau des tissus, la distribution des molécules du CMH de classe I et de classe II, est également similaire entre l'homme et le singe (Salter-Cid and Flajnik, 1995). La présence de groupes sanguins chez le singe est une autre caractéristique importante, mais il faut être prudent car les groupes sanguins peuvent varier entre différentes espèces de NHP (Moor-Jankowski, 1993). Les

singes du nouveau monde (New World Monkeys - NWM) sont intéressants au niveau de l'aspect de leurs antigènes des groupes sanguins. En plus des antigènes ABO les NWM expriment également l'antigène GALα1,GAL (comme les cochons) et de ce fait permettent d'approcher la xéno transplantation (Collins et al., 1995). Enfin, les singes (comme les hommes) ne sont pas consanguins ce qui permet une distinction par rapport aux transplantations effectuées entre des souches de rongeurs. Il faut dire que le typage du CMH permet l'approche d'une situation clinique de transplantation chez le singe (Penedo et al., 2005). Le travail sur des NHP a de nombreux avantages, en particulier la similarité anatomique, la biodisponibilité des médicaments, et la similarité de la réactivité croisée aux médicaments utilisés en clinique. A ce jour, il existe très peu de protocoles d'induction de la tolérance réussis chez les NHP – la déplétion des lymphocytes, le blocage de la co-stimulation, le chimérisme (pour revue voir (Hale et al., 2005)). Malheureusement, ces différents protocoles peuvent seulement prolonger la survie de l'allogreffe et ne permettent pas d'obtenir un état de tolérance.

Les protocoles d'induction de la tolérance chez le rongeur ont en commun certaines caractéristiques qui concernent leur homogénéité – des âges uniformes, un environnement identique et en particulier le fond génétique. Contrairement aux NHP, les rongeurs possèdent différents patterns d'expression du CMH et un système immunitaire beaucoup mois complexe.

Il existe plusieurs explications au plus grand succès des protocoles d'induction de la tolérance chez les rongeurs. L'exposition environnementale aux différents

types des pathogènes et le développement de la mémoire immunologique sont présents chez l'homme et chez les NHP ce qui n'est pas de cas chez les rongeurs. Les cellules mémoires constituent un risque pour la survie de l'allogreffe car elles peuvent «cross-réagir» avec des alloantigènes (Adams et al., 2003). Les protocoles d'induction de la tolérance chez les NHP ciblent la réponse immunitaire adaptative, alors que chez les rongeurs, il existe certaines études qui prennent également en compte l'immunité innée (naturelle) et son impact dans le rejet d'allogreffe (Maier et al., 2001) (Ogasawara et al., 2005).

Il est important de préciser que des souches «knock-out» pour certains gènes ne sont disponibles que chez les rongeurs, et pour être plus précis chez la souris. L'existence de souris KO (knock-out) permet de mieux comprendre les mécanismes immunologiques et l'importance de certains compartiments immunologiques dans la tolérance d'allogreffe (Lakkis et al., 2000). L'avantage le plus important lorsque l'on travaille chez le rat est la taille des organes qui permet des procédures rapides et relativement simples par rapport aux mêmes techniques effectuées chez la souris. Le modèle de rat est également fiable et reproductible et la bonne combinaison entre les différentes souches peut conduire à une réponse forte contre les antigènes du donneur. Plusieurs protocoles d'induction de la tolérance existent chez le rat dont certains sont discutés en Annexe 1 «Transfer of tolerance to heart and kidney allografts in the rat modèle » (Jovanovic et al., 2007). Cependant, dans ma thèse je me concentrerai plutôt sur les protocoles utilisés dans notre laboratoire.

IV.2. Les protocoles d'induction de tolérance chez le rat

Dans notre laboratoire les greffes du cœur et du rein sont réalisés entre les CMH complètement différentes souches de rat - LEW.1A (RT1a) et LEW.1W (RT1u). Les LEW.1A sont utilisés comme les donneurs des organes et LEW.1A comme les receveurs. Le greffe du rein a été effectué a la manier suivant : Un rein de LEW.1A est remplacé par le rein de LEW.1W en façon orthopique et la nephrectomie de deuxième rein naïf est effectué sept jours plus tard. La survie de l'animal greffé dépend de la fonction normale d'allogreffe (Soulillou et al., 1976). La fonction du greffé de rein est évaluée par la mesure du niveau d'urée et de créatinine dans le sang. Le greffe de cœur est heterotopique, et il est réalisé par le technique décrit par Ono et Lindsey (Ono and Lindsey, 1969). La fonction du greffé de cœur est évaluée par la palpation et le rejet est défini en jour de greffe arrêt.

IV.2.1. Les protocoles de l'induction de la tolérance de la greffe de rein

IV.2.1.1. Le blocage du signal co-stimulateur en utilisant des anticorps anti-CD28

Le blocage sélectif de CD28 par les anticorps (Ac) anti-CD28 a mis en évidence l'activité biologique et inhibé l'activation et la prolifération de cellules T en MLR (mixed lymphocyte reaction) (Vanhove et al., 2003). In vivo l'application de 4mg/kg/jour d'anti-CD28 Ac (de jour 0 à jour 7) empêche le rejet aigu et le rejet chronique et induit la tolérance (Haspot et al., 2005). Cette étude a montré que le blocage de CD28 produit des cellules B7+ non-Treg et la tolérance dépend de

l'activité de l'indoleamine 2,3-dioxygenase (IDO) et d' inducible nitric oxide synthase (iNOS). Une caractéristique importante de ce protocole est que les receveurs tolérants restent immunocompétents. Une hypothèse propose que les anticorps inhibent la molécule CD28 et l'activation des cellules T mais la fonction inhibitrice CTLA4-B7-1/2 reste intacte (Dong et al., 2002).

IV.2.1.2. Induction de la tolérance en utilisant le sérum contre le CMH de classe II du donneur (le protocole anti-classe II)

Les anticorps dirigés contre le CMH de classe II du donneur peuvent induire la tolérance spécifique de greffe de rein (Soulillou et al., 1976) (Degauque et al., 2006). Le protocole anti-classe II a deux avantages: il est actif au moment de l'injection et seuls les antigènes du donneurs sont touchés.

La préparation de sérum contre le CMH de class II du donneur commence avec l'hyper immunisation des rats LEW.1A avec la greffe de peau de LEW.1W. Trois jours après la greffe de peau les cellules de LEW.1W (originaires de la rate, du thymus, des ganglions) sont injectées en IV (intra-venus) et cette procédure est refaite pendant deux mois tous les 14 jours. Apres l' hyper immunisation, les rats sont sacrifiés et du sang est récupéré. A la fin, le sérum est décomplementé et dépleté des antocorps de classe I sur les globules rouges de LEW.1W (Gagne et al., 2001).

Les rats greffés avec un rien et traités suivant le protocole anti-classe II sont tolérants et la tolérance est donneur spécifique (les animaux tolérants acceptent la greffe de peau de LEW.1W mais rejettent la peau de rat Brown Norway) (Degauque et al., 2006). Les splenocytes originaires de rats tolérants transfèrent

la tolérance sans trace de signe de rejet chronique sur plus de deux générations. Les résultas montrent que la coopération des cellules dendritiques $CD103^+$ avec les cellules T est nécessaire pour le transfert de la tolérance (Degauque et al., 2006).

Quand on utilise le protocole anti-class II avec la même combinaison de souches de rat mais avec une greffe de cœur, des résultats intéressants sont obtenus. Dans cette situation les anticorps dirigés contre le CMH de classe II du donneur peuvent juste prolonger la survie du greffon mais ils n'induisent pas la tolérance (Gagne et al., 2001). La déplétion des DC dans le greffon (avec cyclophosphamide, 5 jours avant la transplantation) va écourter la survie de la greffe de cœur par rapport aux receveurs traités avec le sérum anti-classe II. Ce résultat suggère que les anticorps dirigés contre le CMH de classe II vont interagir avec les DC du donneur et prolonger la survie de greffon (Roussey-Kesler et al., 2005).

Les analyses ont montré une augmentation du niveau de l'ARNm du TGF-β1 après la transplantation chez deux groupes: les animaux non traités et les rats traités avec le sérum dirigé contre le CMH de classe II du donneur. Les résultats in vivo montrent que l'application des anticorps anti-TGF-β (injectés de jour 0 à jour 4) écourtent de façon significative la survie de greffon chez les animaux traités avec le sérum anti-classe II. Ce résultat suggère que TGF-β joue un rôle de stimulateur des mécanismes d'induction de tolérance et n'est pas un mécanisme en lui-même (Gagne et al., 2001).

Les effets bénéfiques du sérum anti-classe II sur la survie de greffe de cœur ont été montrés en utilisant des exosomes (Peche et al., 2003). Les exosomes, originaires des DC de moelle osseuse, présentent une grande quantité de molécules CMH de classe I et de classe II et de molécules co-stimulatrices et ils peuvent imiter les cellules du donneur et moduler la réponse immunitaire. Le traitement avec 10 µg d'exosomes originaires des DC de moelle osseuse du donneur (injectés 14 et 7 jours avant la transplantation) résulte en une prolongation de la survie de la greffe cardiaque. Apres la transplantation, les receveurs prétraités avec les exosomes, augmentent la quantité des anticorps anti-CMH de classe II et le rejet de greffe est retardé. En conclusion on peut dire que les exosomes ont induit l'inhibition de rejet de greffe de cœur (Peche et al., 2003).

Différents hypothèses tentent d'expliquer le mode d'action des anticorps dirigés contre le CMH de classe II du donneur. Dans le cœur naïf, les seules cellules qui expriment les molécules CMH de classe II sont les DC (Spencer and Fabre, 1990) (Forbes et al., 1986) et les anticorps peuvent jouer de manière passive et inhiber la reconnaissance directe entre les DC de LEW.1W et les cellules T de LEW.1A. Cette hypothèse correspond au résultat d'une étude où il a été montré que l'interaction des cellules CD4+ du receveur avec les molécules CMH de classe II du donneur a été critique pour le rejet (Campos et al., 1995). Différentes expériences discutées précédemment indiquent l'importance de la présence des cellules DC en protocole anti-classe II. Notre hypothèse est que les anticorps dirigés contre le CMH de classe II du donneur vont jouer un rôle actif et induire

des signaux régulateurs après l'interaction avec des DC du donneur. Aussi le sérum anti-classe II peut opsoniser les DC du donneur et les cibler pour le processus de phagocytose. Finalement les DC tolérogéniques peuvent induire l'anergie des cellules T effectrices et/ou la transformation des cellules T effectrices en Treg (Mahnke et al., 2002).

IV.3.2. Les protocoles de l'induction de la tolérance de la greffe de coeur

IV.3.1.1. L'induction de la tolérance par la transfusion spécifique du donneur (donor specific transfusion - DST)

En utilisant la même combinaison des souches, LEW.1W et LEW.1A, la survie du greffon à long terme peut être obtenue par DST à 14 et 7 jours avant la transplantation (Soulillou et al., 1984). Les publications précédentes de notre laboratoire élucident les mécanismes et les caractéristiques du protocole DST. «Priming» avec le sang du donneur influence la fonction des cellules Th (T helper) (Bugeon et al., 1992) (Josien et al., 1995) et nécessite la présence de DC intactes du donneur au moment de la transplantation (Josien et al., 1998b). Une production précoce de TGFβ (Josien et al., 1998a) ainsi qu'une différentiation de Treg CD8+ (Douillard et al., 1999) (Vignes et al., 2000) sont aussi impliquées. Les greffons à long terme montrent les signes de rejet chronique (Lair et al., 2007) et les cellules T $CD25^-$ originaires de la rate ont des capacités régulatrices. Les cellules T $CD25^-$ des rats tolérants accumulent l'ARNm de l'IFN-γ, l'IL10 et de FoxP3. L'activité suppressive des $CD25^-$ dépend du contact avec les cellules (cell – cell interaction) et de facteurs solubles comme IL10 ou IFN-γ. Aussi, les

$CD25^-$ ont la capacité de transférer la tolérance (Degauque et al., 2006). Ces résultats montrent que le CD25 n'est pas forcement un marqueur des Treg en périphérie (Stephens and Mason, 2000). Pour appuyer cette conclusion, d'autres études montrent que les cellules $CD4^+CD25^+$ après cinq divisions n'expriment plus le CD25 mais conservent une capacité suppressive importante (Gavin et al., 2002). Chez lez animaux tolérants à long terme, notre dernière étude a caractérisé différents compartiments immuns et leur capacité de transférer la tolérance (Lair et al., 2007). Les splenocytes et les cellules T de la rate transfèrent la tolérance à long terme à chaque fois tandis que le sang la transfère dans la moitié des cas et les cellules T du sang ne la transfèrent pas. Ce résultat suggère donc que le sang n'est pas un réservoir de Treg (Lair et al., 2007).

Les mécanismes immunologiques de la tolérance, induite par la DST, posent toujours beaucoup de questions. Les mécanismes peuvent inclure la délétion clonale (Terasaki, 1984), l'anergie (Dallman et al., 1991), la génération de Treg (Bushell et al., 2003), la régulation de la production des cytokines (Yang et al., 1998) ou le microchimérisme (Wood, 2003). Une caractéristique importante de ce protocole est la présence des signes de rejet chronique. En DST, le «switch» des cytokines Th1 (importantes dans l'induction de tolérance) à Th2 (associées avec le développement du rejet chronique) a été identifié comme étant important (Pirenne et al., 2005). Une autre hypothèse suggère que dans ce modèle de tolérance le nombre de cellules $CD4^+CD25^+$ est insuffisant pour protéger le greffon du rejet chronique.

IV.3.1.2. L'induction de tolérance par le LF15-0195

La tolérance de l'allogreffe de cœur peut être induite par vingt jours de traitement avec LF15-0195, analogue de la deoxyspergualine (DSG) (Chiffoleau et al., 2002). Ce protocole est caractérisé par l'accumulation de cellules T $CD4^+CD25^+$ dans le greffon et dans le thymus (Chiffoleau et al., 2002) (Chiffoleau et al., 2002) (Heslan et al., 2005). Dans le greffon, différents types d'analyses ont confirmé l'accumulation des molécules ayant un rôle cytoprotecteur comme l'Indoleamine 2, 3-dioxygenase (IDO), Nitric Oxide Synthase (NOS) et Heme-Oxygenase-1 (HO-1) (Heslan et al., 2006)). Une accumulation dans le greffon de cellules $CD4^+CD25^+FoxP3^+$ et $CD4^+CD25^+FoxP3^-$ ainsi qu'une stimulation des cellules endothélialles par des mécanismes IDO et IFN-γ dépendant sont observées dans le transfert de tolérance avec les cellules $CD4^+$. Ces résultats suggèrent l'interaction entre les Treg, les cellules T effectrices et les APC dans le greffon. Ces interactions vont conduire à une situation de tolérance (Thebault et al., 2007).

IV.3.1.3. L'inhibition de la co-stimulation CD40-CD40L

Le transfert de gène pour CD40Ig induit une survie de greffe de cœur indéfinie entre les souches LEW.1W et LEW.1A (Guillet et al., 2002). Une étude récente de notre laboratoire montre que le blocage CD40-CD40L induit le développement des Treg $CD8^+CD45RC^{low}$ permettant la survie du greffon (Guillonneau et al., 2007). Les expériences de transfert de $CD8^+CD45RC^{low}$ confirment la capacité régulatrice de ce type cellulaires (Guillonneau et al., 2007).

Dans ce modèle, IDO est fortement exprimée dans les cellules endothéliales du greffon. Le rôle d'IDO est déjà confirmé dans la tolérance. IDO dégrade le tryptophane et les produits de dégradation peuvent induire l'apoptose des cellules T ou l'anergie (Mellor and Munn, 2004) (Munn et al., 2005). IDO peut être produite par différents types cellulaires dont les DC et les cellules endothéliales. Cette production est stimulée principalement par l'IFN-γ (Mellor and Munn, 2004) (Beutelspacher et al., 2006). Dans le protocole du transfert de gène de CD40Ig, la neutralisation de l'IFN-γ ou d'IDO conduit à l'absence de tolérance (Guillonneau et al., 2007).

V. La tolérance opérationnelle en clinique

Aujourd'hui, la transplantation est le traitement de choix pour les patients dont les organes se trouvent dans une phase de dysfonction terminale. Il y a deux facteurs principaux qui limitent la survie du greffon: le rejet chronique irréversible et les effets secondaires des médicaments immunosuppressifs. Cependant, dans certains cas très rares, il est possible d'obtenir une fonction normale et stable du greffon en absence totale d'immunosuppresseur. En clinique, un patient greffé qui est ***tolérant***, ne prenant aucun traitement immunosuppresseur, montre une fonction normale du greffon (avec l'histologie normale) et il conserve une réponse immunitaire intacte (Tisone et al., 2007). Malheureusement, on a très peu de connaissance pour expliquer quels sont les éventements, au niveau du système immunitaire, importants pour établir un état de tolérance (VanBuskirk et al., 2000). Il est très difficile d'identifier un seul médiateur ou type cellulaire nécessaire pour les phénomènes immunologiques comme le rejet ou la tolérance. Les facteurs tels que le type d'organe transplanté, la sensibilisation du receveur causée par des infections précédentes ou un traitement d'immunosuppresseur peuvent influencer la survie du greffon (Martinez and Rosen, 2005).

Il faut bien distinguer la tolérance opérationnelle en clinique de la tolérance tenue par immunosuppression minimale - tolérance «prope». Le mot «prope» vient de Latin et le terme «prope = presque» signifie les patients greffés, avec une fonction et une histologie normale, sous immunosuppression minimale (Calne, 2004).

La tolérance opérationnelle en clinique a été rapportée plus souvent chez les patients ayant subi une greffe du foie que pour d'autres types d'organes. Les études cliniques (Takatsuki et al., 2001) (Martinez-Llordella et al., 2007) (Koshiba et al., 2007) (Tisone et al., 2007) et les expériences in vivo (Kamada and Calne, 1983) (Qian et al., 1994) (Kataoka et al., 2003) ont caractérisé le foie comme étant un organe immuno-privilègié. En clinique, les derniers résultats montrent que l'arrêt progressif d'immunosuppression est possible chez 20% de patients greffés hepatiques (Martinez-Llordella et al., 2007).

La tolérance opérationnelle chez les patients greffés rénaux est très rare (Roussey-Kesler et al., 2006). Dans ce groupe de patients, l'arrêt progressif d'immunosuppression est dû à des effets secondaires des médicaments ou à la non complaisance des patients (Roussey-Kesler et al., 2006). Pendant les trente dernières années, plusieurs études ont été publiées sur la tolérance opérationnelle d'allogreffe rénale (Zoller et al., 1980) (Owens et al., 1975) (Uehling et al., 1976) (Christensen et al., 1998). Malheureusement, on ne peut pas dire que le bon protocole immunosuppressif, induisant une tolérance opérationnelle, existe.

Cependant, les derniers résultats obtenus avec le Belatacept sont plutôt encourageant. Le Belatacept est un médicament bloquant simultanément le CD80 et le CD86 à la surface des APC. Les dernières études montrent que Belatacept peut préserver le taux de la filtration glomérulaire et réduire le taux de CAN (chronic allograft nephropathy) (Vincenti et al., 2005).

VI. Biomarqueur de la tolérance en transplantation

Le «Biomarquers Definitions Working Group» a défini le biomarqueur comme une caractéristique mesurée et évaluée objectivement et utilisée comme un indicateur d'un processus biologique normal, un processus pathologique ou pour suivre la réponse pharmacologique après intervention (2001). Un bon biomarqueur doit avoir la capacité de discriminer différents états de maladie par rapport à un état de référence (dans la plupart des cas l'état de référence représente l'état sain). Les biomarqueurs traditionnels sont le glucose pour le diabète (Miedema, 2003), le cholestérol pour les maladies cardiaques (Hawkins, 2004) ou la troponine spécifique des infarctus (Eisenman, 2006).

En transplantation, l'identification de biomarqueurs peut aider à mettre en évidence l'échec ou la réussite d'un traitement immunosuppresseur. Une bonne sélection des biomarqueurs doit permettre l'optimisation de la stratégie immunosuppressive (Ashton-Chess, in press). Aujourd'hui, les biopsies représentent le meilleur standard «the gold standard» pour les biomarqueurs. Les nouvelles recherches pour les biomarqueurs vont inclure des méthodes moins invasives pour l'obtention de biomatériaux: les fluides en contact avec le greffon (l'urine par exemple) ou du sang dans le cas idéal. En effet, les biofluides qui sont en contact étroit avec le site de la maladie, représentent les meilleurs échantillons biologiques pour les biomarquers (Ashton-Chess, in press).

VI.1. Les échantillons biologiques

VI.1.1. L'urine

L'urine est un très bon échantillon biologique que l'on peut utiliser pour la surveillance de la fonction de greffe rénale. Granzyme B et FoxP3 sont utilisés comme les biomarqueurs dans le monitorage du rejet de greffe rénale (Veale et al., 2006) ou pour prévoir les résultats de rejet aigu de greffe rénale (Muthukumar et al., 2005)

VI.1.2. Le sang périphérique

Le sang périphérique représente un autre biomarqueur facilement accessible. Le profil d'expression des PBMC (peripheral blood mononuclear cells) a été utilisé pour distinguer les patients avec une greffe cardiaque en état «d'inactivité» (le rejet du greffon au niveau 0) des patients qui sont en état de rejet modéré à sévère. Dans cette étude, 11 gènes ont été sélectionnés pour prévoir le devenir du greffon (Deng et al., 2006). L'équipe de Martinez-Liordella a analysé trois groupes de patients transplantés hépatiques: 16 patients avec une tolérance opérationnelle, 16 patients sous immunosuppresseur et 10 individus sains. En utilisant la technique des puces à ADN, ces auteurs ont identifié certains gènes (gènes codant les transcrits de cellules T γδ et de cellules NK) qui distinguent les patients tolérants des patients sous immunosuppresseurs - (Martinez-Llordella et al., 2007).

