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Analyse de la différenciation et de la régulation des lymphocytes B chez des patients tolérant un greffon rénal

JURY

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La recherche est un processus sans fin dont on ne peut jamais dire comment il évoluera.
L'imprévisible est dans la nature même de la science.

François Jacob Biologiste, Médecin, Physiologiste, Scientifique (1920 - 2013)

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Sommaire

Liste des abréviations

AD	Atopic Disease ou maladie atopique
AICD	Activation-Induced Cell Death ou mort cellulaire induite par l'apoptose
B10	Lymphocytes B sécrétant de l'IL-10
BAFF	B cell Activating Factor ou facteur d'activation des lymphocytes B
BAFF-R	B cell Activating Factor – Receptor ou récépteur du facteur d'activation des lymphocytes B
BANK-1	B cell adaptor protein with ankyrin repeats-1
BCL6	B-Cell Lymphoma 6 protein
Cellules NK	Cellules Natural Killer ou cellules tueuses naturelles
CSR	Class Switch Recombination ou commutation de classe
DCs	Dendritic Cells ou cellules dendritiques
DCf	Dendritic Cell follicular ou cellule dendritique folliculaire
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor, ou facteur de stimulation de colonies de granulocyte--macrophages
HMS	HyperMutation Somatique
HV	Healthy Volunteers ou volontaires sains
IDO	Indoleamine 2, 3-dioxygenase
IFN-γ	InterFeroN gamma
IL	InterLeukine
IRF4	Interferon Regulatory Factor 4 ou facteur de régulation de l'interféron n° 4
LB	Lymphocytes B
MPR	Mannose-6-Phosphate Receptor ou récepteur du manose-6-phosphate
ODN	OligoDeoxyNucleotides
PAX5	Paired Box Gene 5
PBMCs	Peripheral Blood Mononuclear cells ou cellules mononuclées du sang périphérique

PRDM1	PR Domain Containing 1
STA	Patients Stables
Tfh	Lymphocyte T folliculaire helper
TGF-β	Transforming Growth Factor beta ou facteur de croissance transformant beta
TNSF TNF	TNF ligand superfamily member ou membre de la superfamille des ligands du
TOL	Patients Tolérants
TRAIL TNF	TNF ligand superfamily member ou membre de la superfamille des ligands du
Treg	Lymphocyte T régulateur
XBP1	X-box Binding Protein 1

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Introduction

I- La transplantation rénale

A- Généralités

Le rein est un organe qui possède différentes fonctions, il filtre le sang de ses déchets via la production d'urine. Il possède également un rôle hormonal, via la production d'érythropoïétine ; hormone qui stimule la production des globules rouges dans la moelle osseuse et la production de rénine qui joue un rôle essentiel dans la régulation de la pression artérielle. Le rein a également une fonction « enzymatique » intervenant dans le métabolisme phospho-calcique par l'activation de la vitamine D.

En cas d'insuffisance rénale terminale, la transplantation rénale est le seul traitement qui permet d'éviter la dialyse à vie. La transplantation rénale permet aussi une meilleure qualité de vie et une meilleure espérance de vie (Wolfe et al., 1999) en échappant notamment à la dialyse très contraignante. Greffés, les patients retrouvent une vie normale et une liberté de mouvement. Il y a en France environ 3000 transplantations rénales par an, c'est la transplantation la plus fréquente (Agence de la biomédecine).

A ce jour, en France, environ 35000 malades souffrent d'insuffisance rénale chronique au stade terminal (environ 4000 personnes sur liste d'attente de greffe rénale). Les principales causes de l'insuffisance rénale chronique sont :

- L'athérosclérose c'est-à-dire les maladies cardio-vasculaires telles que l'hypertension artérielle, les hyperlipidémies, le tabagisme.

- Le diabète et l'hyperglycémie prolongée.

- Les glomérulonéphrites.

- La polykystose rénale.

La transplantation rénale peut s'effectuer à partir d'un rein d'un donneur en état de mort cérébrale ou à cœur arrêté (NLD Non Living Donor ou donneur mort) ou bien, à partir d'un donneur vivant.

Bien que la transplantation rénale soit le traitement de choix de l'insuffisance rénale terminale, elle ne représente pas une solution définitive. En effet, l'introduction d'un organe étranger induit l'activation de la réponse immunitaire du receveur envers l'organe transplanté ce qui conduit à son rejet. A ce jour, et grâce aux progrès réalisés dans l'immunosuppression, 90 à 95 % des reins greffés fonctionnent toujours après 12 mois et 80 % des greffons sont encore parfaitement fonctionnels 5 ans après la greffe (Agence de la biomédecine). Ces dernières années, les traitements immunosuppresseurs se sont perfectionnés et individualisés pour en limiter les effets secondaires. Aujourd'hui, en 2014, la demi-vie d'un greffon rénal est d'environ 20 ans pour un greffon issu d'un donneur vivant, contre 13 ans pour un rein provenant d'un donneur décédé (Agence de la biomédecine).

B- Le rejet de greffe

Le rejet est lié à l'activation des cellules du système immunitaire (Lymphocytes T (LT), lymphocytes B (LB), cellules mononuclées…..) par des alloantigènes du donneur (présent dans le greffon). L'activation d'une réponse immunitaire envers le greffon conduira à sa destruction (rejet) plus ou moins rapide en l'absence de traitements immunosuppresseurs. Il existe différents types de rejet en fonction des mécanismes de réponse immunitaire mis en jeu.

B-1- Le rejet hyper aigu

Il survient dans les minutes ou les heures qui suivent la transplantation. Il est lié à l'existence d'anticorps préexistants qui se fixent sur l'endothélium du greffon lors de la revascularisation et entraînent la fixation et l'activation du complément et l'activation de l'endothélium qui exprime à sa membrane des molécules d'adhérence et des molécules pro-coagulantes (Cai and Terasaki, 2005a, 2005b). La conséquence au niveau du rein est la thrombose des artères et la nécrose hémorragique du greffon nécessitant la transplantectomie d'urgence. Il n'existe pas de traitement curatif au rejet hyper aigu. Le seul traitement est préventif. Comme la grande majorité des anticorps responsables du rejet hyper aigu sont les anticorps anti HLA, la prévention repose sur la recherche d'anticorps anti

HLA chez les patients en liste d'attente et la réalisation d'un cross-match juste avant la transplantation (test de cytotoxicité entre les lymphocytes d'un ganglion du donneur et le sérum du receveur potentiel). L'existence de tels anticorps est due à une réaction immunologie antérieure (transfusion sanguine, grossesse, transplantation). Ce type de rejet est très rare de nos jours.

B-2- Le rejet aigu

Le premier épisode de rejet aigu apparaît dans les semaines ou les mois qui suivent la transplantation. Il peut être dû à deux mécanismes immunologiques différents ; le rejet aigu humoral qui est lié à l'apparition d'anticorps spécifiques du donneur et le rejet aigu cellulaire qui est principalement lié à une réaction LT. Les LT du receveur reconnaissent des antigènes allogéniques du donneur. Les lymphocytes vont alors s'activer, proliférer et envahir le greffon ce qui entraine sa perte de fonction et sa destruction. Actuellement, grâce aux améliorations réalisées sur les traitements immunosuppresseurs, l'incidence du rejet aigu a fortement baissé et survient dans moins de 20% des transplantations et n'entraînent que des altérations modestes de la fonction des organes, généralement bien contrôlées par une modification du traitement immunosuppresseur. Le rejet aigu cellulaire survient essentiellement dans les 3 premiers mois avec un pic de fréquence dans le premier mois. Néanmoins, il peut s'observer à tout moment en cas d'arrêt du traitement immunosuppresseur (non-compliance). Il est diagnostiqué par la biopsie de l'organe greffé et les lésions observées font l'objet d'une classification internationale (classification de Banff) (Marks and Finke, 2006).

B-3- Le rejet chronique

Le rejet chronique décrit en 2007 (Solez et al., 2007) correspond à la perte progressive et irréversible des fonctions normales du greffon. La perte du greffon est associée à la survenue d'une fibrose et d'une atteinte des vaisseaux artériels dont la lumière se rétrécit progressivement (vasculopathie du transplant). Les mécanismes moléculaires de ce type de rejet sont mal connus.

C- La réponse Alloimmune

L'utilisation des traitements immunosuppresseurs permet aujourd'hui une survie du greffon en nette augmentation, notamment par une diminution très importante du nombre

de rejets aigus. Malgré le développement de nouveaux traitements immunosuppresseurs de plus en plus efficaces, leurs utilisations s'accompagnent d'effets secondaires néfastes : infection, cancer, néphrotoxicité… (Fishman, 2007; Nankivell et al., 2004; Roberts et al., 2002). Il existe différentes classes d'immunosuppresseurs, ceux utilisés comme traitements d'induction, administrés au moment de la transplantation et dans les jours qui suivent. Ces traitements bloquent la prolifération des LT et provoque un état de non-réponse immunitaire envers le greffon. Il existe également des traitements d'entretien qui sont utilisés au long cours afin de prévenir les rejets aigus. Ces traitements visent à bloquer les interactions entre lymphocytes et cellules présentatrices d'antigènes. Les traitements immunosuppresseurs vont principalement viser à bloquer l'activation lymphocytaire. Pour comprendre leurs modes de fonctionnement, il est donc important de rappeler les mécanismes d'activation de la réponse alloimmune.

La réponse alloimmune aboutit à l'activation lymphocytaire et nécessite la mise en place de trois signaux (Figure 1). Le premier signal est transmis par la reconnaissance d'un déterminant antigénique du donneur par le récepteur T (TCR) du LT naïf. Ce premier signal entraîne, par différentes voies de signalisation, l'activation de facteurs de transcription tels que NFAT, NFκB et AP-1 (Diehn et al., 2002; Michel et al., 2000; Rincón and Flavell, 1994). Ces facteurs de transcription vont notamment induire l'expression de CD154 (CD40L) et d'IL-2 qui permet d'initier le second signal.

Le second signal correspond à l'engagement des molécules CD40-L et CD28 qui permet de renforcer le premier signal. L'absence de ce second signal ne permet pas une activation totale des lymphocytes qui deviennent anergiques. Le renforcement du signal induit l'expression de récepteurs de haute affinité à l'IL-2 qui conduit au troisième signal.

Le troisième signal est induit par la liaison de l'IL-2 à son récepteur l'IL-2R et entraîne la prolifération cellulaire, l'expression de gènes anti-apoptotiques et la sécrétion de cytokines et chimiokines qui permettent aux LT de quitter le ganglion pour rejoindre le tissu cible (figure 1).

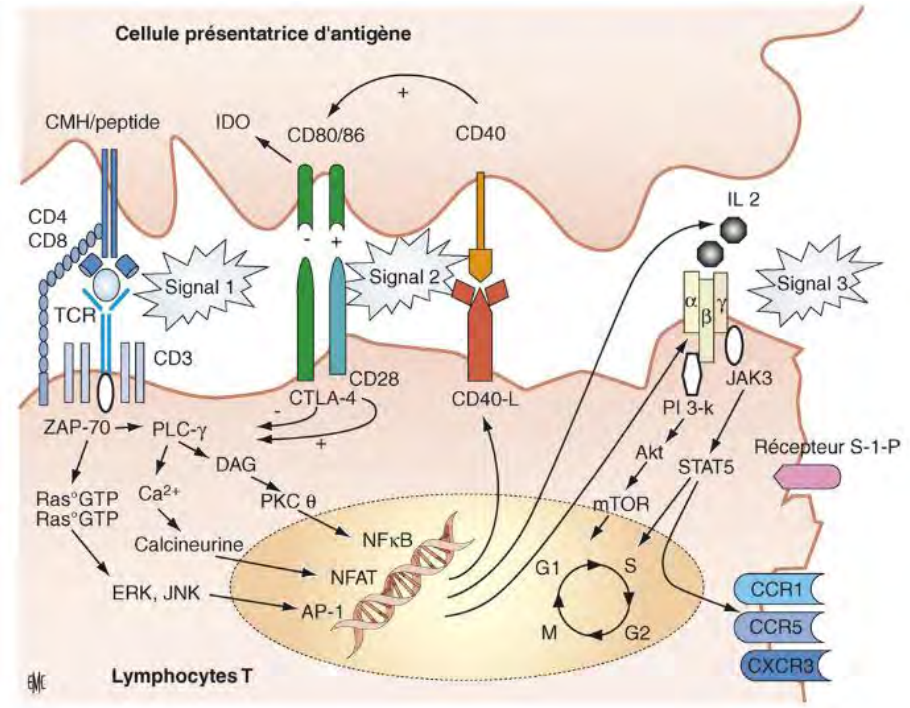

Figure 1 *:* Schéma représentant les 3 signaux de la réponse allo-immune

Le premier signal correspond à la reconnaissance d'un déterminant antigénique avec le TCR du lymphocyte T, ceci entraine l'activation de facteurs de transcription NFκB, NFAT et AP-1. Le deuxième signal correspond à l'engagement des molécules de co-signal (CD28, ICOS-L, CD40-L) qui permet le renforcement du premier signal. Enfin, le troisième signal correspond à la fixation de l'IL-2 à son récepteur l'IL-2R qui aboutit à la prolifération cellulaire des lymphocytes T, à l'expression de gènes anti-apoptotiques et à la sécrétion de cytokines et chimiokines. (Legendre et al., 2007)

D- Les traitements immunosuppresseurs

Les traitements immunosuppresseurs peuvent être classés en fonction de leurs cibles, en effet ceux-ci peuvent cibler les différents signaux de la réponse alloimmune (Figure 2).

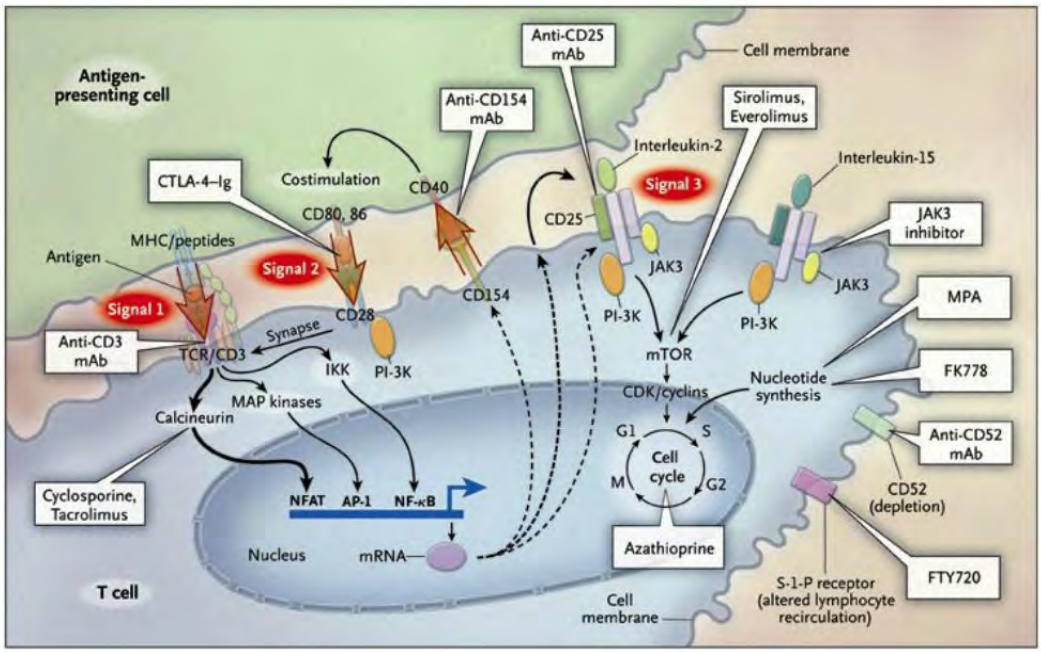

Figure 2 : Les différents immunosuppresseurs et leurs sites d'action

Ce schéma représente les différents immunosuppresseurs et leurs cibles au sein de la réponse alloimmune (Halloran, 2004).

D-1- Les corticostéroïdes

Les corticostéroïdes sont des analogues d'hormones naturelles. Ils possèdent des propriétés anti-inflammatoires et immunosuppressives importantes (Rhen and Cidlowski, 2005). Ils inhibent la transcription de cytokines pro-inflammatoires, l'expression de molécules d'adhésion diminuant la migration des cellules immunes au site d'inflammation, la prolifération, la fonction effectrice, suppressive et cytolytique des LT (Vacca et al., 1992). Les corticostéroïdes sont utilisés à forte concentration juste après la transplantation mais diminués au fil du temps en raison du nombre important d'effets secondaires.

D-2- Les traitements visant le premier signal

La cyclosporine et le tacrolimus sont des inhibiteurs de la calcineurine (Clipstone and Crabtree, 1992). Le blocage de la calcineurine inactive le facteur de transcription NFAT, qui joue un rôle sur la transcription de gènes pro-inflammatoires (IL-2, IL-4, IFN-γ, TNF-α, GM-CSF, IL-2-R), bloquant ainsi leurs expressions, ce qui inhibe donc l'activation des LT.

L'anticorps anti-CD3 humanisé (hOKT3γ1), joue un rôle dans le premier signal. Il bloque l'activation des LT. De nos jours, il est uniquement utilisé pour le traitement des rejets sévères chez les patients résistant aux corticostéroïdes, en raison de l'importance des effets secondaires liés à ce traitement (Legendre et al., 1992).

D-3- Les traitements visant le second signal

Le LEA29Y (bélatacept) est une protéine de fusion entre CTLA-4 (CD152) et la région (Fc) d'une IgG1 humaine. Cette protéine entre en compétition avec la molécule CD28 ce qui a deux effets ; bloquer l'activation du LT et provoquer soit son apoptose, soit son anergie. Le bélatacept entraîne moins d'effets secondaires que les inhibiteurs de la calcineurine qui eux, induisent une augmentation de la glycémie et de la lipidémie (Larsen et al., 2005).

D-4- Les traitements visant le troisième signal

Les anticorps dirigés contre le récepteur à l'IL-2 (CD25), les inhibiteurs de mTOR (Sirolimus ou Rapamicine) et les inhibiteurs de JAK3 visent le troisième signal. Ces inhibiteurs du troisième signal empêchent la prolifération lymphocytaire. Ils ont un effet antiprolifératif, réduisent le taux de cytokines et chimiokines (Webster et al., 2004).

D-5- Les traitements visant une déplétion lymphocytaire

L'alemtuzumab est un anticorps humanisé spécifique de CD52 qui est une molécule exprimée par toutes les cellules mononuclées sanguines. Son utilisation va donc induire une déplétion du compartiment lymphocytaire massive et durable, on parle de Pan-déplétant (Hanaway et al., 2011). L'OKT3, va se fixer au complexe CD3 et induire une déplétion des LT (Norman et al., 2000). Le rituximab est un anticorps humanisé spécifique du CD20, il va induire une déplétion des LB naïfs et mémoires, mais n'a pas d'effet sur les plasmocytes qui n'expriment pas CD20 (Becker et al., 2004).

D-6- Les traitements visant les lymphocytes en multiplication

À l'inverse des anti-calcineurines, les inhibiteurs de l'inosine 5' monophosphate déshydrogénase (IMPDH) sont dénués de néphrotoxicité et n'induisent pas de troubles métaboliques. L'Azathioprine est un anti métabolite, analogue à la purine, il inhibe la synthèse de l'ADN cellulaire (Lennard, 1992). L'Azathioprine diminue le nombre de LT et LB circulant, réduit la synthèse d'Ig et la sécrétion d'IL-2 (Röllinghoff et al., 1973).

L'acide mycophénolique est un puissant inhibiteur sélectif, non compétitif et réversible de l'inosine monophosphate déshydrogénase ; il inhibe la synthèse de novo des nucléotides guanine (sans être incorporé à l'ADN) (Eugui and Allison, 1993). Comme la prolifération des LT et LB est essentiellement dépendante de la synthèse de novo des purines (Allison et al., 1977), alors que d'autres types cellulaires peuvent utiliser d'autres voies métaboliques, l'acide mycophénolique inhibe la prolifération des LT et des LB (Remuzzi et al., 2004).

Le léflunomide et le FK778 sont deux inhibiteurs de l'enzyme DHODH (Dihydroorotate déshydrogénase), impliqués dans la synthèse de novo des pyrimidines (Williamson et al., 1995). On leur prête un effet antiprolifératif sur les LT et L B *in vitro* (Waldman et al., 1999).

D-7- Les traitements visant la migration lymphocytaire

FTY 720 cible les mécanismes de trafic des lymphocytes, *in vivo*, le FTY 720 est rapidement phosphorylé, ce qui donne un composé actif, le FTY 720-P. Le FTY 720-P bloque le récepteur S1P1, qui est internalisé, ce qui provoque une séquestration des lymphocytes dans les ganglions (Brinkmann et al., 2004). Cependant ce traitement n'est pas beaucoup utilisé car il entraine des complications rétiniennes.

D-8- Les principales stratégies d'immunosuppression

En transplantation rénale le principe de l'immunosuppression consiste en un traitement fort, qui permet une prévention du rejet aigu. Ensuite il y a mise en place d'une immunosuppression de maintenance qui permet de contenir les risques de néphropathie d'allogreffe. Les traitements d'induction sont utilisés, soit de principe, soit en cas de reprise retardée de la fonction du greffon, ou encore en cas de risques immunologiques. Les sérums polyclonaux sont employés en cas de risques immunologiques élevés et les anticorps monoclonaux en cas de risques faibles. Les traitements d'immunosuppression d'entretien associent en général, un inhibiteur de la calcineurine (ciclosporine) et un inhibiteur de l'IMPDH (acide mycophénolique) avec ou sans stéroïdes.

Les progrès réalisés au cours de ces 20 dernières années sont principalement liés à une diminution significative des rejets aigus, grâce à l'efficacité des traitements immunosuppresseurs. Cependant même si l'effet à court terme des immunosuppresseurs est vérifié, les problèmes liés à l'immunosuppression à long terme subsistent et sont notamment

dus au déficit immunitaire chronique qui peut entraîner des infections (Fishman, 2007; Soulillou and Giral, 2001), des complications cardiovasculaires (Roberts et al., 2002), de la néphrotoxicité (Nankivell et al., 2004), des cancers… d'où l'intérêt de trouver d'autres thérapies.

II- La tolérance

Normalement un organisme est tolérant à ses propres constituants, on appelle cela la tolérance au soi ou l'auto-tolérance (Goodnow et al., 2005). Dans le cadre de la transplantation d'organes, on cherche à établir une tolérance envers un greffon, on souhaite donc une « non-réponse » envers les antigènes du donneur. Dans cette partie, je vais décrire rapidement les mécanismes de tolérance immunitaire T et B afin de comprendre les mécanismes mis en jeu dans la tolérance immunitaire.

A- La tolérance immunitaire T

Cette auto-tolérance fait intervenir deux grands types : la tolérance centrale et celle, périphérique.

A-1- La tolérance centrale

La tolérance centrale intervient en premier, elle correspond à la mort par apoptose des lymphocytes auto réactifs, c'est-à-dire, les lymphocytes reconnaissant les antigènes du soi. Cette tolérance centrale a lieu dans le thymus pour les LT, elle permet de sélectionner uniquement les lymphocytes ne reconnaissant pas les auto-antigènes (Starr et al., 2003). Bien que la majorité des lymphocytes autoréactifs soit supprimée par les mécanismes de tolérance centrale, certains lymphocytes peuvent échapper à cette sélection dite négative, également appelée délétion clonale et pourraient induire des réactions auto-immunes en l'absence de mécanismes de tolérance périphérique (Khan et al., 2013).

A-2- La tolérance périphérique

La tolérance périphérique fait intervenir quatre mécanismes différents, l'ignorance immunologique, l'anergie lymphocytaire, la délétion clonale périphérique et la suppression par des cellules régulatrices.

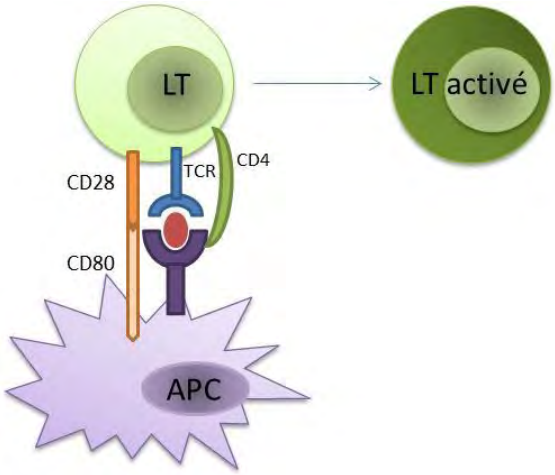

Figure 3 : Schéma d'activation normale d'un LT par une cellule présentatrice d'antigène.

A-2-1- L'ignorance immunologique

C'est l'ignorance des lymphocytes envers un antigène. En effet, tous les auto-antigènes n'étant pas représentés dans le thymus, les lymphocytes T autoréactifs à cet auto-antigène ne seront pas supprimés par les mécanismes de tolérance centrale et vont migrer dans le sang. Or tant que ces lymphocytes n'ont pas rencontré leur auto-antigène, ils ne sont pas activés. Ce sont donc des lymphocytes T naïfs qui circulent uniquement dans le sang, la rate et les ganglions (Alferink et al., 1998).