La dernière étude réalisée au sein de notre laboratoire a identifié un panel de biomarqueurs qui sont associés avec la tolérance opérationnelle chez des

patients avec une allogreffe rénale. 75 patients ont été étudiés et le profil d'expression des gènes originaires des PBMC a été comparé aux situations suivantes: la tolérance opérationnelle, le rejet chronique/aigu, les greffe stables sous l'immunosuppression et 16 individus sains. La comparaison des échantillons du sang entre les patients tolérants,les patients présentant des signes de rejet chronique et les patients avec fonction de greffon stable, trois groupes de gènes ont été identifiés. Le groupe 1 suggère une activation du système immunitaire réduite chez les patients tolérants. Le groupe 2 comprend deux catégories de gènes sous-exprimés: les gènes codant pour les molécules de fixation à l'ARN et les gènes impliqués dans la traduction des signaux. Finalement, Le groupe 3 contient les gènes qui régulent le cycle cellulaire (les gènes qui sont exprimés pendant la mitose ou les gènes impliqués dans les processus énergétiques). Plus précisemment, cette étude indique les gènes cibles du TGF-β. L'expression du TGF-β est équivalente entre les patients tolérants et les patients présentant des signes de rejet chronique. Cependant, le TGF-β a été découvert comme un régulateur de 27% des gènes identifiés dans le sang périphérique qui sont différemment exprimés entre les patients tolérants et les patients présentant des signes de rejet chronique.

VI.1.3. Les biopsies de greffon

Les biopsies présentent une autre possibilité de surveiller la fonction de la greffe et la question qui se pose aujourd'hui est «faire les biopsies ou non» (Thaunat et al., 2007). Cependant, l'intervention semble relativement sûre, et avec cette technique on peut détecter les changements précocement et protéger la fonction

du greffon (Wilkinson, 2006). Les biomarqueurs, provenant d'une biopsie, sont définis par rapport à la classification de Banff et présent aujourd'hui le meilleur standard diagnostic pour déterminer l'état de l'organe greffé (Solez et al., 2007). Grâce aux biopsies on peut identifier le rejet aigu et le rejet chronique au moment où est encore possible de sauver l'organe transplanté. Les biopsies donnent des résultats intéressants, parce qu'avec cette méthode de surveillance les signes de rejet sub-clinique ont été détectés chez 30% de greffés rénaux au cours des six premiers mois post-transplantation (Jain et al., 2000) (Roberts et al., 2004). Le diagnostic précoce de CAN peut être confirmé grâce aux biopsies (Dimeny et al., 1995) (Seron et al., 2000) et cette méthode a été efficace chez les greffés rénaux pédiatriques (Fujisawa et al., 1999). Chez les patients à risques (par exemple les patients avec une fonction retardée de greffe rénale) la biopsie est une méthode importante pour la protection du greffon. Les études montrent que la moitié des épisodes de rejet aigu, chez les patients avec une fonction retardée de greffe rénale, sont détectés juste par les biopsies (Jain et al., 2000). Chez les patients ayant subi une greffe du foie le processus de fibrose est surveillée par les biopsies. La corrélation positive entre la fibrose aigue et la septicémie est établie, parce que la septicémie représente la cause principale de mort chez les patients ayant une greffe de foie (Yilmaz et al., 2007).

VI.2. L'identification de biomarqueurs

Aujourd'hui plusieurs techniques d'identification de biomarqueurs existent et ces techniques peuvent être utilisées pour prévoir le devenir d'un greffon. L'analyse de la dynamique d' accumulation de cytokines intra greffe représente la limite

fine entre le rejet et l'acceptation de greffe de cœur chez les rongeurs (Holzknecht and Platt, 2000). Le polymorphisme génétique pour les différentes cytokines est corrélé avec la réussite de greffe de foie chez les enfants. Un niveau bas de TNF-α et un niveau intermédiaire ou haut pour IL-10 a été montré chez les patients tolérants ou chez les patients «prope» par rapport à des malades sous immunosuppression standard (Mazariegos et al., 2002). Le ratio entre les différents précurseurs des cellules dendritiques est un autre indicateur du devenir de l'allogreffe. DC monocytoide (DC1) et DC plasmocytoide (DC2) polarisent la réponse de cellules T vers le phénotype immunogénique (Th1) ou le phénotype tolérogénique (Th2). Chez l'homme et chez le rat, le ratio DC1/DC2 peut être utilisé pour prévoir le devenir du greffon (pour la revue cf (Coates and Thomson, 2002)). ELISPOTassay est une autre technique pour déterminer la fréquence de l'IFN-γ et de l'IL-10 dans cellules réactives contre le donneur. Les cellules viennent du sang du receveur et cette technique a été proposée comme un marquer subrogé d'alloréponse, essentiellement en transplantation rénale (Augustine et al., 2005) (Hricik et al., 2003). Pour la cytometrie de flux, on peut dire que c'est une technique la plus souvent utilisée en terme de nombre de patients (Ashton-Chess, in press). Plusieurs études ont identifié les $CD4^{+}CD25^{high+}$ circulantes dans le maintien de la tolérance opérationnelle chez les patients pédiatrique (Li et al., 2004) et adultes (Martinez-Llordella et al., 2007) ayant subi une greffe du foie. Les résultats qui viennent des patients avec des signes de rejet chronique de greffe de rein montrent un nombre réduit de $CD4^{+}CD25^{high+}$ par rapport aux patient tolérants, patients sous immunothérapie

et individus sains (Louis et al., 2006). Aussi les marqueurs comme CD27+ et CD28+ ont été étudiés pour la prédiction du devenir de la greffe rénale chez l'homme (Baeten et al., 2006).

Chez les rongeurs l'attention a été focalisée sur des cellules qui ont la capacité de transferer la tolérance. Le succès a été confirmé pour CD4+CD25+ et CD4+CD25- (Kitade et al., 2005), CD25- (Degauque et al., 2007), CD8+CD45RClow (Guillonneau et al., 2007) et pour CD8+CD45RC- (Kitade et al., 2005). L'importance de CD103+ DC en transfert de tolérance a également été confirmé (Degauque et al., 2006). Les autres types cellulaires sont discutés plus en détaille en l'Anexe1 dans l'article «Transfer of tolerance to heart and kidney allografts in the rat modèle»(Jovanovic et al., in press).

Pendant la réalisation de ma thèse, les puces à ADN et la PCR quantitative ont beaucoup été utilisées pour identifier les gènes qui sont importants pour comprendre les mécanismes de tolérance dans le modèle de greffe de rein chez le rat «modèle l'anti-class II». Pour cette raison, ces deux techniques sont discutées plus en détail.

VI.2.1. La technique des puces à ADN – l'outil pour chercher des biomarqueurs en transplantation

Cette technique représente un système de dépistage à grande échelle qui mesure simultanément l'expression de milliers de gènes dans un certain échantillon (Ashton-Chess, in press). Cette méthode peut être utilisée pour identifier des «empreintes moléculaires» d'un état biologique particulier. En immunologie de transplantation, la puce à ADN est utilisée pour rechercher des

biomarqueurs du rejet chronique chez les patients ayant subi une greffe du rein (Flechner et al., 2004), (Mas et al., 2007) (Sarwal et al., 2003) ou de poumon (Gimino et al., 2003). Chez les patients tolérants avec une greffe du foie (Martinez-Llordella et al., 2007) ou du rein (Brouard et al., 2007) la puce à ADN a également été utilisée. Les analyses à grande échelle doivent permettre d'identifier les gènes candidats pour la prédiction du devenir du greffon. Cette technique va permettre de personnaliser l'immunothérapie, d'indiquer les possibilités d'arrêt du traitement immunosuppressif ou de donner le temps de réagir aux patients subissant un rejet.

Chez les rongeurs, les puces à ADN ont été utilisées pour identifier des gènes et des voies de signalisation impliqués dans la tolérance et le rejet du greffon. Les études détaillées ont été faites sur un modèle de greffe de cœur chez le rat (Heslan et al., 2006) (Kitagawa-Sakakida et al., 2005) (Liu et al., 2005) (Garin et al., 2007), un modèle de greffe de rein chez le rat (Kusaka et al., 2007) ou sur un modèle de greffe de cœur chez la souris (Saiura et al., 2001).

VI.2.2. L'application de la PCR quantitative en prédiction du devenir de l'allogreffe

De la même façon que les techniques de dépistage à grande échelle, on peut aussi utiliser les techniques de dépistage à petite échelle pour rechercher des biomarqueurs en transplantation – par PCR quantitative par exemple. La PCR quantitative a été utilisée dans plusieurs études chez l'homme. Chez les patients transplantés rénaux, la mesure de l'ARNm de FoxP3 dans l'urine peut aider dans la prédiction du rejet aigu (Muthukumar et al., 2005). Dans les biopsies rénales,

Fas ligand a été aussi utilisé comme biomarqueur d'un rejet aigu résistant à la thérapie (Nickel et al., 2001). Dans le même type de biopsie, le niveau d'expression des gènes codant pour Fas ligand, perforine, granzyme B a été utilisé comme biomarqueur (Strehlau et al., 1996). Chez les patients ayant eu une greffe rénale, la comparaison du niveau d'ARN dans les biopsies et dans le sang montre que l'expression de Granzyme B et du HLA-DRA est un biomarqueur du rejet de la greffe rénale (Sabek et al., 2002). Tous ces résultats identifient précisément des biomarqueurs de rejet du greffon. L'identification de biomarqueur, dans les matériaux obtenu par les techniques non invasives (prélèvement du sang ou d'urine par exemple), est très encourageante et donne l'espoir d'améliorer les méthodes existantes.

Quelques résultats intéressants sont aussi obtenus chez le rat. Dans le greffe du cœur chez la souris et le greffe du reine chez le rat pour les gènes TOAG-1 (tolerance associated gene 1) et α-1,2 une forte expression a été détectés pendant l'induction et le maintien de l'acceptation de greffon. Pour ces deux gènes, une sous régulation a été identifié pendant la phase de rejet (Sawitzki et al., 2007). Les études de notre laboratoire identifient une accumulation de transcrits d'IDO et de FoxP3 dans les greffes tolérantes du cœur et du rein chez le rat (Degauque et al., 2006) (Guillonneau et al., 2007) (Heslan et al., 2006). Ces études montrent un état d'accommodation de greffes tolérantes et suggèrent un rôle important d'IDO et d'FoxP3 dans le maintien de la tolérance à long terme. Les études du développement de CAN chez la souris montrent une surexpression de CTGF (connective tissue growth factor). Ces résultats

suggèrent que CTGF est potentiellement un biomarqueur de CAN (Cheng et al., 2006). Dans les greffes de la voie respiratoire chez la souris, une surexpression d'ARNm d'IL-13 a été corrélée avec le développement de lésions obstructives (Lama et al., 2006). Globalement, les études discutées précédemment doivent nous aider à mieux comprendre quels processus sont derrière les états de tolérance ou de rejet de greffon. Tous nos efforts doivent aboutir à la découverte de biomarqueurs fiables de la tolérance, de biomarqueurs communs entre les différentes espèces qui vont permettre de prévoir le rejet d'allogreffe.

Résultats

Article 1

L'implication de Matrix Matalloprotéinase 7 et la voie de signalisation non-classique WNT dans le modèle de la tolérance de greffe du rein induite par l'injection des anticorps contre le MHC de classe II du donneur

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Implication of matrix metalloproteinase 7 and the noncanonical wingless-type signaling pathway in a model of kidney allograft tolerance induced by the administration of anti-donor class II antibodies.

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In rats, tolerance to MHC-incompatible renal allografts can be induced by the administration of anti-donor class II Abs on the day of transplantation. In this study we explored the mechanisms involved in the maintenance phase of this tolerance by analyzing intragraft gene expression profiles by microarray in long-term accepted kidneys. Comparison of the gene expression patterns of tolerated to syngeneic kidneys revealed 5,954 differentially expressed genes ($p < 0.05$). Further analysis of this gene set revealed a key role for the wingless-type (WNT) signaling pathway, one of the pivotal pathways involved in cell regulation that has not yet been implicated in transplantation. Several genes within this pathway were significantly up-regulated in the tolerated grafts, particularly matrix metalloproteinase 7 (MMP7; fold change > 40). Analysis of several other pathway-related molecules indicated that MMP7 overexpression was the result of the noncanonical WNT signaling pathway. MMP7 expression was restricted to vascular smooth muscle cells and was specific to anti-class II Ab-induced tolerance, as it was undetectable in other models of renal and heart transplant tolerance and chronic rejection induced across the same strain combination. These results suggest a novel role for noncanonical WNT signaling in maintaining kidney transplant tolerance in this model, with MMP7 being a key target. Determining the mechanisms whereby MMP7 contributes to transplant tolerance may help in the development of new strategies to improve long-term graft outcome.

IMPLICATION OF MATRIX METALLOPROTEINASE 7 AND THE NON-CANONICAL WNT SIGNALING PATHWAY IN A MODEL OF KIDNEY ALLOGRAFT TOLERANCE INDUCED BY THE ADMINISTRATION OF ANTI-DONOR CLASS II ANTIBODIES

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Abbreviations

MMP7 - matrix metalloproteinase 7

DKK3 - dickkopf homolog 3

NFATc1 - nuclear factor of activated T-cells cytoplasmic 1

MHC - major histocompatibility complex

WNT - wingless-type

LEW - lewis

DST - donor-specific blood transfusion

FC -fold change

PCR - polymerase chain reaction

HPRT - hypoxanthine phosphoribosyltransferase

FITC - fluorescein isothiocyanate

TNFα - tumor necrosis factor alpha

TGFβ - transforming growth factor β

SD – standard deviation

AXIN - axis inhibitor

APC - adenomatous polyposis coli

CK1 - casein kinase 1

GSK3β – glycogen synthase kinase 3β

LRP - low-density lipoprotein receptor related protein

PLC - phospholipase C

PKC - protein kinase C

ADAM - adisintegrin and metalloprotease

SMC – smooth muscle cells

Abstract

In rats, tolerance to MHC incompatible renal allografts can be induced by administration of anti-donor class II antibodies on the day of transplantation. Here we explored the mechanisms involved in the maintenance phase of this tolerance by analyzing intra-graft gene expression profiles by microarray in long-term accepted kidneys. Comparison of the gene expression patterns of tolerated to syngeneic kidneys revealed 5954 differentially expressed genes ($p<0.05$). Further analysis of this gene set revealed a key role for the WNT signaling pathway, one of the pivotal pathways involved in cell regulation that has not yet been implicated in transplantation. Several genes within this pathway were significantly up-regulated in the tolerated grafts, particularly Matrix Metalloproteinase 7 (Fold Change>40). Analysis of several other pathway-related molecules indicated that MMP7 over-expression was the result of the non-canonical WNT signaling pathway. MMP7 expression was restricted to vascular smooth muscle cells (SMC) and was specific to anti-class II antibody-induced tolerance as it was undetectable in other models of renal and heart transplant tolerance and chronic rejection induced across the same strain combination. These results suggest a novel role for non-canonical WNT signaling in maintaining kidney transplant tolerance in this model, with MMP7 being a key target. Determining the mechanisms whereby MMP7 contributes to transplant tolerance may help in the development of new strategies to improve long-term graft outcome.

Introduction

Despite improvements in renal allograft survival over the last three decades, the half-life of renal allografts has increased only marginally, largely due to late graft injury resulting from drug-related nephrotoxicity and chronic rejection (1-4). Such chronic injury is poorly influenced by currently used immunosuppressors. Moreover, life-long immunosuppression puts transplant patients at a higher risk of infection and malignancy (5). Thus, inducing donor-specific tolerance, i.e. indefinite survival of a well-functioning graft in an immunocompetent adult host in the absence of immunosuppression, is a major goal in transplantation. A number of tolerance trials are currently taking place, but there are still many challenges to face before tolerance can be achieved routinely in clinic practice (6).

In rodents, a variety of maneuvers can induce donor-specific allograft tolerance, including pre-transplantation priming with donor MHC antigens (blood or splenocytes) (2) (7) (8) (9-11), transfected cells expressing donor MHC antigens (12, 13), MHC gene transfer (14, 15) or DNA vaccination (16).

We have previously shown that donor-specific tolerance to MHC mismatched renal allografts in adult rats can also be induced by administration of antibodies directed against donor MHC class II to recipients on the day of transplantation (17) (18, 19). These animals display normal and stable kidney function and their grafts display normal histology with no signs of chronic rejection (19). Although numerous studies have shown the importance of MHC II signaling in the regulation of APC activity and fate (20), the mechanisms responsible for maintaining this anti-MHC II antibody-induced tolerant state remain to be fully elucidated. Here we took advantage of genome-wide transcriptome analysis to further explore these mechanisms. By analyzing intragraft gene expression and networks 100 days after transplantation in tolerated kidneys compared to syngeneic controls, we were able to identify a major involvement of several gene pathways, including that of WNT signaling, a pathway

known to control a variety of processes such as cell migration, polarity or differentiation (21). Deregulation of this pathway has been reported in numerous human diseases (21) but to date it has never been implicated in the field of immune tolerance. One of the targets of this pathway, Matrix Metalloproteinase 7 (MMP7, also known as Matrilysin, MAT, MPMM, MPSL1 and PUMP-1), was explored further in tolerated and rejecting rat heart allografts. We show that in rodents, MMP7 upregulation is governed by a non-canonical WNT pathway and is specific to renal transplant tolerance induced by anti-donor class II treatment, where it is located on vascular smooth muscle cells within the graft. These results reveal a novel role for MMP7 in allograft responses. Determining the mechanisms whereby MMP7 contributes to transplant tolerance may help in the development of new strategies to improve long-term graft outcome.

Materials and Methods

Rodent transplant models

Surgical procedures, treatments and experimental groups

Inbred male adult rats (200–250g) of the LEW.1A (RT1a) and LEW.1W (RT1u) congenic strains were purchased from Janvier (Le Genest-Saint-Isle, France) and maintained in an animal facility under standard conditions according to the European and Institutional Guidelines.

Kidney transplantation: The model used was that of kidney allotransplantation from LEW.1W donors to MHC-mismatched LEW.1A recipients receiving 0.5 mL IV of anti-LEW.1W class II alloimmune serum (prepared as described (18) on the day of transplantation (n=12)). At day 100 post transplantation, these animals tolerate a skin graft from the same donor and their kidney grafts show no histological signs of chronic humoral rejection (19). Controls included a group of LEW.1A recipients of LEW.1A kidneys (syngeneic transplants; n=5) and a group of untreated LEW.1A recipients of LEW.1W kidneys (allogeneic, untreated; n=3). Kidney transplantations were performed aseptically and a binephrectomy was performed 7 days after transplantation as previously described (17). Rejection, indicated by the death of the binephrectomized rat, was confirmed by histology. Blood urea and creatinine and urine protein: creatinine ratios were measured throughout the post-transplant period. Blood urea <8 mmol/L and blood creatinine <40 mmol/L were considered as normal (19).

Heart transplantation: The model used was that of heart allotransplantation from LEW.1W donors to MHC-mismatched LEW.1A recipients receiving either a donor-specific blood transfusion (DST) 7 and 14 days prior to transplantation (2) or a 20-day course of the deoxyspergualin analogue LF015-095 (22). At day 100 post transplantation, the grafts of

DST-treated animals show clear-cut histological signs of chronic humoral rejection (chronic transplant vasculopathy) (10) whereas those of LF015-095-treated animals do not and are thus tolerated (22, 23). Analyses were performed on DST-treated (n=3) and LF-treated recipients (n=3) at day 100 post transplantation. Syngeneic transplants (LEW.1A donors to LEW.1A recipients) were performed as controls (n=3). Heterotopic cardiac allografts were performed as previously described (24).

Sample preparation

Organs were harvested at day 100 (tolerant/syngeneic kidney allografts) or 1 day before rejection (acutely rejected kidney allografts). Blood was collected by cardiac puncture into a heparinized syringe and peripheral blood mononuclear cells were separated on a Ficoll® gradient then stored in TRIzol® reagent (Invitrogen, Cergy Pontoise, France). Sections of the spleen and kidney graft were either snap frozen in liquid nitrogen for future RNA extraction, or embedded in O.C.T. compound (Tissue Tek; Miles Laboratories, Elkhart, IN) and snap-frozen in liquid nitrogen for future immunohistochemistry analysis. Blood cell contamination was avoided by perfusing the organ with PBS. All samples were stored at –70°C until use.

RNA extraction and reverse transcription

Rat samples: Total RNA from rat kidney grafts and PBMC was prepared using the TRIzol® (Invitrogen, Cergy Pontoise, France) extraction method. RNA quantity and quality were systematically determined using a nanodrop spectrophotometer and an Agilent 2100 bioanalyzer. Ten µg of total RNA was set aside for microarray analyses (see below). The remaining RNA was treated with DNase (Roche, Indianapolis, USA) to remove genomic DNA and reverse transcribed into first strand cDNA using polydT oligonucleotide and Moloney murine leukemia virus reverse transcriptase (Invitrogen).

Microarray experiments

RNA preparation, amplification and hybridization: 10 µg of total RNA were cleaned up using Rneasy columns (QIAGEN). RNA quantity and quality were determined using a nanodrop spectrophotometer and an Agilent 2100 bioanalyzer. A rRNA 28S/18S ratio of 1.0 ±0.1 was considered as acceptable for RNA amplification. The Applied Biosystems (Applied Biosystems, Foster City, CA, USA) rat Genome Survey Microarray (P/N 4337467) used contained 26,857 60-mer oligonucleotide probes representing 27,088 individual rat genes. An additional 3400 control spots were present on the chip to cover various steps in the hybridization process. Digoxigenin-UTP labeled cRNA was generated and amplified from 0.5 µg of total RNA from each sample using an Applied Biosystems Chemiluminescent NanoAmp RT-IVT Labeling Kit (P/N4365715). Array hybridization was performed for 16 hours at 55°C. Chemiluminescence detection, image acquisition and analysis were performed using the Applied Biosystems Chemiluminescence Detection Kit (P/N 436875D), Analyzer (P/N 4338036) and v-1.1 analyzer software (P/N 4336391) according to the manufacturer's protocol.

Microarray analysis: Microarray raw data were analyzed by R - language and environment for statistical computing and graphics. Genes were identified using the Panther™ Protein Classification System Probe ID database. Raw data were analyzed according to p value and Fold Change (FC). FC corresponds to the ratio of the signal intensity of the gene in tolerant rats to the signal intensity of the gene in syngeneic rats. FC was calculated for genes that fulfilled the criterion of significance at $p<0.05$ (Figure 1A) and the genes were then processed using software available on www.pantherdb.org. Genes were classified according to their signaling pathway, molecular function or biological process. For each category, the

first twenty up-regulated and down-regulated genes were chosen and matched whenever they belonged to the same group (Table I A, B and C).

Real-time quantitative PCR

Real-time quantitative PCR was performed in an Applied Biosystems GenAmp 7700 Sequence Detection System using SYBR-Green PCR Core Reagents as previously described (19) or TaqMan® Gene Expression Assays according to the manufacturer's instructions (Applied Biosystems). Rat primer and probe sets were either designed: rTCF-7 Up CCAGAACTCATCCTTCAGCATTT, rTCF-7 Lo GGAACCATGTCCACCACAAAG; rβ arrestine1,2 Up ACTATGCCACAGACGACGACAT, rβ arrestine1,2 Lo CCCTCTTCCTAGCAGAACTGGT; rHPRT Up CCTTGGTCAAGCAGTACAGCC, rHPRT Lo CGCTGATGACACAAACATGA; NFATc1 Up GGAGATGGAAGCGAAAACTGA, NFATc1 Lo TAAGTCCCTGGCTCATTGGTC, or purchased from Applied Biosystems: DKK3 Rn00593415_m1, AXIN2 Rn00577441_m1, CADHERIN13 Rn00594145_m1, HPRT Rn01527838_g1, MMP7 Rn00563467_m1. HPRT was used as an endogenous control gene to normalize varying starting amounts of RNA. Relative expression between a given sample and a control sample, used for all experiments, was calculated with the $2^{-\Delta\Delta Ct}$ method (ABI PRISM 7900 user bulletin, PE Applied Biosystems, Foster City 2:11–24, 1997). All samples were analyzed in duplicate. Expression of genes of interest was compared between tolerated animals and syngeneic controls.

Immunohistochemistry

Cryostat sections of 5 µm were cut, air-dried and fixed in acetone for 10 min. Sections were then either labeled by a two-step immuno-fluorescent technique, using rabbit anti-rat MMP7 (Calbiochem, Darmastad, Germany) as the primary antibody and donkey anti-rabbit FITC

(Jackson ImmunoResearch, Baltimore, USA) as the secondary antibody or by a three-step indirect immuno-peroxidase technique as described in (25) with the same primary and secondary antibodies. In order to confirm MMP7 expression by smooth muscle cells we performed double staining using a two-step immuno-fluorescent technique using mouse anti-rat alpha smooth muscle Actin (Abcam, Cambridge, UK) and rabbit anti-rat MMP7 (Calbiochem, Darmastad, Germany) as primary antibodies. Anti-rabbit FITC (Jackson ImmunoResearch, Baltimore, USA) and goat anti-mouse Alexa Fluor® 568 (Invitrogen, Oregon, USA) were used as a secondary antibodies.

Statistical analysis

The non parametric Mann-Whitney and Kruskal-Wallis tests were used to compare two or three or more groups, respectively. Differences were defined as statistically significant when $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

Results

Differential gene expression patterns in tolerated vs. syngeneic kidney grafts 100 days after transplantation

We have previously shown that a single administration of anti-donor MHC class II alloimmune serum on the day of transplantation results in indefinite survival of MHC-mismatched kidney grafts. The kidney grafts of tolerated animals display normal function and have no histological evidence of chronic rejection (19). Here the gene expression patters of 3 tolerated and 3 syngeneic kidney allografts were analyzed using pangenomic Genome Survey Microarrays (P/N 4337467). Of the approximately 27000 genes present on the microarray, 26857 genes were found to be expressed and were subsequently analyzed by R software. Of these 26857 expressed genes, 5954 were found to be significantly differentially expressed between the two biological situations ($p<0.05$; Figure 1). Analysis of these 5954 genes using the Probe ID database from the Panther™ Protein Classification System (www.pantherdb.org) enabled their organization according to signaling pathways (Table IA), molecular function (Table IB) and biological process (Table IC). According to this analysis, the most significantly up and down regulated genes in tolerance belonged to the categories of inflammation chemokine and cytokine-mediated signaling pathway, integrin signaling, the WNT signaling pathway, PDGF signaling, angiogenesis, as well as EGF receptor, interleukin and apoptosis signaling (Table IA). Certain major categories consisted of genes that were up-regulated only e.g. T cell activation, and G-protein, TLR and B cell signaling (Table IA). The majority of the up- and down-regulated genes belonged to the molecular function categories of nucleic acid binding, receptors, transcription factors and regulatory molecules, whereas again, certain functional categories were up-regulated only, e.g. defense/immunity proteins, signaling molecules and ribosomal proteins (Table IB). The highest ranking biological

processes were signal transduction, protein/nucleic acid metabolism and immunity/defense (Table IC), suggesting that tolerance is correlated with an active intragraft immune response (19).

Signaling pathways distinguish tolerated from syngeneic kidneys: the WNT pathway and its downstream molecular target MMP7

The two highest ranking pathways were that of inflammation and integrin signaling, whose roles in transplantation are already well known. The third highest ranking pathway, however, has to our knowledge, never been described in transplantation. This WNT signaling pathway implicated 45 up-regulated and 26 down-regulated genes, of which three molecules were expressed at least 10 fold more in tolerated vs. syngeneic kidneys: MMP7 (matrix metalloproteinase 7; FC>40), TCF7 (transcription factor 7, T-cell specific; FC>15) and DKK3 (dickkopf homolog 3; FC>12). DKK3 and TCF7 are molecules involved in WNT signal transduction whereas MMP7 is a metalloproteinase whose expression is regulated by this pathway (26). Although several other members of the MMP family (MMP2, MMP12, MMP16, MMP19, and MMP23) were also up-regulated in the tolerated kidneys, the biggest fold change was observed for MMP7 (FC>40; Figure 2A). Significant upregulation ($p<0.05$) of MMP7 in tolerance was confirmed by quantitative PCR performed on a further 12 tolerated kidneys (>100 days post transplantation), 5 syngeneic grafts (>100 days post transplantation), as well as 3 naive kidneys that expressed no MMP7 and 3 acutely rejected kidneys at day 11 ±1 post transplantation that expressed MMP7 only moderately (Figure 2B).

Involvement of the non canonical WNT signaling pathway in the kidney grafts of tolerated recipients

We next set out to understand the mechanisms governing MMP7 expression in our model. Previous studies have shown that MMP7 expression can be controlled by the canonical WNT signaling pathway (27, 28) (illustrated in Figure 3A and B). Briefly, in the absence of WNT, or if the WNT pathway is inhibited by DKK, β-catenin becomes phospohorylated by destruction complex and degraded by the proteasome. When WNT is present, it binds to Frizzled receptor and the co-receptors, preventing β-catenin degradation. β-catenin enters the nucleus and binds TCFs (and additional nuclear proteins) and thereby activates target genes, including MMP7. We thus analyzed the expression of several molecules involved in the canonical WNT signaling cascade. We thereby identified upregulation of genes that block the canonical WNT pathway (WNT inhibitor-DKK3, the members of the destruction complex - Axin2 and β-arrestin) and genes whose expression is typical of activated canonical WNT signaling (TCF7 and the target gene MMP7). Interestingly, in addition to MMP7, all of these molecules, which are members of the canonical WNT signaling pathway, were significantly increased in the tolerated kidneys according to both microarray (Figure 3C) and quantitative PCR (Figure 3D). Moreover, β-catenin, a key molecule in regulating MMP7 expression, was undetectable. The fact that the level of WNT pathway inhibitors was increased as well as MMP7 indicates the involvement of the non canonical WNT signaling pathway.

Regulation of MMP7 by the non-canonical WNT signaling pathway in tolerated kidneys

In addition to the canonical WNT pathway, there is also a so-called non-canonical pathway (illustrated in Figure 4A). This pathway is activated by ligand binding to the Frizzled receptor, leading to release of intracellular calcium, possibly via G-proteins. Activation of this pathway in our model of tolerance is coherent with the fact that G-protein signaling

pathways were one of the major pathways upregulated in tolerated kidneys, accounting for 52 genes (Table IA). This pathway involves activation of phospholipase C (PLC) and protein kinase C (PKC). Elevated Ca^{2+} can activate the phosphatase calcineurin, which leads to dephosphorylation of the transcription factor NFATc1 and its accumulation in the nucleus (29, 30). Most components of the non-canonical WNT signaling pathway were detected by the microarray, including WNT6 (FC>3), Frizzled6 (FC>2), Frizzled2 (FC>2.5) and NFATc1 (FC>3) (Figure 4B). Up-regulation of one of the key molecules in the non-canonical WNT signaling pathway, NFATc1 (which enters the nucleus and activates c-myc (31)), was additionally confirmed by quantitative PCR (Figure 4C). Thus, altogether our data suggest that the high MMP7 expression (FC>40) in tolerated kidney grafts is the result of non-canonical WNT signaling.

Regulation of MMP7 in the tolerated kidneys and hearts in the same strain combination following different protocols of tolerance induction

To determine whether MMP7 up-regulation was tissue-specific, we next compared MMP7 expression in kidney grafts and spleens from tolerant animals and syngeneic controls. As shown in Figure 5A, MMP7 transcripts were only detected in tolerated kidney grafts, being very weak in the other compartments (spleen and blood). Kinetic studies also showed that MMP7 expression in tolerant kidneys was time dependent with a high level of transcript as soon as day 25 after transplantation (24.8±8.7 fold tolerant vs syngenic) (*data not shown*). We then asked whether MMP7 up-regulation was specific to tolerated kidneys or could also be relevant to tolerated hearts. We therefore compared MMP7 expression in tolerated kidneys to that observed in long-surviving heart grafts (day 100 post transplantation) transplanted in the same allogeneic strain combination (LEW.1W to LEW.1A). To induce heart allograft tolerance, we used different protocols: donor-specific blood transfusion (DST)

7 and 14 days prior to transplantation (grafts showing clear histological signs of chronic rejection) (10) or a 20-day course of the deoxyspergualin analogue LF015-095 (grafts showing no signs of rejection i.e. tolerated) (22). The results clearly show an absence of MMP7 expression in heart grafts from both DST-treated (chronic rejection) and LF-treated (tolerated) recipients at this time (Figure 5B), suggesting that MMP7 expression is specific to kidney graft tolerance induced by the anti-donor MHC class II protocol.

MMP7 expression by tolerated kidney vascular smooth muscle cells

Localization of MMP7 was next assessed by immunostaining of syngeneic vs. tolerated kidneys using immunoperoxidase (Figure 6A, 6B) or immunofluorescence (Figure 6C) techniques. Within the tolerated kidney allografts, MMP7 protein was found to be expressed primarily by the smooth muscle cells of blood vessels (Figure 6B and Figure 6C). This was confirmed using double staining for MMP7 and alpha actin as a marker for the smooth muscle cells. Photos of red fluorescence (alpha actin staining, Figure 6D) and green fluorescence (MMP7 staining, Figure 6E) were superimposed (Figure 6F) and orange fluorescence indicated that MMP7 was expressed by the smooth muscle cells.

Discussion

In this study, we further explored the mechanisms involved in the maintenance phase of tolerance to kidney allografts induced by administration of anti-donor class II serum to rat recipients of renal allografts on the day of transplantation (19). By analyzing intragraft gene expression profiles of tolerated compared to syngeneic kidneys 100 days post transplantation using microarray technology, we show that the maintenance phase of tolerance brings into play multiple gene pathways and biological processes. In particular, our data reveal an potentially important role for a signaling pathway that has not previously been described in transplantation, that of WNT signaling, as well one of its key targets, MMP7 (26), which was up-regulated 40 fold in tolerated kidneys.

WNT signaling, which is initiated by binding of members of the WNT family of glycoproteins to their respective receptors (also known as Frizzled proteins), is known to be involved in the regulation of cell growth and differentiation (28). Two main WNT signaling pathways exist, the so-called canonical and the less classical non-canonical pathways. The canonical pathway, when activated, results in a complex signaling cascade ultimately leading to β-catenin-mediated gene transcription. In this pathway's resting state, β-catenin is degraded by the proteasome. The non-canonical pathway is a Ca^{2+}-dependant pathway thought to act through G-protein-coupled receptors. Activation of this pathway results in gene transcription via the transcription factor NFATc 1 (nuclear factor of activated T-cells cytoplasmic 1). Although the precise and relative roles of both pathways in mammals are largely unknown, deregulation of WNT signaling has been reported in a number of human pathologies, from cancer to cardiovascular disease (32) (33) (34). So far, its role in transplantation is unknown. However, our findings of an upregulation of the potential WNT inhibitor DKK3 (35) as well as other inhibitory molecules acting downstream within the canonical pathway, such as Axin2 (36), together with the absence of β-catenin, suggest that

this pathway was not responsible for MMP7 up-regulation in the tolerated kidneys. On the other hand, the over-expression of components of the non-canonical pathway such as, Frizzled 6, Frizzled 2 and NFATc1, in tolerated kidneys indicates that MMP7 expression was driven by this alternative pathway. These results thus imply a role for non-canonical WNT signaling in maintaining kidney transplant tolerance in this model, with MMP7 being a key target.

MMP7 is the smallest matrix metalloproteinase (37), a family of enzymes classically described as being responsible for the turnover and degradation of connective-tissue proteins (38, 39) and for the turn-over, degradation, catabolism and destruction of the extracellular matrix (40, 41). The time-dependant upregulation of MMP7 transcripts in tolerated kidneys (no expression on day 0 and increasing expression on day 25 to day 100 after transplantation compared to syngeneic or acutely rejected grafts) suggests that this molecule may play a role in maintaining tolerance. Interestingly, this role appears to be specific to the anti-donor class II model of kidney transplantation as no up-regulation of MMP7 was observed in other models of kidney transplant tolerance in the same strain combination (anti-CD28 tolerance induction protocol (42) - *data not shown*) or in models of heart tolerance or chronic rejection (LF015-095 and DST protocols) (22) (43) (10). We have already shown in the laboratory that different mechanisms may operate following different tolerance induction protocols, even in the same genetic LEW.1W to LEW.1A background (9). Indeed, whereas the anti-MHC II antibody-induced tolerant state can be successfully transferred to naïve recipients by both T cells and $CD103^+$ cells (19), transfer failed in the same kidney allograft model when tolerance was induced using anti-CD28 antibodies (42). In the heart allograft model, Degauque et al showed that in donor specific transfusion-induced tolerance, spleen $CD25^+$ T cells from tolerant rats were unable to transfer tolerance into naïve rats (9), whereas Chiffoleau et al provided evidence that 20-day treatment with LF15-0195, a deoxyspergualin analogue,

induced spleen $CD4^+CD25^+$ cells able to transfer tolerance (44). Finally, in the same heart allograft model, when recipients were treated with CD40Ig, only the adoptively transferred $CD8^+CD45RC^{low}$ subset resulted in donor-specific long-term survival, whereas $CD8^+CD45RC^{low}$ T cells from naive animals did not (45). All these examples show that, in the same allograft model, different protocols lead to specific and distinct mechanisms of tolerance induction and maintenance.

The mechanisms whereby MMP7 contributes to tolerance maintenance require further investigation. One possibility is that MMP7 helps to maintain tolerance through protective effects, since MMPs have been shown to contribute to tissue repair (46). Another possibility is that MMP7 acts by regulating cellular transmigration to the graft. Indeed, MMPs can act on non-matrix proteins, such as cytokines and chemokines, to potentate their activity (47). As such, MMPs have been shown to regulate inflammatory cell influx by determining bioavailability of chemokines (47, 48). Thus, MMP7-deficient mice are more sensitive to acute lung injury after administration of a chemotactic peptide (49). Although the tolerated grafts display a larger infiltrate than their syngeneic counterparts (19), MMP7 may serve to regulate the type of cells entering the graft, perhaps favoring a "friendly" infiltrate, as previously suggested (19). Along these lines, MMP7 has been shown to direct cell migration rather than just movement per-se (49). Interestingly, the regulation of cell transmigration has been reported to be not only due to the MMP7 proteinase but also to other proteinases such as MMP2, MMP12 and the ADAM17 molecule (50) which were up-regulated (>11 fold, >8 fold, and >2.8 fold respectively) in the tolerated kidney grafts vs. syngeneic controls.

Another hypothesis is that MMP7 cleaves particular substrates releasing molecules with tolerogenic activity. Along these lines, MMP7 deficient mice have been reported to reject a skin allograft significantly earlier than wild-type mice, possibly through its cleavage of FasL and TNFα (Herbert et al., communication at the European Congress in Immunology, 2006).

Perhaps even more relevant to our findings, however, is the fact that MMP7 has been described as one of the most potent MMPs in its ability to degrade the ubiquitous proteoglycan Decorin, thereby releasing TGF-β1 (51), a cytokine known for a long time for its immunomodulatory capacities (52) (53) and its involvement in transplant tolerance (54). Supporting this hypothesis, in the same LEW.1W to LEW.1A strain combination, we have previously shown that TGF-β1 is significantly increased in rat heart allografts with prolongation of graft survival in the first days following transplantation (18). TGF-β1 was also upregulated in this present study, in the tolerated rat kidney allografts >100 days post transplantation (*data not shown*). Furthermore, in the same model of tolerance induction to mismatched kidney grafts, anti-TGF-β mAb breaks the tolerance process (18). Finally, it is important to note that MMP7 has already been detected in SMC in the media and in the stenotic region in patients with supravalvular aortic stenosis and Williams Beuren syndrome and that remodeling of the stenotic part of the aorta has been shown to be an adaptative mechanism to preserve a correct function of the aorta (55).

To conclude, we report here, for the first time, a role for non-canonical WNT signaling in the state of kidney allograft tolerance induced by administration of anti-donor class II antibodies in rats. We also report on a new potential role for MMP7, a target of this pathway, in maintaining tolerance in this model. Determining the underlying mechanisms of this pathway and MMP7 may help in the design of strategies to improve long-term graft outcome.

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Legends to Figures

Figure 1. Microarray assessment of differential gene expression between tolerant and syngeneic kidney allografts. Visualization of the 5954 differentially expressed genes according to microarray analysis between 3 tolerant (recipients treated with anti-donor MHC class II alloimmune serum) and 3 syngeneic kidney allografts at >100 days post transplantation. Statistical analyses were performed on Fold Change (FC), which corresponds to the ratio of the signal intensity of the gene in tolerant rats to the signal intensity of the gene in syngeneic rats. Statistically significant ($p<0.05$) FC were calculated and are represented as Log2 as a function of the average Log2 signal. The color code represents degree of FC: light blue = 1.0 – 1.2; green = 1.2 – 1.6; dark blue = 1.6 – 2.0; black = 2.0 – 4.0; red = >4.16.

Figure 2. Differential expression of MMP7 and other MMP family members in rat kidney allografts. A. Fold change in expression of MMP-2, MMP-7, MMP-12, MMP-16, MMP-19 and MMP-23 in tolerant (n=3) vs. syngeneic (n = 3) kidney allografts 100 days post transplantation according to microarray. **B.** MMP7 mRNA accumulation in naive, syngeneic (Day 100), tolerant (Day 100) and acutely rejecting (Day 11) rat kidney allografts, as measured by quantitative PCR. Results are expressed as mean ±SD of the $2^{-\Delta\Delta Ct}$ values for each group of samples. * indicates $p<0.05$; ** $p<0.01$.

Figure 3. Regulation of MMP7 by the canonical WNT signaling pathway. A. The inhibited canonical WNT signaling pathway. In the absence of WNT, β-catenin associates with a multiprotein destruction complex consisting of the tumour-suppressor gene products AXIN (axis inhibitor) and APC (adenomatous polyposis coli) and the serine/threonine kinases CK1 (casein kinase 1) and GSK3β (glycogen-synthase kinase 3β). β-Catenin is

phosphorylated by CK1 and GSK3β leading to its recognition by β-TRCP (β-transducin-repeat-containing protein), its ubiquitylation and its subsequent degradation by the proteasome. Members of the TCF (T-cell factor)/LEF (lymphocyteenhancer- binding factor) family are blocked in the nucleus by the repressors and are inactive. DKK3 (Dickkopf homolog 3) can also take part in the inhibition of the WNT canonical signaling pathway. **B.** The activated canonical WNT signaling pathway. WNT proteins bind their receptors, frizzled proteins, in addition to a co-receptor LRP5 or LRP6, resulting in the inactivation of GSK3β by Disheveled. β-Catenin then migrates to the nucleus where it binds TCFs to activate target genes. **C.** Expression of various molecules implicated in the canonical WNT pathway in tolerant and syngeneic kidneys 100 days post transplantation according to microarray. Results are expressed as fold change. **D.** Expression of various molecules implicated in the canonical WNT pathway, in tolerant and syngeneic kidneys 100 days post transplantation according to quantitative PCR. Results are expressed as means ±SD of $2^{-\Delta\Delta Ct}$ values. * $p<0.05$, ** $p < 0.01$.