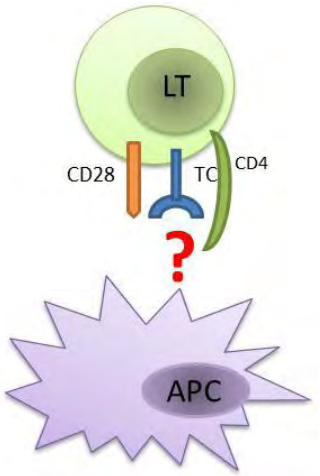

Figure 4 : Schéma représentant l'ignorance immunologie.

A-2-2- L'anergie lymphocytaire

C'est l'inactivation fonctionnelle des LT. Elle apparait lorsque les LT reconnaissent des antigènes en l'absence de costimulation d'intensité suffisante (second signal) (DeSilva et al., 1991; Harding et al., 1992), nécessaire à l'activation complète des LT. Cette anergie se traduit par un état de non-réponse du lymphocyte T qui ne prolifère pas et ne sécrète pas d'IL-2 en présence de l'antigène.

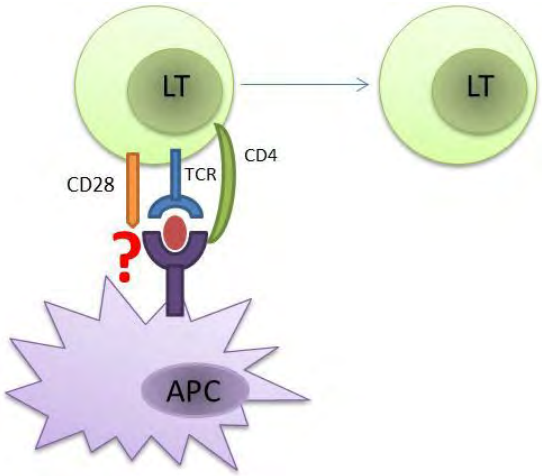

Figure 5 : Schéma représentant l'anergie lymphocytaire.

A-2-3- La délétion clonale périphérique

C'est la mort par apoptose des LT autoréactifs. Il existe deux mécanismes possibles de mort des LT induits par des auto-antigènes. Un mécanisme d'apoptose actif ; en présence d'une trop forte dose d'auto-antigène, tous les précurseurs lymphoïdes T spécifiques vont s'activer, proliférer et sécréter de l'IL-2. L'IL-2 peut entraîner l'expression de Fas à la surface des LT activés (Li et al., 2000). FasL va se lier à Fas exprimé à la surface des LT et induire un signal suicidaire de mort et provoquer l'activation de caspases. On appelle ce processus, la « mort cellulaire induite par l'activation » (AICD) (Brunner et al., 1995). Les LT peuvent également mourir par un mécanisme d'apoptose passif, lorsque les lymphocytes sont privés de facteurs de croissance (IL-2, IL-4, IL-9, IL-15, IL-21), il n'y aura plus d'activation des facteurs anti-apoptotiques tels que Bcl-2 et Bcl-xl, entrainant ainsi leur apoptose (Lechler et al., 2003).

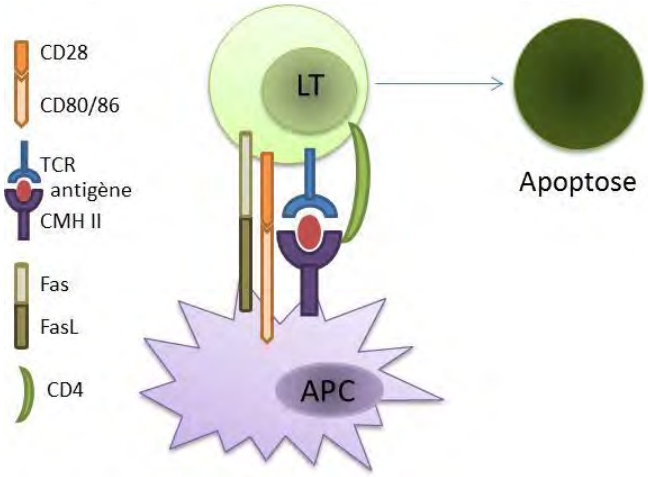

Figure 6 : Schéma représentant la délétion clonale périphérique

A-2-4- La suppression

Les lymphocytes T régulateurs peuvent se développer dans le thymus ou dans les tissus lymphoïdes périphériques. Ces LTreg peuvent bloquer l'activation des lymphocytes potentiellement dangereux et spécifiques des auto-antigènes. C'est en 1970 qu'est apparu le concept de suppression induit par les lymphocytes régulateurs (Gershon and Kondo, 1970). Ces lymphocytes T régulateurs vont inhiber la réponse immunitaire par la sécrétion de cytokines anti-inflammatoires (IL-10, TGF-β) et/ou par contact cellulaire.

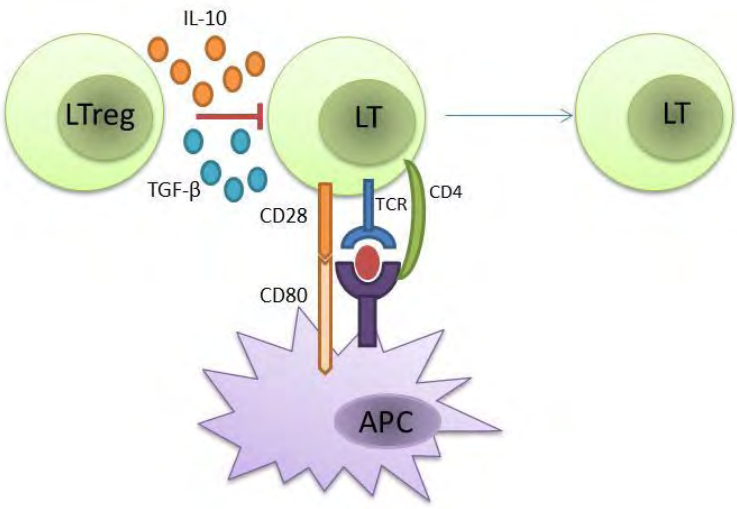

Figure 7 : Schéma représentant la suppression

B- La tolérance Immunitaire B

Comme pour la tolérance T, Les LB sont contrôlés par deux grands mécanismes : la tolérance centrale qui a lieu dans la moelle osseuse et la tolérance périphérique qui permet de contrôler les clones B autoréactifs.

.B-1- La tolérance centrale

Il existe plusieurs mécanismes impliqués dans la tolérance centrale des LB : la réédition du récepteur pour l'antigène appelé « receptor editing » et la délétion clonale sont les deux mécanismes majeurs de la tolérance centrale B. La délétion clonale est la mort par apoptose des LB autoréactifs (figure 8). Lorsque l'antigène induit une forte agrégation des récepteurs membranaires ou en cas de forte affinité pour l'antigène, l'activation de signaux intracellulaires va favoriser les mécanismes de « receptor editing » (modification de la spécificité du BCR) et de délétion clonale. En revanche si le seuil d'affinité n'est pas assez fort ou si l'antigène agrège moins de BCR (dans le cas d'antigènes solubles par exemple), il y aura tolérisation par anergie. Dans le cas où l'antigène n'est pas ou peu présent dans la moelle osseuse, les clones B autoréactifs échappent à la tolérance centrale et quittent la moelle osseuse par ignorance clonale (Goodnow, 1992).

Le receptor editing

La rencontre d'un LB autoréactif avec son auto-antigène dans la moelle osseuse n'aboutit pas toujours à la délétion de celui-ci. Au contraire, dans la majorité des cas, il y a réarrangement des gènes d'immunoglobulines, ce qui va permettre de modifier la spécificité antigénique du BCR et éviter ainsi la reconnaissance d'auto-antigènes (Brack et al., 1978; Weigert et al., 1978). Le « receptor editing » touche la région V (Variable) spécifique de l'auto-antigène. Celle-ci va être échangée avec un segment génique différent de la région V à l'aide d'une recombinaison V(D)J, qui va produire un récepteur moins autoréactif et permettre la survie de la cellule (Gay et al., 2011; Tiegs et al., 2011).

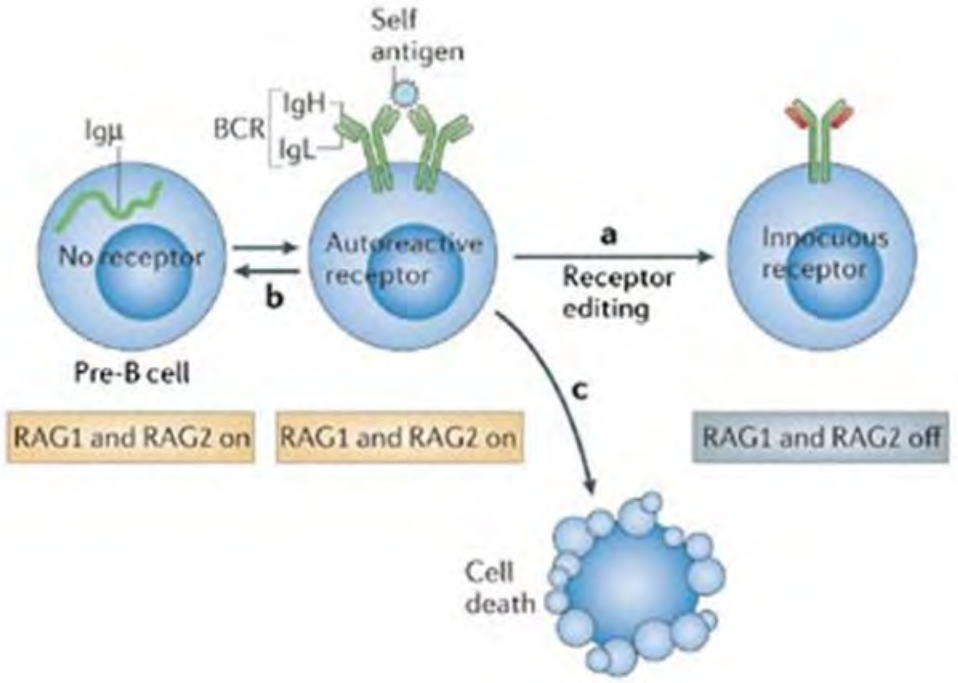

Figure 8 : Tolérance centrale des LB au niveau de la moelle osseuse.

Les précurseurs des LB (Pre-B) expriment un BCR avec une chaine lourde H réarrangée, en cas de reconnaissance avec les auto-antigènes, la chaine légère L sera réarrangée afin d'obtenir une expression de chaine d'IgL qui ne reconnaîtra plus l'auto-antigène (a) ce qui conduit à l'inactivation des gènes RAG1 et RAG2. Une mauvaise recombinaison entrainera une perte du récepteur de surface (b). La correction infructueuse d'un récepteur autoréactif mène à la mort cellulaire (c) (Nemazee, 2006).

B-2- La tolérance périphérique B

Une fois sortie de la moelle osseuse, les LB immatures deviennent des LB transitionnels qui doivent maturer en périphérie. Pour cela, le LB transitionnel passe d'abord dans la rate puis dans les organes lymphoïdes secondaires. Dans ces organes les LB rencontrent des auto-antigènes qui n'étaient pas présents dans la moelle osseuse. Il existe donc des mécanismes de tolérance périphérique qui permettent d'éliminer l'activation de ces LB autoréactifs (Melchers et al., 1995).

B-2-1- L'anergie

Les LB autoréactifs qui expriment une faible affinité pour un auto-antigène entrent en anergie. Cet état se caractérise par une perte de la signalisation du BCR et donc une perte de la réponse B ainsi qu'une incapacité à présenter l'antigène ou à produire des anticorps (Cambier et al., 2007; Goodnow et al., 2009). Ces cellules B anergiques ont une espérance de vie raccourcie , une migration altérée , une incapacité à interagir avec les LT helper et nécessitent une forte quantité de facteur de BAFF pour survivre (Lesley et al., 2004; Thien et al., 2004).

B-2-2- L' ignorance

L'ignorance est liée à la compartimentation. Les LB autoréactifs ne rencontrent pas leur auto-antigène. Ou bien, des LB qui expriment des récepteurs de faible affinité échappent aux processus de sélection négative de la moelle osseuse et peuvent arriver en périphérie (Shlomchik, 2008). Une fois en périphérie, le signal induit par l'auto-antigène est trop faible pour entrainer une réponse : c'est l'ignorance clonale (Aplin et al., 2003; Hannum et al., 1996; Shlomchik et al., 1993)

B-2-3- Le receptor editing

Lorsque le LB présente une forte affinité pour un auto-antigène cela entraine le « receptor editing » par expression des gènes RAG 1 et 2 et le réarrangement des gènes de la chaine légère pour modifier la spécificité du BCR (Pelanda and Torres, 2006)

B-2-4- La délétion clonale

Lorsque les LB autoréactifs présentent une très forte affinité pour l'auto-antigène cela entraîne la délétion rapide de LB autoréactifs Bim-dependant (Enders et al., 2003)(mort par apoptose).

III- La tolérance en transplantation rénale

Le concept de la tolérance a émergé au siècle dernier avec Billingham, Brent et Medawar, qui ont montré qu'il était possible d'induire une tolérance de greffe de peau chez la souris par inoculation de tissus cellulaires homologues étrangers pendant la période fœtale (Billingham et al., 1955). Ainsi, l'induction de tolérance permet la transplantation d'organes ou de cellules d'un individu étranger sans administration de traitements immunosuppresseurs tout en conservant une immunocompétence du receveur. La tolérance en transplantation représente donc le Graal en transplantation. De nombreuses études ont été réalisées dans des modèles animaux dans lesquels les protocoles d'induction sont décrits pour obtenir une tolérance (Jovanovic et al., 2008; Sakaguchi et al., 2006; Wood and Sakaguchi, 2003). Mais malheureusement, beaucoup de ces modèles ne sont pas transposables chez l'Homme. Chez l'Homme, en transplantation rénale, on distingue deux principaux types de tolérance : la tolérance induite et la tolérance spontanée.

A- La tolérance induite en transplantation rénale

L'induction de tolérance en transplantation rénale est principalement obtenue par chimérisme hématopoïétique. Cette induction de tolérance est obtenue par une greffe de moelle osseuse ou injection de cellules souches hématopoïétiques du donneur au receveur. Ainsi, le patient transplanté dispose de ses propres cellules hématopoïétiques et de celles du donneur. Le chimérisme prévient du rejet chronique et induit la survie du greffon dans plusieurs modèles animaux (Huang et al., 2000; Ildstad and Sachs, 1984). Cette induction de tolérance par chimérisme a été observée chez des patients ayant reçu une greffe de moelle osseuse comme traitement de maladie hématologique et qui, par la suite, ont reçu une greffe de rein provenant du même donneur que la greffe de moelle osseuse (Dey et al., 1998; Helg et al., 1994; Jacobsen et al., 1994; Sayegh et al., 1991). Des études plus récentes ont confirmé la capacité de cette technique à être transposée chez l'Homme (Kawai et al., 2008; Sachs et al., 2011; Scandling et al., 2008). Cependant dans ces études, soit le chimérisme n'était pas durable (Kawai et al., 2008; Sachs et al., 2011), soit il était durable mais limité à des transplantations HLA identiques (Millan et al., 2002; Scandling et al., 2008). Une étude récente décrit l'induction de chimérisme à long terme en transplantation rénale (Leventhal et al., 2012). Dans cette étude, ils associent un conditionnement myélo-ablatif de faible intensité

(irradiation et cyclophosphamide avant transplantation), suivi de la transplantation rénale puis d'une greffe de cellules souches hématopoïétiques (CSH) dérivées du donneur. Cette greffe est combinée avec des cellules facilitantes (cellules proches des pDCs qui vont aider la greffe de CSH et éviter la maladie du greffon contre l'hôte (GvHD)). La greffe est suivie d'un traitement immunosuppresseur d'entretien. Ce protocole a permis d'obtenir un chimérisme durable chez la plupart des receveurs HLA incompatibles qui permet un sevrage de toutes immunosuppressions (Leventhal et al., 2012). Toutefois, les problèmes majeurs de l'induction de tolérance par chimérisme hématopoïétique sont les risques de GVHD et les problèmes liés au traitement de conditionnement (myélo-ablation) (Bölling et al., 2011) donné au receveur avant la greffe de moelle osseuse.

B- La tolérance spontanée en transplantation rénale

Comme son nom le suggère, à l'inverse de la tolérance induite, la tolérance spontanée ne provient pas d'un protocole d'induction de tolérance et est observée dans de rare cas lorsque les patients transplantés ne prennent plus leur traitement immunosuppresseur et ne rejettent pas leur greffon. Les patients spontanément tolérants arrêtent leur traitement pour deux raisons principales, pour non-compliance ou sur décision médicale due notamment aux complications liées aux traitements immunosuppresseurs tels que des infections qui peuvent conduire à des maladies lymphoprolifératives après transplantation (PTLD - post transpant lymphoproliferative disorder) (Singh et al., 2014). La PTLD survient souvent après infection une EBV.C'est une complication grave de la transplantation (Caillard et al., 2006; Opelz and Döhler, 2004). Dans de rares cas, malgré l'arrêt des traitements immunosuppresseurs, quelques patients conservent une bonne fonction de leur greffon et une résistance envers les infections (Ballet et al., 2006). Le terme 'Opérationnelle' est souvent utilisé pour caractériser ces patients, puisque leur état de tolérance est uniquement défini par la fonction du greffon, en raison de l'absence de biopsie pour confirmer une histologie normale (Brouard et al., 2012; Roussey-Kesler et al., 2006). Bien que la tolérance opérationnelle spontanée soit plus souvent observée chez les transplantés hépatiques (Ashton-Chess et al., 2007; Lerut and Sanchez-Fueyo, 2006; Turka and Lechler, 2009), elle est également observée dans quelques rares cas de transplantation rénale. La tolérance opérationnelle en transplantation rénale a

été définie par une fonction stable du greffon (créatinine inférieure à 150µmol/l et une protéinurie inferieure à 1g/24H) en l'absence de traitement immunosuppresseur depuis plus d'un an (Orlando et al., 2010; Roussey-Kesler et al., 2006).

C- Signature B

En transplantation, le rôle des LB dans le rejet ou l'acceptation du greffon est d'un intérêt majeur. La présence d'anticorps spécifiques du donneur chez le patient transplanté est souvent associée à une baisse de la fonction du greffon et à un rejet (Lee et al., 2002; Willicombe et al., 2012; Worthington et al., 2003), et démontre la fonction pathogénique des LB en transplantation (Clatworthy, 2011; Noorchashm et al., 2006). Néanmoins, nous allons voir que plusieurs études portant chez des patients transplantés rénaux avec une tolérance opérationnelle, présentent un rôle potentiel des LB dans le maintien de la tolérance (tableau 1). Dans le chapitre suivant, nous constaterons également que plusieurs études chez l'Homme et la souris confirment une fonction régulatrice des LB.

En 2006, Louis et al ont démontré pour la première fois une augmentation du nombre de LB chez les patients tolérants. Suite à cette étude, différentes équipes se sont intéressées aux LB dans la tolérance en transplantation rénale. Une signature de 49 gènes a été retrouvée chez les patients tolérants avec notamment des gènes liés aux LB tels que CD79a,CD79b, CD19 et CD20 (Brouard et al., 2007). Puis, l'étude de Newell et al a décrit une signature B associée aux patients tolérants (Newell et al., 2010). Pallier et al en 2010 montrent une augmentation du nombre de LB dans le sang des patients tolérants et un enrichissement en LB transitionnels par rapport aux patients stables. Cette augmentation en LB transitionnels va être confirmée dans différentes études (Chesneau et al., 2013a; Newell et al., 2010; Sagoo et al., 2010). De plus, il a été prouvé que les LB transitionnels ($CD19^{+}CD24hiCD38hi$) possèdent des fonctions régulatrices notamment via leurs capacités à sécréter de l'IL-10 (Blair et al., 2010; Das et al., 2012; Flores-Borja et al., 2013). Ainsi, une augmentation du nombre de LB avec des capacités régulatrices pourrait être impliquée dans la tolérance en transplantation rénale. L'étude de Pallier et al montre également une diminution du ratio CD32a/CD32b (FcgRIIa/FcgRIIb) chez les patients tolérants. CD32a et CD32b sont des récepteurs Fcg de faible affinité, le premier activateur et l'autre inhibiteur (Veri et al., 2007). Cette diminution du ratio des CD32a activateurs par rapport aux CD32b inhibiteurs chez les patients tolérants

suggère une diminution de l'activation des LB chez ces patients. De plus, les patients tolérants présentent une surexpression de ratio BAFF-R/BAFF en périphérie, impliquée dans la maturation des LB et dans le maintien du nombre de LB en périphérie (Schiemann, 2001; Stadanlick and Cancro, 2008; Yang et al., 2014). Chez les patients tolérants il a également été observé une augmentation de BANK-1 dans les PBMCs des patients tolérants comparés aux patients stables. Cette tendance est également retrouvée dans les LB purifiés, montrant qu'il ne s'agit pas seulement d'un effet « nombre » (plus de LB chez les patients tolérants donc augmentation des molécules exprimées par LB dans les PBMCs des tolérants). BANK-1 est une protéine adaptatrice fortement exprimée par les LB périphériques (Yokoyama et al., 2002) qui régulent les réponses B hyperactives en inhibant l'activation via CD40 par inhibition de l'activation par AKT (Aiba et al., 2006). Cette étude a également montré une plus grande fréquence de LB $CD5^+$ et $CD1d^+$. Toutefois, les LB $CD5^+$ $CD1d^{hi}$ ont été décris comme une sous-population de LB régulateurs chez la souris appelée lymphocytes B10 (Yanaba et al., 2008). Les études de Sagoo et al et Newell et al confirment cette idée d'une augmentation du nombre de LB chez les patients tolérants avec un potentiel rôle régulateur (plus de LB transitionnels sécrétant de l'IL-10) (Newell et al., 2010; Sagoo et al., 2010). Plus récemment l'étude de Silva a rapporté que les patients tolérants ont un répertoire du BCR conservé. Mais les patients en rejet chronique ont un défaut dans leur capacité à activer les B reg par phosphorylation de STAT3. De fait, cette étude suggère que la tolérance B serait davantage liée à une homéostasie des LB conservée plutôt qu'à une différence réelle de la fonction des LB (Silva et al., 2012).

Il est très intéressant de voir qu'une partie de ces résultats a pu être vérifiée dans un modèle de tolérance d'allogreffe cardiaque chez le rat (Le Texier et al., 2011). En effet, dans cette étude il a été montré chez des rats tolérants qu'il y a une augmentation du nombre de LB dans les PBMC de ces animaux et une surexpression de FcgRIIb et BANK-1.

Chez l'Homme, les LB TGF-β^+ joueraient un rôle dans le cadre de la tolérance en transplantation rénale (Haynes et al., 2012). De façon intéressante, le TGF-β chez les patients tolérant une greffe rénale avait déjà été mis en avant dans une étude de Danger et al. Une surexpression du miR142-3p a été retrouvée dans les PBMC et dans les LB des patients tolérants. Or, le miR142-3p est impliqué dans la voie du TGF-β. Dans cette étude, il a

également été montré une augmentation de l'expression du TGF-β1 dans les LB des patients tolérants (Danger et al., 2012).

Reference	Groups studied	Implication of B cells
Louis et al. (44)	TOL/STA/CR	TOL display more circulating B cells compared to STA and CR
Brouard et al. (45)	TOL/STA/CR/HV	Transcriptional signature in OT patients. Footprint of 49 genes, several genes implicated B cells, CD79a,b, CD19, CD20
Pallier et al. (46)	TOL/STA/CR/HV	Higher absolute number and frequency of total B cells in blood. Increased expression of BANK-1, CDld, CD5, FCγRIIb in TOL vs. STA
Newell et al. (4)	TOL/STA/HV	B-cell signature in TOL patients, increase of naives B cells and increase of IL-10 expression in TOL vs. STA
Sagoo et al. (5)	TOL/STA/CR/HV	Increase number of B cells and TGF-b producing cells in TOL vs. STA and CR
Danger et al. (47)	TOL/STA	Over-expression of miR142-3p in B cells and increase of TGF-BI expression in B cells from TOL vs. STA
Silva et al. (48)	TOL/STA/CR/HV	Transitional B cells from TOL preserved ability to activate the CD40/STAT3 signaling pathways in transitional B cells in contrast with CR
Haynes et al. (49)	TOL/STA/CR	Increase of circulating naive B cells in TOL vs. STA and CR. Higher POT score ("probability of being tolerant": score including B-cell parameters and direct pathway T-cell parameters) in TOL vs. STA and CR
Chesneau et al. (50)	TOL/STA/HV	Less plasma cells in TOL vs. STA. *In vitro* their is a default in B-cell differentiation and an increase B-cell sensitivity to apoptosis in late step of differentiation of B cells from TOL vs. STA. Increase of IL-10 expression by activated B cells in TOL vs. STA

Tableau 1 : Études réalisées chez les patients tolérant une greffe rénale et montrant une implication des LB (Chesneau et al., 2013b).

IV- Les lymphocytes B

Les LB sont le support de l'immunité humorale adaptative dont les effecteurs terminaux sont les anticorps ou Immunoglobulines (Ig). Les LB ont pour fonction de « neutraliser/détruire » les éléments étrangers et pathogènes (Slifka and Ahmed, 1998). Les LB représentent 5-15% des lymphocytes sanguins. Ils reconnaissent les antigènes (Ag) grâce à leur récepteur de surface spécifique de l'Ag : BCR induisant, le plus souvent, l'activation et la différenciation des LB en plasmocytes ou en LB mémoires.

A- Origine et développement

La maturation du LB passe par plusieurs stades évolutifs. Il existe deux grandes étapes de maturation du LB ; une étape indépendante de l'Ag, la lymphopoïèse qui a lieu au niveau de la moelle osseuse et une étape dépendante de l'Ag ou immunopoïese qui se déroule dans les organes lymphoïdes périphériques (Banchereau et al., 1994; Clark et al., 2014).

A-1- La lymphopoïèse

C'est dans la moelle osseuse que se trouvent les cellules souches hématopoïétiques (HSC), qui sont à l'origine des différentes populations cellulaires du système immunitaire et notamment des LB (Baba et al., 2004; Hardy and Hayakawa, 2001; Nagasawa, 2006). C'est également dans la moelle osseuse qu'auront lieu la prolifération et la maturation des LB. Au contact des cellules de soutien de la moelle osseuse (les cellules stromales) et sous l'influence de l'IL-7 (Clark et al., 2005; Herzog et al., 2009), les cellules pro-B (progéniteurs B) se différencient en cellules pré-B (Précurseurs B) puis en cellules B immatures. Au cours de cette différenciation au sein de la moelle osseuse, il y a réarrangement des segments de gènes des immunoglobulines qui expriment un BCR unique ainsi que des protéines de surface (Ghia et al., 1996; LeBien and Tedder, 2008). Les LB immatures exprimant un BCR-IgM vont subir une première phase de sélection contre les Ag médullaires du soi, puis les LB survivants - appelés LB transitionnels de type 1 (T1) - entrent dans la circulation et migrent vers la zone marginale de la rate où ils poursuivent leur maturation en LB transitionnels T2 (T2) (Loder et al., 1999) (figure 9).

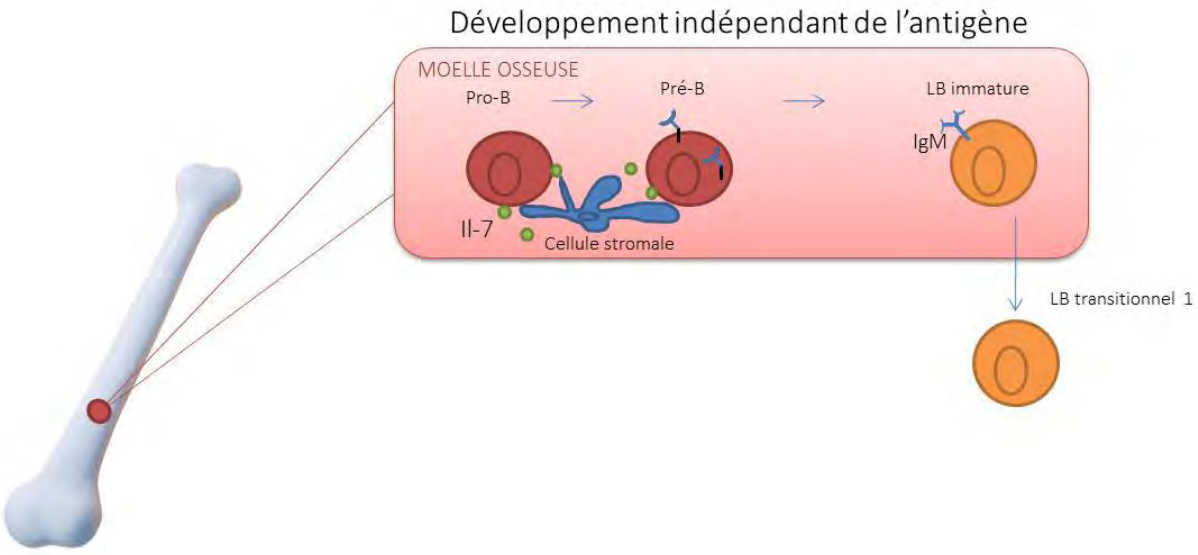

Figure 9 : Schéma représentant la lymphopoïèse B.

A-2- L'immunopoïese

A-2-1- Éducation splénique (tolérance périphérique) :

Dans la zone marginale de la rate (ZMG), les interactions BCR-Ag splénique « du soi » déterminent si un LB T2 va se différencier en LB mature naïf folliculaire ($IgM^{+}IgD^{++}$) ou en LB de la ZMG ($IgM^{++}IgD^{+}$). Ces deux sous-populations de LB possèdent des fonctions différentes. Les LB de la ZMG sont responsables de la réponse dite thymo- ou T-indépendante (TI) alors que les LB matures naïfs folliculaires sont responsables de la réponse thymo- ou T-dépendante (TD). En l'absence de rencontre avec l'antigène, les LB matures naïfs ne survivront pas au-delà de quelques jours-semaines et entreront en apoptose (Lagresle et al., 1996).

4-2-2- La réponse thymo-indépendante (TI)

Contrairement à l'activation thymo-dépendante, l'activation thymo-indépendante ne nécessite pas l'aide des LT helper (LTh) pour produire les anticorps (figure 10). On les classe en 2 catégories :

- L'activation thymo-indépendante de type 1 entraîne une stimulation polyclonale des LB. Cette activation ne passe pas par le BCR mais par des récepteurs communs à tous les LB qui reconnaissent les pathogènes que l'on appelle des mitogènes (Coutinho et al., 1974).

- L'activation thymo-indépendante de type 2 entraîne une stimulation monoclonale des LB (Mond et al., 1995). Cette activation passe cette fois-ci par le BCR qui reconnaît des déterminants sucrés répétitifs. On observera cependant essentiellement une production d'IgM.

Suite à une activation thymo-indépendante, les LB de la zone marginale peuvent se différencier en LB mémoire à IgM de membrane et donner des plasmocytes sécrétant des IgM de faible affinité pour l'antigène et à courte durée de vie (Liu and Arpin, 1997; MacLennan, 1994).

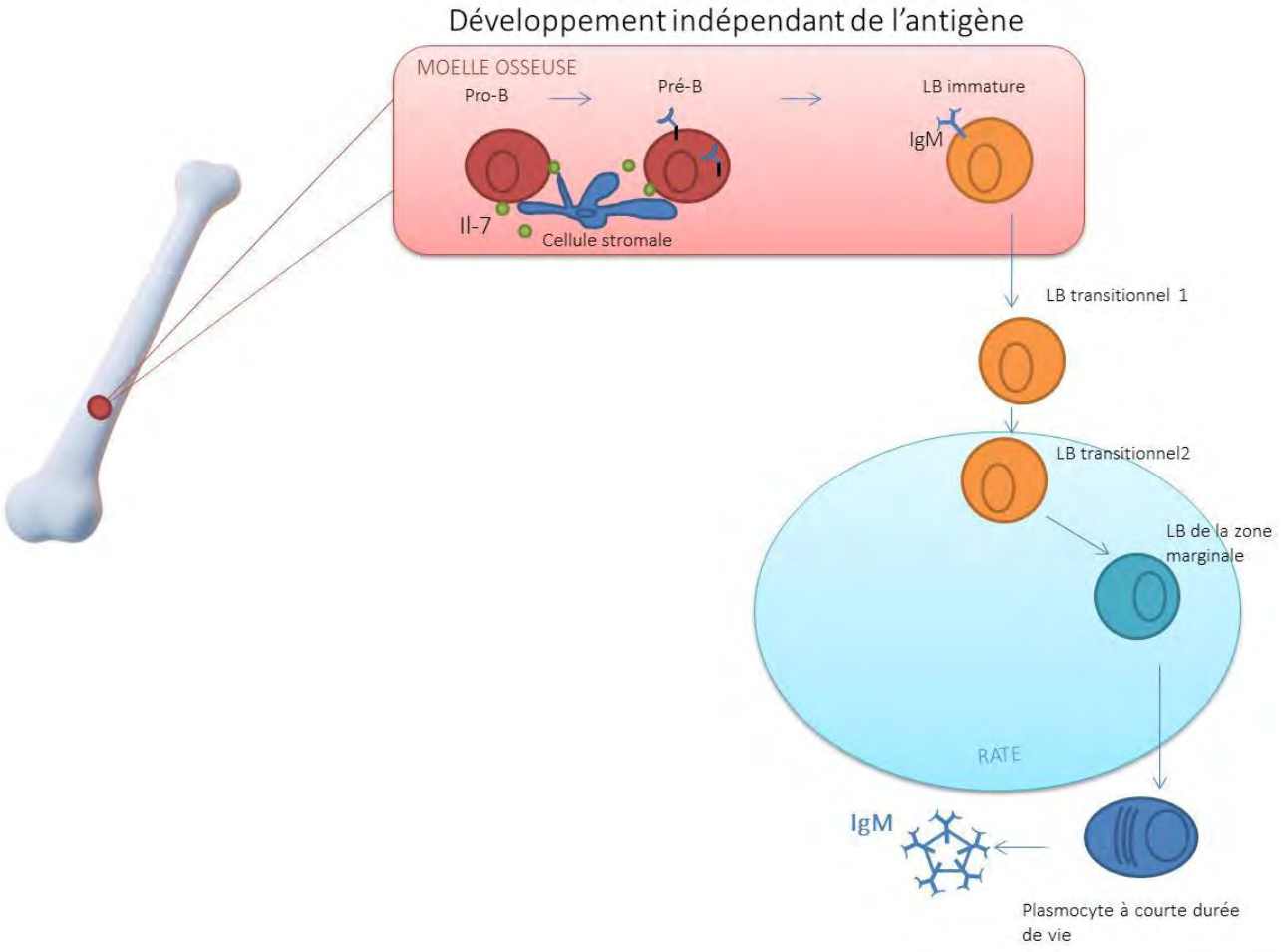

Figure 10 : Schéma représentant la réponse thymo-indépendantes des LB

4-2-3- La réponse thymo-dépendante (TD)

Les LB folliculaires circulent en permanence dans le sang et les zones B des organes lymphoïdes secondaires jusqu'à ce qu'ils rencontrent leurs antigènes (Okada and Cyster, 2006; Tew et al., 1997). Soit les LB migrent vers une zone extra folliculaire, puis les LB

prolifèrent et se différencient en plasmocytes à courte durée de vie sécrétant des anticorps, soit les LB migrent vers le follicule primaire (agrégat cellulaire des organes lymphoïdes secondaires) prolifèrent et forment des centres germinatifs (Cyster, 2010). Le centre germinatif est le résultat d'une prolifération des LB actifs et apparaît dans les 1-3 semaines après la reconnaissance de l'antigène TD. Le centre germinatif est le site de prolifération importante et de différenciation en LB mémoires ou en plasmocytes (Goodnow et al., 2010). L'architecture du centre germinatif permet un contact privilégié et prolongé entre les LB et LT ayant la même spécificité antigénique (Carter and Myers, 2008).

A-2-4- La différenciation des LB

Dans le centre germinatif, les LB subissent des processus d'hypermutation somatique (HMS) et de commutation isotypique, avant de se différencier en LB mémoires ou en plasmocytes à longue durée de vie (Smith et al., 2000).

A-2-4-1- L'hypermutation somatique (HMS)

C'est un processus qui introduit des mutations ponctuelles dans les régions variables des chaines lourdes (IgH) et légères IgL) du BCR suite à son activation par liaison à l'antigène (Peled et al., 2008). Même si ces mutations se font au hasard, on distingue quatre types de mutations : silencieuse, neutre, délétère et positive. Seules les mutations délétères et positives auront des effets sur l'affinité de l'antigène pour son BCR (Dörner et al., 1998). La délétion entrainera une diminution de l'affinité de l'antigène pour le BCR et sera responsable de la mort cellulaire. Alors que la mutation positive augmente l'affinité de l'antigène pour le BCR et n'entrainera pas la mort de la cellule (Rajewsky, 1996). L'intérêt de l'HMS est de sélectionner les LB qui auront la meilleure affinité pour l'antigène donc d'aboutir à des anticorps plus efficaces et des cellules mémoires plus spécifiques dans le cas de nouvelle rencontre avec l'antigène (Berek et al., 1991; Jacob et al., 1991).

A-2-4-2- La commutation isotypique

La commutation isotypique est également appelée commutation de classe ou CSR (pour «Class Switch Recombination»). Il existe cinq classes d'immunoglobulines qui sont différentes au niveau des variations de leur région constante (Stavnezer and Schrader, 2014): IgM, IgD, IgG, IgE, IgA. Pour chacun de ces isotypes il existe un gène de région constante : Cμ (pour les IgM), Cδ (pour les IgD), Cγ (pour les IgG), Cε (pour les IgE), Cα (pour les IgA). Tous ces

isotypes reconnaissent le même antigène, car seules les régions constantes les différencient les uns des autres. La commutation de classe est donc le remplacement du locus Cμ par un autre locus (Cμ étant le premier gène exprimé puisque les LB expriment en premier l'IgM) pour exprimer une immunoglobuline d'un autre isotype. Le choix de l'isotype se fait en fonction de la réponse immunitaire voulue (Coffman et al., 1993). Dans le centre germinatif, les LB folliculaires activés peuvent se différencier en centroblastes qui prolifèrent et changent l'affinité de leurs immunoglobulines par hypermutation somatique (SHM) (Berek et al., 1991). Les centroblastes se situent dans la zone sombre du centre germinatif. Ils migrent ensuite dans la zone claire où ils vont modifier la partie constante de leurs immunoglobulines (class switch) et deviennent des centrocytes. Les centrocytes ayant des Ig de forte affinité pour l'antigène vont être sélectionnés (Lam et al., 1997). A contrario, les clones autoréactifs ou ceux qui possèdent une faible affinité vont mourir par apoptose ou retourner dans la zone sombre où ils subiront de nouveau des hypermutations somatiques. Les clones sélectionnés reçoivent de l'aide des LTh ou des cellules dendritiques folliculaires (DCf) pour subir une commutation de classe (CSR) et démarrer leur différenciation en LB mémoires ou en plasmocytes (Phan 2006) (Figure 11).

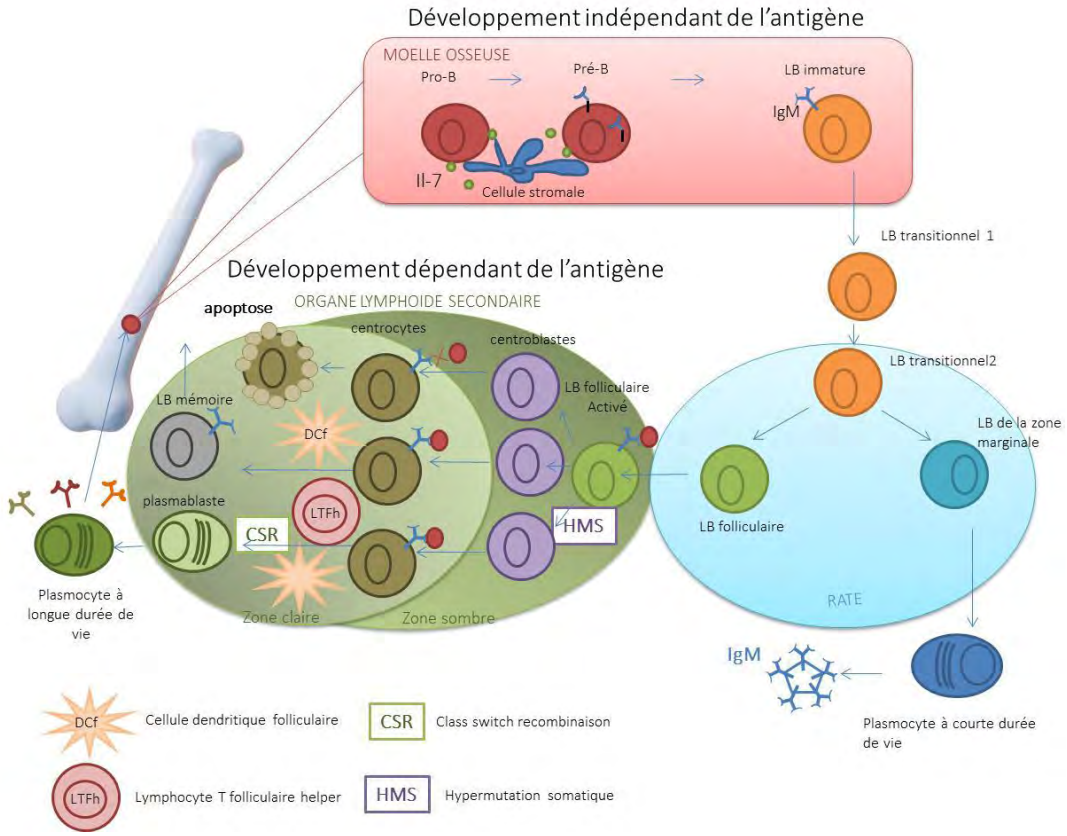

Figure 11 : Schéma représentant la lymphopoïèse et l'immunopoïese B.

A-2-5 Facteurs de transcriptions impliqués dans la différenciation:

Les centrocytes seraient donc les précurseurs des LB mémoires et des plasmocytes (Victora and Nussenzweig, 2012). L'inactivation de PAX-5 (paired box protein 5) par un stimulus et un mécanisme encore inconnu semble être la première étape de la différenciation en plasmocytes (Alinikula and Lassila, 2011). PAX-5 est un inhibiteur transcriptionnel qui joue un rôle important dans le maintien de l'identité des LB matures, mais également des LB naïfs du centre germinatif et des LB mémoires (Cobaleda et al., 2007). L'inhibition de l'expression de BCL-6 serait le deuxième signal nécessaire à la différenciation en LB mémoires ou plasmocytes (Angelin-Duclos et al., 2000; Falini et al., 2000). Plusieurs signaux peuvent induire l'inhibition de BCL-6 comme l'activation du BCR et du CD40 qui inhibe l'activation de BCL-6 via IRF4 dans les centrocytes qui à son tour lève l'inhibition de BLIMP1 (Shaffer et al., 2000). XBP1 est un facteur de transcription nécessaire pour le phénotype sécréteur des plasmocytes ; il interviendrait en aval de BLIMP-1 (Reimold et al., 2001; Shaffer et al., 2004). L'expression de BLIMP1, IRF4 et XBP1 est régulée de façon indépendant, mais les trois facteurs sont nécessaires pour obtenir une différenciation complète en plasmocytes (Kallies et al., 2007) (Figure 12). Les étapes menant à la différenciation des centrocytes en LB mémoires sont moins connues. STAT5 semblerait jouer un rôle dans la différenciation en LB mémoires et non en plasmocytes par la régulation de l'expression de BCL-6 (Scheeren et al., 2005). Cependant le rôle de STAT5 doit encore être clarifié. Comme les LB mémoires continuent d'exprimer PAX-5 contrairement aux plasmocytes, Il semblerait que la non-inactivation de PAX-5 pourrait jouer un rôle dans la différenciation en LB mémoires. La stimulation de CD40, semble également jouer un rôle primordial dans la différenciation en LB mémoires vs plasmocytes. En effet Arpin et al en 1995 ont montré in vitro que des LB du centre germinatif purifiés et cultivés sur des fibroblastes présentant CD154 (CD40) avec IL-2 et IL-10 acquièrent un phénotype de LB mémoires alors que sans CD154 les LB acquièrent un phénotype de plasmocytes (Arpin et al., 1995). Récemment, il a été montré que l'activation de CD40 induit l'inhibition de BLIMP1 nécessaire à la différenciation en plasmocytes et ce par des mécanismes indépendants de PAX-5 et BCL6 (Upadhyay et al., 2014). Reste à définir ce qui rompt la signalisation de CD40 pour la différenciation en plasmocytes.

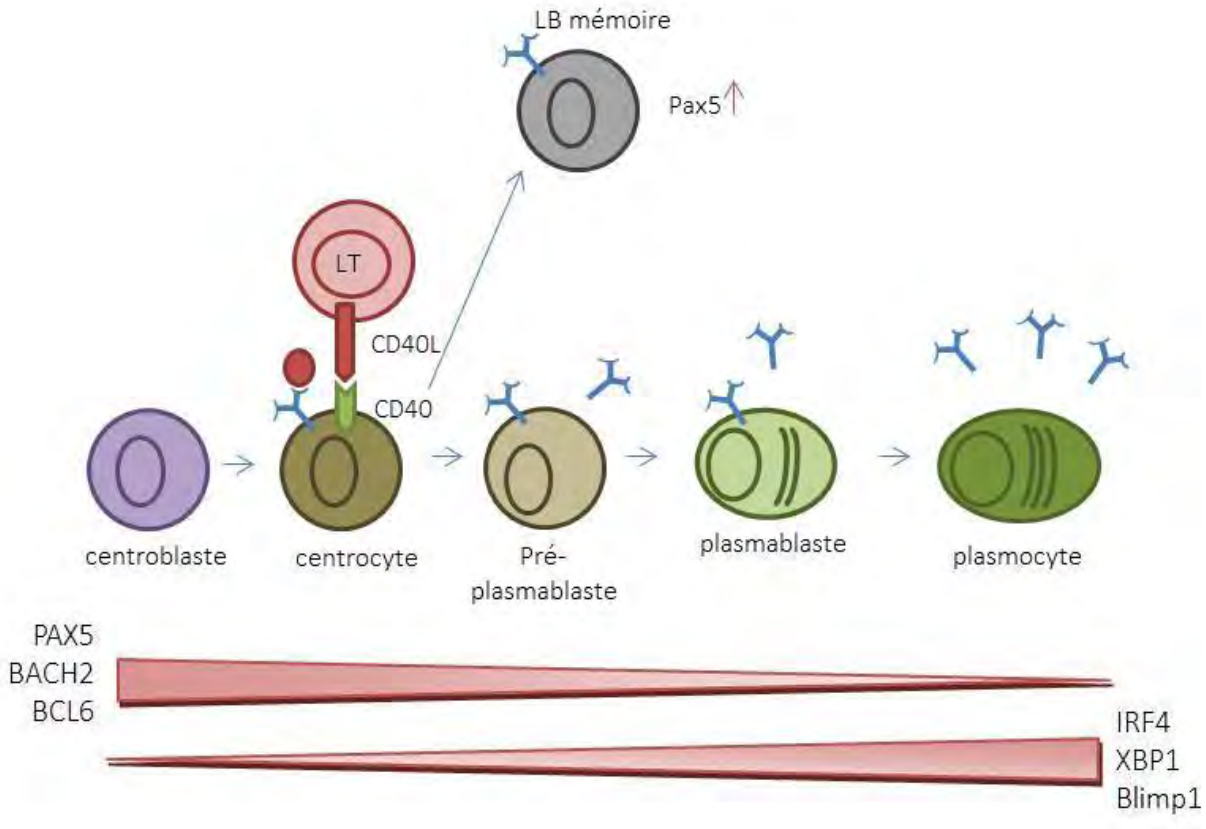

Figure 12 : Schéma représentant l'expression des facteurs de transcription au cours de la différenciation des centrocytes en plasmocytes

A-2-6- Les lymphocytes B en périphérie

La survie des LB en périphérie dépend de l'expression d'un BCR fonctionnel et des signaux générés par BAFF/BAFF-R (Thompson et al., 2001). Au cours de la maturation des LB, BAFF est exprimé d'abord par les LB immatures dans la moelle osseuse. En plus de BAFF-R, BAFF peut également se fixer sur deux autres récepteurs TACI et BCMA (Vincent et al., 2013). Ces trois récepteurs forment un système ligand récepteur qui inclut le ligand APRIL. APRIL qui contrairement à BAFF, se lie seulement à TACI et BCMA et pas à BAFF-R (Vincent et al., 2013).

L'expression de BAFF-R est augmentée lorsque les LB transitionnels se différencient en LB de la zone marginale ou en LB folliculaires. Mais BAFF-R n'est pas exprimé par les plasmocytes à longue durée de vie, qui expriment BCMA, alors que TACI est exprimé par les LB MZ et les LB mémoires « switchés ». Le rôle de BAFF-R sur la survie des LB a d'abord été démontré chez la souris (Sasaki et al., 2004; Thompson et al., 2001). Le rôle principal de BAFF-R est d'apporter des signaux de survie aux LB immatures et matures. Les souris déficientes pour BAFF et BAFF-R ont une diminution du nombre de LB et un développement bloqué au

stade transitionnel T2 (Sasaki et al., 2004). Chez l'Homme, des individus déficients en BAFF-R ont été découverts chez des patients atteints d'Immunodéficience Commune Variable (CVID) avec un faible taux de LB (Warnatz et al., 2009). En plus d'une forte lymphopénie B, l'étude des LB dans le sang périphérique révèle une augmentation du nombre de LB transitionnels. Une forte diminution du nombre de LB mémoires et de LB mémoires switchés est accompagnée d'une diminution du taux d'IgM et IgG sériques.