Figure 4. The non-canonical WNT signaling pathway. A. Non-canonical WNT signaling leads to release of intracellular calcium, possibly via G-proteins. This pathway involves activation of phospholipase C (PLC) and protein kinase C (PKC). Elevated $Ca2^{+}$ can activate the phosphatase calcineurin, which leads to dephosphorylation of the Nuclear Factor of Activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFATc1) and its accumulation in the nucleus. NFATc1 activates c-myc, resulting in the induction of MMP7. **B.** Analysis of various molecules implicated in the non-canonical WNT signaling pathway in tolerant vs. syngeneic kidney allografts according to microarray (results expressed as fold change; n = 3 per group). **C.** Analysis of NFATc1 in tolerant (n = 12) vs. syngeneic (n = 5) kidneys

according to quantitative PCR (results expressed as mean +/SD of $2^{-\Delta\Delta Ct}$ values).* $p<0.05$, ** $p < 0.01$.

Figure 5. Expression of MMP7 in different immunological compartments of anti-class II-tolerant rats and grafts from other models of allograft tolerance or rejection in the same rat strain combination. A. MMP7 transcription in tolerant kidney (n = 12) at > 100 days post transplantation was compared to: syngeneic kidney day 100 (n=5), syngeneic spleen day 100 (n=3), tolerant spleen day 100 (n=5), syngeneic blood day 100 (n=3), tolerant blood day 100 (n=3). Results are expressed as mean ±SD of $2^{-\Delta\Delta Ct}$ values; *** $p < 0.001$ (Kruskal-Wallis test). **B.** MMP7 transcription in syngeneic (n = 5) vs. tolerant (n = 12) kidneys compared to syngeneic (n = 3), tolerant (n = 3) or chronically rejected (n = 3) heart allografts in the same strain combination (see M&M for details of induction protocols). Results are expressed as mean ±SD of $2^{-\Delta\Delta Ct}$; *** $p < 0.001$ (Kruskal-Wallis test).

Figure 6. Expression of MMP7 protein in tolerant kidney allografts. Staining of MMP7 in kidney grafts of syngeneic **(A)** and tolerant **(B)** animals > 100 days post transplantation using a three-step indirect immuno-peroxidase technique (X20 and X40 magnification) and of **(C)** tolerant allografts by immunofluorescence (X40 magnification). In order to confirm MMP7 expression by the smooth muscle cells, double staining for alpha actin (D) and MMP7 (E) was performed and images were superimposed (F).

Figure1

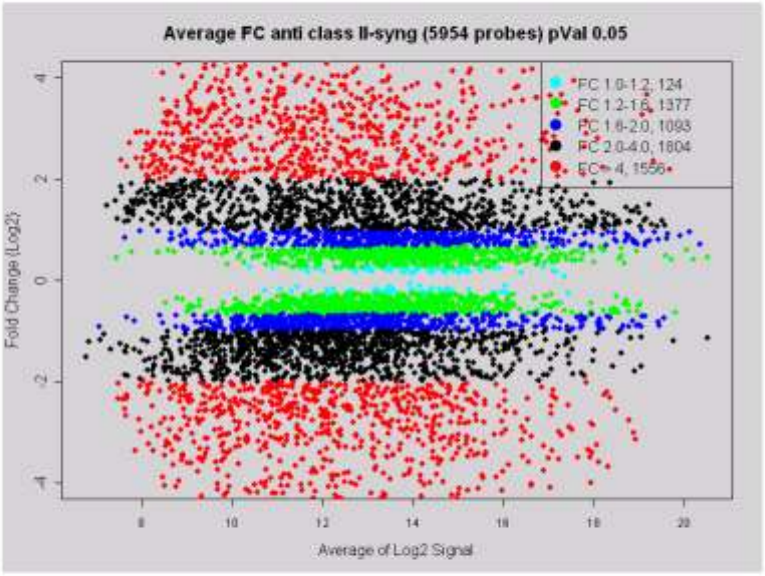
Average FC anti class II-syng (5954 probes) pVal 0.05
FC 1.0-1.2, 124
FC 1.2-1.6, 1377
FC 1.6-2.0, 1093
FC 2.0-4.0, 1804
FC > 4, 1556
Fold Change (Log2)
Average of Log2 Signal

Figure2

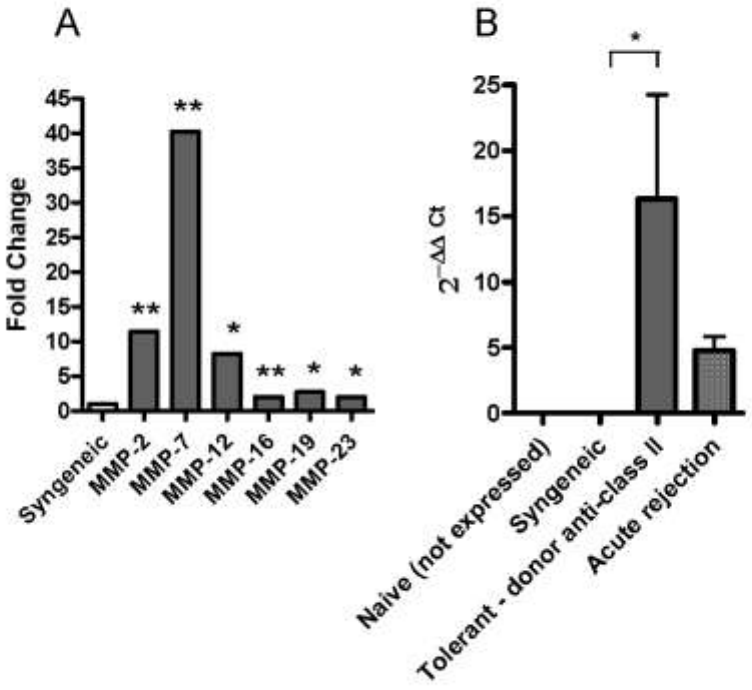
A
Fold Change
45
40
35
30
25
20
15
10
5
0
**
**
*
**
*
*
Syngeneic
MMP-2
MMP-7
MMP-12
MMP-16
MMP-19
MMP-23
B
$2^{-\Delta\Delta}$ Ct
25
20
15
10
5
0
*
Naive (not expressed)
Syngeneic
Tolerant - donor anti-class II
Acute rejection

Figure 3

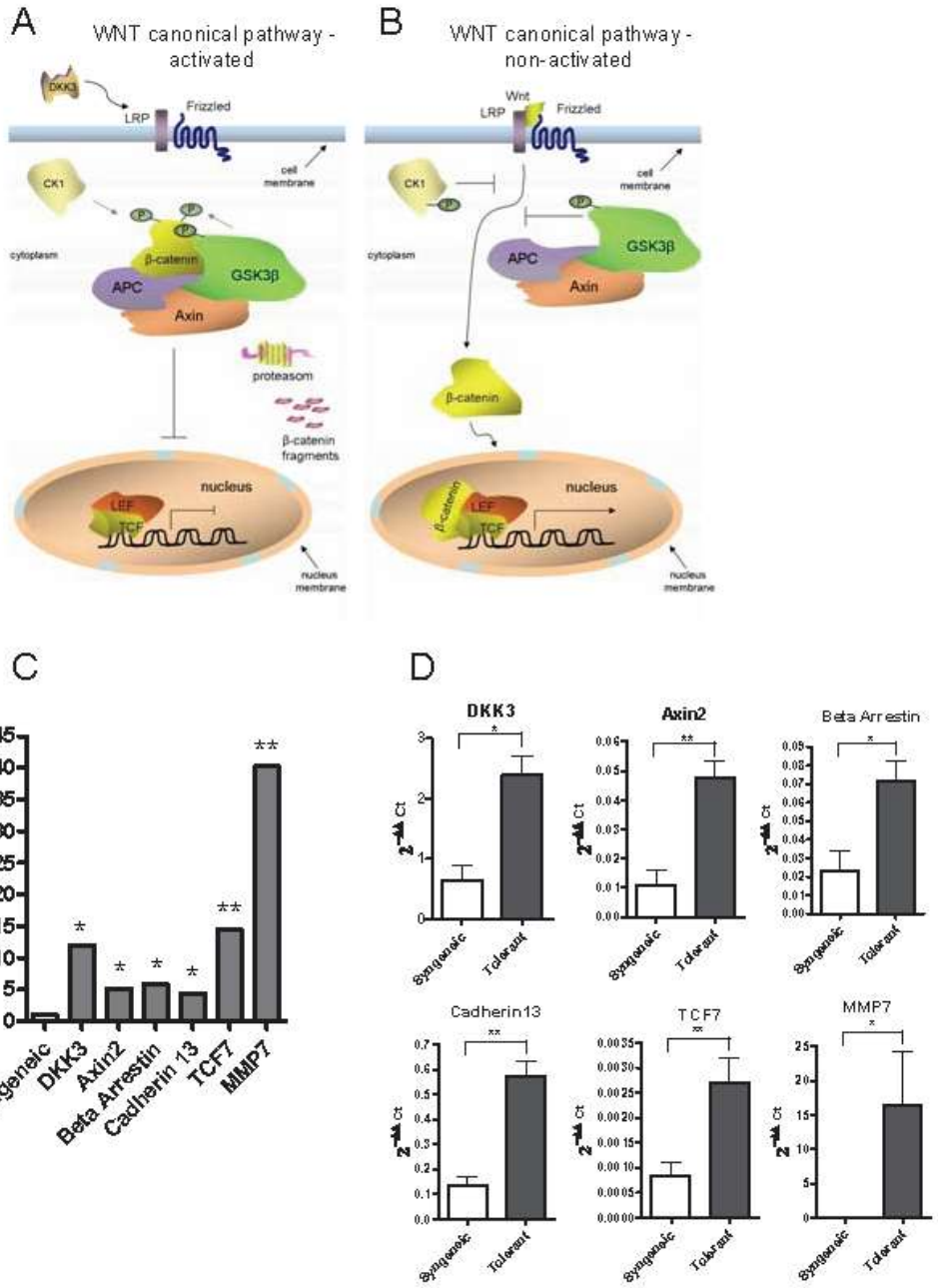
A
WNT canonical pathway - activated
DKK3
LRP
Frizzled
cell membrane
CK1
cytoplasm
β-catenin
GSK3β
APC
Axin
proteasom
β-catenin fragments
nucleus
LEF
TCF
nucleus membrane
B
WNT canonical pathway - non-activated
Wnt
LRP
Frizzled
cell membrane
CK1
cytoplasm
APC
GSK3β
Axin
β-catenin
nucleus
β-catenin
LEF
TCF
nucleus membrane
C
Fold Change
Syngeneic
DKK3
Axin2
Beta Arrestin
Cadherin 13
TCF7
MMP7
D
DKK3
Axin2
Beta Arrestin
Cadherin13
TCF7
MMP7
Syngeneic
Tolerant

Figure 4

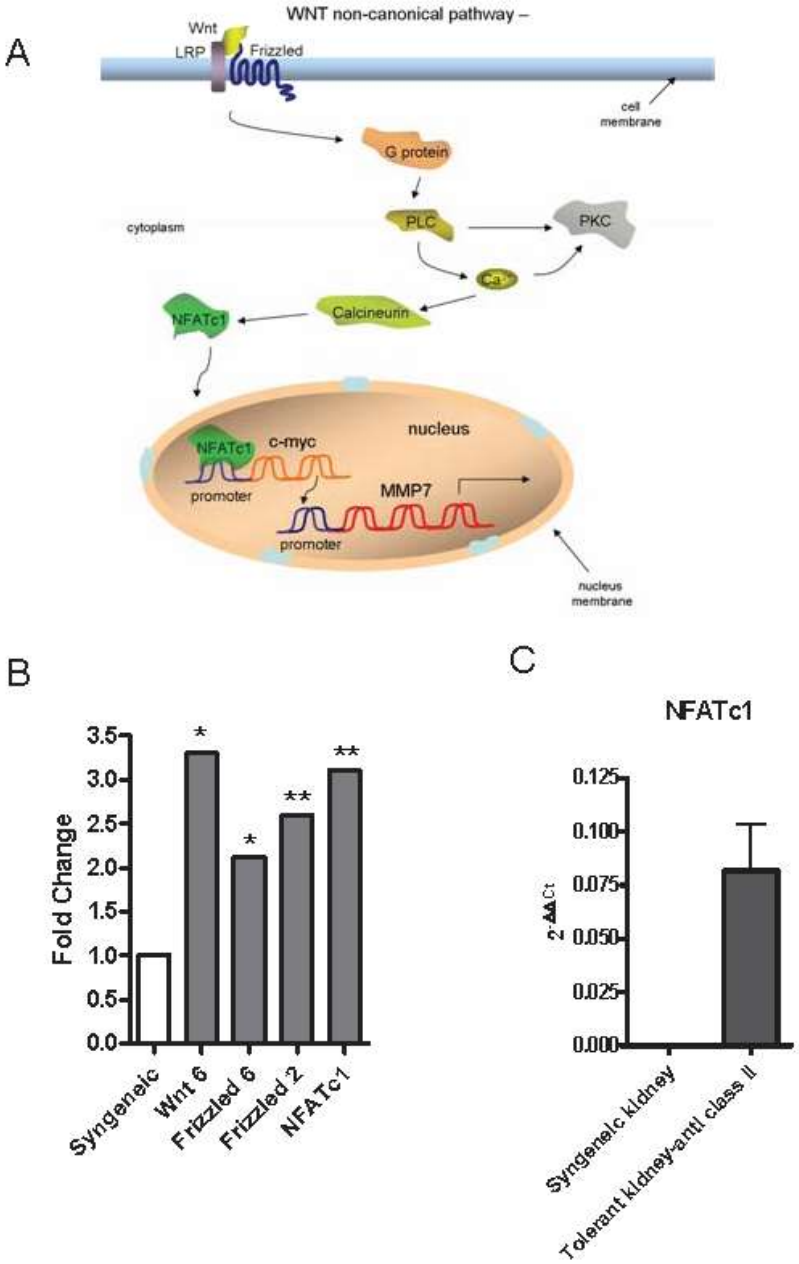
A
WNT non-canonical pathway –
Wnt
LRP
Frizzled
cell membrane
G protein
cytoplasm
PLC
PKC
Ca2+
Calcineurin
NFATc1
nucleus
NFATc1
c-myc
promoter
MMP7
promoter
nucleus membrane
B
Fold Change
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
**
**
Syngeneic
Wnt 6
Frizzled 6
Frizzled 2
NFATc1
C
NFATc1
2-ΔΔCt
0.125
0.100
0.075
0.050
0.025
0.000
Syngeneic kidney
Tolerant kidney-anti class II

Figure 5

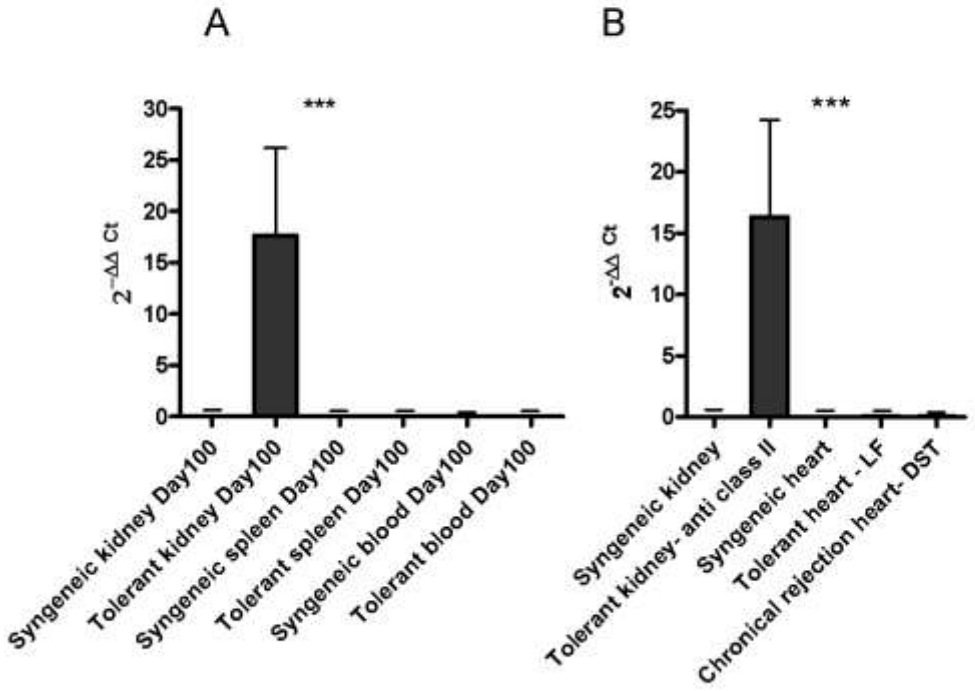
A
B
***
***
$2^{-\Delta\Delta}$ Ct
30
25
20
15
10
5
0
Syngeneic kidney Day100
Tolerant kidney Day100
Syngeneic spleen Day100
Tolerant spleen Day100
Syngeneic blood Day100
Tolerant blood Day100
Syngeneic kidney
Tolerant kidney- anti class II
Syngeneic heart
Tolerant heart - LF
Chronical rejection heart- DST

Figure 6

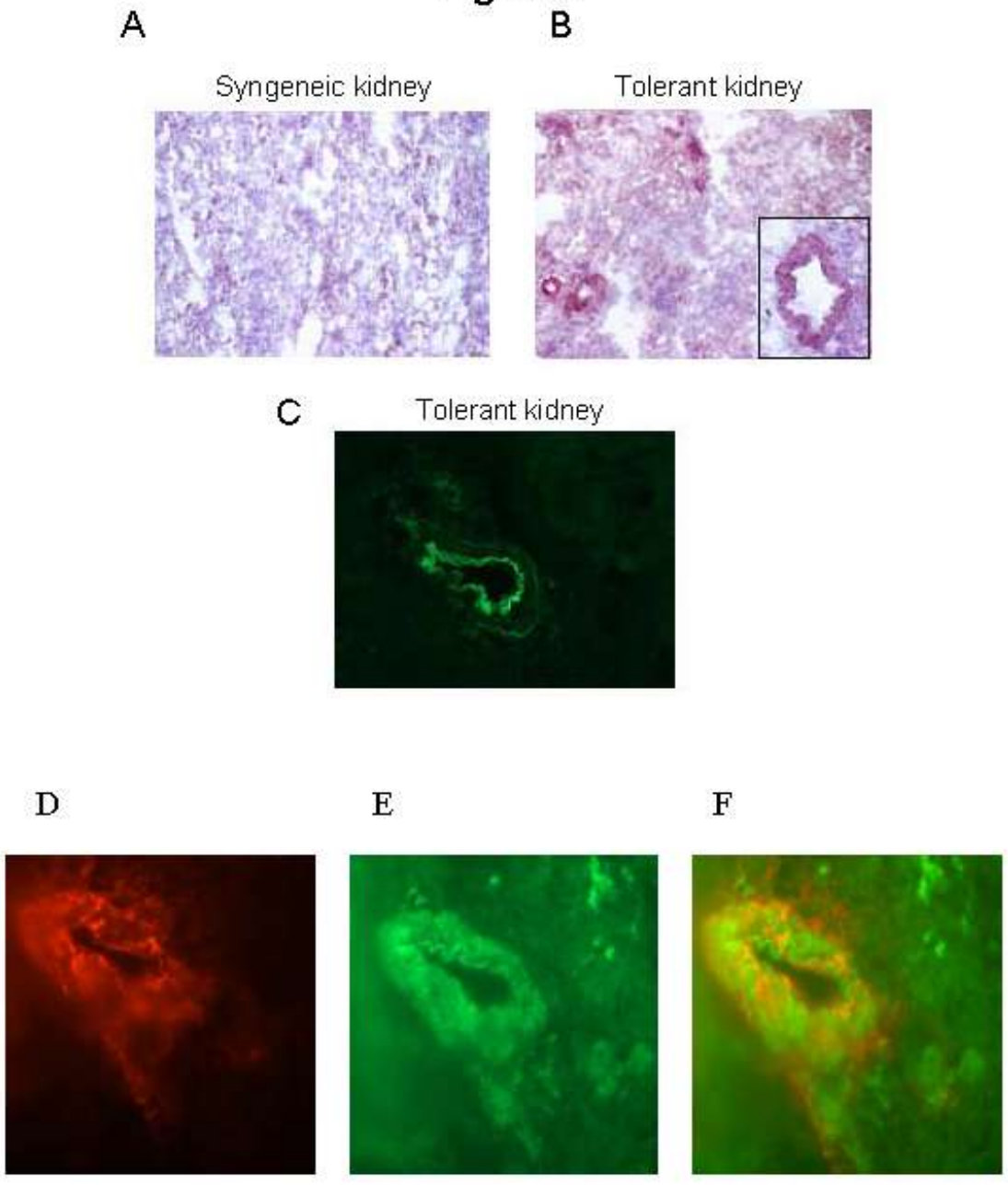

Table I. Organization of differentially expressed genes according to signaling pathways (A), molecular function (B) and biological process (C).