B- Les lymphocytes B régulateurs

Les capacités régulatrices des LB ont été décrites dans les années 1970 dans un modèle d'hypersensibilité retardée chez le cochon d'inde (Neta and Salvin, 1974), puis en 1996 dans un modèle d'EAE (encéphalomyélite *auto-immune* expérimentale) ((Wolf et al., 1996). Si aujourd'hui les LB régulateurs ne sont pas encore bien décrits et ce, en particulier, chez l'Homme, la fonction régulatrice des LB est principalement associée à la capacité qu'ont ces LB à sécréter de l'IL-10 (Blair et al., 2010; Das et al., 2012; Flores-Borja et al., 2013; Watanabe et al., 2010). Cependant, les mécanismes de régulation des LB semblent aujourd'hui plus complexes et des fonctions régulatrices indépendantes de l'IL-10 ont été décrites. On note par exemple l'expression de FasL, Granzyme B (GzmB) ou la production de TGF-β qui ont également été attribués aux LB régulateurs.

B-1- Les lymphocytes B régulateurs chez la souris

Chez la souris comme chez l'Homme, la fonction régulatrice des LB est principalement associée à la capacité qu'ont ces LB à produire de l'IL-10 (Fillatreau et al., 2002). Plusieurs phénotypes ont été décrits montrant qu'il n'y aurait pas un seul type de LB régulateurs mais plusieurs. Les principaux phénotypes actuellement chez la souris sont les LB $CD5^{+}$ $CD1d^{++}$ décrits comme ayant une fonction suppressive importante dans des modèles d'eczéma de contact, d'EAE et de lupus (Matsushita et al., 2010a; Watanabe et al., 2010; Yanaba et al., 2008). Cette population est appelée B10 du fait de leur fonction régulatrice exercée via la sécrétion d'IL-10. Les précurseurs B de la zone marginale (T2-MZP) ont également un rôle régulateur. Ils ont été décrits par Evans et al dans un modèle de CIA (modèle d'arthrite induit par la collagénase) où ils inhibent la réponse Th1 via notamment la sécrétion d'IL-10 (Evans et al., 2007). Une étude plus récente a montré que cette même population de T2-MZP isolée de rate de souris convalescentes d'arthrite par transfert adoptif possède la capacité de restaurer

le pourcentage de Treg dans une souris µMT et ce de façon IL-10 dépendante (Carter et al., 2011). Plus récemment, Montandon et al ont également décrit une autre population de LB régulateurs les Innate Pro-B dans un modèle de diabète de type 1, cette population de LB pro-B innés, possède des fonctions suppressives après activation par le TLR9, en supprimant les cellules effectrices pathogènes, en inhibant leur production d'IL-21 et en induisant leur apoptose via FasL (Montandon et al., 2013). Ces populations régulatrices se caractérisent par des phénotypes, des modes d'activation et d'action différents. Les différentes sous-populations de LB régulatrices décrites sont référencées dans le tableau 2.

	phenotype	modèle	mode d'activation	mode d'action	rérérence
Souris	NC	modèle de diabète de type1 NOD	LPS	expression de FasL et sécrétion de TGF-β par les LB stimulés, apoptose des Th1 et inhibition fonction CPA par le TGF-β	Tian *et al.*, 2001
	surexpression de CD1d	modèle murin d'inflammation instesinal	environnement inflammatoire	production d'IL-10, inhibition de la cascade inflammatoire associée à la surexpression d'IL-10 et à l'activation de STAT3	Mizoguchi *et al.*, 2002
	NC	modèle murin d'arthrite	CD40L	le transfert des LB activés qui produisent de l'IL-10 induit une inhibition de la reponse Th1 pathogénique = diminution de la maladie	Mauri *et al.*, 2003
	NC	-	LPS	augmentation de TGF-β1 à la surface des LB ce qui induit l'anergie des LT CD8+	Parekh *et al.*, 2003
	CD19+CD21++CD23+ CD24++CD93+	modèle d'arthite induite par collagène (CIA)	BCR	production d' IL-10 qui inhibibe la réponse Th1	Evans *et al.*, 2007
	NC	modèle d'EAE	-	induction de Treg via contact LB/LT(B7)	Mann *et al.*, 2007
	NC	modèle d'allergie des voies aériennes	-	Les LB sécrétent du TGF-β et induisent la convertion de LT effecteurs CD4+ CD25- en LT régulateurs fonctionnels	Singh *et al.*, 2008
	NC	modèle d'EAE	activation LB par TLR	inhibition de la réponse Th1 et Th17	lampropoulou *et al* ., 2008
	B10 CD1d++CD5+	modèle de maladies autoimmunes	LPS+CD40L	production IL-10. L'autoimmunité induit le developpement des B10	Yanaba *et al.*, 2009
	cellules B10 (CD1d++CD5+)	modèle d'EAE	CD40/LPS	production d'IL-10 et inhibition de la sécrétion d'IFN-γ et de TNF-α par LT sans effet in vitro sur prolifération des LT CD4+ et diminution des capacités de présentation antigénique des DCs. Ce qui va inhiber la prolifération des LT CD4+	Matsushita *et al.*, 2010
	T2-MZP	modèle d'arthrite	-	Les LB T2-MZP induisent les T reg et inhibent la réponse Th1 et Th17 via IL-10	Carter *et al.*, 2011
	CD19+CD25++CD69+ +CD81++CD86++	souris 4T1 modèle de cancer du sein	LPS	convertion des LT CD4+ en Treg FoxP3+ dépendamment du TGF-β	Olkhanud *et al.*, 2011
	NC	modèle d'EAE	interaction CD40 et IL-21	sécrétion d'IL-10 par les LB, inhibition de la maladie après transfert de LB IL-10+	Yoshizaki *et al.*, 2012
	NC	modèle d'EAE	-	B reg induisent T reg via GITR et IL-10 et B7 independant	Ray *et al.*, 2012
	Innate Pro-B	modèle de diabète de type 1 (NOD)	activation TLR9	diminution de la sécrétion d'Il-21 par les cellules effectrices et induction de l'apoptose via FasL	Montanton *et la.*, 2013
	NC	modèle de transplantation d'ilôts pancréatiques	LPS	*in vivo* les LB reg induisent des LTreg et prolongent la survie du greffon de façon TGF-β dépendantes	Lee KM *et al.* , 2014
	CD138+CD22-	EAE/ infection bacterienne (Salmonela)	TLR4/CD40	sécrétion d'IL-35 par les LB, IL-35 inhibe la fonction CPA des LB et inhibe les LT inflammatoires	Shen *et al.*, 2014
	CD19+IL-35+	EAU modèle d'Uvéite Expérimentale Autoimmune	IL-35	IL-35 induit la différencition des LB reg qui produisent de l'IL-10 et de l'IL-35. Breg IL-35+ inhibent Th17 et Th1 et induisent des LTreg	wang *et al.*, 2014

Tableau 2 : Les différents types de LB régulateurs décrits chez la souris.

En transplantation, le rôle des LB a été mis en évidence dans différents modèles animaux. La première preuve montrant l'implication des LB dans la tolérance à l'allogreffe a été rapportée par Parker et al. Dans un modèle d'allogreffe d'îlots pancréatiques, la prolongation de la survie des animaux greffés est obtenue après injection des lymphocytes délétés en LT CD4+ avec un CD40L pour bloquer les interactions LT/LB (Parker et al., 1995). Ensuite plusieurs études ont montré l'implication des LB dans la survie du greffon, via une injection de LB (Niimi et al., 1998) ou l'immunosuppression via le blocage du CD45 (Deng et al., 2007; Huang et al., 2008; Zhao et al., 2010). Le CD45 fait partie de la famille des protéines tyrosines phosphatases transmembranaires impliquées dans le développement et l'activation des lymphocytes (Koretzky et al., 1991). L'étude de Deng et al a montré qu'une administration de courte durée d'anti-CD45-RB aux jours 0, 3, 5 et 7 après transplantation prévient efficacement le rejet d'allogreffe cardiaque chez la souris C3H sur B6 (Deng et al., 2007). Dans ce modèle, la tolérance induite par l'anti CD45-RB est perdue dans un modèle murin B6µMT-/-, qui est un modèle transgénique de souris sans anticorps et sans LB. La tolérance est restaurée après transfert de LB dans les souris B6µMT-/-, montrant que l'efficacité tolérogène du traitement anti CD45-RB nécessite la présence des LB de l'hôte. Dans cette même étude, ils ont également démontré qu'en plus de la présence des LB de l'hôte, la tolérance induite par l'anti CD45-RB est médiée par l'interaction des molécules de costimulation CD80-86 sur les LB et T (Moore et al., 2004). L'implication des LB sécréteurs d'IL-10 a aussi été démontrée dans un modèle de tolérance d'allogreffe d'Îlots pancréatiques chez la souris, induite par un court traitement immunosuppresseur avec anti-CD45-RB et anti TIM-1. Dans ce modèle, la survie du greffon est obtenue par le transfert de LB WT mais pas lors de transferts de LB déficients en IL-10 (Lee et al., 2012). Ainsi ces différentes études montrent l'implication des LB dans la tolérance à l'allogreffe et ce, notamment, via la sécrétion d'IL-10 (tableau 3).

Reference	Animal model	Modality of tolerance	Implication of B cells
Parker et al. (35)	Mouse pancreatic islet allografts	Treatment with allogenic small lymphocyte or T-depleted small lymphocytes plus blocking antibody to CD40L	Increase survival of recipients treated with T-depleted small lymphocytes plus CD40L
Niimi et al. (36)	Mouse model of cardiac allograft	Resting B cells plus blocking antibody to CD40L	Tolerance induced by B cells involves the CD40 pathway
Yan et al. (37)	Rat model of kidney allograft	I.V. injection of donor B cells at time of transplantation	B cells induce more efficiently long-term acceptance of graft than T cells
Deng et al. (38)	Mouse model of cardiac allograft	Anti-CD45-RB therapy	Anti-CD45-RB is not efficient in transgenic mouse without B cells
Huang et al. (39)	Mouse model of cardiac allograft	Treated with anti-CD45-RB, anti-ICAM, anti-LFA or combination of these agents	Expression of ICAM-1 by B cells and interaction with LFA-1 form a central aspect of transplantation tolerance induced by CD45-RB therapy
Zhao et al. (40)	Mouse model of cardiac allograft	Anti-CD45-RB therapy	IL-10 expressed by B cells inhibits B-cell-mediated tolerance induction in cardiac allograft model
Ding et al. (41)	Mouse model of islet allograft	Anti-TIM-1 therapy	TIM-1 B cells are regulatory and transfer donor-specific long-term graft survival
Le Texier et al. (42)	Rat model of cardiac allograft	Short-term immunosuppression	Accumulation of B cells in PBMC of tolerant recipients and a phenotype of inhibited B cells partially blocked at their IgM to IgG switch and over expressing the inhibitory receptor Fcgr2b
Lee et al. (43)	Mouse model of islet allograft	Anti-CD45-RB and anti-TIM-1 therapy	Combined anti-CD45-RB and anti-TIM-1 treatment induced allograft survival that is B-cell dependent, dependent on B-cell production of IL-10, and is associated with up-regulation of TIM-1 on B cells

Tableau 3 : Les différentes études montrant l'implication des lymphocytes B dans des modèles animaux de tolérance d'allogreffe (Chesneau et al., 2013b)

B-2- Mécanismes d'action des LB régulateurs chez la souris

B-2-1- La sécrétion de cytokines

Lorsque l'on parle de LB régulateurs, on pense immédiatement à « Sécrétion d'IL-10 ». Comme nous l'avons vu précédemment, les LB régulateurs ont la capacité de produire de l'IL-10. En effet, le transfert adoptif de LB sécréteurs d'IL-10 ou de LB T2-MZP préalablement stimulés *ex-vivo* inhibe la prolifération des LT et leur production d'IFN-γ et de TNF-α par les LTh1 (Evans et al., 2007; Yanaba et al., 2008; Yang et al., 2010). La sécrétion d'IL-10 par les LB peut également conduire à la conversion de LT effecteurs (pro-inflammatoires) en LT régulateurs (Carter et al., 2011). Toutefois, la conversion de LT effecteurs en T régulateurs via l'IL-10 par les LB est controversée et pourrait être dépendante du modèle de maladie (Hoehlig et al., 2012).

En plus d'inhiber la différenciation des LTh1, l'IL-10 produit par les LB peut également inhiber la différenciation en LTh17. *In vitro* les LB via leur production d'IL-10 inhibent la

différenciation Th17 en inhibant la phosphorylation de STAT3 ce qui inhibe le facteur de transcription RORgT (Yang et al., 2012). L'IL-10 produit par les LB induit la sécrétion d'IL-4 et inhibe la sécrétion d'IL-12 ce qui affecte la balance Th1/Th2 (Moulin et al., 2000). De plus, l'IL-10 inhibe la capacité de présentation antigénique des DCs qui conduit à une baisse de la prolifération des LT CD4+ (Matsushita et al., 2010b).

Néanmoins, la sécrétion d'IL-10 n'est pas toujours associée à un effet bénéfique. Dans un modèle de tolérance d'allogreffe cardiaque induit par l'anti CD45RB, l'IL-10 empêche la tolérance dans ce modèle. De plus, l'utilisation d 'un anticorps neutralisant l'IL-10 ou le transfert de LB déficients pour l'IL-10 inhibe la survenue de vasculopathie d'allogreffe chronique et la production d'anticorps réactifs envers le greffon (Zhao et al 2010). Ainsi, la fonction régulatrice des LB dans la tolérance en transplantation peut agir de façon différente des autres modèles.

La régulation par les LB n'est pas seulement liée à la sécrétion d'IL-10. En effet, de nombreuses études montrent que l'IL-10 n'agit pas seul, mais nécessite en plus un contact avec la cellule cible ou agit par d'autres mécanismes que je vais décrire.

Les LB ont également la capacité de sécréter du TGF-β. Chez la souris uniquement, il a été montré que les LB activés par LPS ont la capacité d'inhiber la réponse Th1 en induisant leur apoptose et/ou en inhibant la fonction présentatrice d'antigène des LB par leur sécrétion de TGF-β (Tian et al., 2001). Les LB ont également la capacité de présenter sur le CMH de classe 1 des antigènes aux LT CD8+ et ainsi d'induire une réponse cytolytique via les T $CD8^+$. Le traitement des LB avec LPS entraîne une augmentation de l'expression de TGF-β de surface qui induit l'anergie des LT $CD8^+$ (Parekh et al., 2003). Enfin, dans des modèles d'allergie, il a été démontré que les LB TGF-β^+ joue un rôle dans la conversion des LT effecteurs $CD4^+CD25^-$ en LT régulateurs fonctionnels (Natarajan et al., 2012).

Récemment, il a été démontré que les LB ont la capacité de sécréter de l'IL-35. L'IL-35 est un membre de la famille des IL-12. Cette cytokine avait déjà été décrite pour ses propriétés régulatrices dans les LTreg naturels (nTreg) L'IL-35 induit des Breg qui sécrètent eux-mêmes de l'IL-35 et de l'IL-10 et possèdent la capacité de supprimer les uvéites auto-immunes par l'intermédiaire de leurs sécrétions d'IL-35 et d'IL-10 (Wang et al., 2014). L'activation par LPS et CD40 induit l'expression d'IL-35 par les LB (Shen et al., 2014; Wang et

al., 2014). L'IL-35 serait produite principalement par les plasmocytes CD138+. En effet, dans l'étude de Shen ils regardent l'expression d'IL-10 et d'IL-35 dans les plasmocytes plus ou moins différenciés qu'ils analysent en fonction de leur phénotype $CD138^{-}$ / $CD138^{int}CD22^{+}$ / $CD138^{++}CD22^{+}$ / $CD138^{++}CD22^{-}$ du moins au plus différencié. Ils démontrent que ce sont les populations les plus différenciées qui produisent le plus d'IL-10 et d'IL-35. Il a également été démontré que L'IL-35 inhibe la fonction de présentation antigénique des LB (Shen et al., 2014).

B-2-2- L'induction de l'apoptose

Plusieurs études montrent que l'expression de FasL et l'induction de mort par les LB peuvent également jouer un rôle important dans le maintien ou l'induction de la tolérance (Minagawa et al., 2004). Certaines études réalisées chez la souris, ont montré que les LB régulateurs exercent en partie leur fonction régulatrice via la mort cellulaire induite par l'activation ou AICD induisant l'apoptose des LT effecteurs. L'AICD est induite par l'interaction du récepteur de Fas (CD95) et de son ligand FasL (CD95L). Les LB ont la capacité de sécréter FasL (Tanner et al 1999). Elles sont donc des cellules effectrices de l'AICD. Une étude récente a prouvé que la stimulation avec un ligand du TLR-9 favorise l'émergence de lymphocytes proB-innés qui peuvent protéger les souris NOD (Non Obese Diabetic) du développement du diabète de type 1 (Montandon et al., 2013). Dans cette étude en réponse à l'IFN-γ sécrété par les LT effecteurs cette population de pro-B-innée augmente leur expression de FasL, ce qui leur permet de tuer les LT effecteurs par apoptose. De plus, les pro-B (eux-même FasL+) se différencient en outre en cellules B matures diverses mais toutes caractérisées par un haut niveau de FasL (supérieur à celui des LB du receveur dans les mêmes tissus où les cellules injectées ont migré) et toujours capable d'induire l'apoptose des LT effecteurs, ce qui induit un contrôle à long terme par l'apoptose des LT effecteurs (Montandon et al., 2013). L'existence de LB exprimant FasL a également été démontrée dans des modèles d'allergie. (Klinker et al., 2013; Lundy and Boros, 2002).

B-3- Les lymphocytes B régulateurs chez l'Homme

La fonction régulatrice des LB est moins décrite chez l'Homme que chez la souris. Comme pour la souris, différentes sous-populations de LB ont été décrites chez l'Homme pour avoir des capacités régulatrices (tableau 4). Parmi les populations décrites on trouve les

LB transitionnels $CD19^{+}CD38^{++}CD24^{++}$ (Blair et al., 2010; Lemoine et al., 2011). Après stimulation par le CD40L, ces LB transitionnels bloquent la différenciation des LT en Th1. Cette fonction régulatrice contact dépendante, est en partie exercée via la sécrétion d'IL-10. Une autre population de LB régulatrices - appelées B10 - a été décrite par le groupe de Tedder. Cette population est caractérisée par sa capacité à sécréter de l'IL-10 après 5H de stimulation *ex vivo*, alors que les progéniteurs B10 (B10pro) nécessitent 48H de stimulation avant d'acquérir la capacité de sécréter de l'IL-10 (Iwata et al., 2011). La fonction régulatrice des LB ne passe pas uniquement par la sécrétion d'IL-10. En effet Morva et al ont démontré que les LB stimulés via CD40 et ODN inhibent la prolifération des LT via les cellules dendritiques (Morva et al., 2012). Les LB $IgD^{faible}CD38^{+}CD24^{faible}CD27^{-}$ stimulés inhibent *in vitro* la maturation des cellules dendritiques, entrainant une diminution de la production de molécules de costimulation (CD80/CD86) et de la sécrétion d'IL-12p70 responsable de la polarisation Th1. Cette inhibition de la maturation des DCs est dépendante de l'IL-10 et du TGF-β mais requiert un contact LB /DCs. Comme pour les LB reg chez la souris, la fonction régulatrice des LB a principalement été décrite dans des modèles de maladie auto-immune. Cependant, plusieurs études en transplantation rénale et notamment chez les patients opérationnellement tolérants ont montré un rôle potentiel des LB dans le maintien de la tolérance (tableau 1) (Voir chapitre signature B).

Ainsi les différentes études portant sur les modèles animaux et chez l'Homme montrent que les LB peuvent avoir des fonctions régulatrices. L'hétérogénéité des phénotypes décrits à la fois chez l'Homme et chez la souris laisserait à penser que différentes sous-populations de LB pourraient avoir des fonctions régulatrices, avec peut-être une induction d'un profil régulateur lié à un environnement particulier (cytokinique, protéinique, inflammatoire…).

Homme	CD24++CD38++	lupus	CD40L	via la sécrétion d'IL-10 et contact cellulaire : inhibition de la différenciation Th1	Blair *et a.,* 2010
	CD24++CD27+	HV et patients avec maladies auto-immunes	CD40L/ LPS	la production d'IL-10 inhibe la sécrétion de cytokines par les monocytes	Iwata *et al.,* 2011
	$CD19^{+}IgD^{faible}CD38^{+}CD24^{faible}CD27^{-}$	-	CD40L/ ODN	inhibe la prolifération des LT via les DCs en inhibant la maturation des DCs et leur expression de molécules de costimulations (CD80/86) dependant du contact cellulaire mais pas de l'IL-10 ni TGFb	Morva *et al.,* 2011
	CD19+CD5+	lupus	IL-21 + stimulation du BCR	induction de la sécrétion de GzmB par les LB	Hagn *et al.,* 2010
	CD19+CD38+CD1d+IgM+CD147+	cancer	IL-21 /ODN	inhibition de la prolifération des LT via la dégradation du TcR zeta par le GzmB	Lindner *et al.,* 2013
	CD24++CD38++	patients avec arthrite rhumatoïde	-	inhibe la polarisation TH1 et TH17 et la prolifération des LT CD4+ et induit des LT reg via l'IL-10 et le contact (par CD80/86)	Flores Borja *et al.,* 2013
	CD19+CD25++CD71++CD73- (Br1)	apiculteurs	TLR9-L	la production d'IL-10 inhibe la prolifération des LT. Les BR1 produisent des anticorps non inflammatoires IgG4	Van de Veen *et al.,* 2013
	CD19+ CD25++Foxp3+	sclerose en plaque	-	expression de perforine/GzmB (et LAMP-1/2) par les LB regulateurs du LCR des patients MS	Clara de Andrès *et al.,* 2014

Tableau 4 : Les différents types de LB régulateurs décrits chez l'Homme.

B-4- Mécanismes d'action des LB régulateurs chez l'Homme

B-4-1- La sécrétion de cytokines

Comme pour la souris, les LB régulateurs chez l'Homme sont principalement connus pour leur fonction régulatrice induite par la sécrétion d'IL-10 (Blair et al., 2010; Flores-Borja et al., 2013; Iwata et al., 2011; van de Veen et al., 2013).L'IL-10 sécrétée par les LB chez l'Homme possède donc la capacité d'inhiber la différenciation des LTh1 et Th17 (Blair et al., 2010; Flores-Borja et al., 2013), mais également d'inhiber la prolifération des LT CD4+ (van de Veen et al., 2013). L'étude de Iwata et al montre que les LB $CD24^{++}CD27^{+}$ sécrètent de l'IL-10 après stimulation CD40/ODN et qu'*in-vitro* ces LB ont la capacité d'inhiber la réponse immunitaire innée en inhibant la sécrétion de cytokines TNF-α par les monocytes et ce de façon IL-10 dépendante. Cependant, dans cette étude, ils analysent la sécrétion de TNF-α par les LT, celle-ci est inhibée en présence de LB mais pas plus en présence de $CD24^{++}CD27^{+}$ qu'en présence de $CD24^{++}CD27^{-}$. Ainsi la sécrétion d'IL-10 n'interviendrait pas dans l'inhibition de la sécrétion de TNF-α par les LT (Iwata et al., 2011). Récemment l'équipe de Claudia Mauri a montré que les LB $CD24^{++}CD38^{++}$ ont la capacité d'inhiber la sécrétion de cytokines pro-inflammatoires IFN-γ et TNF-α par les LT effecteurs et également la différenciation des LTh1 et Th17 via la sécrétion d'IL-10 et contact CD80/86. De plus, les LB $CD24^{++}CD38^{++}$ induisent

des LT régulateurs, là encore, via la sécrétion d'IL-10 (Flores-Borja et al., 2013). L'équipe d'Akdis a également montré que les LB $CD73^{-}CD25^{+}CD71^{+}$ inhibent la prolifération des LT via la sécrétion d'IL-10 (van de Veen et al., 2013). Bien que plusieurs études chez l'Homme montrent la capacité qu'ont les LB à sécréter de L'IL-10 (Amel Kashipaz et al., 2003; Díaz-Alderete et al., 2004; Duddy et al., 2007, 2004), toutes n'ont pas un rôle régulateur de l'IL-10 sur les LT effecteurs chez l'Homme. En effet même si la plupart des sous-populations de LB ont la capacité de sécréter de l'IL-10 après stimulation CD40/ODN, peu d'études démontrent une capacité régulatrice de ces sous-populations via la sécrétion d'IL-10.

Parmi les cytokines sécrétées par les LB ayant un rôle régulateur, le TGF-β a été décrit chez la souris comme une des voies de régulation des LB (Tian et al., 2001) (Natarajan et al., 2012) (Parekh et al., 2003), mais aucune étude réalisée chez l'Homme ne montre l'implication du TGF-β dans la fonction régulatrice des LB.

Comme il l'est précisé dans le paragraphe sur le mécanisme d'action des LB chez la souris et la sécrétion de cytokines, l'IL-35 a été récemment décrit pour ses fonctions régulatrices. L'existence de cette population de LB $IL\text{-}35^{+}$ a été confirmée chez l'Homme (Wang et al., 2014).

B-4-2- L'induction de l'apoptose

Contrairement à la souris, chez l'Homme, aucune étude n'a encore mis en évidence l'induction de l'apoptose des LT effecteurs par l'expression de FasL par les LB.