Table I A

Signaling Pathway	Number of up-regulated genes	Number of down-regulated genes
Inflammation mediated by chemokine and cytokine signaling pathway	76	24
Integrin signaling pathway	57	21
Wnt signaling pathway	45	26
PDGF signaling pathway	42	22
Angiogenesis	38	18
T cell activation	35	
EGF receptor signaling pathway	34	16
Apoptosis signaling pathway	31	15
Interleukin signaling pathway	31	13
Huntington disease	29	15
Heterotrimeric G-protein signaling pathway -Gi alpha and Gs alpha mediated pathway	27	
Heterotrimeric G-protein signaling pathway -Gq alpha and Go alpha mediated pathway	25	
Toll receptor signaling pathway	24	
PI3 kinase pathway	23	12
B cell activation	23	
FGF signaling pathway	22	16
Cytoskeletal regulation by Rho GTPase	22	
Parkinson disease	21	11
Ras pathway	18	
Endothelin signaling pathway	18	

Nicotinic acetylcholine receptor signaling pathway		14
Ubiquitne proteasome pathway		13
Adrenaline and noradrenaline biosynthesis		13
Cadherin signaling pathway		13
5HT2 type receptor mediated signaling pathway		13
De novo purine biosynthesis		12
Alzheimer disease-presenilin pathway		11
TCA cycle		11

2

Table I B

Molecular function	Number of up-regulated genes	Number of down-regulated genes
Nucleic acid binding	459	255
Receptor	220	116
Transcription factor	212	137
Select regulatory molecule	184	158
Defense/immunity protein	159	
Signaling molecule	129	
Ribosomal protein	129	
Cytoskeletal protein	126	81
Miscellaneous function	104	103
Transferase	93	162
Kinase	93	101
Protein kinase	83	
Protease	81	85
Hydrolase	79	158
Acid binding cytoskeletal protein	75	
Other transcription factor	75	
Extracellular matrix	66	
Other miscellaneous function protein	65	67
Transporter	62	176
G-protein modulator	62	
Oxidoreductase		238

Discussion

"*Implication of Matrix Metalloproteinase 7 and the non-canonical WNT signaling pathway in a model of kidney allograft tolerance induced by the administration of anti-donor class II antibodies*".

Précédemment nous avons montré que chez le rat, la tolérance des allogreffes rénales CMH-incompatibles peut être induite par l'administration le jour de la greffe d'anticorps anti-CMH II du donneur (Degauque et al., 2006) (Gagne et al., 2001) (Soulillou et al., 1976). Dans cette étude nous avons exploré quels mécanismes peuvent être impliqués dans la phase de maintien de la tolérance d'allogreffe.

En utilisant des puces à ARN exhaustives, nous avons analysé l'expression des gènes de greffons rénaux tolérés avec les greffons syngénique 100 jours après la transplantation. 5954 gènes, différentiellement exprimés ($p<0.05$), ont été identifiés et ces gènes ont été organisés en fonction de leur fonction moléculaire, de la voie de signalisation ou processus biologique dans lesquels ils sont impliqués. Avec cette stratégie, parmi les voies les plus altérées dans ce modèle de tolérance, nous avons identifié la voie de signalisation WNT. Plusieurs gènes appartenant à cette voie ont été significativement surexprimés dans les greffons tolérés, en particulier la protéine MMP7 (Matrix Metalloproteinase 7) – augmenté de 40 fois. De plus, dans notre modèle nous avons identifié suffisamment d'évidences impliquant que l'expression de MMP7 est réglée par, ainsi nommée, la voie de signalisation non-classique WNT. En générale, la voie de signalisation WNT est principalement impliquée dans le développement embryonnaire et la

voie de signalisation non-classique WNT est responsable des mouvements cellulaires et de la polarité des tissus (Katoh and Katoh, 2007). Une altération de la voie WNT est normalement corrélée avec la cancerogenèse (Lee et al., 1995) (Katoh and Katoh, 2006) (Satoh et al., 2000) (Lipkin and Afrasiabi, 2007). Les résultats de notre étude peuvent ouvrir un nouveau chapitre de la compréhension de la voie WNT, parce qu'à ce jour, le WNT a jamais été impliqué dans la tolérance d'allogreffe.

Outre MMP7, dans les greffons tolérants, nous avons identifié une sur expression de quelques autres membres de la famille MMP (MMP2, MMP12, MMP16, MMP19 et MMP23). Ces résultats peuvent élucider d'autres fonctions des MMP et des études récentes montrent que les MMP peuvent être avoir un rôle dans le raffinement postnatal, le remodeling et la neoangiogenèse (pour la revue cf (Page-McCaw et al., 2007)).

Pour MMP7 on peut dire qu'il a un double rôle. L'expression de MMP7 est corrélée avec les maladies de cancer (Liu et al., 2007) (Vairaktaris et al., 2007) (Kaneko et al., 2007) (Lynch et al., 2007), mais MMP7 est aussi impliqué dans les phénomènes d'immunité innée (naturel) (Lopez-Boado et al., 2000) et dans la cicatrisation de blessures (McGuire et al., 2003). En immunologie, MMP7 a sa propre place et cette enzyme peut réguler la migration cellulaire (Li et al., 2002). En fait, dans notre modèle les greffons montrent un infiltrat plus important que le contrôle syngénique (Degauque et al., 2006) et MMP7 peut également être impliqué dans la régulation des types cellulaires infiltrant le greffon et peut-être favoriser l'infiltrat «amical» (Degauque et al., 2006). Traditionnellement, les MMP

sont considérés comme les enzymes qui dégradent la matrice extracellulaire. MMP7 a été décrit comme étant une enzyme avec une grand habilité à dégrader la protéine proteoglycane Decorine et à libérer le TGF-β1 (Imai et al., 1997). Cette cytokine est déjà connue pour ses capacités immunomodulatoires (Wahl et al., 1989) et son engagement dans la tolérance en transplantation (Josien et al., 1998a). Dans notre laboratoire a été montré que l'expression élevée de TGF-β1 dans les greffes de cœur, est corrélée avec la prolongation de la survie du greffon les quelques premiers jours après la transplantation (Gagne et al., 2001). Les expériences avec le protocole «anti-classe II» mettent en évidence que l'induction de la tolérance est interrompue après l'application des anticorps anti-TGFβ1 (Gagne et al., 2001).

Le fait que dans notre modèle MMP7 soit exprimé par les cellules musculaires lisses indique que l'on a un processus de remodelage en cours et que ce processus appartient aux mécanismes adaptifs (Dridi et al., 2005). A notre connaissance, une seule étude confirme la corrélation entre le MMP7 et la tolérance d'allogreffe. Pour les souris MMP7 KO (knock-out) il a été confirmé qu'elles rejettent la greffe de peau plus tôt que les souris naives probablement grâce a clivage de FasL et TNFα (Herbert et al., les résultas présentées pendant le « European Congress in Immunology, 2006 »).

Finalement, notre étude montre que l'expression de MMP7 est spécifique du protocole et de l'organe. Les transcrits MMP7 ont été détectés uniquement dans les greffons ayant subi le protocole «anti-classe II» et pas dans le protocole «anti-CD28» (Vanhove et al., 2003). De la même façon, l'expression de MMP7

n’est pas détectée dans les greffes de cœur où la tolérance a été induite par le LF15-0195, un analogue de la deoxyspergualine (DSG) (Chiffoleau et al., 2002). Nous avons testé l’expression de MMP7 dans les différents compartiments chez les rats soumis au protocole «anti-classe II» et la présence de MMP7 a été confirmée dans le greffon mais pas dans le sang, dans la rate ou dans les cellules infiltrant le greffon (GIC – graft infiltrating cells).

Tous les résultats que l’on a obtenus peuvent apporter de nouveaux indices sur la voie de signalisation non-classique WNT et MMP7 dans la tolérance de greffe de rein. MMP7 et quelques autres molécules de a voie de signalisation non-classique WNT peuvent être utilisées comme les biomarqueurs de la tolérance dans les échantillons originaires de biopsies.

Enfin, je voudrais dire que cet article est le résultat d’une collaboration. ASD, JAC, JMH, AP m’ont aidé à faire les puces à ADN. Certains matériaux utilisés dans cette étude ont été fournis par mes collègues qui travaillent sur les différents protocoles d’induction de tolérance ND, DL, EC. Les procédures microchirurgicales ont été faites par CU et HS et les analyses histopathologiques par AM. MG, BV, JPS et SB m’ont aidé dans la préparation de ce manuscrit. Néanmoins, le travaille sur ce projet m’a donné l’opportunité d’appliquer un grand nombre de techniques de laboratoire et d’agrandir mes connaissances théoriques et pratiques.

Conclusions et perspectives

Chez le rat, la tolérance spécifique du donneur à la greffe de rein peut-être induite par l'injection d'anticorps dirigés contre le CMH de classe II du donneur le jour de la transplantation (Degauque et al., 2006) (Gagne et al., 2001) (Soulillou et al., 1976). Dans ce projet, nous avons identifié pour la première fois quelles voies de signalisation et quelles molécules sont impliquées dans le maintien de la tolérance. En utilisant des puces à ADN exhaustives, nous avons comparé les profils d'expression des gènes du greffon entre des reins tolérés et des reins syngéniques. Nous avons identifié des altérations dans la voie de signalisation non-classique WNT et la surexpression de MMP7. Plusieurs gènes impliqués la voie de signalisation non-classique WNT, dont MMP7, ont été confirmés par PCR quantitative. De plus, par l'utilisation de techniques immunohistologiques, nous avons confirmé l'expression de MMP7 au niveau de la protéine et nous avons montré que les cellules qui expriment MMP7 dans notre modèle sont les cellules musculaires lisses. Les aspects futurs de ce projet vont inclure des expériences *in vivo* et *in vitro*.

Les expériences *in vivo*

Ces expériences incluent deux stratégies:

1) La surexpression de MMP7 par un AAV8 (Adeno-Associated Virus serotype 8) chez les rats receveurs d'une greffe de rein (sans aucun traitement supplémentaire). Si avec cette stratégie nous parvenons à une prolongation de la survie du greffon, cela constituera un indice fort

pour un rôle important de MMP7 dans la tolérance de greffe rénale. Cette expérience nous permettra de confirmer qu'une expression précoce de MMP7 est nécessaire pour l'induction de la tolérance dans ce modèle.

2) Les expériences supplémentaires vont inclure des stratégies qui permettront de « casser » la tolérance induite par le protocole anti-classe II. L'activité de MMP7 sera inhibée par des inhibiteurs chimiques pouvant provoquer le rejet du greffon.

Les expériences *in vitro*

Les expériences *in vitro* incluent la stimulation de cellules musculaires lisses par le sérum «anti-classe II». Notre but est de vérifier si le sérum « anti-classe II » a un effet direct sur les cellules musculaires lisses et sur l'expression de MMP7. Cependant, les résultats obtenus dans les cellules musculaires lisses chez l'homme montrent que ce type de cellule exprime les antigènes de CMH de classe II dans la vasculitite expérimentale et dans la maladie athéromateuse (Stemme et al., 1990). La transplantation constitue une situation non naturelle au cours de laquelle la réponse immunitaire est provoquée et où les antigènes de CMH de classe II peuvent être exprimés par les cellules musculaires lisses. Il sera intéressant de tester l'expression du CMH de classe II sur les cellules musculaires lisses dans notre modèle et de tester l'effet direct du sérum anti-classe II TGF-β ou OX3 (anticorps anti-CMH II) sur les cellules

musculaires lisses. La présence du TGF-β a déjà été confirmée dans le modèle de tolérance induite par le sérum anti-classe II (Gagne et al., 2001). Cette expérience nous permettra de mieux comprendre l'effet ultime du TGF-β sur les cellules musculaires lisses. La présence des transcrits de MMP7 dans les cellules musculaires lisses stimulées sera également testée.

PSMB – Sous unités du protéasome (prosome, macropain)

Les expériences suivantes seront dirigées vers une compréhension plus profonde du rôle du protéasome dans notre modèle de tolérance. Les résultats des puces à ADN montrent une augmentation de l'expression des trois sous-unités du protéasome (prosome, macropain) PSMB8, PSMB9, PSMB10.

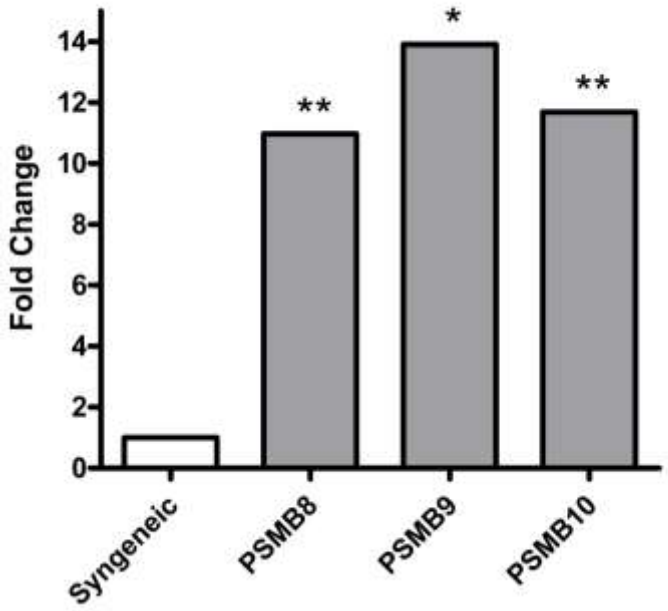

Figure 4: Surexpression des PSMB dans la greffe de rein (protocole anti-classe II).

Le rôle du protéasome a déjà été montré dans la transplantation de moelle osseuse et ce rôle est souvent corrélé avec les problèmes provoqués par la réaction du greffon contre l'hôte (Graft-versus-host disease, GVHD) (Fowler, 2006). De plus, le rôle du protéasome est également impliqué dans le processus de présentation croisée (Palmowski et al., 2006). Cependant, il sera intéressant d'aller plus loin et de mieux comprendre le rôle du protéasome dans la tolérance d'allogreffe, comme nos résultats le suggèrent. Afin d'obtenir de nouveaux éléments confirmant le rôle des PSMB dans la tolérance, les expériences suivantes seront réalisées : Tester l'expression des PSMB dans les différents protocoles d'induction de la tolérance (LF15-0195 et DST). La cinétique d'expression de PSMB8/9/10

sera étudiée dans la greffe de cœur. La présence de PSMB sera testée dans les différents compartiments (le sang, la rate, le thymus) et dans des situations différentes (greffes syngéniques, rejet chronique, rejet aigu, tolérance). De plus, l'expression de PSMB8/9/10 sera analysée dans le panel de lymphocytes activés ou au repos ($CD4^+CD25^+$, $CD4^+CD25^-$, $CD8^+$, cellules B, monocytes). Ces expériences doivent permettre de mieux comprendre le rôle des PSMB dans les phases d'induction et de maintien de la tolérance. En fonction des résultats obtenus pour les expériences *in vitro*, des expériences *in vivo* seront organisées.

Liste des figures

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Annexes

Annexe 1

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Transfer of tolerance to heart and kidney allografts in the rat model.

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Long-term allograft acceptance can be induced in the rat using a variety of maneuvers. One of the cardinal features of some models of tolerance is that once the tolerance state has been established, it can be perpetuated to naive recipients by the adoptive transfer of donor-specific regulatory cells. Such adoptive transfer studies have also addressed the capacity of T-cell subpopulations and non-T cells to transfer tolerance. However, tolerance cannot be transferred in all models. The underlying reasons for this are unclear with some studies pointing towards dose-dependent aspects and timing of expansion of T regulatory cells following tolerance transfer. Further exploration of this phenomenon will help us to understand better the mechanisms upon which allograft tolerance is based, and will provide new perspectives for further experimental studies.

TRANSFER OF TOLERANCE TO HEART AND KIDNEY ALLOGRAFTS IN THE RAT MODEL

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Key words: allograft, rat model, tolerance induction, tolerance transfer

Abstract

Long-term allograft acceptance can be induced in the rat using a variety of maneuvers. One of the cardinal features of some models of tolerance is that once the tolerance state has been established, it can be perpetuated to naive recipients by the adoptive transfer of donor-specific regulatory cells. Such adoptive transfer studies have also addressed the capacity of T cell subpopulations and non-T cells to transfer tolerance. However, tolerance cannot be transferred in all models. The underlying reasons for this are unclear with some studies pointing towards dose-dependent aspects and timing of expansion of T regulatory cells following tolerance transfer. Further exploration of this phenomenon will help us to better understand the mechanisms upon which allograft tolerance is based, and will provide new perspectives for further experimental studies.

Despite the introduction of new and potent immunosuppressive agents and the better control of acute rejection, transplant patients still require non-specific immunosuppressive therapy that is associated with major side effects (drug toxicity, infections and malignancies) (1). Thus, achieving tolerance in the clinic remains a major objective in transplantation. Since the experiments by Medawar and colleagues in the 1950s (2), tolerance, which is defined as "*the acceptance of a transplanted organ without indefinite immunosuppression*", has been a major goal in organ transplantation (3). In the context of solid organ transplants, two types of tolerance can be distinguished: central and peripheral tolerance. Central tolerance includes central thymic deletion, which requires the establishment of a chimeric immune system such that recipient T cells are educated by recipient (or donor) thymic tissue to donor antigens (Ags), resulting in the deletion of potentially alloreactive T cells. Peripheral tolerance can be achieved via a multitude of mechanisms including anergy (functional inactivity of T cells), ignorance (absence of reactivity to the donor alloantigen), peripheral deletion of alloreactive T cells by apoptosis and suppression of T cell activity (by T regulatory cells) (4). However, what actually differentiates central from peripheral tolerance is still unclear. In animal models, the phenomenon of allograft tolerance is classically characterized by donor specificity (tolerant recipients accept a secondary donor-specific allograft but reject third-party allografts (5) (6)), as well as by the absence of chronic rejection (based on the histological analysis of grafts surviving long-term (6) (7)). Finally, in many instances, once induced, peripheral tolerance can be maintained and perpetuated into naive recipients by regulatory cells, a phenomenon termed "infectious tolerance" (8). Infectious tolerance implies the ability to transfer tolerance to unmanipulated recipients over

multiple generations by cell transfer. In the infectious tolerance phenomenon, regulatory properties of cells from tolerant recipients can be transferred to a naive cell population, converting them into regulatory cells. Tolerance to a given alloantigen is spread to other alloantigens present on the same target cells. Originally described in the mouse (8), infectious tolerance was first described in the rat by Kupiec-Weglinski's group who demonstrated that CD4-targeted therapy leads to permanent acceptance of cardiac allografts in presensitized rat recipients, and that donor-specific tolerance can be transferred by spleen cells in an infectious-type fashion (9).Our current knowledge of the mechanisms that enable the transfer of tolerance to an allograft are based on *in vivo* experiments in animal transplantation models, where, once a tolerant state is established, it can be perpetuated by the adoptive transfer of cells to secondary naive recipients over multiple generations.

In this paper, focusing on the rat model, we will review how tolerance transfer may be influenced by the tolerance induction protocols and highlight the different organ compartments and cell subtypes able to transfer this tolerance state.

We would like to add, that at some points was necessary to mention the results obtained in the mouse model. Experimental work in the mouse model has advantages compared to the rat model (for example, the availability of transgenic and "knockout" mice). Also, studies in mice are made easier by a greater availability of experimental tools such as monoclonal antibodies. However, here we focus on the rat model and will only refer to

murine models when data provided in these models provide a better explanation for some of the phenomena identified in the rat.

Differential capacity of immune compartments to transfer tolerance

The spleen is the most frequently used compartment to test the adoptive transfer of tolerance in experimental models. One of the pioneering experiments where spleen allografts were used to induce tolerance and where spleen cells where used to transfer tolerance to heart allografts was performed by Stepkowski et al. (10) Regardless of the protocol used for tolerance induction and the type of organ transplanted, in various strain combinations splenocytes have been identified as robust and reliable cells for tolerance transfer. In the LEW.1W to LEW.1A congenic MHC incompatible rat strain combination (11), adoptive transfer demonstrated the presence of potent donor-specific regulatory cells in splenocytes. In this model, tolerance induction to an MHC-mismatched heart allograft ($RT1^u$ to $RT1^a$) can be achieved by treating recipients with a deoxyspergualine (DSG) analogue, LF15-0195, for 20 days following transplantation, with the tolerated grafts showing no signs of chronic rejection (12). Tolerance is uniformly transferred with spleen cells from LF15-0195-treated recipients 100 days after transplantation. In the same strain combination, heart allograft tolerance can be induced by donor-specific blood transfusion to the recipient, 14 and 7 days before transplantation and tolerance can be transferred with $50x10^6$ splenocytes to a secondary rat recipient (13). Finally, again in the LEW.1W to LEW.1A strain combination, tolerance to heart allografts can be induced by treatment of recipients with AdCD40Ig or AdCD40Ig and anti-ICOS (14). In the latter

model, adoptive transfer of $50x10^6$ splenocytes was again sufficient to induce significant prolongation of allograft survival (14). Splenocytes have been shown to transfer tolerance in numerous other models and in different rat strain combinations. In the LEW to DA heart allograft model where recipients were treated with anti-rat lymphocyte serum and intrathymic injection of LEW donor antigens, $50x10^6$ spleen cells from tolerant DA rats conferred unresponsiveness to donor alloantigen *in vivo* and transferred tolerance to secondary untreated rat recipients (15). In the same strain combination, but this time in DST induced tolerance, adoptive transfer was achieved using $100x10^6$ spleen cells (16). In the inverse rat strain combination (DA to LEW), treatment of Lewis recipients of a DA cardiac allograft with a combination of AdCTLA4-Ig and anti-ICOS antibody induced tolerance, which was transferable with $50x10^6$ spleen cells (17). Thus, the spleen provides an abundant source of cells able to transfer tolerance. Nevertheless, there are several models of tolerance that are not associated with the capacity of splenocytes to transfer long-term graft prolongation to secondary hosts (*see below*).