B-4-3- La sécrétion de GzmB

Certains mécanismes décrits chez la souris ne le sont pas chez l'Homme et c'est également le cas dans l'autre sens. Certains mécanismes n'existent pas chez la souris et sont décrits chez l'Homme. C'est notamment le cas du GzmB. En effet, la sécrétion de GzmB par les LB est de plus en plus décrite. Le GzmB est un composé enzymatique des granules cytotoxiques produit principalement par les LT $CD8^{+}$ cytolytiques (CTL) et les cellules Natural Killer (NK). Il induit la mort des cellules cibles par l'intermédiaire de la perforine (Ewen et al., 2012). En 2006 Jahrsdörfer et al ont décrit pour la première fois que les LB ont la capacité de sécréter du GzmB. Cette étude portait sur des patients atteints de leucémie lymphoïde chronique (Jahrsdörfer et al., 2006). Les LB de ces patients mis en culture avec de l'IL-21 et une stimulation du TLR-9 (ODN) induit la sécrétion de GzmB par les LB et leur mort par

apoptose dépendant de la sécrétion de GzmB. Ce même groupe a montré que chez des patients atteints de lupus, la sécrétion de GzmB est principalement observée dans la sous-population de LB $CD5^+$ (sous-population surexprimée dans les maladies autoimmunes) (Hagn et al., 2010) et que l'IL-21 induit directement la sécrétion de GzmB par les LB $CD5^+$ en combinaison avec une stimulation via le BCR. De plus, il existe une corrélation entre le niveau d'IL-21 et de GzmB dans le sérum des patients atteints de lupus, ainsi que de l'expression du récepteur à l'IL-21 sur les cellules $CD5^+$ (Hagn et al., 2010). Les LB pourraient induire via le GzmB la mort cellulaire par apoptose (AICD). Cependant dans ces études, la sécrétion de GzmB n'est pas liée à la sécrétion de perforine, nécessaire à la fonction cytolytique du GzmB. Néanmoins, la régulation de la prolifération des LT via l'expression de GzmB de façon dépendant de la sécrétion de perforine a déjà été décrite. En effet, les cellules dendritiques plasmacytoïdes (pDC) ont également la capacité de sécréter du GzmB. Les pDC sont connues pour leur capacité à réguler la réponse T et il a été montré que les pDcs peuvent être une source abondante de GzmB et que ces pDCs $GzmB^+$ suppriment la prolifération des LT et ce de façon dépendant du GzmB et de la sécrétion de perforine (Jahrsdörfer et al., 2010). Il est intéressant de voir que les LT régulateurs possèdent également des capacités régulatrices via la sécrétion de GzmB et par l'intermédiaire de la sécrétion de perforine (Gondek et al., 2005). Bien que la sécrétion de GzmB par les LB ne soit pas associée à une sécrétion de perforine, les LB $GzmB^+$ peuvent tout de même avoir une fonction cytolytique (Hagn et al., 2012). La fonction du granzyme B est souvent associé à la sécrétion de perforine qui permet la formation de pores à la surface des cellules cibles et donc l'entrée du GzmB dans celles-ci (Browne et al., 1999). Mais, il a été démontré que le GzmB peut pénétrer dans les cellules sans présence de perforine (Pinkoski et al., 1998; Shi et al., 1997) et ce, par sa fixation au récepteur MPR (Manose-6-phosphate receptor) (Trapani et al., 2003). Il a notamment été montré qu'une surexpression de ces récepteurs rend les cellules plus sensibles à l'apoptose induite par le GzmB (Motyka et al., 2000). Parmi les fonctions non cytolytiques du GzmB, les LB activés via IL-21 et BCR inhibent la prolifération des LT $CD4^+$ par dégradation du TCR zeta via le GzmB. Le TCR zeta étant un substrat direct du GzmB (Lindner et al., 2013; Wieckowski et al., 2002).

Une étude récente réalisée chez des patients atteints de sclérose en plaque montre que les LB $GzmB^+$ peuvent également avoir une fonction cytolytique via la sécrétion conjointe

de perforine. En effet chez ces patients atteints de sclérose en plaque, une sous-population de LB $CD19^{+}CD25^{++}FoxP3^{+}$ a été retrouvée principalement dans le liquide céphalorachidien (LCR). Cette population possède la capacité de sécréter de la perforine et du granzyme en plus d'exprimer à leur surface CD107a+b (LAMP-1 et 2). Ce qui montre que ces LB $CD25^{++}Foxp3^{+}$ ont des capacités cytolytiques probablement liées à l'inhibition de la prolifération des LT *in vitro* (de Andrés et al., 2014).

L'IL-21 semble avoir un rôle essentiel dans l'induction de LB GzmB+. L'Il-21 est principalement sécrété par les LTfh, les Th17 et les cellules NKT. L'IL-21 peut avoir différents effets sur les LB. Tout d'abord en l'absence de stimulation via le BCR et sans aide des LT, les LB vont entrer en apoptose (Jin et al., 2004). En présence de CD40L et avec une stimulation TLR ou via BCR, L'IL-21 va induire la différenciation des LB en LB mémoires à longue durée de vie ou en plasmocytes (Spolski and Leonard, 2008a, 2008b). Enfin l'IL-21 peut induire l'expression de GzmB par les LB en l'absence de stimulation par CD40L (Hagn et al., 2010, 2012)(Figure 13).

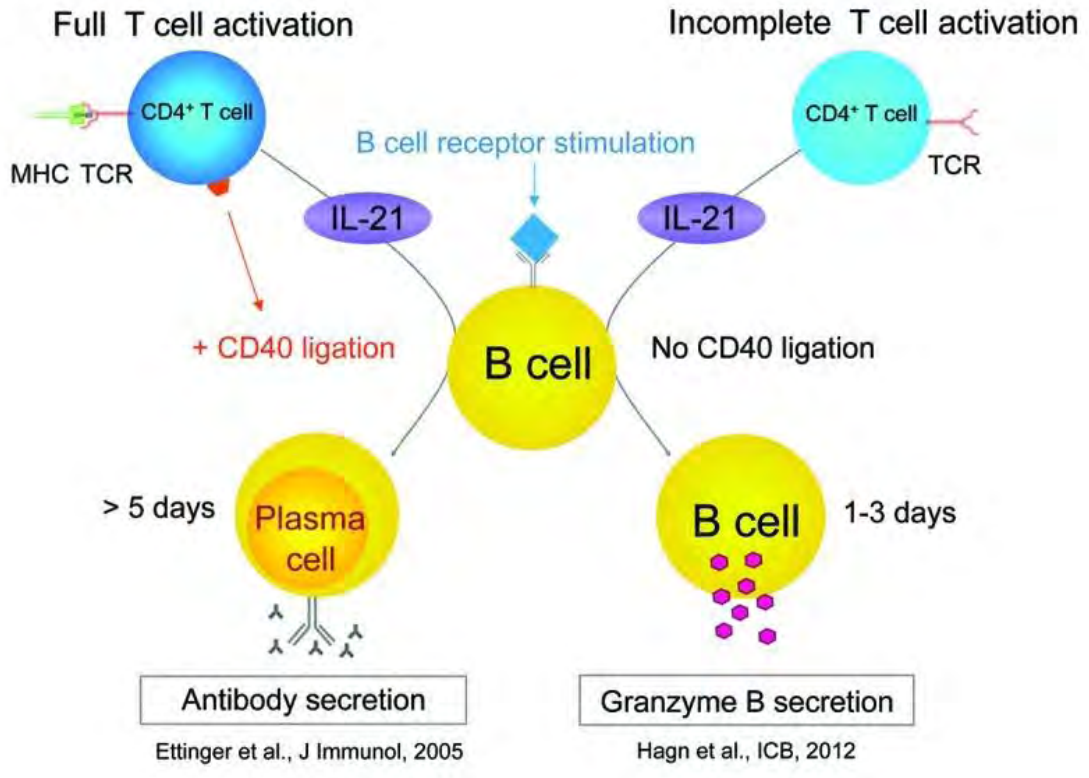

Figure 13 : Schéma représentant les deux voies menant à la différenciation des LB en plasmocytes ou en LB sécréteurs de GzmB (Hagn and Jahrsdörfer, 2012).

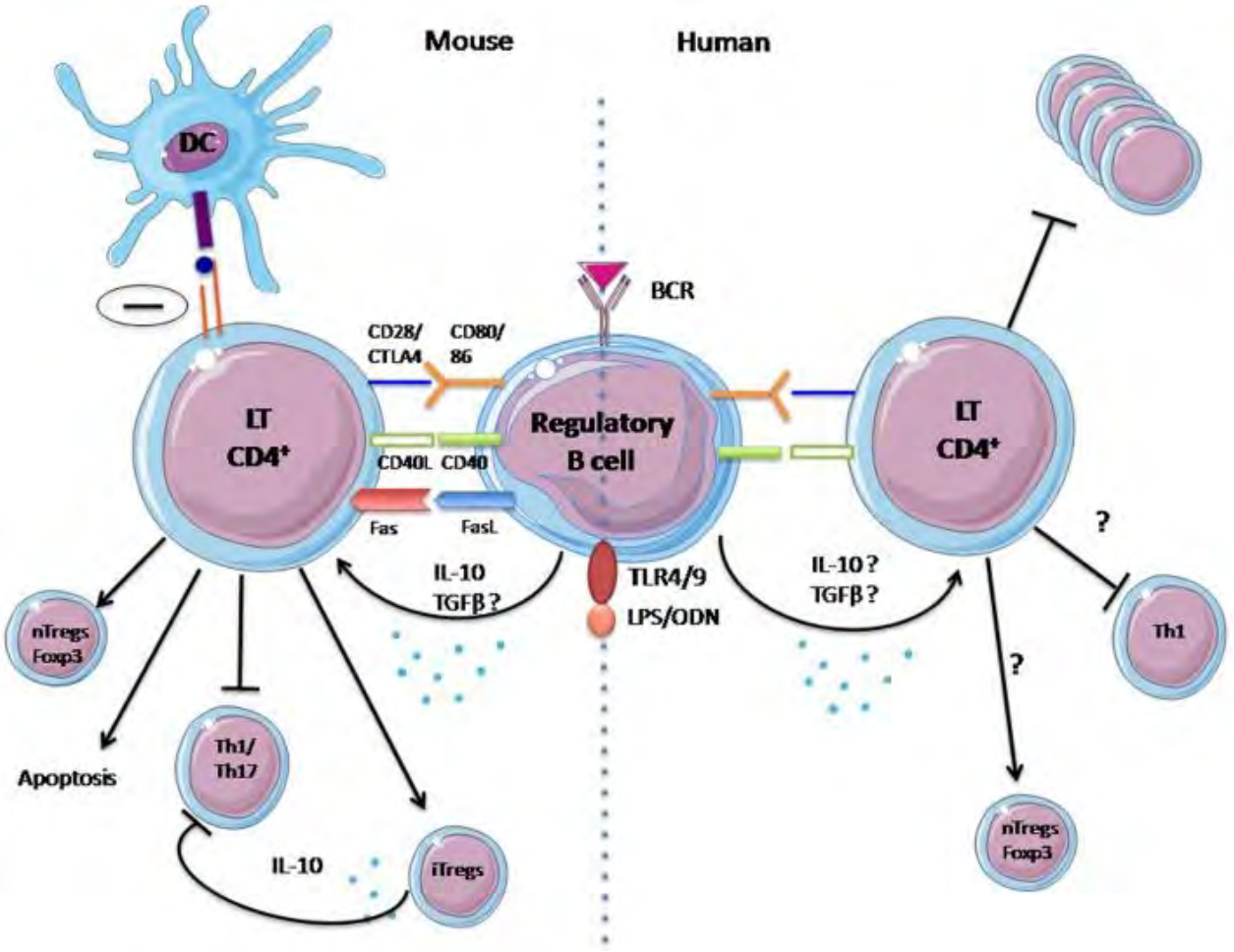

Figure 14 : Mécanismes de suppression des Lymphocytes B identifiés chez l'Homme et l'animal (Chesneau et al., 2013b).

Résultats 1

Les patients tolérant une greffe rénale ont un profil de différenciation des lymphocytes B unique.

Chesneau M, Pallier A, Braza F, Lacombe G, Le Gallou S, Baron D, Giral M, Danger R, Guerif P, Aubert-Wastiaux H, Néel A, Michel L, Laplaud DA, Degauque N, Soulillou JP, Tarte K, Brouard S.

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Résumé de l'article

Nous avons dans un premier temps démontré que les patients tolérants ont plus de LB transitionnels et naïfs et moins de plasmocytes que les patients stables. Suite à cette première observation nous avons émis l'hypothèse d'un défaut de la différenciation des LB chez les patients tolérants qui pourrait expliquer cette déficience de plasmocyte au profit des LB transitionnels et naïfs. Afin d'étudier la différenciation des LB, nous avons utilisé un protocole de différenciation *in vitro* déjà décrit (Gallou et al., 2012). Ce protocole est un protocole en deux étapes, une première étape qui permet d'activer les LB et une deuxième qui induit la différenciation des LB en plasmocytes.

Ce protocole a tout d'abord permis de voir que les LB des patients tolérants *in vitro* ne présentent pas de défaut au d'activation et de prolifération *in vitro*. Néanmoins, ce protocole met en évidence un défaut de la différenciation des LB en phase tardive. En effet, bien que l'on n'observe ni de différences au niveau du nombre de plasmocytes $CD138^+$ générés en fin de culture entre les trois groupes (TOL, STA et HV), ni de défaut de la production d'IgM et d'IgG, l'analyse transcriptionnelle des gènes impliqués dans la différenciation des LB (BCL-6, BACH-2, PAX-5, IRF-4, PRDM1, XBP1) montre un défaut de l'expression des gènes impliqués dans la phase tardive de différenciation des plasmocytes (IRF-4, PRDM1, XBP1) chez les TOL comparé aux STA. De plus, l'analyse de la sécrétion de cytokines par les LB activés montre une forte expression d'IL-10 chez les TOL comparé aux STA et HV.

Ces deux observations sont en accord avec une balance tolérogène des LB chez les TOL avec plus de LB de type « régulateurs » et moins de plasmocytes pathogéniques. Enfin, l'analyse de l'apoptose des LB, montre que les LB des STA sont moins sensibles à l'apoptose que les LB des TOL. Ceci pourrait être expliqué par une adaptation des LB des STA à l'apoptose afin de résister au traitement immunosuppresseur.

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Unique B Cell Differentiation Profile in Tolerant Kidney Transplant Patients

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Operationally tolerant patients (TOL) display a higher number of blood B cells and transcriptional B cell signature. As they rarely develop an allo-immune response, they could display an abnormal B cell differentiation. We used an *in vitro* culture system to explore T-dependent differentiation of B cells into plasma cells. B cell phenotype, apoptosis, proliferation, cytokine, immunoglobulin production and markers of differentiation were followed in blood of these patients. Tolerant recipients show a higher frequency of $CD20^{+}CD24^{hi}CD38^{hi}$ transitional and $CD20^{+}CD38^{lo}CD24^{lo}$ naïve B cells compared to patients with stable graft function, correlating with a decreased frequency of $CD20^{-}CD38^{+}CD138^{+}$ differentiated plasma cells, suggestive of abnormal B cell differentiation. B cells from TOL proliferate normally but produce more IL-10. In addition, B cells from tolerant recipients exhibit a defective expression of factors of the end step of differentiation into plasma cells and show a higher propensity for cell death apoptosis compared to patients with stable graft function. This *in vitro* profile is consistent with down-regulation of B cell differentiation genes and anti-apoptotic B cell genes in these patients *in vivo*. These data suggest that a balance between B cells producing IL-10 and a deficiency in plasma cells may encourage an environment favorable to the tolerance maintenance.

Keywords: Apoptosis, B cells, cytokine, differentiation, tolerance, transplantation

Abbreviations: Ab, antibody; BCR, B cell receptor; BrdU, 5-brom-deoxyuridine; CBA, cytometric bead array; CD, cluster of differentiation; CD40L, cluster of differentiation 40 ligand; Csa, cyclosporin A; DA, atopic dermatitis patients; DMSO, dimethyl sulfoxide; FACS, fluorescence-activated cell sorting; HV, healthy volunteers; Ig, immunoglobulin; IL, interleukin; PBMCs, peripheral blood mononuclear cells; PCR, polymerase chain reaction; STA, stable patients; TGF, transforming growth factor; TLR, Toll-like receptor; TOL, tolerant patients

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Introduction

A number of rodent and human studies have demonstrated the possibility of inducing tolerance by manipulating host immunological mechanisms (1–5). However, most attempts at clinical implementation have so far proven unsuccessful.

In humans, long-term acceptance of mismatched kidney allograft after immunosuppressive drug withdrawal, defined as operational tolerance, can occasionally be observed (6,7); however, the mechanisms behind this are as yet unknown. Recently, we have highlighted a higher number of B cells in the blood of these tolerant individuals, associated with an enriched B cell gene profile related to B cells (8–12), and whereas Newell et al showed that tolerant patients (TOL) display a higher number of IL-10 expressing blood transitional B cells compared to stable patients (STA) (11,12), we report on an inhibitory B cell phenotype associated with a lack of $CD19^{+}CD38^{+}CD138^{+}CD20^{-}$ plasma cells in these patients (10). As they only rarely develop an allo-immune response (8) and may not display an ''optimal'' response to alloantigens, we hypothesize that control of this response may take place and/or that their B cells may have a differentiation defect. Using an *in vitro* model of blood B cell differentiation (13), we analyze the profile of blood B cells from tolerant individuals compared to patients with stable graft function under immunosuppression and healthy volunteers (HV). While B cells from operationally tolerant individuals proliferate normally, they produce more IL-10 than B cells from patients with a stable graft function and HV. We also show that plasma cells from the former exhibit normal cell death susceptibility compared to HV but increased compared to patients with stable graft function and a lack of some molecules of transcription

in late stage differentiation compared to patients with stable graft function and HV. These data were confirmed in the transcriptional pattern of purified B cells from these patients *in vivo*.

Materials and Methods

Primary B cell purification

Peripheral blood mononuclear cells (PBMCs) were obtained after Ficoll density centrifugation (Sigma–Aldrich, St. Louis, MO). B cells were purified using negative selection on magnetic columns according to the manufacturer's instructions (B cell isolation Kit II; Miltenyi Biotech, Gladbach, Germany).

Immunophenotyping of B cells

At days 0, 2, 4 and 6, B cells were stained with following anti-human mAbs: anti-IgD PE or FITC, anti-IgM APC, anti-CD20 FITC, anti-CD19 PeCy7, anti-CD24 PE, anti-CD138 PE (BD Biosciences, San Diego, CA), anti-CD27 PeCy7, anti-CD38 PeCy5 (Beckman Coulter, Marseille, France) and analyzed by flow cytometers (LSR II, BD Biosciences). Fluorescence minus one (FMO) was used as negative control (14). Dead cells were excluded from the analysis.

Cell cultures

Cultures were performed in complete medium RPMI 1640 (Invitrogen, Carlsbad, CA). For the activation phase, purified human B cells were cultured at 3.75×10^5 cells/mL and activated as previously described (13). Day-4-activated B cells were washed and cultured at the same concentration for 2 days with 50 U/mL IL-2, 10 ng/mL IL-10 (R&D Systems, Minneapolis, MN) and 10 ng/mL IL-4 (R&D Systems).

Apoptosis and proliferation assays

Apoptosis and proliferation were analyzed at days 2, 4 and 6 using a PE-conjugated anti-active caspase-3 apoptosis kit (BD Biosciences) and an APC-conjugated anti-BrdU kit (BD Biosciences), following the manufacturer's instructions. Caspase-3 and BrdU were analyzed on all B cells at days 2, 4 and 6, and at day 6 in the $CD20^+CD38^+$, $CD20^{lo}CD38^{hi}$ B cell, $CD27^-CD38^+$ and $CD27^{hi}Cd38^{hi}$ B cell subpopulations generated during the differentiation phase.

Cytokine and immunoglobulin measurements

Cytokine production was measured from culture supernatants at day 4. IL-10 and IL-6 levels were determined using Flow Cytomix technology as recommended (Bender Med Systems, Vienna, Austria). IL-10 secretion was confirmed by intracellular staining. Immunoglobulin (Ig) production was measured from culture supernatant at day 6 using BD CBA Human Immunoglobulin IgG and IgM flex set as recommended by the manufacturer (BD Biosciences).

Extraction and preparation of cDNA

RNA was extracted and purified from B cells frozen at days 0, 4 and 6 (RNeasy micro kit; Qiagen, Hilden, Germany). RNA concentration was calculated using a Nanodrop ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), amplified and reverse transcribed into cDNA using a Quantitect Whole Transcriptome Kit (Qiagen) following the manufacturer's instructions.

Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (PCR) was performed in an Applied Biosystems Viia 7 (Foster City, CA) using primers and probe sets purchased from Applied Biosystems for Pax5 (Hs00172003-m1), BACH2 (Hs00222364-m1), BCL-6 (Hs00277037-m1), PRDM1 (Hs00153357), XBp1 (Hs00231936) and IRF-4 (Hs 01056533-m1). The housekeeping genes: Glyceraldehyde-3-phosphate dehydrogenase GAPDH (Hs99999905-m1) and CD19 (Hs 99999192-m1) were used as endogenous controls. The relative expression between a given and a reference sample was calculated using the $2^{-\Delta\Delta Ct}$ method after normalization to GAPDH and CD19 means.

Microarrays

Human gene microarray (8×60 k; Agilent Technologies, Inc., Palo Alto, CA; part number: G4851A) was performed on purified B cells from 10 TOL, 12 STA and 10 HV. Fluorescence signals were normalized using a Lowess (locally weighted scatterplot smoothing) procedure (15). Probe conversion was performed using the MADGene tool (16). Selection of genes was based on a greater than 95 probability of being differentially expressed between TOL and STA (Student's t-test, $p < 0.005$). Expression data were $\log_2$ transformed and median-centered using Cluster and visualized using TreeView (17). Functional interpretation was performed using GoMiner software (18).

Statistical analyses

Statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc., LaJolla, CA), and p-values were calculated using the Mann–Whitney test ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

Results

Tolerant recipients and patients with stable graft function under immunosuppression showed different B cell subset frequencies

CD19, CD20, CD24, CD27, CD38 and CD138 expression was monitored by flow cytometry to analyze transitional B cells ($CD20^+CD24^{hi}CD38^{hi}$), naïve B cells ($CD20^+CD24^{lo}CD38^{lo}$), memory B cells ($CD20^+CD27^+$) and plasma cells ($CD38^+CD138^+$) from 9 TOL, 15 STA and 18 HV (Table 1). Representative gating schemes are shown in Figure 1A. Whereas B cell phenotype from TOL did not differ from that of HV, TOL showed a significantly higher frequency of $CD20^+CD24^{hi}CD38^{hi}$ transitional B cells ($p < 0.05$) and $CD20^+CD24^{lo}CD38^{lo}$ naïve B cells ($p < 0.05$) (Figure 1B and C) and a significantly lower frequency of $CD19^+CD38^+CD138^+$ differentiated plasma cells compared to STA (Figure 1E) ($p < 0.01$). No significant difference was observed for memory B cells (Figure 1D).

Validation of experimental design to differentiate $CD20^+$ B lymphocytes into $CD138^+$ plasma cells: a two-step model of B cell differentiation

We explored the T-dependent B cell terminal differentiation of blood B cells using a two-step culture model allowing the differentiation of naïve B cells in addition to memory B cells (13). Blood $CD20^+$ B cells from 18 HV were cultured as described in Materials and Methods section. During the activation phase (days 0–4), a proliferation burst was observed in about 33% of B cells at day 4 ($p < 0.001$, Figure 2A) while only 3.3% of B cells showed caspase-3 positive staining ($p < 0.001$, Figure 2B). At day 6, B cells stop proliferating and enter cell death apoptosis ($p < 0.001$, Figure 2A and B). The B cells acquired the CD38 and CD27 markers during the activation phase at day 4 (Figure 2E) and

Table 1: Summary of clinical data for TOL, STA and HV

Patient group	Age (years)	Gender	Donor (living vs. deceased)	Number of HLA mismatches	Time between graft and analysis (months)	Creatinemia (μmol/L)	Proteinuria (g/24 h)	Time between immunosuppression withdrawal and analysis (years)
HV								
(n = 18)		5F/13M						
Median	43							
Min.	23							
Max.	60							
IQT	12.5							
TOL								
(n = 9)		4F/5M	3LD/6NLD					
Median	57.19			2.00	226.00	95.00	0.06	9.00
Min.	41.08			0.00	114.43	60.00	0.00	5.00
Max.	83.61			4.00	343.80	152.00	0.26	15.00
IQT	11.79			4.00	140.37	47.00	0.11	2.00
STA								
(n = 15)		6F/9M	0LD/15NLD					
Median	49.96			4.00	133.17	104.00	0.12	
Min.	19.19			2.00	48.60	72.00	0.04	
Max.	81.68			6.00	294.23	145.00	0.29	
IQT	31.83			1.00	123.85	26.00	0.14	

HV, healthy volunteers; IQT, interquartile; LD, living donor; NLD, nonliving donor; STA, stable patients; TOL, tolerant patients.