In addition to the spleen, other compartments of the immune system (e.g. lymph nodes) have also been reported to contain cells able to transfer tolerance. Indeed, in the RA to PVG (Kitade et al) (18) and LEW to DA (Kataoka et al) (16) rat strain combinations, heart allograft tolerance was shown to develop after one-step DST priming with blood 12 days before transplantation (18) or splenocytes 7 days (16) before transplantation. In both models, donor-specific tolerance was successfully transferred to secondary recipients using $100x10^6$ lymph node cells or $100x10^6$ splenocytes. Contrasting with these data, Zhai et al. report that, in the $LBNF_1$ to LEW model of heart allograft tolerance, only splenocytes successfully transferred tolerance ($100x10^6$), whereas the same number of

lymph node cells failed to do so (19). This difference may reflect the different methods of primary tolerance induction since Zhai et al exposed the recipient rat to a skin graft 1 week before heart transplantation under the cover of 10 injections of anti-CD4 mAbs from the day of skin grafting to 3 weeks after heart transplantation. Further explanations of these discordant results can be found in a mouse model of heart allotransplantation. Lakkis et al showed that in alymphoplastic (*aly/aly*) mice, which lack lymph nodes and Peyer's patches, rejection of cardiac allografts was considerably delayed in comparison to the wild-type and heterozygous (*aly/+*) recipients, which had normal secondary lymphoid organs. Splenectomized *aly/aly* mice accepted their cardiac allografts indefinitely (20). These findings indicate that fully vascularized allogeneic organ transplants do not induce a productive alloimmune response in the absence of secondary lymphoid tissue. In the context of tolerance transfer, lack of secondary lymphoid organs also abrogates generation of cells with regulatory capacity, which is crucial for tolerance transfer (20).

One could ask whether primary and secondary lymphoid organs are equally important in the induction and maintenance of tolerance. Some experiments have addressed the importance of primary and secondary lymphoid organs in tolerance development and transfer. Kitade et al examined the effect of thymectomy or splenectomy on graft survival and on the generation of Tregs in DST-treated rats. These authors showed that the thymus and spleen are required for the generation of Tregs but not for their expansion (18). In agreement with this observation, Onodera et al showed that thymectomy prevents induction but not maintenance of infectious tolerance in CD4 mAb-treated rat recipients pre-sensitized with donor skin grafts (21). Chiffoleau et al documented conflicting results

to the previously mentioned studies. In their experimental model, where tolerance was induced with the DSG analogue LF15-0195, the thymus was neither required to induce allograft tolerance nor to induce and expand regulatory cells in the periphery capable of transferring tolerance (12). These results imply that the thymus is critical for generation of regulatory T suppressor cells whenever recipients are pre-challenged with donor antigens. In such a situation, one can hypothesize that recipient T cells are educated by recipient thymic tissue to donor Ags, resulting in the deletion of potentially alloreactive T cells.

Regarding the importance of different compartments in tolerance induction, followed by adoptive transfer, it is important to mention the presence of the graft itself. Kataoka et al showed that without the presence of a heart allograft, DST alone was ineffective in generating the regulatory cells capable of transferring tolerance (16).

Investigations have also been undertaken to determine whether the blood or graft infiltrating cells (GIC) have a role in tolerance transfer. The first evidence that blood can also transfer tolerance came from studies by Bektas et al (22). In a LEW.1W to LEW.1A heart graft model, using two-step DST tolerance induction, these authors showed that 1ml of full blood transfers tolerance. However, in the same strain combination, using the same protocol, Lair et al showed that peripheral blood mononuclear cells (PBMC) are less efficient in transferring tolerance than spleen cells. In the latter study, adoptive transfer of 100×10^6 splenocytes from DST-treated recipients indefinitely prolonged graft survival in all recipients whereas $20\text{-}40\times10^6$ PBMC from the same animals induced long-term

graft survival in only 50% of secondary recipients (13), although higher doses of PBMC were not tested.

Another cell population studied for their ability to transfer tolerance is graft infiltrating cells (GIC). Zhou et al reported that, after oral administration of donor splenocytes, $70x10^6$ renal allograft GIC (harvested 5 days after transplantation) could adoptively transfer tolerance to a naive animal in a BN to LEW strain combination (23). Another indication that GIC are powerful in transferring tolerance was provided by Kataoka and colleagues (16) who reported that, in the LEW to DA strain and 60 days after transplantation and DST induction (seven days prior to the heart transplantation), 0.3-$30x10^6$ GIC transferred tolerance in 40% of secondary recipients. In the same experiment, $30x10^6$ splenocytes from long-term surviving recipients were necessary to transfer tolerance, suggesting that GIC were 10 to 100 times more effective in transferring tolerance (16). In discordance with this observation, $5x10^6$ GIC from two-step DST-induced heart allograft tolerance in the LEW.1W to LEW.1A strain combination could not transfer tolerance (24).

Why is irradiation prior to tolerance transfer important?

In numerous studies where the capacity of different organ compartments or T-cell subpopulations to transfer tolerance was tested, sub-lethal whole body irradiation (3 to 5 Gy) of secondary recipients appeared to be a necessary experimental step. An explanation for this lies in a fact that, whenever T-cells participate in tolerance transfer, preservation

of the homeostasis of T cell numbers is critical. The immune system tends to maintain its structure and function by establishing dynamic equilibriums controlled by multiple regulatory mechanisms. These mechanisms participate in the homeostatic control of T cell numbers and population distribution (25) (26). If a population of regulatory T cells is introduced into a secondary recipient, the T-cells expand to reach steady state numbers. In other words, different sub-populations of lymphocytes are in "competition to occupy specific niches". Irradiation of the secondary recipient prior to cell transfer would ensure that certain "niches" are empty and that there is no competition for them. In a situation such as this, the regulatory properties of cells from tolerant recipients can be transferred to a naive cell population, thus converting them into regulatory cells without competition with host cells.

However, in the model described by Degauque et al (induction using anti-donor MHC class II serum (6)), an irradiation step was not necessary for successful tolerance transfer. In this model, T cells alone were not sufficient to transfer a state of dominant tolerance, but required the presence of $CD103^+$ DC which, in concert with T cells from tolerant recipients, educated naive host T cells (6) without competition for "specific niches". Bektas et al (using two-step DST tolerance induction) also reported tolerance transfer through full blood without exposing the secondary recipients to irradiation (22). Tolerance may thus also function through the education of naive host T cells and not through their expansion after irradiation.

Adoptive transfer identifies different T-cell subtypes as key players in tolerance transfer

$CD4^+$ T cells are powerful in transferring tolerance. Various types of cells have been described to transfer tolerance to naive syngeneic hosts when injected at the time of transplantation. The most studied populations are $CD4^+$ and $CD8^+$ T-cells. Sometimes, even within the same strain combination, different tolerance induction protocols provide contradictory results as regards the capacity of certain cell sub-populations to transfer tolerance.

In the PVG to DA strain combination, following tolerance induction by anti-CD4 mAb therapy, 100 days post transplantation, tolerance to heart transplants can be transferred with $20x10^6$ $CD4^+$ T cells, whereas the same number of $CD8^+$ T cells is ineffective (5). In the RA to PVG rat strain combination, where heart allograft tolerance develops after one-step DST priming with blood 12 days prior to transplantation (18), Kitade et al showed that $CD4^+$ cells are more powerful than $CD8^+$ cells in transferring tolerance to a heart allograft. Similarly, regulatory cells develop after DST-induced acceptance of a LEW heart transplanted into a DA rat and Kataoka et al identified $CD4^+$ cells that uniformly transferred tolerance, as regulatory cells. However, in this study, at day 60 the same number of $CD8^+$ cells ($10x10^6$) showed suppressive activity and transferred tolerance in 62% of grafts (16). Heart transplantation performed in the same strain combination, where tolerance was induced using DST with splenocytes, again showed that $10x10^6$ of $CD4^+$ cells fully transferred tolerance, whereas $10x10^6$ of $CD8^+$ T-cells transferred tolerance with limited capacity (16). Finally, in the Lewis to DA combination, both

$10x10^6$ of $CD4^+$ or $CD8^+$ T-cells transfer tolerance to heart allografts. In this study, and in general, both $CD4^+$ and $CD8^+$ populations appeared to have regulatory activities, although the $CD4^+$ population played the dominant role (27).

Liu et al reported that in their model of heart allotransplantation, where tolerance was induced in ACI recipients by multiple transfusions of UVB-irradiated blood from Lewis heart donors, $CD8^+$ T cells from tolerant ACI rats expressed FOXP3 and $25x10^6$ of $CD8^+$ T cells transferred tolerance to naive secondary hosts and induced the up-regulation of the paired immunoglobulin-like receptor (PIR)-B in Lewis dendritic cells and heart endothelial cells (EC) (28). Similarly, Zhou et al reported the generation of $CD8^+$ regulatory GIC in a renal allograft model after oral administration of donor splenocytes in the BN to LEW strain combination and showed that the $CD8^+$ graft infiltrating cells could adoptively transfer allograft tolerance to a naive recipient (23). Finally, in a heart allograft model of AdCD40Ig-induced tolerance, Guillonneau et al. showed that adoptive transfer of 2.5×10^6 $CD8^+CD45RC^{low}$ T cells resulted in indefinite allograft survival, whereas transfer of the same number of $CD8^+CD45RC^{high}$ T cells failed to inhibit early acute rejection (29).

Suppressive activity: $CD4^+CD25^+$ T cells, $CD4^+CD25^-$ T cells, or both?

Among the $CD4^+$ T cell populations, particular attention has been paid to the $CD4^+CD25^+$ regulatory T cell subset. In a rat model of DST-induced graft survival prolongation (the RA to PVG rat strain combination), Pirenne and colleagues published a detailed

description of induced Treg cells (18). They found that both $CD4^+CD25^+$ ($25x10^6$) and $CD4^+CD25^-$ T cells ($25x10^6$) had the ability to transfer tolerance (18).

Studies performed by our own group in the LEW.1W to LEW.1A combination showed that $5x10^6$ of $CD4^+CD25^+$ T cells (of spleen and thymus origin) from animals treated with a DSG derivative were highly efficient in transferring tolerance, whereas the same number of $CD4^+CD25^-$ cells only partially transferred tolerance (12). In contrast, in the same strain combination, when the tolerance was induced by a DST protocol, tolerance transfer with $50x10^6$ $CD25^-$ spleen cells was successful (13), whereas $4x10^6$ of $CD25^+$ T cells from tolerant animals were unable to prolong graft survival following transfer to a naive host. This suggests that, even in the same genetic background and the same strain combination, different mechanisms operate when different tolerance induction protocols are applied. These data also show that CD25 may not be a stable marker for regulatory T cells in the periphery (30). In support of this conclusion, Gavin et al demonstrated that during the homeostatic process, $CD4^+CD25^+$ T cells that had divided more than five times no longer expressed the CD25 marker but remained highly potent in terms of their suppressive capacity (31).

On the subject of $CD4^+$ cells, interesting results have been obtained concerning the $CD4^+CD45RC^+$ and $CD4^+CD45RC^-$ populations. Kitade et al shed new light on the phenotype of Tregs by showing that, in vivo, transfer of tolerance was not associated with the CD25 marker. Whereas only $10x10^6$ $CD4^+CD45RC^-$ cells adoptively transferred tolerance, the same number of $CD4^+CD45RC^+$ cells was unable to do so (18). One conflicting report with these data comes from Zhai et al, who reported that

$CD4^+CD45RC^+$ cells (and not $CD4^+CD45RC^-$) were hyporesponsive to alloantigen and able to suppress normal T cell function in coculture assays (19), although nothing was reported on their *in vivo* properties.

T-cell and non T-cell cooperation in the transfer of tolerance

Several studies have provided evidence that both T-cells and non T-cells are required for the successful transfer of tolerance. In the heart allograft model, where tolerance was induced using AdCD40Ig, subtraction of the T-cell fraction from splenocytes resulted in heart allograft rejection. Moreover, we previously showed that in the LEW.1W to LEW.1A strain combination, kidney allograft tolerance can be induced using anti-donor MHC class II serum. In this model, tolerance was also transferred in an "infectious" manner over several generations using $80x10^6$ spleen cells (6). Moreover, splenocytes depleted of T cells or $CD103^+$ dendritic cells were no longer able to transfer tolerance. These data indicate that, in this model, transfer of tolerance requires the presence of both T and non T-cells. In the same strain combination in a model of DST-induced heart allograft tolerance, Lair et al showed that $20x10^6$ purified blood T cells had no effect in adoptive transfer (24). In contrast, the adoptive transfer of $20x10^6$ blood non-T cells from DST-treated recipients induced an indefinite graft survival in 40% of secondary recipients. The models mentioned above, including the different rat strain combinations, tolerance induction protocols and cell subtypes used to efficiently transfer tolerance, are outlined in Table 1.

Immune tolerance mechanisms involved in Treg development

Several studies have shown the contribution of central and peripheral immune tolerance mechanisms in the development of Treg capable of transfering tolerance. Kataoka et al demonstrated that both LEW DST and a LEW heart allograft were necessary to generate regulatory lymphocytes in DA recipients. The adoptive transfer of cells from DA rats receiving only LEW DST, but no heart transplant, did not lead to LEW heart graft acceptance in irradiated naive recipients (16). In the rat model, there is another piece of evidence showing that continuous presence of alloantigen is a critical factor in the development and maintenance of non-responsiveness to donor antigens. Onodera et al showed that normal LEW rats rejected LBNF1 hearts despite the hearts having been parked for 100 days in CD4 mAb-conditioned LEW hosts. The authors concluded that this tolerant state does not result from "graft adaptation," and regulatory T cells were exposed to continuous stimulation by the donor antigens. Their results from both graft-free and adoptive transfer studies demonstrate that effective memory for suppression in the infectious tolerance pathway depends upon persistent donor-specific alloantigen stimulation and wanes about 3 weeks after allograft removal (21).

The thymus and spleen are also thought to be important in Treg generation. Kitade and colleagues showed that the thymus and spleen are required for the generation of DSBT-Tregs but not for their expansion. Thymectomy or splenectomy, when performed 4 weeks before DST, abrogated heart allograft tolerance . However, the same procedures performed on the day of transplantation did not influence tolerance (18). The same conclusion that thymectomy prevents induction but not maintenance of regulatory T cells capable of transfering tolerance was also documented in Onodera's experiments (21).

Overall, the results of these studies suggest that both central and peripheral mechanisms of tolerance are involved in allograft acceptance and transfer of tolerance.

Timing of Treg expansion

Information about the timing of Treg expansion in the spleen and other compartments (including the graft itself) comes from Kitade et al (18). Using the RA rat strain as a heart donor and the PVG strain as a recipient in the context of a DST protocol, these authors showed that Tregs transferring tolerance are present in the spleen and lymph nodes in tolerant rats as soon as day 5 after transplantation. At 2 and 4 weeks post-transplantation, Tregs expanded and were present in all compartments tested: not only in the spleen and lymph nodes but also in the thymus and the graft itself (18). When comparing the efficiency of $100x10^6$ spleen cells and $100x10^6$ lymph node cells to transfer tolerance 5 and 14 days after transplantation, they showed that 14 days post-transplantation, spleen and lymph node cells transferred tolerance in 100% of cases, whereas Tregs harvested on day 5 transferred tolerance in only ≈ 40% of cases. Moreover, a reduced number of splenocytes (10 and $25x10^6$), which failed to transfer tolerance when taken from tolerant rats at day 5, fully transferred tolerance at day 14. Four weeks post transplantation, Tregs were detected in all compartments analyzed and transferred tolerance with great success: 100% for 10-$100x10^6$ splenocytes, 100% for $100x10^6$ lymph node cells, ≈85% for $100X10^6$ thymocytes and ≈80% for $10x10^6$ graft infiltrating cells (18). These data suggest a progressive expansion of regulatory cells during the development phase of tolerance and their maintenance thereafter. Other data from rat models support these conclusions. Liver transplantation from LEW to DA rats results in spontaneous

acceptance. To detect the presence of cells regulating graft rejection, Kataoka et al performed heart transplantation after adoptive spleen cell transfer from DA recipients who spontaneously accepted LEW hepatic grafts (27). LEW cardiac allografts were rejected when splenocytes were adoptively transferred from DA rats bearing LEW livers for only 30 days. On the other hand, splenocytes from DA rats bearing LEW livers for >60 days completely transferred tolerance and all LEW cardiac allografts were accepted. This result again indicates that time was necessary for the expansion of regulatory cells able to transfer tolerance (27).

Transfer of tolerance is dose-dependent

The dose-dependent aspect of tolerance transfer has been documented in previously mentioned models of spontaneous acceptance of hepatic grafts. Splenocytes ($100x10^6$) from DA tolerant recipients (bearing LEW livers for >60 days) transferred tolerance with 100% of success compared with a 70% success rate for $50x10^6$ splenocytes (27). In the same strain combination, using different protocols for tolerance induction, Kataoka et al provided another example that transfer of tolerance is dose-dependent (16). In the LEW to DA heart allograft model, tolerance was induced by priming with donor splenocytes 7 days before transplantation. In this study, $30\text{-}100x10^6$ splenocytes transferred tolerance to all recipients, but $1x10^6$ and $10x10^6$ splenocytes were insufficient to prevent acute rejection of the cardiac transplant (16).

Is it possible to extrapolate across models?

One of the questions arising at this point is whether it is possible to extrapolate results obtained in rodents to large animal models, or even more, to apply rodent tolerance induction protocols to nonhuman primate models (NHPs). Establishing successful protocols in old world monkeys, such as the rhesus macaque or baboon, would offer the possibility of moving towards transplantation tolerance in the clinic. On the whole, nearly all tolerogenic strategies that are successful in rodents have proven less effective in NHPs (32). This can be explained by the homogeneity of rodent models - uniform age, environmental exposure and, most importantly, genetic background (32). The advantage of performing experimental studies in rodents is that many rat and mouse inbred strains are available, which is not the case with large animals or monkeys. Moreover, tolerance transfer is always performed in the same strain combination, meaning that the cells responsible for transferring tolerance, even in the new recipient, face the same antigens and thus successfully transfer tolerance.

Another huge problem that exists between small animals and NHPs is environmental exposure to different pathogens and the development of heterologous immunologic memory. Memory cells present a clear threat to antigens, they respond vigorously and in certain situations can exert cross-reaction to alloantigen (33). This may explain failure to transfer tolerance in some circumstances. Finally, in large animals and NHPs most tolerance induction protocols include calcineurin inhibitors that may inhibit the development and activation of Treg (34) that have often been identified as key mediators of tolerance transfer in rodents.

Occasionally, some authors have been able to transfer tolerance with anergic T cells generated ex vivo in rhesus monkeys (35) or have been able to prolong graft survival by transferring peripheral blood lymphocytes from tolerant recipients in miniature swine (36). Nevertheless, in this review we wanted to focus on tolerance transfer in rat modelsand for this reason, the results obtained in mice, guinea pigs and large animals have been only mentioned briefly. Overall, translation of tolerance strategies from animals to humans has become increasingly difficult (32).

Conclusion

Tregs are present very early after transplantation in primary and secondary lymphoid organs. This was documented in numerous studies where different organ compartments were used in adoptive transfer experiments. In this way, Tregs can block the initiation of the alloimmune response and participate in the induction of tolerance. Tolerance transfer can also be successfully achieved with graft infiltrating cells, suggesting that Tregs can directly and locally protect the transplanted tissues. Different cell subpopulations have proven their capacity in tolerance transfer. The eesults that are sometimes contradictory, as mentioned by Pirenne, can be explained by mechanisms that may depend upon the strain combination and the species used (18). Finally, both dose and time components can be critical factors in the transfer of tolerance.

Future immunosuppressive strategies should include agents enable expansion of Tregs while preventing innate and adaptive immunity mechanisms and, in this context,

experiments of tolerance transfer may help us to better understand the biology of Tregs. Clinical observations have shown that transplantation tolerance may be lost over time and Treg presence or absence could be used as a useful biomarker in the prognosis of graft survival. It will be very interesting to make correlations between Tregs, proven for their capacity to transfer tolerance, and their possible role in prediction of graft outcome in numerous rat models.

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Table 1

Overview for the tolerance transfer experiments in rat model

Summary of strain combination, graft type, tolerance induction protocols and cell subtypes used in adoptive transfer experiments

Group	*Strain combination*	*Graft type*	*Tolerance induction protocol*	*Cell subtype able to transfer tolerance*	*Long-term transfer success*
Hall	PVG → DA	Heart	anti-CD4 mAb	$CD4^+$ $20x10^6$	6/6
Kataoka	LEW → DA	Heart	DST spleen (D-7)	$CD4^+$ $10x10^6$	3/3
Kataoka	LEW → DA	Heart	DST spleen (D-7)	$CD8^+$ $10x10^6$	5/8
Kataoka	LEW → DA	Heart	spontaneous acceptance of donor liver	$CD4^+$ $10x10^6$	4/5
Kataoka	LEW → DA	Heart	spontaneus acceptance of donor liver	$CD8^+$ $10x10^6$	4/7
Kitade	RA → PVG	Heart	DST blood (D-12)	$CD4^+$ $25x10^6$	5/5
Kitade	RA → PVG	Heart	DST blood (D-12)	$CD4^+CD25^+$ $25x10^6$	5/5
Kitade	RA → PVG	Heart	DST blood (D-12)	$CD4^+CD25^-$ $25x10^6$	5/5
Chiffoleau	LEW.1W → LEW.1A	Heart	LF15-0195	$CD4^+CD25^+$ $5x10^6$	4/4
Degauque	LEW.1W → LEW.1A	Heart	DST blood (D-14, D-7)	$CD25^-$ $50x10^5$	4/9
Kitade	RA → PVG	Heart	DST blood (D-12)	$CD4^+CD45RC^-$ $10x10^6$	6/6
Liu	LEW → ACI	Heart	DST (UVB irradiated blood, D-21,D-14,D-7)	$CD8^+$ $20x10^6$	3/5
Guillonnoeau	LEW.1W → LEW.1A	Heart	AdCD40-Ig	$CD8^+CD45RC^{low}$ $2.5x10^6$	4/4
Lair	LEW.1W → LEW.1A	Heart	DST blood (D-14, D-7)	PBMC non-T cells $20x10^6$	4/10

Annexe 2

Je voudrais préciser ici que ma contribution à l'article suivant s'est située dans la partie expérimentale (le prélèvement des organes, l'extraction des cellules/purification, l'extraction d'ARN, la rétro-transcription et la PCR quantitative. J'ai également participé à l'interprétation des résultats et à l'écriture du manuscrit.