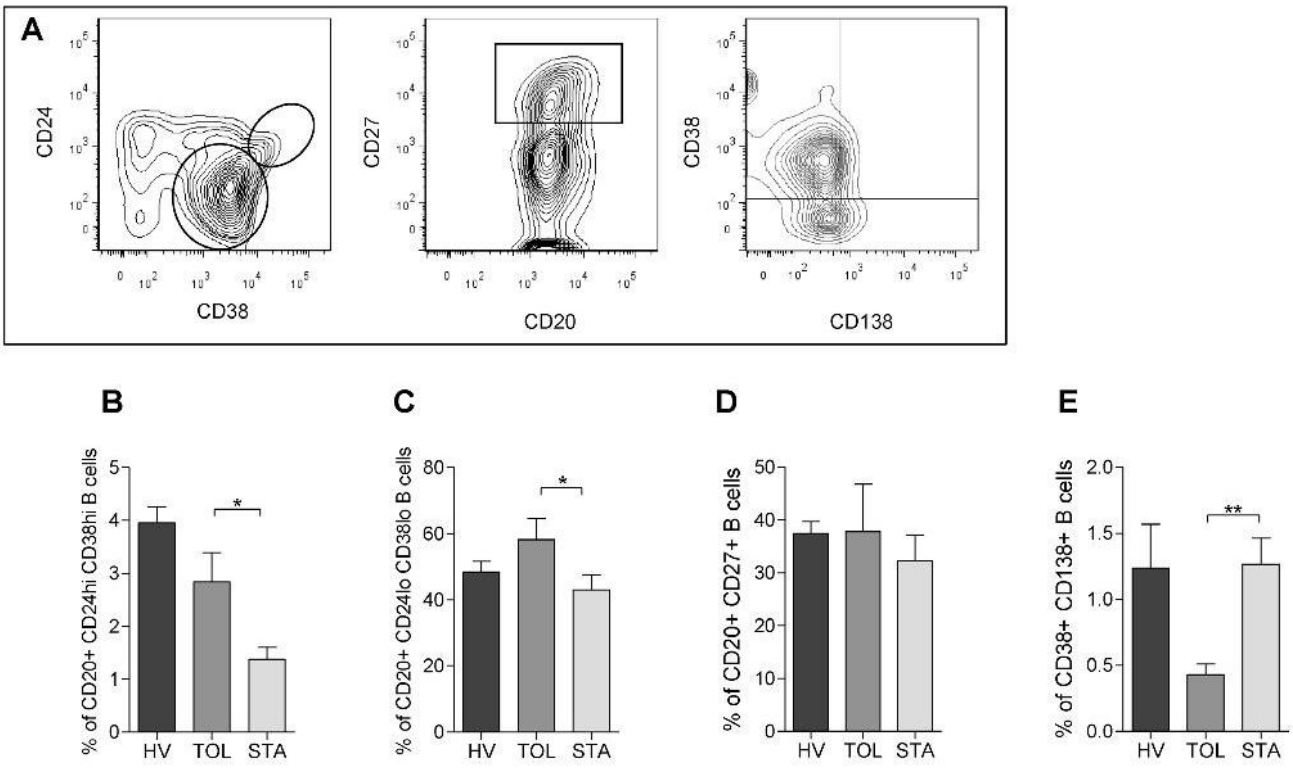


Figure 1: B cell phenotype of tolerant patients. B cell subsets were analyzed by flow cytometry as follows (A). Frequency of transitional B cells $CD20^{+}CD24^{hi}CD38^{hi}$ (B). Frequency of naïve B cells $CD20^{+}CD24^{lo}CD38^{lo}$ (C). Frequency of memory B cells $CD20^{+}CD27^{+}$ (D). Frequency of plasma cells $CD38^{+}CD138^{+}CD19$ B cells (E) (*$p < 0.05$, **$p < 0.01$). HV, healthy volunteers; STA, stable patients; TOL, tolerant patients.

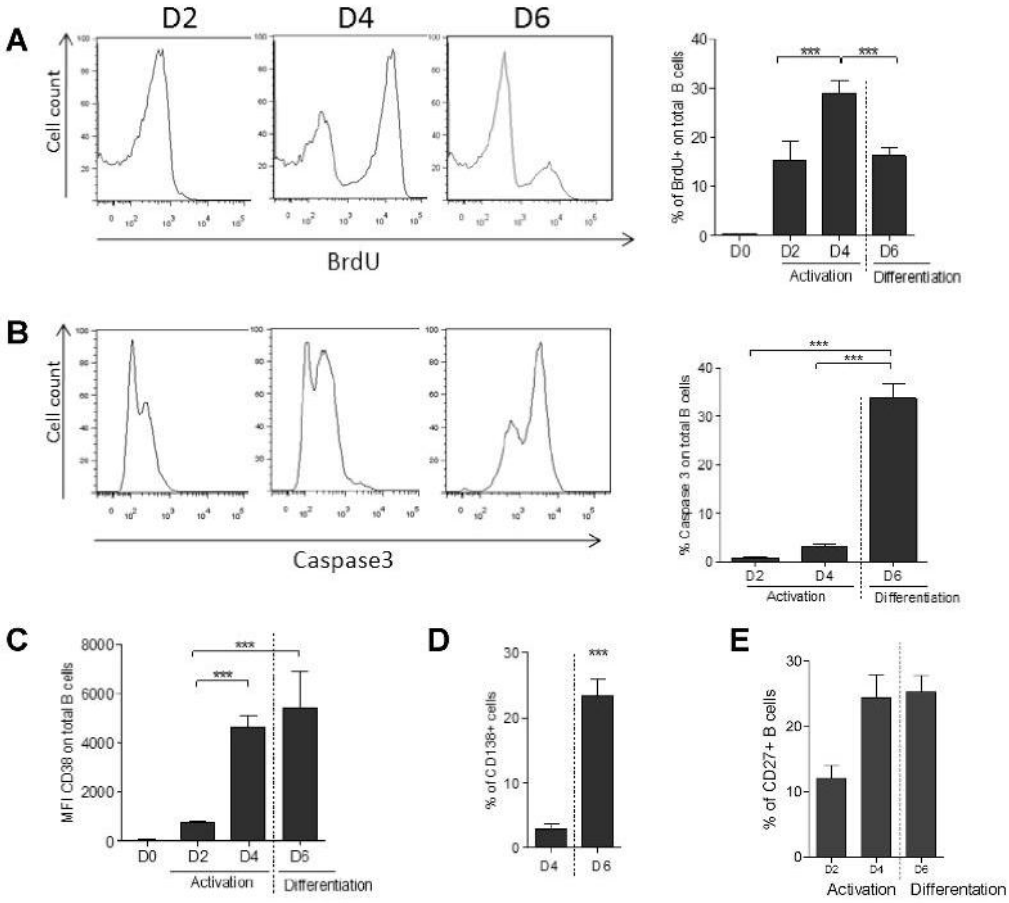


Figure 2: Validation of B cell activation and differentiation model in 18 healthy volunteers. For the activation phase, purified human B cells were cultured at 3.75×10^5 cells/mL and activated with 2 μg/mL F(ab')^2 Fragment Goat anti-Human IgA+ IgG+ IgM+ (H + L) (Jackson ImmunoResearch Laboratories, West Grove, PA), 50 ng/mL recombinant human soluble CD40L, 5 μg/mL cross-linking Ab (R&D Systems), 2.5 μg/mL CpG oligodeoxynucleotide 2006 (InvivoGen, San Diego, CA) and 50 U/mL recombinant IL-2 (SARL Pharmaxie, Aigueperse, France), as previously described (13). Day-4-activated B cells were washed and cultured at the same concentration, at 3.75×10^5 cells/mL for 2 days with 50 U/mL IL-2, 10 ng/mL IL-10 (R&D systems) and 10 ng/mL IL-4 (R&D Systems). B cell proliferation and apoptosis were analyzed by flow cytometry using BrdU (A) and caspase-3 (B) staining. Activation and differentiation are measured through median of fluorescence intensity of CD38 (C), percentage of CD27$^+$ B cells (E) and percentage of CD138$^+$ B cells (D) (***$p < 0.001$).

the CD138 plasma cell expression on day 6, thus confirming the terminal differentiation of B lymphocytes ($p < 0.001$, Figure 2D).

B cells from TOL maturate activate and produce IgM and IgG in vitro

The fact that TOL show a significantly higher frequency of CD20$^+$CD24^{hi}CD38^{hi} transitional B cells and CD20$^+$CD24^{lo}CD38^{lo} unmutated naïve B cells and a significantly lower frequency of CD20$^-$CD38$^+$CD138$^+$ terminally differentiated plasma cells compared to STA raises the question whether B cells from TOL exhibit a defect in the B cell differentiation process. B cells were cultured as previously described. Expression of CD38, CD27 and CD138 did not differ between the three groups of patients on days 2, 4 and 6 (Figure 3A–C). These data show that B cells from TOL activate and mature normally when cultured *in vitro*. This is corroborated to the absence of differences between TOL, STA and HV in the number of B cell subsets during the *in vitro* activation and differentiation (Figure S1). The same frequency of differentiated (CD27^{hi}CD38^{hi} B cells) and nondifferentiated B cells (CD27$^-$CD38$^+$ B cells) at the end step of differentiation confirms the ability to activate and differentiate B cells from TOL *in vitro* (Figure S2B and C). The frequency of Ig class switch (IgM$^+$IgD$^-$CD27$^+$ B cells and IgM$^-$IgD$^-$CD27$^+$ B cells) and unswitched memory B cells (IgM$^+$IgD$^+$CD27$^+$ B cells and IgM$^-$IgD$^+$CD27$^+$ B cells) was analyzed over the culture, with no differences between the three groups of patients (Figure S3), suggesting that there is no difference *in vitro* in the ability to Ig class switch in TOL. Supernatants of B cell cultures were collected on day 6 and assayed using an IgM/IgG FlowCytomix multiplex kit. No significant difference was shown for IgM and IgG production between the total B cells from the three groups of patients (Figure 3D

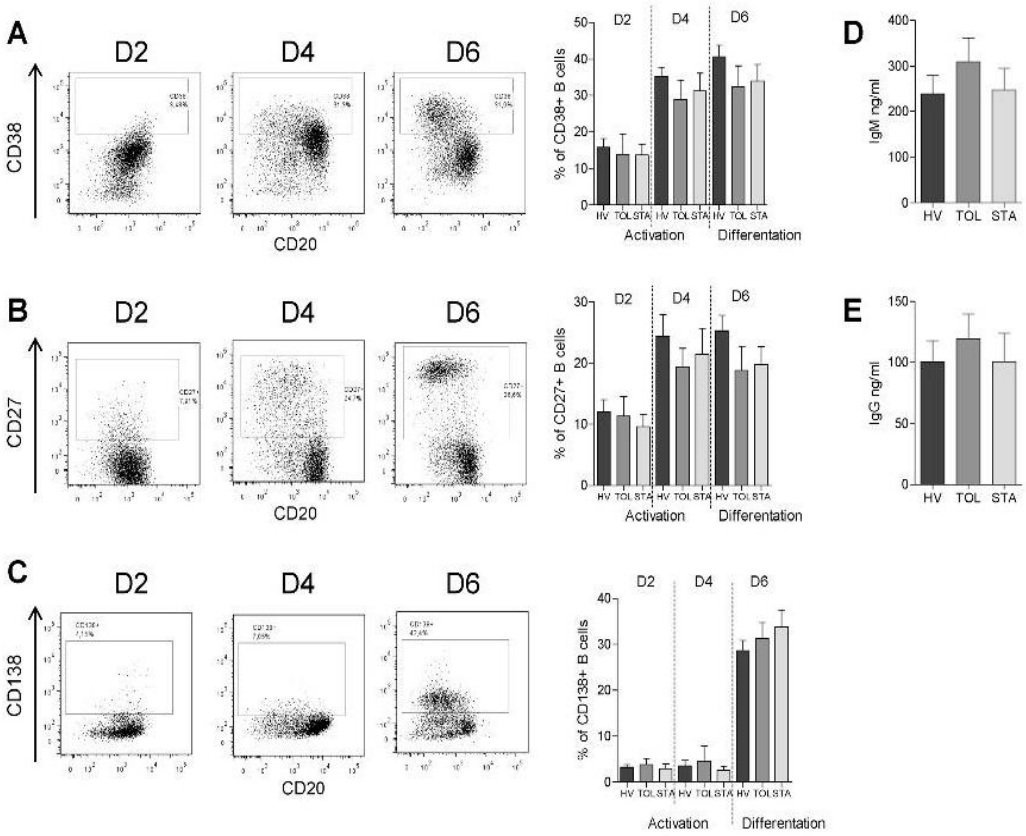


Figure 3: Activation and differentiation into plasma cells. Dot plot and histograms for the generation of $CD20^{+}CD38^{+}$ B cells (A), $CD20^{+}CD27^{+}$ B cells (B) and plasma $CD20^{lo}CD138^{+}$ cells (C) analyzed by flow cytometry on total B cells on days 2, 4 and 6 of the culture. IgM (D) and IgG (E) human immunoglobulin were measured in the supernatant at day 6 of culture using CBA Flex (BD Biosciences). Immunoglobulin secretion is expressed in ng/mL. HV, healthy volunteers; STA, stable patients; TOL, tolerant patients.

and E). Altogether, these data show that *in vitro*, B cells from TOL maturate and, when stimulated, produce normal levels of IgM and IgG.

TOL B cells display defective expression of molecules involved in later stages of differentiation

Gene expression levels of *BCL6*, *BACH2*, *PAX5*, *IRF4*, *PRDM1* and *XBP1* at days 4 and 6 were measured relative to their expression at day 0 (13). As expected, the expression of *BCL6*, *BACH2* and *PAX5* were down-regulated during the differentiation process, showing that cells from TOL, STA and HV completed the first differentiation step (Figure 4A–C) and showed increased CD38 expression (Figure 3A). Whereas the frequency of differentiated $CD20^{lo}CD38^{hi}$ B cells was the same in TOL and STA, *IRF4* and *PRDM1*, implicated in the end step of differentiation into plasma cells, were down-regulated in B cells from TOL at day 6 compared to STA ($p < 0.05$) and *XBP1* was down-regulated in B cells from TOL compared to both STA and HV ($p < 0.05$) (Figures 4D, E, and F and S5). Altogether, these data suggest an *in vitro* defect in the later stages of differentiation in B cells from TOL.

B cells from tolerant recipients show normal B cell proliferation and produce higher levels of IL-10 when stimulated in vitro

We assessed the proliferative capacity of B cells during plasma cell generation by analyzing BrdU incorporation (13). A proliferative burst was observed with more than $19.15\% \pm 6.52\%$ and $22.99\% \pm 9.65\%$ of the B cells in S-phase at day 4 in TOL and STA, respectively (Figure 5A). At day 6, cell proliferation was reduced in all groups (Figure 5A). No difference between the two groups of patients and HV was observed in B cell proliferation capacity from day 0 to 6, which concords with a similar differentiation for a $CD20^{lo}CD38^{hi}$ phenotype (Figure 5A). No difference was observed in the proliferation capacity of $CD20^{lo}CD38^{hi}$ and $CD20^{+}CD38^{+}$ B cell subsets that are late and early differentiated B cells (Figure 5B). Supernatant of B cell cultures were collected on day 4 and assayed using a FlowCytomix multiplex kit to measure the concentration of IL-10, IL-6 and TGF-β. B cells from TOL produced significantly higher amounts of IL-10 compared to STA and HV ($p < 0.05$, Figure 5C), whereas no difference between the three groups was observed for IL-6 (Figure 5D). These data were confirmed by IL-10 intracellular

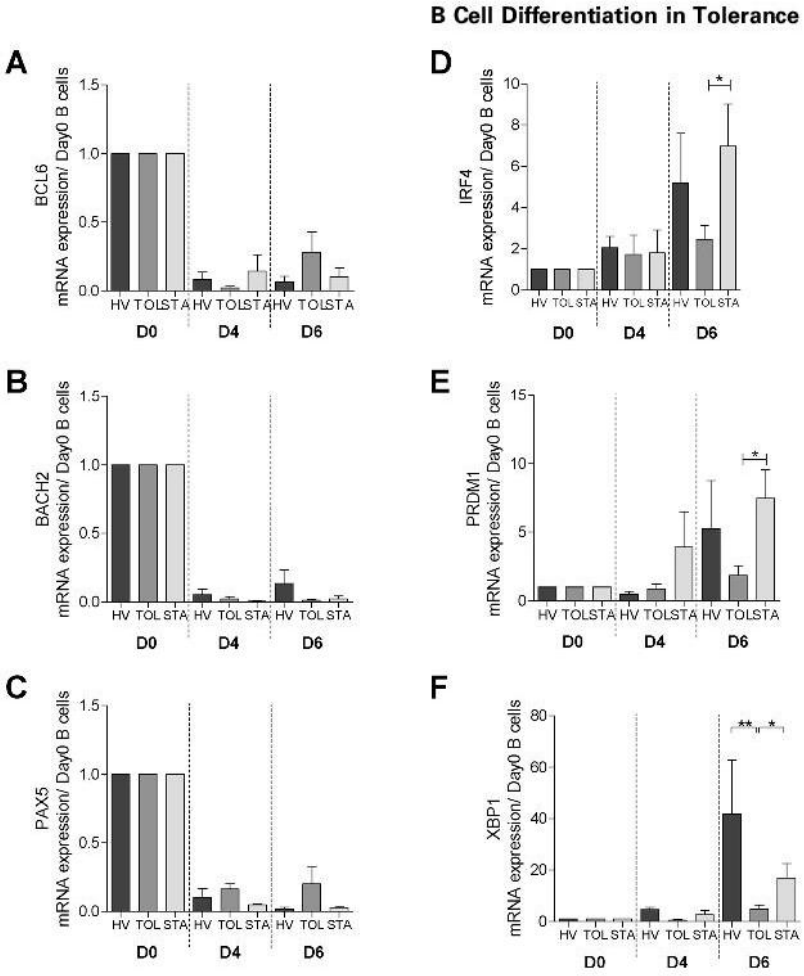


Figure 4: B cell differentiation. *BCL6* (A), *BACH2* (B), *PAX5* (C), *IRF4* (D), *PRDM1* (E) and *XBP1* (F) transcriptional levels in B cells are shown relative to the day of experiment. Results of quantitative polymerase chain reaction are expressed relative to gene expression at day 0. Bars represent mean values ± SEM (*$p < 0.05$, **$p < 0.01$). HV, healthy volunteers; STA, stable patients; TOL, tolerant patients.

staining (Figure 5E). Finally, the amount of TGF-β in the supernatant at day 4 was very low, with no difference between the groups of patients (data not shown). Altogether, these data show that B cells from TOL produce higher levels of IL-10 when stimulated *in vitro*.

Terminally differentiated B cells from tolerant recipients show higher cell death susceptibility than patients with stable graft function in vitro

Knowing that TOL show a lower frequency of circulating $CD20^{-}CD38^{+}CD138^{+}$ plasma cells compared to STA, we investigated if this could be due to an increase in B cell death in their later stage of differentiation. As expected, whereas cell death susceptibility was not different between TOL and HV, B cells (nondifferentiated $CD20^{+}CD38^{+}$ and differentiated $CD20^{lo}CD38^{hi}$) from TOL showed a higher level of cell death apoptosis on day 6 (40.05 ± 19.76) than STA (18.67 ± 8.003, $p < 0.05$) (Figure 6A and B).

B cells from tolerant recipients exhibit a clear down-regulation of B cell differentiation genes and down-regulation of anti-apoptotic genes

As shown in Figure 7 and Table S1, TOL, STA and HV displayed differentiated profiles particularly on genes from the cell cycle, apoptosis, maturation, development and differentiation of B cells, confirming that the *in vitro* profile of B cells from TOL is not an artifact. The majority of genes differentially expressed between TOL and STA are not differentially expressed between STA and HV, suggesting that this is not an effect of immunosuppressive treatment. On the 12 040 annotated genes retained as being reliably expressed across the 32 examined samples, 1333 could be significantly detected as being differentially expressed by pairwise comparisons (i.e. STA vs. HV, TOL vs. HV and TOL vs. STA). They include 420 genes differentially expressed ($p < 0.005$, false discovery rate [FDR] = 14%) between TOL and HV (172 up-regulated and 248 down-regulated in TOL). Two hundred and thirty-nine could also be significantly detected as differentially expressed between TOL and STA ($p < 0.005$, FDR = 25%), including 136 down-regulated and 103 up-regulated genes. Among these genes, a focused analysis (Figure 7) identified genes of B cell activation, differentiation and apoptosis (GO:0042113; GO:0006915). Functional annotation analysis confirms this profile with biological functions related to B cell activation, apoptosis and proliferation to be significantly enriched in TOL ($p < 0.01$, Table S1). In conclusion, among several other genes that were differentially expressed between the three groups of patients, there is a clear down-regulation of genes

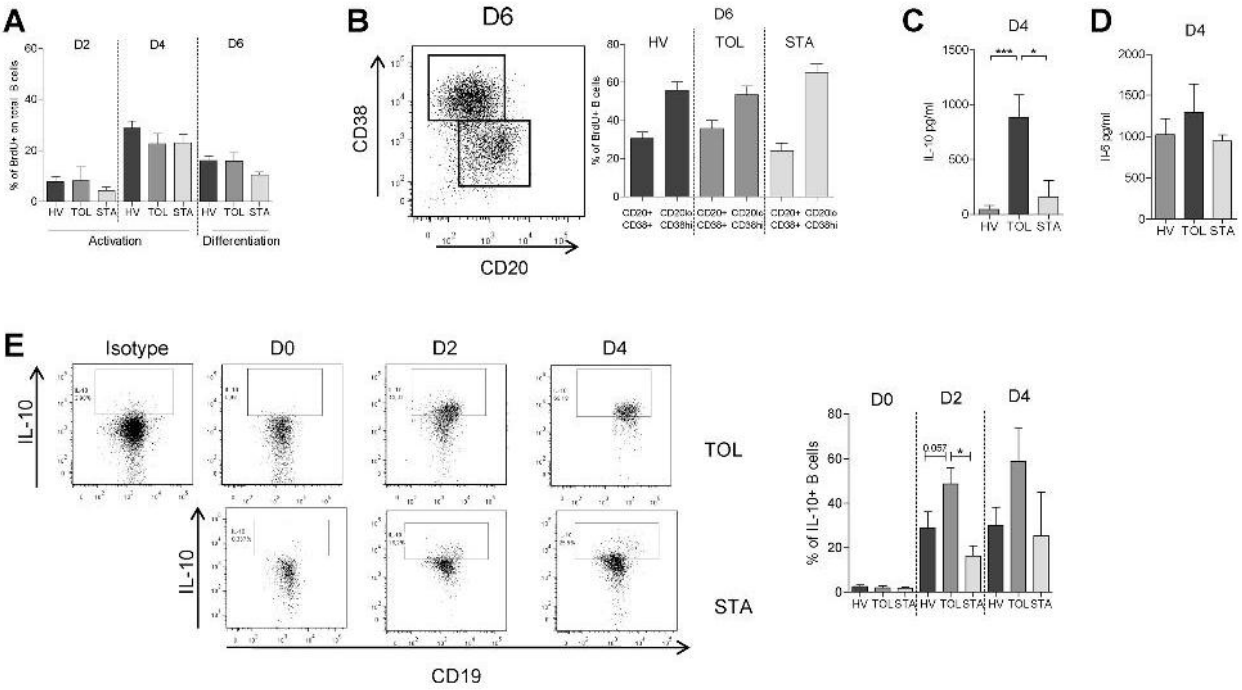


Figure 5: Proliferation and cytokines secretion. Proliferation was analyzed by intracellular staining of BrdU in total B cells at days 2, 4 and 6 (A). Proliferation of B cell subset at day 6: $CD20^{+}CD38^{+}$ B cells and $CD20^{lo}CD38^{hi}$ B cells with the representative dot plot (B). IL-10 (C) and IL-6 (D) are measured in the supernatant at day 4 of the B cell culture using the FlowCytomix multiplex kit (eBiosciences, San Diego, CA). B cells were stained with LIVE/DEAD cells (Invitrogen, Eugene, OR), washed and extracellular staining was performed on them using an anti-CD19 antibody. After washing, B cells were permeabilized (cytofix/cytoperm permeabilization KIT; BD Biosciences) and intracellular staining was performed using anti-IL-10-PE antibody or rat IgG1,k PE isotype as negative control (BD Biosciences). Dot plot of the secretion of IL-10 at days 0, 2 and 4 with isotype control or IL-10 antibody, and secretion variation of cytokines expressed in pg/mL (E). ($^{*}p < 0.05$, $^{***}p < 0.001$). HV, healthy volunteers; STA, stable patients; TOL, tolerant patients.

of B cell differentiation (five gene clustering) and anti-apoptotic genes (six gene clustering) in TOL versus STA, in accordance with their different levels of apoptosis *in vitro* during the differentiation process.

Treatment is not responsible for the B cell differentiation profile observed in operationally tolerant recipients

The effects of treatment on B cell differentiation, cytokine production and apoptosis have been further studied through comparison with a group of atopic dermatitis patients (DA, $n = 4$). These patients received 150–300 mg/day of cyclosporin A, which is comparable with the doses administered to STA. B cell death and IL-10 productions were both increased in B cells from DA compared to STA under similar immunosuppression ($p < 0.05$), suggesting that the characteristics of B cells from TOL are not a result of the absence of immunosuppressive treatment (Figure S4).

Discussion

Over the last decade, significant effort has been made among the transplant community in Europe (Reprogramming the Immune System for Establishment of Tolerance and Indices of Tolerance, http://www.immunetolerance.org/In) and in the United States (Immune Tolerance Network, http://www.risetfp6.org/) to identify biological signatures of organ tolerance in kidney transplantation (9–12,19). The mechanisms for the induction and maintenance of this state of drug-free long-term graft function remain unknown. These patients display a higher number of circulating B cells (8), a whole blood B cell transcriptional profile (9,10,12) and an increased production of IL-10 (11) compared to patients with stable graft function. However, to what extent this B cell footprint and environment are instrumental in tolerance or are merely consequences of the state of drug-free long-term graft function remains to be established.

As these patients only rarely develop an allo-immune response, we hypothesize that their B cells may display some abnormal characteristics during the differentiation process. Besides an overall inhibitory B cell profile (10), TOL display a higher proportion of blood transitional and naïve B cells compared to STA under immunosuppression. These cells are known to have a limited ability to activate T cells, to preferentially expand regulatory T cells (20–24) and to

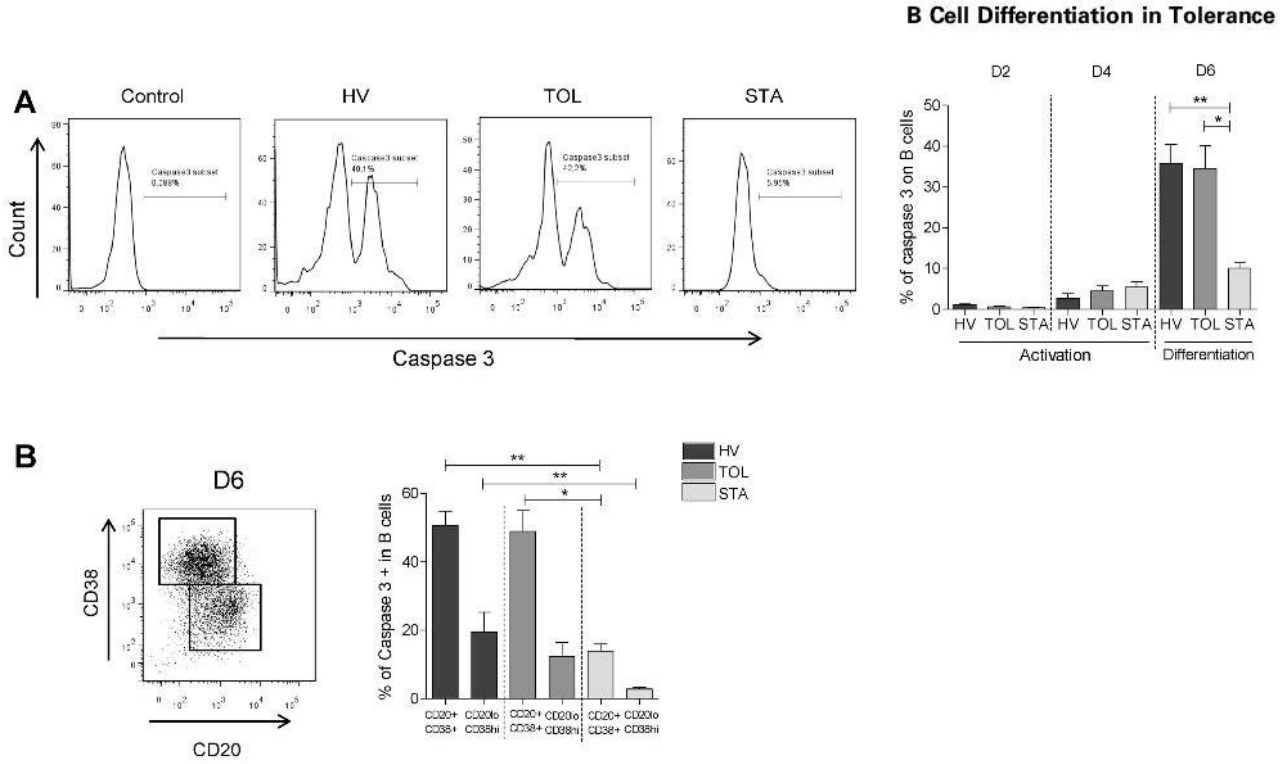


Figure 6: Apoptosis signaling. Apoptosis was analyzed by flow cytometry by measuring caspase-3 staining in total B cells, caspase-3 representative histograms and percentage of caspase-3+ on B cells at days 2, 4 and 6 (A) and in $CD20^{+}CD38^{+}$ and $CD20^{lo}CD38^{hi}$ B cell subsets at day 6 (B) (*$p < 0.05$), **$p < 0.01$). HV, healthy volunteers; STA, stable patients; TOL, tolerant patients.

display regulatory capacities through IL-10 production (25,26). These data give further evidence to the idea that the B cell response in TOL may be more tolerogenic than deleterious. This paper focuses on differentiation of B cells into plasma cells for such patients using a validated primary culture system (13,27,28) combining two steps of activation and differentiation (29). No difference in *in vitro* B cell expansion was observed in tolerant recipients compared to STA and HV, confirming the absence of differences in k-deletion recombination excision circles performed on B cells in these patients (data not shown). Thus, while increased proliferation may not explain the increase in blood transitional and naïve B cells, some alteration or some unidentified events, occurring in the development of B cells in the bone marrow of TOL, cannot be excluded since this naïve/transitional signature was found in patients undergoing depletion strategies (30,31).

More interestingly, whereas B cell cytokine production is mainly dependent upon culture conditions and the stimuli used (32–34), we demonstrate here that B cells from operationally TOL, stimulated using the same method and time course, do produce more IL-10 than STA and HV. Of course, these *in vitro* finding may be different from *in vivo* setting but, in accordance with the *in vivo* data from Newell et al (11), suggest that different regulatory processes may be involved during B cell differentiation in these patients, independent of culture conditions. Interestingly, the higher production of IL-10 by B cells in tolerant recipients is not due to an absence of treatment since they also produce more IL-10 than B cells from HV. This is also probably not due to unspecific B cell activation since B cells from tolerant recipients secrete normal levels of the central effector cytokine IL-6 and no TGF-β (33).

B cells from operationally TOL are able to mature normally *in vitro* and to produce IgM and IgG with normal unswitch/switch B cells populations. These observations, which should be enriched in the future with more specific markers (35), are consistent with the fact that operationally TOL display no overall humoral immunity deficiency (7). These data also show that B cells from operationally tolerant recipients exhibit a higher propensity to cell death apoptosis in their late phase of differentiation, compared to STA but similar to that observed in HV. This observation may be ''not specific'' to TOL but due to a default in B cell apoptosis from STA that would adjust their apoptotic machinery to survive long term in the presence of immunosuppressive drugs and are less sensitive to cell cycle arrest. Nevertheless, we also showed that differentiation of B cells from tolerant recipients is associated with a decreased expression of *IRF4* and *PRDM1*, involved in germinal center fate compared to patients with stable graft function and a decreased expression of *XBP1* compared to both patients with stable graft function and HV, suggesting a partial or incomplete B cell differentiation. These data were reinforced by transcriptional analysis of *in vivo* purified B cells from TOL, which are characterized by a clear down-

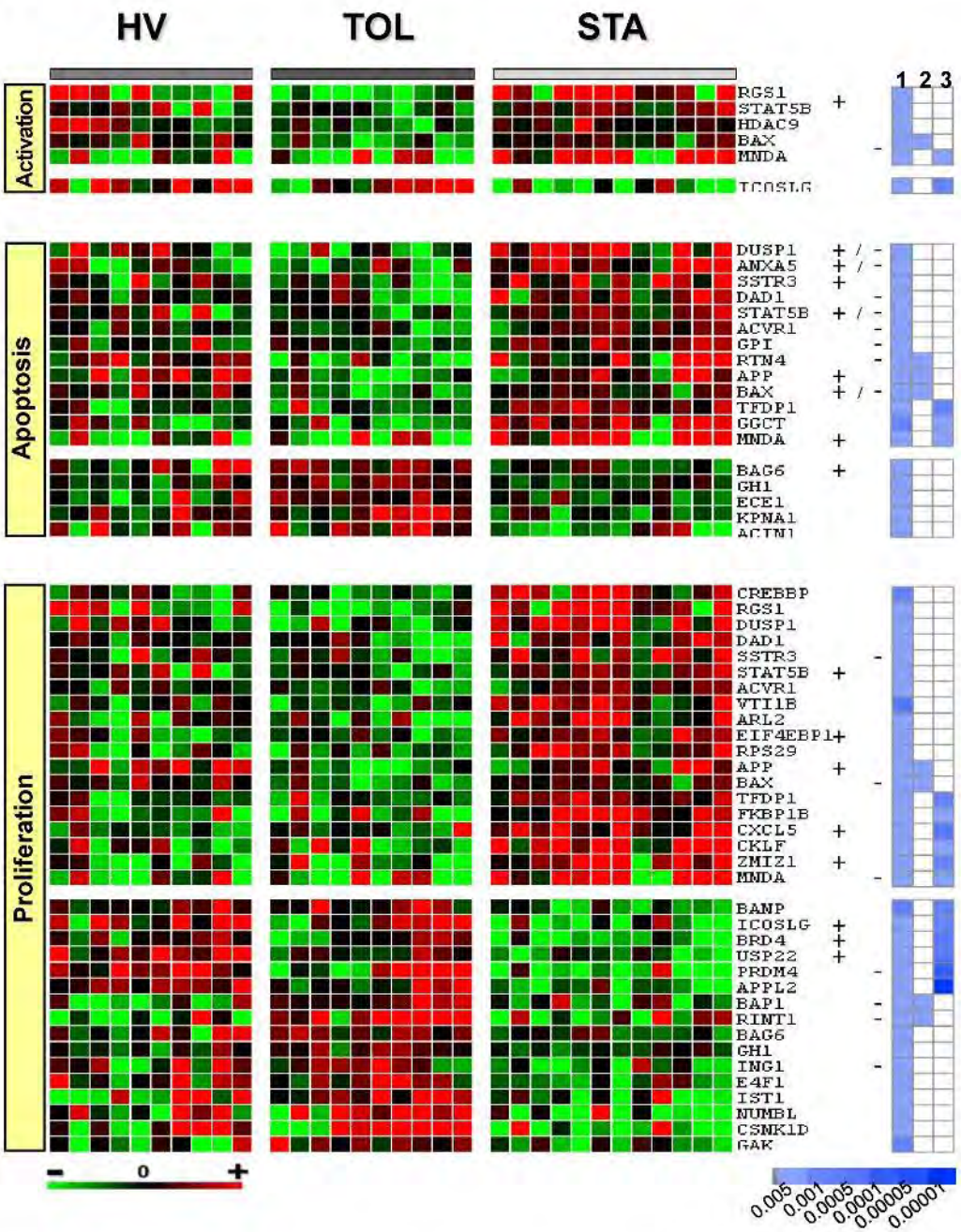


Figure 7: Gene expression of the genes related to B cell activation, apoptosis and proliferation from the tolerant gene signature. Expression values across HV, TOL and STA are presented in heatmaps. Each cell in the matrix corresponds to the expression level of a gene in a sample, with red for over-expression, green for under-expression and black for gene expression close to the median (see color scale). Genes were selected by a t-statistic ($p < 0.005$) as being differentially expressed between TOL and STA groups. For each pairwise comparison (1: TOL vs. STA; 2: TOL vs. HV; 3: STA vs. HV), significance is given for each gene using shades of blue (see color scale). Plus (+) and minus (−) denote the involvement of the gene either in positive or in negative regulation of the biological function analyzed. HV, healthy volunteers; STA, stable patients; TOL, tolerant patients.

regulation of genes of B cell differentiation and antiapoptotic genes. These data, which concord with our previous data on PBMCs (9,10), provide some supporting evidence that this is not only an *in vitro* phenomenon.

Altogether, the lack of expression of some molecules of differentiation and the higher cell death apoptosis level of B cells in the late stage of differentiation compared to patients with stable graft function are concordant with the reduced

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1: Gene ontology (GO) analysis of the gene signature

GO terms related to B cell activation, apoptosis or proliferation and significantly enriched ($p < 0.01$) in either the whole signature or one of the two included subsets (up- and down-regulated genes) were retained.

Figure S1: B cells subset during the activation and differentiation steps. Different B cells subset were analyzed by flow cytometry at days 0, 2, 4 and 6. Frequency of naïve and transitional B cells $CD27^-$ IgD^+ B cells (A). Frequency of memory B cells $CD27^+$ $CD38^+$ $CD19^+$ B cells (B). Frequency of plasma cells $CD38^+$ $CD138^+$ $CD19^+$ B cells (C) ($^*p < 0.05$).

Figure S2: Frequency, proliferation and apoptosis of differentiated and nondifferentiated B cells. Activation and differentiation phenotype following by CD27 and CD38 staining of B cells at days 0, 2, 4 and 6 (A). Day 6 frequency of differentiated B cells $CD27^{hi}CD38^{hi}CD19^+$ cells (B). Frequency of nondifferentiated B cells $CD27^-CD38^+CD19^+$ cell at day 6 (C) ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

Figure S3: Ig class switch memory B cell subset during the activation and differentiation steps. B cell subsets were analyzes by flow cytometry at days 2, 4 and 6. Representative dot plot (A). Frequency of IgM+ IgD+ memory B cells (B). Frequency of IgD only memory B cells (C). Frequency of IgM only memory B cells (D). Frequency of Ig class switch memory B cells (E).

Figure S4: B cell IL-10 secretion and percentage of apoptosis in atopic dermatitis and stable patients. B cell IL-10 secretion analyzed by intracellular staining with an anti-human IL-10-PE antibody (BD Biosciences) at days 0, 2 and 4 of culture (A). Apoptosis was analyzed by flow cytometry by measuring caspase-3 staining in total B cells (B) in AD and STA.

Figure S5: Kinetics of expression of *IRF4*, *PRDM1* and *XBP1* during differentiation process. *IRF4* (A), *PRDM1* (B) and *XBP1* (C) transcriptional levels in B cells are shown relative to the day of experiment. Results of quantitative PCR are expressed relative to gene expression at day 0. Bars represent mean values ± SEM ($^*p < 0.05$, $^{**}p < 0.01$).

Table S1:

	WHOLE SIGNATURE (DOWN AND UP GENES)				UP-REGULATED GENES				DOWN-REGULATED GENES					
	CHANGED GENES #	GENE DESCRIPTION	ENRICHMENT	P-VALUE	CHANGED GENES #	GENE DESCRIPTION	ENRICHMENT	P-VALUE	CHANGED GENES #	GENE DESCRIPTION	ENRICHMENT	P-VALUE	GO ID	GO TERM DESCRIPTION
B-CELL RELATED TERMS	6	BAX, HDAC9, ICOSLG, MNDA, RGS1, STAT5B	3.3901	**0.00883**	1	ICOSLG	1.27905	0.54487	5	BAX, HDAC9, MNDA, RGS1, STAT5B	5.06059	**0.00316**	GO:0042113	B-cell activation
	1	BAX	49.78641	0.02009	0		0	1	1	BAX	89.18261	**0.01121**	GO:0002358	B-cell homeostatic proliferation
	1	BAX	43.78884	0.02271	0		0	1	1	BAX	78.43913	**0.01271**	GO:0002339	B-cell selection
	1	BAX	43.78884	0.02271	0		0	1	1	BAX	78.43913	**0.01271**	GO:0002352	B-cell negative selection
	3	BAX, HDAC9, STAT5B	3.41212	0.05806	0		0	1	3	BAX, HDAC9, STAT5B	6.11214	**0.01311**	GO:0030183	B-cell differentiation
APOPTOSIS RELATED TERMS	3	ACIN1, BAX, KPNA1	7.50666	**0.00734**	2	ACIN1, KPNA1	11.328728	**0.01344**	1	BAX	4.48224	0.20071	GO:0030262	apoptotic nuclear change
	2	BAG6, BAX	19.4617	**0.00443**	1	BAG6	22.02808	0.04450	1	BAX	17.43092	0.05594	GO:0070059	apoptosis in response to endoplasmic reticulum stress
	2	BAX, RTN4	35.03107	**0.00127**	0		0	1	2	BAX, RTN4	62.7513	**0.0004**	GO:0019987	negative regulation of anti-apoptosis
	1	BAX	87.57767	**0.01142**	0		0	1	1	BAX	156.87826	**0.00637**	GO:0046674	induction of retinal programmed cell death
	3	BAX, DUSP1, RTN4	4.95723	0.02263	0		0	1	3	BAX, DUSP1, RTN4	8.8799	**0.00469**	GO:0045767	regulation of anti-apoptosis
	1	SSTR3	49.78641	0.02009	0		0	1	1	SSTR3	89.18261	**0.01121**	GO:0008628	induction of apoptosis by hormones
	1	SSTR3	49.78641	0.02009	0		0	1	1	SSTR3	89.18261	**0.01121**	GO:0035081	induction of programmed cell death by hormones
	6	ANXA5, BAX, DAD1, DUSP1, RTN4, STAT5B	1.7934	0.11982	0		0	1	6	ANXA5, BAX, DAD1, DUSP1, RTN4, STAT5B	3.21252	**0.01123**	GO:0006916	anti-apoptosis
	1	BAX	43.78884	0.02271	0		0	1	1	BAX	78.43913	**0.01271**	GO:0030264	nuclear fragmentation involved in apoptotic nuclear change
PROLIFERATION RELATED TERMS	5	APP, BRD4, EIF4EBP1, STAT5B, USP22	14.12543	**0.00002**	2	BRD4, USP22	12.7905	**0.01064**	3	APP, EIF4EBP1, STAT5B	15.18177	**0.001**	GO:0045931	positive regulation of mitotic cell cycle
	5	APP, BRD4, EIF4EBP1, STAT5B, USP22	4.78716	**0.00376**	2	BRD4, USP22	4.33474	0.07762	3	APP, EIF4EBP1, STAT5B	5.14515	0.02038	GO:0045787	positive regulation of cell cycle
	6	ACIN1, BAX, KLF2, SEC23B, SLC25A38, STAT5B	3.05504	**0.01423**	3	ACIN1, SEC23B, SLC25A38	3.4579	0.05631	3	BAX, KLF2, STAT5B	2.73625	0.0974	GO:0048872	homeostasis of number of cells
	2	ICOSLG, STAT5B	12.5111	**0.01079**	1	ICOSLG	14.16091	0.06837	1	STAT5B	11.20559	0.08567	GO:0042104	positive regulation of activated T cell proliferation
	1	BRD4	43.78884	0.02271	1	BRD4	99.12637	**0.01006**	0		0	1	GO:0010971	positive regulation of G2 M transition of mitotic cell cycle
	1	DAD1	43.78884	0.02271	0		0	1	1	DAD1	78.43913	**0.01271**	GO:0030472	mitotic spindle organization in nucleus
	1	STAT5B	43.78884	0.02271	0		0	1	1	STAT5B	78.43913	**0.01271**	GO:0032817	regulation of natural killer cell proliferation
	1	STAT5B	43.78884	0.02271	0		0	1	1	STAT5B	78.43913	**0.01271**	GO:0032819	positive regulation of natural killer cell proliferation

Figure S1

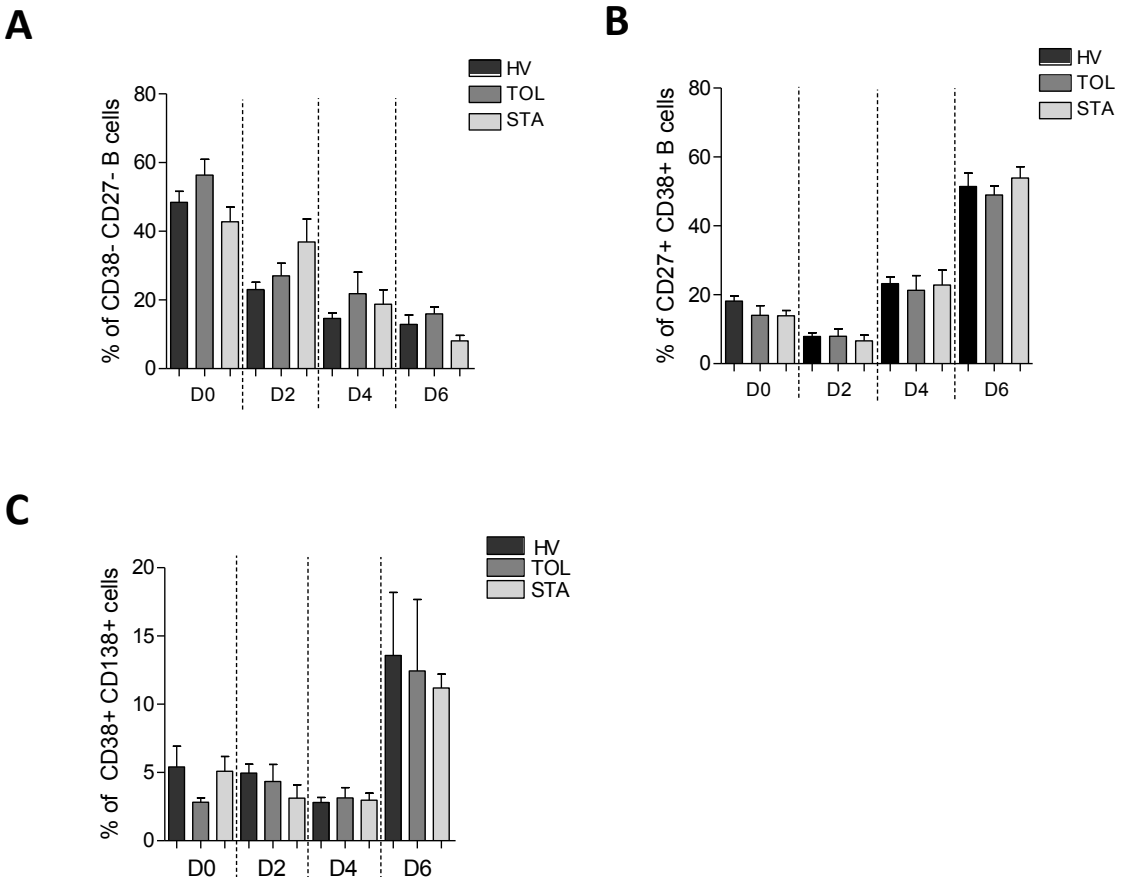


Figure S2

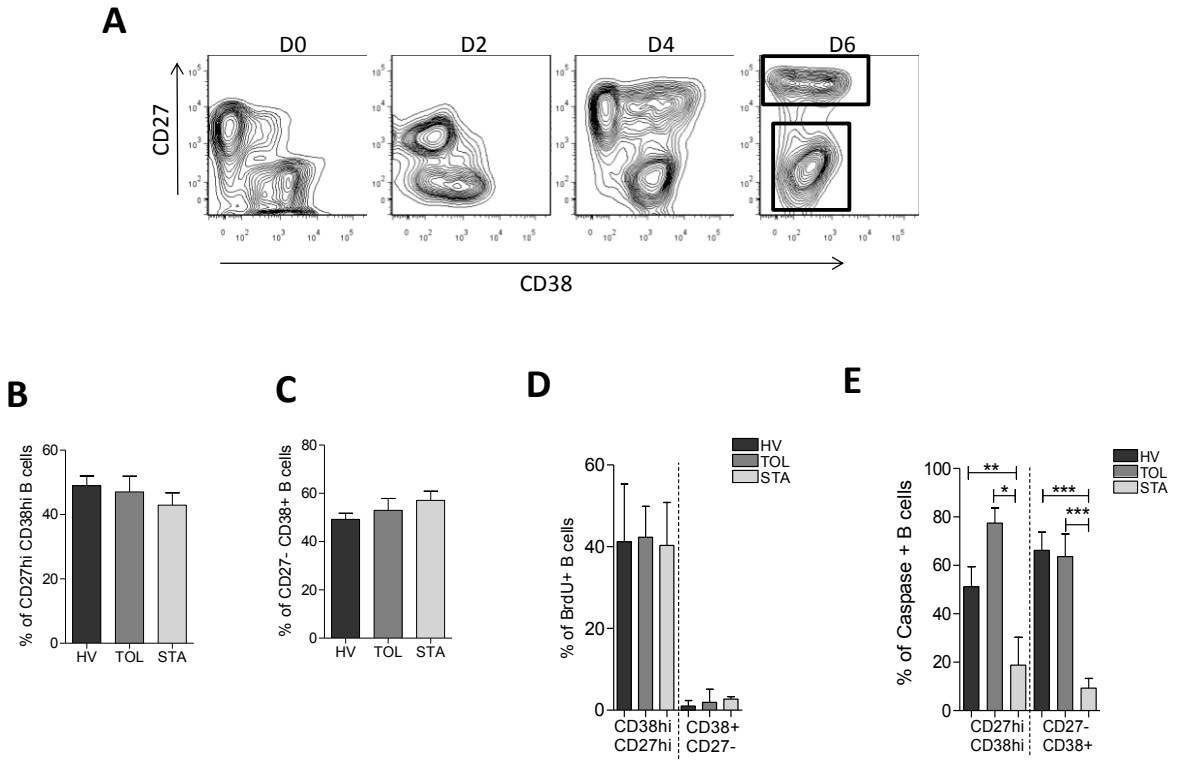


Figure S3

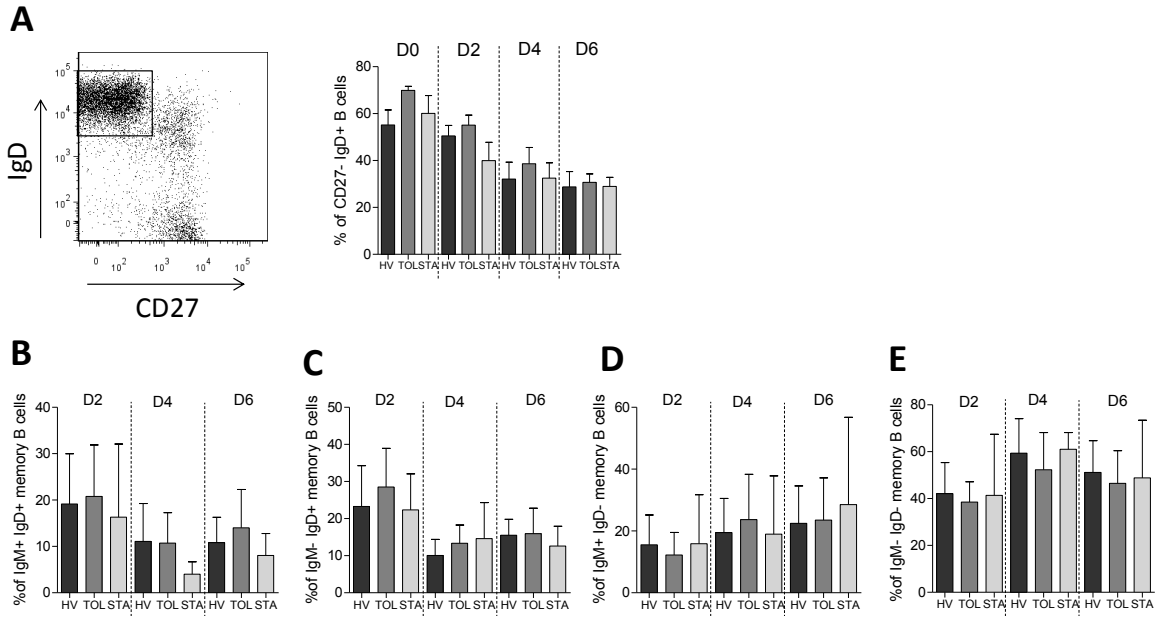


Figure S4

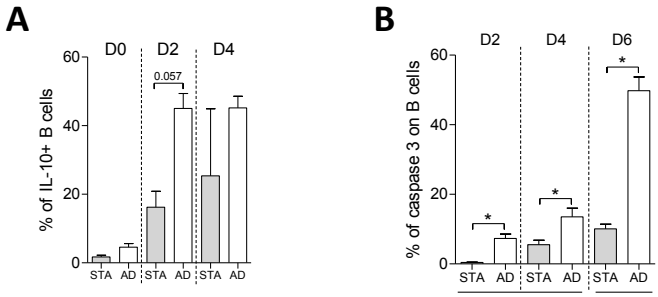
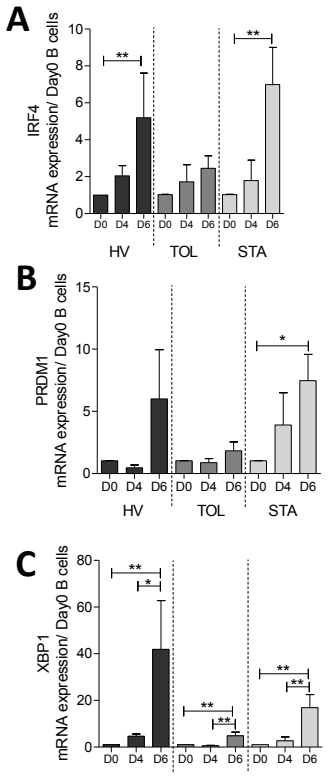