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

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

Key words: Compartment, DST, TCR repertoire, tolerance, transplantation

Abbreviations: LEW.1A, Lewis 1A; LEW.1W, Lewis 1W; BN, Brown Norway; TCR, T-cell receptor; PCR, polymerase chain reaction; PBS, phosphate buffered saline; DST, donor-specific transfusion; FCS, fetal calf serum; I.V., intravenously; PBMC, peripheral blood mononuclear cells; CDR3-LD, complementary determining region 3 length distribution; MLR, mixed leukocyte reaction; Gy, Gray; APC, antigen presenting cells.

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Introduction

A major goal in transplantation is to achieve an immunological state of allo-antigen donor-specific tolerance in the absence of chronic immunosuppression (1). Although this has been achieved in a number of animal models (2–4), it remains difficult to reproduce in large animals (5,6) and in the clinic (7,8). However, understanding the precise cellular and molecular mechanisms of induction and maintenance of immune tolerance to allografts in adult organisms is of paramount importance for the conception of clinical protocols. It is now known that, in addition to clonal anergy, active suppression may largely contribute to graft survival (9). Thus, $CD4^+CD25^+$ T cells with regulatory activities, the most extensively studied population to date, have been shown to play an important regulatory role in several animal models of autoimmune diseases (10) and transplantation (11). $CD4^+CD25^+$ T cells with regulatory activities *in vitro* have also been found to be present in decreased numbers in the peripheral blood of patients suffering from chronic rejection (12–14). Nevertheless, although several studies have defined the cellular mechanisms by which regulation may develop (9,11,12,15–17), few studies have analyzed their anatomic compartmental distribution and where the 'tolerization' phenomenon takes place (18,19).

The efficiency of donor-specific blood transfusion to induce tolerance is well recognized (8,20,21), and several studies have shown the key role of $CD4^+CD25^+$ in this model (2,4,19,22). We used this model to characterize the different immune compartments of long-term allograft survivors and to analyze the capacity of these compartments to transfer tolerance and/or to protect against chronic rejection. We report strongly contrasting properties between spleen, graft and blood cells, suggesting that whereas the spleen may be a reservoir for regulatory T cells, the clonally expanded blood $CD8^+$ T cells are potentially cytotoxic T cells. These data strongly suggest that although blood may

be useful for the identification of new diagnostic tests for tolerance, it may not be informative for the identification of new genes or molecules involved in the mechanisms of tolerance that form the basis for novel therapeutic approaches.

Materials and Methods

Animals

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

Model of DST-induced long-term survival of heart allografts

LEW.1A rat recipients were transfused I.V. with 1 mL of blood from a LEW.1W donor 14 and 7 days before transplantation. Heterotopic LEW.1W cardiac transplantation was performed as previously described (23). Graft function was evaluated daily by abdominal palpation and rejection was defined as the day of cessation of heart beating and confirmed by laparotomy.

Purification of T cells and subsets

Spleen cells were recovered from DST-treated recipients and rejecting recipients 100 days after transplantation. Cells were isolated by passing the spleen through a stainless steel mesh. Erythrocytes were depleted by osmotic shock. Mononuclear cells were washed twice in phosphate-buffered saline (PBS) and resuspended in sterile RPMI 1640 (Life Technologies, Grand Island, NY). T cells were purified from spleen cells using Rat T-cell enrichment columns (R&D Systems, Lille, France). Purity was checked by flow-cytometry and was typically ≥94%. CD4$^+$ or CD8$^+$ cells were enriched by negative selection with purified anti-CD8 mAbs or anti-CD4 mAbs, respectively, followed by incubation with Pan Mouse IgG magnetic Dynabeads® (Dynal, Invitrogen, Cergy Pontoise, France). Negative selection of CD25$^-$ T cells was performed using the OX39 mAb followed by incubation with Pan Mouse IgG Dynabeads® (purity >96%). Graft infiltrating cells were recovered from DST-treated recipients 100 days after transplantation. The heart graft was washed with PBS-SVF-EDTA, cut into pieces, perfused with Collagenase D for 20 min at 37°C and passed through a stainless steel mesh. The cells were collected, washed twice with PBS-SVF-EDTA, resuspended and filtered. The cell suspension was then deposited onto a Ficoll® layer (Amersham Biosciences, Uppsala, Sweden) (4 mL) and were washed twice. Blood from DST-treated recipients was collected in EDTA tubes by cardiac puncture. PBMC were separated on a Ficoll® layer (Amersham Biosciences, Uppsala, Sweden) and T cells were purified using the same protocol as described above. Non-T cells were purified from PBMC: mononuclear cells were isolated as described above and T cells depleted by negative selection with a purified anti-TCRαβ mAb (R7.3) and superparamagnetic beads (Dynal, Oslo, Norway).

Adoptive cell transfer

Spleen cells, spleen T cells, PBMC, blood T cells and graft infiltrating cells from DST-treated recipients, naive LEW.1A rats and rejecting recipients were injected I.V. on the day of transplantation into a subirradiated (4.5 Gy whole body irradiation) LEW.1A secondary naive recipient 3 days before transplantation.

Flow cytometry analysis

Cells from DST-treated recipients, naive rats and rejecting recipients were incubated for 30 min at room temperature with FITC-conjugated W3/25 mAb (anti-CD4) (Serotec Laboratories, Oxford, UK) and the biotinylated antibodies R7.3 (anti-TCRαβ) and OX39 (anti-CD25α chain) (Bioatlantic, Nantes, France), and were subsequently revealed by Streptavidine-PE (Immunotech, Beckman coulter, Marseille, France). Cells were washed twice in PBS and two-color staining analyzed by a FACSCalibur using Cellquest Pro® software (BD Biosciences, Mountain View, CA). The same protocol was used for complete phenotyping of cells from DST-treated recipients, naive rats and rejecting recipients. The FITC-conjugated antibody R7-3 (anti-TCRαβ) (Bioatlantic, Nantes, France) and the following biotinylated antibodies were used: OX34 (anti-CD2), W3/25 (anti-CD4), OX19 (anti-CD5), OX8 (anti-CD8), JJ319 (anti-CD28), OX33 (anti-CD45RA), OX22 (anti-CD45RC), OX85 (anti-CD62L), OX26 (anti-CD71), OX7 (anti-CD90), OX40 (anti-CD134), OX2 (anti-CD200), OX6 (anti-RT1B class II), OX17 (anti-RT1D class II) and OX52 (anti-integrin αE) (Bioatlantic, Nantes, France), and revealed by Streptavidine-PE.

Mixed leukocyte reactions and inhibition assays

Irradiated (35 Gy) antigen-presenting cell-enriched cell populations from donor-type LEW.1W or third-party BN (RT1.n) rats served as stimulator cells. One hundred thousand purified responder T cells from the spleen and blood of DST-treated recipients, naive rats and 2×10^5 irradiated stimulatory cells were plated in 96-well round-bottom plates in triplicate in 200 µL of RPMI 1640 supplemented with 2 mM L-glutamine, 5×10^{-5} M 2-ME, 1 mM sodium pyruvate (Life Technologies), 1% non-essential amino acids, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 10% heat-inactivated FCS (Life Technologies) and for blood cell culture, L-NMMA (N-methyl-L-arginine, Sigma-Aldrich) an iNOS inhibitory was added (2.5%). Cultures were incubated at 37°C, in 5% CO_2, for 5 days and pulsed for the final 8 h with 0.5 µCi of [^{3}H]TdR (Amersham, Les Ulis, France). The cells were then recovered on glass fiber filters and [^{3}H]TdR incorporation was measured using standard scintillation procedures (Packard Institute, Meriden, CT). For inhibition assessment, increasing numbers of CD25$^+$ T lymphocytes purified from naive LEW.1A rats or DST-treated recipients were added to the MLR using naive CD25$^-$ T cells as responder cells. Cultures were pulsed with [^{3}H]TdR for the final 12 h of culture. For the inhibition assay, the results were expressed as specific count per minute [c.p.m. MLR − (c.p.m. DC alone + c.p.m. added cells alone + c.p.m. CD25$^-$ alone)].

Histology analysis

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

RNA extraction and cDNA synthesis

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

TCR repertoire analysis

Complementary determining region 3 length distribution (CDR3-LD) alteration was analyzed using Immunoscope® software (24). Global CDR3-LD alterations were measured according to Gorochov et al. (25). The profiles obtained from 11 naive rats were used as controls. Vβ/HPRT transcriptratios were measured by real-time PCR (26). Data were displayed as a bidimensional TopView or as a tridimentional TcLandscape® (27,28) where the x-axis displays the 21 Vβ families, the z-axis shows the Vβ transcript/HPRT transcriptratios and the y-axis gives the 13 possible CDR3 lengths per Vβ family. Percentages of alterations were represented as a color code, ranging from deep blue (values −30%) to dark red (30%) in the integrated landscapes

(27,28). MatLab® 5.3 software (The MathWorks Inc., Natick, Maryland) was used to compute and display the data.

Cytokine transcript analysis

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

Results were expressed as the intrasample ratio of cytokine to HPRT mRNA copy number. Perforin-1 and granzyme quantification was performed using labeled TaqMan® Gene Expression Assays (granzyme B: assay ID Rn00821152_g1 and perforin-1: Rn00566906_m1, Applied Biosystems).

Statistical analyses

Different statistical approaches were used to compare the CDR3-LD alterations in the blood, spleen and graft of DST-treated recipients, naive rats and rejecting recipients. A box-and-whisker plot representation was used to more easily visualize the global differences between the groups. The Kruskal–Wallis test followed by a Dunn's post hoc test was performed to compare perturbations between two or three groups, respectively. Statistical significance was considered for $p < 0.05$. Graft survival was analyzed using the Kaplan–Meier method. p-values were considered as significant when <0.05. The Mann–Whitney test was performed to compare transcript accumulation. p-values were considered significant when <0.05. Analyses were performed using the STATGRAPHICS PLUS 5.1 software (Rockville, Maryland, Manugistics Inc.).

Results

Numerous reports have shown that regulatory T cells differentiate rapidly in the spleen, graft (19) and lymph node (19,29) after allotransplantation and in long-term recipients (>100 days) (30) following various maneuvers aimed at inducing tolerance. The regulatory mechanisms described have also been reported to be dependant on the strain combinations used (20,31,32). In this study, we investigated the possibility of identifying the regulatory T-cell population in the different compartments from DST-treated recipients through a combined investigation of the T-cell TCR Vβ repertoire, phenotype and function.

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

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

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

The graft from DST-treated recipients displayed a slightly altered TCR repertoire usage (21.2 ± 4.3% alteration) with very low Vβ/HPRT ratios (1 ± 1.2), probably due to the low number of infiltrating T cells within the graft (Figure 2A).

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

To analyze the presence of the same Vβ clones in different animals but also in the different compartments within the same recipient, the graft, blood and spleen were taken from four rats 100 days after transplantation (Figure 2B). We have previously shown that, in a similar strain combination, the induction of tolerance following DST is associated with a public Vβ18Jβ2.7 monoclonal expansion within the graft infiltrating $CD8^+$ T cells (33). Here, day 100 post-transplantation, this particular clone was only observed in the blood of one of the four animals and was only observed in half of the graft of the recipients. CDR3 lengths of altered Vβ families (Vβ5, 6, 8.2, 11 and 14) identified in the blood were next compared between DST-treated recipients. As shown in Figure 4, although recurrent blood Vβ and CDR3 length alterations were found in the DST-treated recipients, no public alteration was reproducibly found in all the animals tested.

Finally, the box-and-whisker plot method was used to globally compare the TCR Vβ repertoire of the blood T cells purified from DST-treated recipients, naive animals and rejecting recipients. This method is helpful for the handling of large data sets. The data in Figure 5 confirm that the TCR Vβ repertoires in the blood T cells of DST-treated recipients were globally more altered than those of naive or rejecting recipients ($p < 0.01$).

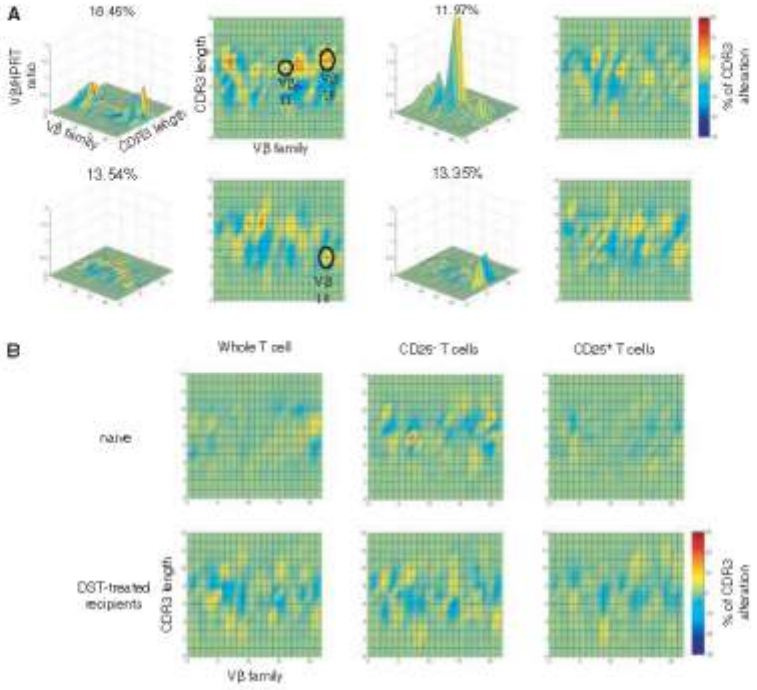

Figure 1: TCR repertoire in different spleen cell subsets of DST-treated recipients. (A) TCR repertoire analysis in spleens (n = 4) of DST-treated recipients (left-hand panel: Tclandscapes; right-hand panel: TopViews). The percentages indicate the global variation of CDR3-LD compared to naive animals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

Altogether, these data showed that DST-treated recipients displayed a highly altered and heterogeneous blood $CD8^+$ TCR repertoire but a Gaussian spleen TCR repertoire suggesting a compartmentalization of TCR repertoire usage. These data also showed that transplantation performed in inbred rat strains differing only in their MHC led to private TCR repertoire usage.

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

Without any treatment, 80% of subirradiated untreated LEW.1A recipients rejected their grafts (Figure 6). Adoptive transfer of 100×10^6 splenocytes from DST-treated recipients indefinitely prolonged graft survival in all subirradiated untreated LEW.1A recipients (Figure 6). Identical results were obtained when purified spleen T cells (30×10^6, n = 6) from DST-treated recipients were transferred to secondary recipients (Figure 6). In contrast, $20–40 \times 10^6$ PBMC from DST-treated recipients induced long-term graft survival in only 50% of secondary recipients (Figure 6). Purified blood T cells (20×10^6, n = 4) had no effect (Figure 6). In contrast, the adoptive transfer of 20×10^6 blood non-T cells (n = 10) from DST-treated recipients induced an indefinite graft survival in 40% of secondary subirradiated LEW.1A recipients. The adoptive transfer of 5×10^6 graft infiltrating cells (n = 2) from DST-treated recipients did not transfer long-term graft survival to secondary subirradiated LEW.1A recipients (*data not shown*).

As a control, transfer of 100×10^6 splenocytes, 40×10^6 purified spleen T cells and 20×10^6 blood non-T cells from rejecting recipients (n = 3) or naive LEW.1A rats (n = 3) had no significant effect (12 ± 1 days and 13 ± 2 days, respectively) on graft survival prolongation. Altogether, these data showed that long-term graft survival may be induced in 100% of subirradiated secondary graft recipients by splenocytes and spleen T cells and in 50% of animals by PBMC from DST-treated recipients only. Blood T cells had

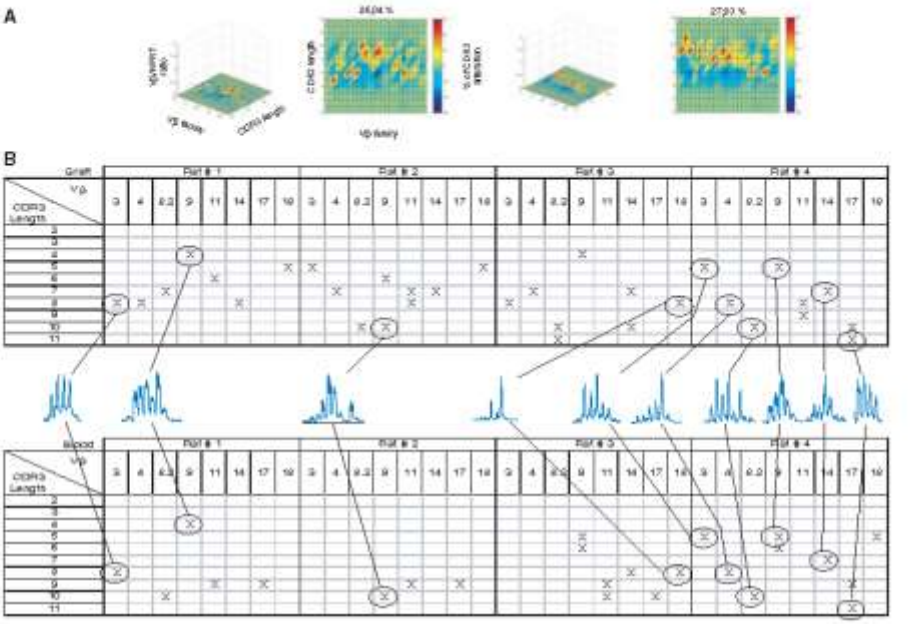

Figure 2: TCR repertoire analysis of the graft infiltrating cells and blood of the same DST-treated recipients. (A) TCR repertoire analysis of the graft infiltrating cells of DST-treated recipients (n = 2; left-hand panel: Tclandscapes; right-hand panel: TopViews). The percentages indicate the global variation of CDR3-LD compared to naive animals (average of 11 CDR3-LD naive profiles). (B) Recurrent Vβ and CDR3 length distributions within the blood and graft from same DST treated recipients (n = 4).

no effect, suggesting that the blood T cells with an oligoclonal repertoire were not selected regulatory T cells.

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

DST is known to result in a complex state where histological lesions of chronic graft rejection coexist with mechanisms controlling acute host antidonor immune responses (34). Thus, the possible occurrence of chronic graft rejection lesions was also analyzed in the secondary recipients that survived 100 days after splenocyte or PBMC cell transfer from DST-treated recipients (Figure 7). All long-term surviving grafts from secondary recipients that had received blood or spleen cells displayed histological lesions of chronic rejection (Figure 7A and B) with inflammatory vasculitis and interstitial fibrosis (Figure 7C).

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

In the DST-treated recipients, regulatory T cells differentiated in different compartments, as assessed by their various capacities to transfer long-term survival to secondary recipients. To investigate the regulatory mechanisms taking place, we compared the alloreactive response of T cells from DST-treated recipients taking into account their origin (blood or spleen). When cultured against donor APC, purified spleen T cells from DST-treated recipients proliferated significantly less ($p < 0.05$) than those from naive rats (Figure 8A). In contrast, no difference was observed when purified blood T cells from DST-treated recipients or naive LEW.1A rats were cultured with naive donor APC (Figure 8A).

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

An increase in the percentage of regulatory $CD4^+CD25^+$ T cells has been described in various tolerance-related models (2). We performed an exhaustive cell phenotype analysis in the spleen and blood of DST-treated recipients compared to naive and rejecting recipients (Figure 8B). A significant increase in $CD4^+CD25^+$ T cells was observed in the spleen of DST-treated recipients (10.8 ± 2.8%) as

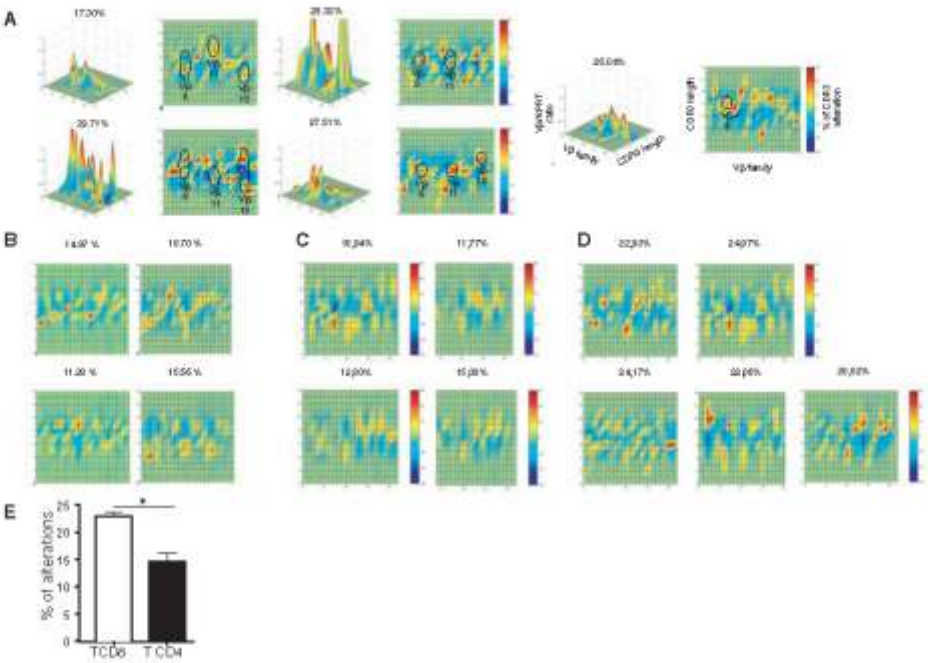

Figure 3: TCR repertoire in the PBMC of DST-treated recipients and rejecting recipients and in CD4$^+$ and CD8$^+$ T cells of DST-treated recipients. (A) TCR repertoire analysis in the blood of DST-treated recipients (n = 5; left-hand panel: Tclandscapes; right-hand panel: TopViews). The percentages indicate the global variation of CDR3-LD compared to naive animals (average of 11 CDR3-LD naive profiles). Circles indicate recurrent Vβ families. (B) TCR repertoire analysis in the blood (TopViews) of rejecting recipients (n = 4). (C) CD4$^+$ (n = 4) and (D) CD8$^+$ (n = 5) T-cell TCR repertoire analysis TopViews in the blood of DST-treated recipients. The percentages indicate the global variation of CDR3-LD compared to naive animals (average of 11 CDR3-LD naive profiles). (E) Percentage of alteration in CD8$^+$ and CD4$^+$ T cells in the blood of DST-treated recipients.

compared to naive rats (5.3 ± 0.8%, $p < 0.05$) and rejecting recipients (6.5 ± 3.6%). In contrast, no such difference was observed in the blood of DST-treated recipients where, if anything, there was a tendency toward decreased CD4$^+$CD25$^+$ T cell numbers (3.9 ± 1% vs. 6.3 ± 1.2% in naive rats and 10.5 ± 3.8% in rejecting recipients (Figure 8B). No difference was observed for any of the other markers tested (see Materials and Methods) in the spleen or the blood from the different groups (*data not shown*).

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

Because DST-treated recipients exhibit an increased percentage of CD4$^+$CD25$^+$ spleen T cells, we assessed whether their ability to inhibit proliferation of naive CD25$^-$ T cells was also increased. Proliferation of naive CD25$^-$ T cells against LEW.1W APCs was tested with or without CD4$^+$CD25$^+$ spleen T cells from either DST-treated recipients or naive rats. CD4$^+$CD25$^+$ spleen T cells from both DST-treated recipients and naive rats significantly and dose-dependently inhibited the alloreactive response of naive CD25$^-$ T cells in the coculture system (Figure 8C). Thus, DST-treated recipients exhibited an increased number of CD4$^+$CD25$^+$ spleen regulatory T cells with intact suppressive function on a cell per cell basis.

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

The Cβ, IL-2, IFN-γ, IL-10, IL-13, TGF-β1 and TNF-α transcript levels were compared in the spleen (n = 3), blood (n = 5) and graft (n = 4) of DST-treated recipients (Figure 9A) using quantitative RT-PCR. The blood of DST-treated recipients showed consistently higher levels of Cβ, IL-2, IFN-γ, IL-10, IL-13, TGF-β1 and TNF-α cytokines than splenocytes

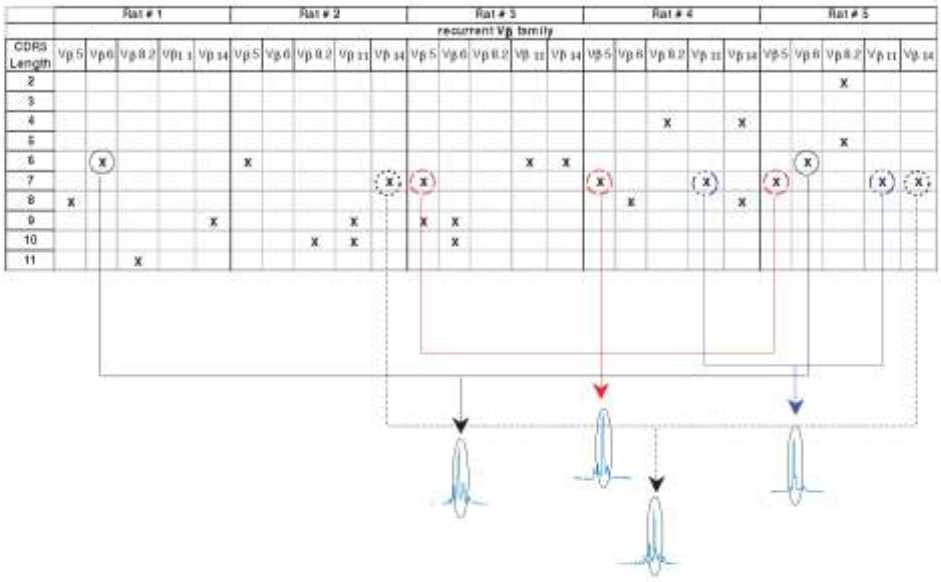

Figure 4: Recurrent Vβ and CDR3 length alterations in the blood of DST-treated recipients.

and grafts, although statistical significance was reached only for IFN-γ ($p < 0.05$) (Figure 9A). This suggests an active phenotype of blood cells from DST-treated recipients. Moreover, low transcript levels of perforin and granzyme were observed in the spleen and the graft from DST-treated recipients 100 days after transplantation whereas the blood accumulated significantly higher levels of cytotoxic transcripts ($p < 0.05$; Figure 9A and B).

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

Discussion

Tolerance in transplantation is associated with inactivation of the recipient's immune response to donor antigens, while retaining normal responsiveness to other unrelated antigens (35). In humans, the beneficial effect of DST on allograft survival was recognized several decades ago (8,20,21) and DST has become a classical model to induce tolerance or long-term graft acceptance in both experimental rodent models and humans (36–38). Nevertheless, the immunological mechanisms of the 'DST effect' are only poorly understood, although it is clear that leukocyte-depleted blood transfusions are ineffective (39). The mechanisms underlying the beneficial effects of DST may include clonal deletion (40), anergy (15), generation of suppressive/regulatory cells (2,9,37), regulation of cytokine production (34,41,42), microchimerism (43,44) or a combination thereof (32). The presence of donor-committed cytotoxic T lymphocyte infiltration in allografts early after DST does not favor a major role for clonal deletion of alloreactive cells (4,41,45–47). Moreover, once established, DST-induced tolerance can be perpetuated by adoptive transfer of regulatory cells into secondary naive recipients (2,4). This phenomenon, which is linked to the 'education' of the naive recipient's immune cells and can be transferred over multiple generations to secondary recipients (2,4), is referred to as 'infectious tolerance' (48–50) and implicates the presence and role of regulatory/suppressive cells.

There is now a large body of evidence showing that, whereas $CD8^+$ T cells may play a role (17,51–53), $CD4^+$ T cells are the major regulatory population involved in the DST effect (2,4,22). Nevertheless, their mode of action, their compartmentalization and the extent to which they

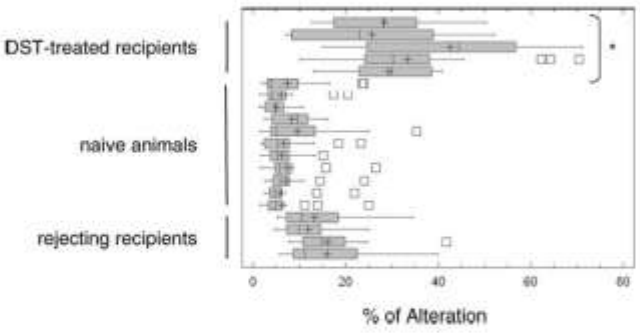

Figure 5: Synthetic representation of the percentages of Vβ family alterations in the blood T cells of DST-treated recipients, naive recipients and rejecting recipients represented per individual by the box-and-whisker plot method. The median of the data is represented by a vertical line. The two whiskers represent the lower and the upper quartiles. Points beyond the whiskers are considered as outliers (squares). The mean is indicated by a cross.

efficiently inhibit effectors or cytotoxic cells, thereby preventing chronic rejection, remains obscure (34,54). Moreover, it has been clearly shown that some genes found in the blood of renal transplant patients may serve as biomarkers and may not be found in the graft (55).

The presence of regulatory cells in the different immune compartments of DST-treated recipients was reported by several studies showing that tolerance can be transferred with splenocytes, lymph nodes, graft infiltrating cells or even blood (4,22,56–59). In this study, we showed that both spleen cells and peripheral blood mononuclear cells from DST-treated recipients were able to transfer long-term graft survival to 100% and 50% of naive host recipients, respectively. We also showed that the nature of the regulatory cells differs between the two compartments. Whereas T cells purified from the spleen were able to transfer long-term survival, purified blood T cells failed to do so and long-term graft survival was reached only after transfer of blood non-T cells. Moreover, T cells varied in their phenotypic and functional characteristics between these two compartments: blood T cells had an activated phenotype with increased perforin/granzyme transcript levels and a preserved proliferative response to donor antigens. These cells also exhibited a highly altered TCR repertoire, which is neither related to the DST priming (*data not shown*) nor consecutive to previous acute rejection episodes. T cells from splenocytes, on the other hand, displayed an increased proportion of $CD4^+CD25^+$ T cells with suppressive properties *in vitro*, a decreased proliferative response to donor antigen and an unaltered TCR repertoire.

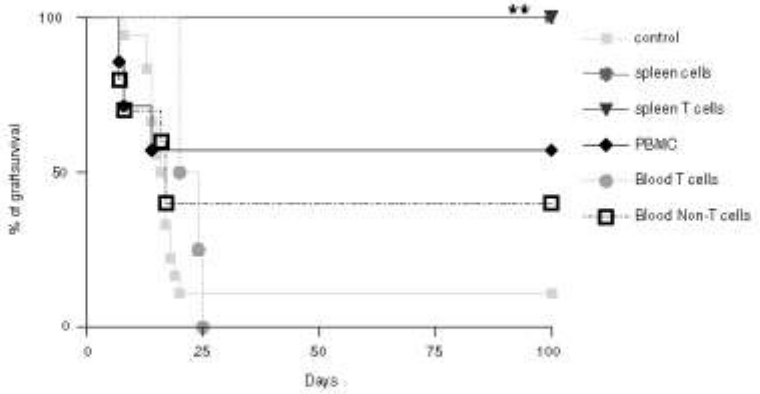

Figure 6: Graft survival after cell transfers into naive secondary LEW.1W recipients. Spleen (total and purified T) cells, blood (total, purified T and non T) cells from DST treated recipients 100 days after transplantation were transferred to secondary irradiated rat recipients. As a control, transfer of 100×10^6 splenocytes or 40×10^6 purified spleen T cells from rejecting recipients (n = 3) or naive LEW.1A rats (n = 3) had no significant effect (12 ± 1, 13 ± 2 days, respectively) on graft survival prolongation.

A

B

C

		mononuclear cell interstitial inflammation	interstitial fibrosis	vasculitis	vascular narrowing	% of pathological arterial sections per heart
Transfer of splenocytes	#1	1	1	2	1	10
	#2	1	1	2	1	6
Transfer of PBMC	#1	2	1	2	3	50
	#2	2	1	2 and 3	3	80
	#3	2	1	1	1	50
	#4	4	3	2	1	50

Figure 7: Histological analysis of grafts from secondary rat recipients after spleen cell transfer (A) and PBMC transfer (B) from DST-treated recipients (C). The table shows Banff scores for mononuclear cell interstitial inflammation, interstitial fibrosis, vasculitis, vascular narrowing and percentage of pathological arterial sections per heart.

Although the contribution of regulatory T cells to the transfer and maintenance of long-term graft acceptance in this model is well established (4,60), the manner in which regulatory T cells control effector mechanisms and the degree to which they protect against (or contribute to) chronic rejection is unclear. We showed that spleen T cells, which displayed a regulatory-like profile and transferred long-term graft survival to secondary recipients, were unable to prevent the onset of chronic rejection. Several other studies have shown that histological signs of chronic rejection

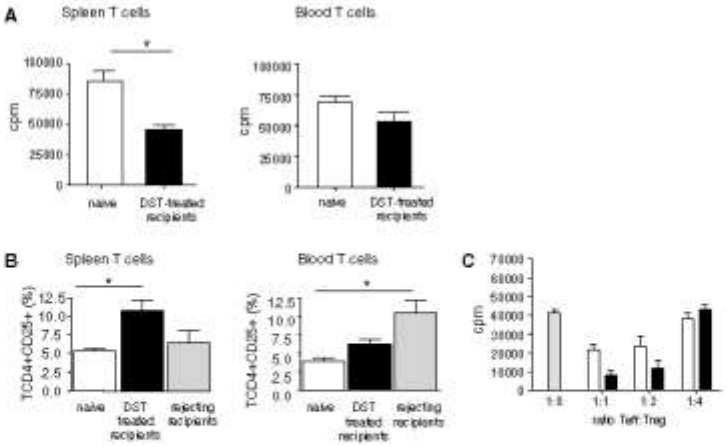

Figure 8: (A) Proliferative response of spleen T cells and blood T cells from DST-treated recipients or naive LEW.1A rats against LEW.1W APC. (The results of one experiment representative of four are shown.) (B) Percentage of $CD4^+CD25^+$ T cells in the spleen and blood of naive LEW.1A rats, DST-treated recipients and untreated recipients recovered on day 100 post-transplantation. Results are representative of four independent experiments and bars represent mean ± SEM. Statistical significance was assessed using the Mann–Whitney test. (C) No increase in suppressive function of the spleen $CD4^+CD25^+$ T cells in DST-treated recipients: various ratios of spleen $CD4^+CD25^+$ T cells from DST-treated recipients (black bar) or naive (white bar) LEW.1A rats, were added to naive $CD25^-$ T cells from LEW.1W rats in MLRs. Bars represent mean ± SEM and the results are representative of four independent experiments.

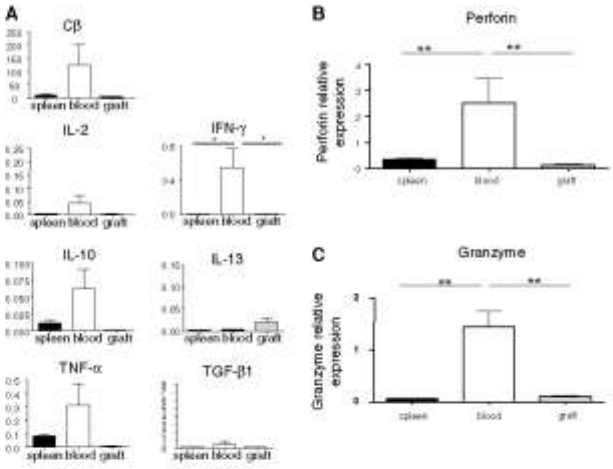

Figure 9: (A) Cytokine transcript accumulation in the spleen (n = 5), blood (n = 5) and graft (n = 4) of DST-treated recipients. Bars represent mean ± SEM. Statistical significance was assessed using the Mann–Whitney test. (B) Perforin and (C) granzyme transcript levels in the blood, spleen and graft of DST-treated recipients. Bars represent mean ± SEM. Statistical significance was assessed using the Mann–Whitney test.

develop in apparently tolerant animals, in models of both heart (34,42) and kidney (61) transplantation, whereas chronic rejection was absent in syngeneic grafts (34). In the present study, both DST-induced tolerant recipients and secondary recipients displayed severe signs of chronic rejection. This observation suggests that the potentially regulatory T cells are not sufficient to protect the graft from chronic rejection. However, we cannot exclude the possibility that regulatory T cells able to inhibit acute rejection (as shown by the effect of DST itself or by the transfer of cells from DST-treated recipients) can also contribute to chronic rejection. Indeed, tolerance has been shown to be an 'active' phenomenon associated with cytotoxic cells directed against donor cells (62) and has also been shown to develop in the context of a Th2 polarization, which has itself been associated with the development of chronic rejection lesions in functioning grafts (34,42). In our model, no Th1/Th2 deviation was observed in DST-treated recipients thus suggesting that the potentially regulatory T cells are probably incapable of protecting the graft from chronic rejection following DST induction.

Altogether, we showed that DST treatment results in the differentiation of regulatory cells in spleen T cells and in the non-T cell compartment in the blood, since blood T cells did not transfer graft survival prolongation to secondary recipients. These data suggest that blood is not a reservoir for regulatory T cells and that the selected clonal expansions within the TCR Vβ repertoire (particularly CD8 T cells) represent potentially cytotoxic T cells rather than regulatory T cells.

Interestingly, the Vβ18 clonotype previously found in the grafts of DST recipients as early as day 5 post-transplantation (33) was found in the graft and blood of 2/4 and 1/4 DST-treated recipients at day 100 post-transplantation in this study. However, these data do not contradict those from our previous studies (33) since in the previous studies only grafts were analyzed and the analyses were performed during the induction phase of tolerance at day 5 post-transplantation. The present analysis was performed at day 100 post-transplantation, that is, during the maintenance phase of tolerance. The fact that these two processes (induction and maintenance) may result in different patterns is likely due to the different mechanism involved, for instance direct or indirect pathways. However, one must keep in mind that the recipients displayed different and individual TCR repertoires despite being inbred over multiple generations and despite the donors and recipients differing only in MHC class I and II molecules. The effect of environmental factors on this phenomenon remains questionable since all donors and recipients were confined in EOPS pathogen-free conditions. The fact that TCR biases remained mainly private suggests that a similar determinant can be recognized by different types of clones which have been selected over time. This is consistent with several lines of data showing that a variety of peptides can interact with the same TCR, and that different TCRs can interact with the same peptide (63–65). These data also suggest that the blood, the main compartment analyzed in the clinic in the search for new biomarkers, may provide a poor representation of the true immunological mechanisms involved in tolerance induction and maintenance. As suggested in previous studies, the presence of certain bio-markers identified as 'potential tolerance markers' may also be a consequence of activation status (66) and may even be specific to the compartment studied (55). Finally, these data question the pertinence of studying

tolerance mechanisms in rodent models as a means to identify new molecules in the blood and to apply them to the clinic.

Acknowledgments

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Analyse des mécanismes de la tolérance à l'allogreffe de rein chez le rat suite à l'administration de sérum anti-CMH de classe n du donneur

RESUME

Chez le rat, la tolérance des allogrcffes rénales CMH-incompatibles peut être induite par l'administration le jour de la greffe d'anticorps anti-CMH II du donneur. Au cours de ce travail, nous avons étudié les mécanismes impliqués dans la phase de maintien de cette tolérance, grâce à l'analyse par microarray des profils d'expression des gènes de greffons rénaux tolérés de longue date. La comparaison des pattems d'expression entre les greffons tolérés et les greffons syngéniques a permis de révéler 5954 gènes différentiellement exprimés ($p<0.05$). Une analyse plus approfondie de ce panel de gènes a montré un rôle clé de la voie de signalisation WNT, l'une des voies principales impliquées dans la régulation cellulaire, et qui, jusqu'à présent, n'avait pas été décrite comme impliquée dans le cadre de la transplantation. Plusieurs gènes appartenant à cette voie ont été signifîcativement surexprimés dans les greffons tolérés, en particulier la protéine MMP7 (Matrix Metalloproteinase 7). L'analyse de plusieurs autres molécules relatives à cette voie a pu montrer que la surexpression de MMP7 était la conséquence d'une activation de la voie de signalisation non-classique WNT. L'expression de MMP7 était restreinte aux cellules musculaires lisses des vaisseaux (SMC). En effet, MMP7 n'a pas été détecté ni dans d'autres modèles de tolérance de greffons rénaux ou cardiaques, ni dans les modèles de rejet chronique obtenus dans les mêmes souches de rats. Déterminer les mécanismes de tolérance par lesquels MMP7 contribue au maintien de la tolérance pourrait permettre le développement de nouvelles stratégies visant à améliorer la tolérance du greffon à long terme.

Mots clés: transplantation, tolérance, allogreffè. rein, coeur, rat. MMP7, WNT

Analysis of the mechanisms of tolerance to kidney allograft in the rat following administration of anti-donor MHC class II serum

ABSTRACT

In rats, tolerance to MHC incompatible renal allografts can be induced by administration of anti-donor class II antibodies on the day of transplantation. Here we explored the mechanisms involved in the maintenance phase of this tolerance by analyzing intra-graft gene expression profiles by microarray in long-term accepted kidneys, Comparison of the gene expression patterns of tolerated to syngeneic kidneys revealed 5954 differentially expressed genes ($p<0.05$). Further analysis of this gene set revealed a key role for the WNT signaling pathway, one of the pivotal pathways involved in cell regulation that bas not yet been implicated in transplantation. Several genes within this pathway were significantly up-regulated in the tolerated grafts, particularly Matrix Metalloproteinase 7 (Fold Change>40). Analysis of several other pathway-related molecules indicated that MMP7 over-expression was the result of the non-canonical WNT signaling pathway. MMP7 expression was restricted to vascular smooth muscle cells (SMC) and was specific to anti-class II antibody-induced tolerance as it was undetectable in other models of renal and heart transplant tolerance and chronic rejection induced across the same strain combination. These results suggest a novel role, for non-canonical WNT signaling in maintaining kidney transplant tolerance in this model, with MMP7 being a key target. Determining the mechanisms whereby MMP7 contributes to transplant tolerance may help in the development of new strategies to improve long-term graft outcome.

Key words:
transplantation, tolerance, allograft, kidney, heart, rat, MMP7, WNT

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