Figure S5



number of circulating plasma cells in operational TOL. The fact that we looked at the blood compartment and that we have no access to the biopsy make speculation on the mechanisms involved in tolerance difficult. Nevertheless, it is likely that the regulation mechanisms of B cells act in secondary lymphoid organs where the immune response occurs. We hypothesize that this default of differentiation and higher cell death apoptosis, linked to a higher naïve/plasma cell ratio and increased IL-10 production, may participate in the pro-tolerogenic balance of tolerance maintenance.

A recurrent issue in such a study is the effect of immunosuppressive treatments. Different immunosuppressive drugs are potent inhibitors of human B lymphocyte function and activation (36). Nevertheless, none of our patients was under these particular drugs and the decreased expression of *XBP1* in late differentiated B cells from tolerant recipients was not shared by HV, suggesting that the effect of treatment was not involved in this process. Moreover, the significant differences observed between patients with AD and STA, who received comparable treatments, in B cell pattern show that the characteristics of B cells from tolerant recipients are not a result of the absence of immunosuppressive treatment. Nevertheless, it is possible that the immunosuppressive treatment may impair their regulatory B cell function and/or B/T cell interaction, which may contribute to tolerance induction and/or maintenance. We have previously reported that CD40L expression is lower in B cells from patients stable under immunosuppressive treatment compared to TOL (10), a phenomenon that may be due to the immunosuppressive treatment itself (37). We might hypothesize that the reinforced CD40L–CD40 interaction in patients with operational tolerance may counterbalance some regulatory processes and that some change in immunosuppressive therapies may drive the B cell compartment toward a pro-tolerogeneic environment (30,38). Haynes et al (39) suggest that tolerance results from a better control of indirect pathway by regulatory Th3, Tr1 and Breg in tolerant recipients. This remains hypothetical at present but we clearly show that operationally TOL also exhibit higher levels of regulatory T cells (8) and lower levels of cytotoxic CD8+ T cells (40) compared to patients with stable function and the fact that they also exhibit higher levels of B cells with a specific pattern suggests that B cells integrate a complex regulation network that is likely influenced by the microenvironment in a particular pathological or physiological context (41).

To conclude, *in vitro*, B cells from tolerant recipients do not fully terminally differentiate into plasma cells that show a higher propensity for cell death apoptosis than patients with stable graft function. This correlates *in vivo* with a decreased number of circulating $CD138^{+}$ plasma cells. In addition, B cells from tolerant recipients produce more IL-10 compared to both STA and HV. But, interestingly, B cell phenotypes and kinetics from TOL share some common points with B cells from HV (level of transitional and naïve B cells, as well as susceptibility for apoptosis) suggesting that tolerance may be the result of a straight control to keep a healthy regulatory/inflammatory balance. Such results were confirmed by the V. Coelho's group, who report on a preserved $CD19^{+}CD24^{high}CD38^{high}$ Breg compartment, a conserved capacity to activate the CD40-STAT3 signaling pathway and preserved B cell repertoire diversity in these patients (42). These data suggest that there is perhaps not a ''signature'' of tolerance but that TOL manage to maintain a healthy and homeostatic profile and that regulatory/effectory balance may be a key point in the comprehension the tolerance. In contrast, patients with stable graft, probably because of their immunosuppressive treatment, would not be able to maintain such a beneficial B cell balance. To what extent this pro-tolerogeneic environment, with an IL-10 B cell profile and lower frequency of differentiated plasma cells, is a driver of the B cell phenotype in tolerance is yet to be established.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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Discussion 1

Au cours de la dernière décennie, de nombreux efforts ont été réalisés par la communauté scientifique de transplantation en Europe (Reprogramming the immune System for Establishment of Tolerance and Indices of Tolerance, http://www.immunetolerance.org/In) et aux Etats Unis (Immune Tolerance Network, http://www.risetfp6.org/) pour identifier des signatures biologiques de la tolérance en transplantation rénale (Brouard et al., 2007; Lozano et al., 2011; Newell et al., 2010; Pallier et al., 2010; Sagoo et al., 2010). Les mécanismes de l'induction et le maintien de cet état de fonction à long terme du greffon sans traitement restent inconnus. Ces patients ont plus de LB circulant (louis 2006), un profil transcriptionnel B au niveau du sang total (Brouard et al., 2007; Pallier et al., 2010; Sagoo et al., 2010) et une augmentation de la production d'IL-10 (Newell et al., 2010) comparé aux patients avec une fonction stable de leur greffon sous immunosuppression. Cependant, il reste encore à définir dans quelle mesure cette « empreinte » et cet environnement contribuent à la tolérance et s'ils ne sont pas simplement les conséquences d'un état de fonction à long terme du greffon sans médicaments.

Comme ces patients développent seulement rarement une réponse allo-immune, nous formulons l'hypothèse que leurs LB peuvent montrer quelques défauts pendant le processus de différenciation. En plus d'un profil inhibiteur global des LB (Pallier et al., 2010), les patients tolérants ont dans leur sang une plus grande proportion de LB transitionnels et naïfs comparé aux patients stables sous immunosuppression. Ces cellules sont connues pour leur capacité limitée à activer les lymphocytes T et pour préférentiellement induire des lymphocytes T régulateurs (Chen and Jensen, 2007; Chung et al., 2003; Eynon and Parker, 1992; Fuchs and Matzinger, 1992; Reichardt et al., 2007). Ces cellules sont également reconnues comme ayant des capacités régulatrices via la production d'IL-10 (Blair et al., 2009; Lemoine et al., 2011). Ces données vont dans le sens d'une réponse B qui serait plus tolérogénique que délétère chez les patients tolérants. Ce premier travail se concentre sur la différenciation des LB en plasmocytes de patients tolérants en utilisant un système de culture primaire déjà validé (Gallou et al., 2012; Huggins et al., 2007; Jourdan et al., 2011) qui combine deux étapes ; d'activation et de différenciation (Choe et al., 1996). Dans ce système, les LB sont dans un premier temps purifiés à partir de sang. Ensuite, les LB sont cultivés à

3.75*10^5 cellules/ml pendant 4 jours avec du CD40L, de l'ODN, de l'IL-2 et de l'anti(Fab)'2 en milieu RMPI complet ce qui va induire l'activation des LB. Puis, les LB sont lavés et remis en culture à la même concentration pendant 2 jours avec de l'IL-2, de l'IL-4 et de l'IL-10, ce qui va induire la différenciation des LB en plasmocytes. Aucune différence n'a été montrée dans l'activation des LB *in vitro* chez les patients tolérants, comparé aux patients stables et aux témoins sains. Ainsi , alors que l'augmentation de la prolifération ne pourrait pas expliquer l'augmentation les LB transitionnels et naïfs dans le sang, des altérations ou des événements non identifiés , survenant dans le développement des LB dans la moelle osseuse des tolérants , ne peuvent être exclus puisque cette signature naïve/transitionnelle a été trouvée chez les patients subissant des stratégies de déplétion (Heidt et al., 2012; Knechtle et al., 2009).

De façon plus intéressante, bien que la production de cytokines soit dépendante des conditions de cultures et des stimuli utilisés (Barr et al., 2012; Lampropoulou et al., 2008; Pasare and Medzhitov, 2005), on démontre ici que les LB des patients opérationnellement tolérants , stimulés par la même méthode et pendant la même durée , produisent vraiment plus d'IL-10 que les STA et les HV. Bien sûr, cette découverte *in vitro* serait peut- être différente *in vivo*, mais elle est en accord avec les données *in vivo* de Newell et al (Newell et al., 2010), qui suggèrent que différents procédés régulateurs peuvent être impliqués pendant la différenciation des LB chez ces patients, de façon dépendant des conditions de cultures. De façon intéressante, la forte production d'Il-10 par les LB chez les patients tolérants n'est pas due à une absence de traitement puisque les LB produisent également plus d'Il-10 que les LB des HV. Cela n'est également probablement pas dû à une activation non spécifique puisque les LB des patients tolérants sécrètent un niveau normal de cytokines effectrices IL-6 et pas de TGF-β (Barr et al., 2012).

Les LB des patients tolérants sont capables de maturer normalement *in vitro* et de produire IgM et IgG avec une proportion entre des populations B ayant ou non vécu le switch. Ces observations pourront être enrichies par la suite avec plus de marqueurs spécifiques (Kaminski et al., 2012) et sont cohérentes avec le fait que les tolérants ne montrent aucun défaut au niveau de leur immunité humorale (Brouard et al., 2012). Ces résultats montrent également que les LB des patients tolérants ont une plus forte sensibilité à la mort cellulaire par apoptose en phase tardive de différenciation, comparé aux patients stables, mais de manière identique aux HV. Ces observations, peuvent être « non

spécifiques » aux TOL mais due à un défaut d'apoptose des LB chez les STA, qui pourraient ajuster leur machinerie apoptotique pour survivre aux traitements immunosuppressifs à long terme et seraient donc, moins sensibles à l'arrêt du cycle cellulaire. Néanmoins, nous montrons également que la différenciation des LB des patients tolérants est associée à une diminution de l'expression d'IRF4 et de PRDM1, impliqués dans la formation du centre germinatif comparé aux patients stables et à une diminution d'XBP-1 comparé aux patients stables et aux témoins sains. Ce qui suggère, une différenciation des LB partielle ou incomplète. Ces données sont renforcées par des analyses transcriptionnelles réalisées sur des LB isolés de patients tolérants, qui sont caractérisés par une nette diminution, de l'expression de gènes de la différenciation des LB et de gènes anti-apoptotiques. Ces données sont en accord aves les données précédentes sur les PBMCs (Brouard et al., 2007; Pallier et al., 2010) et apportent des preuves supplémentaires qu'elles ne reflètent pas seulement des phénomènes *in vitro*. Dans l'ensemble, le manque d'expression de certaines molécules de différenciation et le plus haut niveau de mort cellulaire par apoptose des LB en phase tardive de différenciation comparé aux patients avec une fonction stable de leur greffon, sont en accord avec la diminution du nombre de plasmocytes circulant chez les patients tolérants. Le fait que l'on regarde dans le compartiment sanguin et que nous n'ayons pas accès à des biopsies rend difficile les spéculations sur les mécanismes impliqués. Néanmoins, il est probable que les mécanismes de régulation des LB se déroulent dans les organes lymphoïdes secondaires, là où la réponse immunitaire se produit. Nous formulons l'hypothèse que ce défaut de différenciation et cette plus forte sensibilité à l'apoptose, sont liés à un ratio LB naïf/plasmocytes plus élevé et une augmentation de la production de l'IL-10, qui pourrait participer à la balance pro-tolérogènique du maintien de la tolérance.

Une question récurrente dans de telles études, se pose quant à l'effet des traitements immunosuppresseurs. Plusieurs médicaments immunosuppresseurs sont de puissants inhibiteurs de la fonction et de l'activation des LB (Matz et al., 2012). Néanmoins, aucun de nos patients n'étaient sous ces traitements en particulier et la diminution de l'expression de XBP1 en phase tardive de différenciation des LB chez les patients tolérants n'est pas observée chez les HV. Ce qui suggère que l'effet du traitement n'est pas impliqué dans ce processus. De plus, les différences significatives observées entre patients avec maladie atopique (AD) et STA, qui reçoivent des traitements comparables, montrent que les caractéristiques des LB des

patients TOL ne sont pas un résultat de l'absence de traitement immunosuppresseur. Ceci dit, il est possible que le traitement immunosuppresseur puisse détériorer la fonction régulatrice des LB et/ou l'interaction T/B, ce qui pourrait contribuer à l'induction de la tolérance et ou à son maintien. Nous avons précédemment rapporté que l'expression du CD40L est plus faible chez les patients STA sous immunosuppression comparé aux patients TOL (Pallier et al., 2010), un phénomène qui pourrait être dû au traitement immunosuppresseur lui-même. Nous pourrions formuler l'hypothèse que le renforcement de l'interaction CD40-CD40L chez les patients tolérants peut contrebalancer des processus régulateurs et que quelques changements dans les thérapies immunosuppressives peuvent mener le compartiment cellulaire B vers un environnement pro-tolérogènique (Heidt et al., 2012; Liu et al., 2007). Tout ceci suggère que la tolérance résulte d'un meilleur contrôle de la voie indirecte par les Th3, Th1 régulateurs et par les LB reg chez les patients tolérants. Cela reste hypothétique mais nous constatons clairement que les TOL montrent également un niveau plus élevé de LTreg (Louis et al., 2006) et un niveau plus faible de lymphocytes T $CD8^+$ cytotoxiques (Baeten et al., 2006) comparé aux patients avec une fonction stable de leur greffon. Par ailleurs, le fait qu'ils ont également un niveau plus élevé de LB avec un profil particulier suggère que les LB intègrent un réseau régulateur complexe qui est probablement influencé par le microenvironnement dans un contexte pathologique ou physiologique particulier.

Pour conclure, *in vitro*, les LB des patients tolérants ne se différencient pas entièrement en plasmocytes et montrent une plus grande sensibilité pour la mort cellulaire par apoptose que chez les patients avec une fonction stable de leur greffon. Cela corrèle *in vivo* avec une diminution du nombre de plasmocytes $CD138^+$ circulant. De plus, les LB des TOL produisent plus d'IL-10 comparé aux STA et HV. Mais de façon intéressante, phénotype et cinétique des LB des TOL partagent des points communs avec les LB des HV (taux de LB transitionnels et naïfs, aussi bien que leur susceptibilité à l'apoptose), suggérant que la tolérance pourrait être le résultat d'un contrôle direct pour garder une balance régulateur/inflammatoire saine. De tels résultats ont été confirmés par le groupe de V. Coelho qui rapporte un compartiment B régulateur $CD19^+CD24^{++}CD38^{++}$, une capacité conservée à activer la voie de signalisation CD40-STAT3 et à préserver la diversité du répertoire B chez ces patients (Silva et al., 2012). Ces données suggèrent qu'il ne s'agit peut-être pas d'une « signature » de tolérance mais que les tolérants réussissent à garder un profil homéostatique

et sain et que la balance régulateur/effecteur peut être un point-clef dans la compréhension de la tolérance. A l'inverse, les patients avec une fonction stable de leur greffon, probablement due à leur traitement immunosuppresseur, ne seraient pas capables de maintenir une telle balance des LB bénéfiques. La question qui se pose actuellement est : dans quelle mesure cet environnement pro-tolérogènique, avec un profil B IL-10 et une plus faible fréquence de plasmocytes différenciés, serait le moteur du phénotype B dans la tolérance ? Ceci reste à démontré.

Résultats 2

Les patients tolérant une greffe rénale possèdent des LB qui ont un phénotype de type plasmocyte, expriment du GzmB et ont des propriétés régulatrices.

Chesneau M, Michel L, Dugast E, Chenouard A, Baron A, Pallier A, Durand J, Braza F, Guerif P, Laplaud D-A, Soulillou J-P, Giral M, Degauque N, Chiffoleau E, Brouard S.

Papier accépté dans JASN

Résumé de l'article

Afin d'analyser la fonction régulatrice des LB chez les patients tolérants, nous avons utilisé un protocole de coculture de LB activés (CD40L/ODN) avec des LT effecteurs $CD4^+$ $CD25^-$ autologues activés (CD3/CD28).

L'analyse de la prolifération des LT $CD4^+CD25^-$ a dans un premier temps confirmé l'effet régulateur des LB qui inhibent la prolifération des LT effecteurs. De plus les LB induisent également l'apoptose des LT effecteurs. Cependant, l'inhibition de la prolifération et l'induction de l'apoptose des LT ne sont pas associées à une diminution de la sécrétion des cytokines pro-inflammatoires (IFN-γ et TNF-α).

L'utilisation de différents anticorps bloquants ou inhibiteurs a permis de définir que la fonction régulatrice des LB est dépendante de la sécrétion de GzmB et du contact LT/LB. Nous montrons que les TOL ne présentent pas de différence dans leur fonction régulatrice comparée aux STA et HV. Cependant, in vivo, les TOL ont plus de LB $GzmB^+$ circulant que les STA et HV. Et *in vitro* les LB $GzmB^+$ agissent de manière doses dépendantes sur l'inhibition de la prolifération des LT effecteurs.

L'analyse du phénotype de ces LB $GzmB^+$ a montré que ces LB ont un phénotype de type plasmocytes puisqu'ils expriment $CD138^+$, $CD27^+$, $CD38^+$ et aussi $CD5^+$ tout en étant IgD^-

Récemment, il a été démontré que les LB ont la capacité de sécréter du GzmB notamment après stimulation avec l'IL-21. De façon intéressante, dans cette étude, l'ajout de LB des TOL en coculture diminue le taux de LT sécrétant de l'IL-21. Et nous avons également montré que l'IL-21 sécrété par les LT régule le nombre de LB $GzmB^+$.

Ainsi, cette étude montre une fonction régulatrice des LB chez les patients tolérants via la sécrétion de GzmB et également dépendant du contact cellulaire. Les patients tolérants présentent une plus forte proportion *in vivo* de LB $GzmB^+$, qui ont une fonction dose dépendante sur l'inhibition des LT effecteurs.

BASIC RESEARCH www.jasn.org

Tolerant Kidney Transplant Patients Produce B Cells with Regulatory Properties

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ABSTRACT

Whereas a B cell–transcriptional profile has been recorded for operationally tolerant kidney graft patients, the role that B cells have in this tolerance has not been reported. In this study, we analyzed the role of B cells from operationally tolerant patients, healthy volunteers, and kidney transplant recipients with stable graft function on T cell suppression. Proliferation, apoptosis, and type I proinflammatory cytokine production by effector $CD4^+CD25^-$ T cells were measured after anti-CD3/anti-CD28 stimulation with or without autologous B cells. We report that B cells inhibit $CD4^+CD25^-$ effector T cell response in a dose-dependent manner. This effect required B cells to interact with T-cell targets and was achieved through a granzyme B (GzmB)–dependent pathway. Tolerant recipients harbored a higher number of B cells expressing GzmB and displaying a plasma cell phenotype. Finally, $GzmB^+$ B-cell number was dependent on IL-21 production, and B cells from tolerant recipients but not from other patients positively regulated both the number of $IL\text{-}21^+$ T cells and IL-21 production, suggesting a feedback loop in tolerant recipients that increases excessive B cell activation and allows regulation to take place. These data provide insights into the characterization of B cell–mediated immunoregulation in clinical tolerance and show a potential regulatory effect of B cells on effector T cells in blood from patients with operationally tolerant kidney grafts.

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Tolerance in transplantation is defined as the maintenance of long-term, good, stable graft function in the absence of immunosuppression.[1,2] Numerous studies have demonstrated that tolerance can easily be achieved in rodent models,[3–5] including models for renal transplant.[6] However, while it remains rare in human renal transplant it does exist, current estimates report roughly a hundred cases, mainly patients not compliant with their immunosuppressive regimens.[1,2,7] These patients, defined as "operationally tolerant," are healthy, do not exhibit more infections or malignancies than healthy volunteers (HVs), and do not display clinical evidence of immune incompetence.[2,8] Specific patterns have been associated with this operational tolerance; in particular, a B cell transcriptional signature that correlates with an increase in peripheral B cells has been reproducibly found.[9–14] Several of these B cell markers are being currently tested and validated in multicenter studies around the world to predict patients who may benefit from

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immunosuppression withdrawal. In addition, some of these B cell markers are being examined for their involvement in the tolerance process; however, to date, none have been shown to have a role in tolerance induction or maintenance, and whether B cells are involved, or even have a potential role in tolerance, remains to be determined.

In transplantation, B cells are mainly known for their capacity to differentiate into plasma cells and produce antibodies that may be deleterious for the graft.[15,16] However, B cells also have antibody-independent functions. They are able to produce cytokines and to present antigens, and thus to initiate or maintain an immune response.[17,18] More recently, populations of regulatory B cells (Bregs) able to dampen the immune response have been highlighted as a "driving force" in autoimmune diseases, cancers, and transplantation.[19–23] However, their nature, origin, phenotype, and mode of action in humans remain little known.

Q:4 We previously reported that B cells from tolerant patients (TOLs) do not fully differentiate into plasma cells and that, during their differentiation, B cells from tolerant recipients produce higher levels of IL-10,[24] suggesting that an imbalance between a lower number of deleterious plasma cells and a higher level of Bregs producing IL-10 may exist in these patients. In this article, we investigate the role of B cells in blood from this cohort of operationally TOLs, from patients with stable graft function under classic immunosuppression (STAs), and from HVs. We report that tolerant recipients exhibit a higher number of IL-21–dependent peripheral B cells that express granzyme B (GzmB), display a specific phenotype, and exhibit a contact- and GzmB-dependent, IL-10– and TGF-β–independent inhibition of effector T cell response. These results provide novel insights into the characterization of B cell–mediated immunoregulation in tolerance in the clinic.

RESULTS

CpG-CD40 Prestimulated B Cells Inhibit $CD4^+CD25^-$ T Cell Proliferation

Human B cells have been shown to exhibit regulatory activity through the inhibition of various cell types, in particular through the inhibition of T cells. $CD4^+CD25^-$ T cells were cocultured with CpG-CD40 stimulated or unstimulated autologous B cells, after polyclonal anti-CD3 and anti-CD28 activation, for 3 days and T cell proliferation was then analyzed, using CellTrace Violet staining. We found that prestimulated B cells from HVs, TOLs, and STAs significantly inhibit autologous $CD4^+CD25^-$ T cell proliferation (Figure 1A), whereas unstimulated B cells have a lesser effect (Figure 1B). No difference was found in the number of total B cells and $GzmB^+$ B cells or for B cell inhibition between men and women.

CpG-CD40 Prestimulated B Cells Induce T Cell Apoptosis But Have No Effect on Proinflammatory Cytokine Production

Using Annexin V staining, apoptosis of $CD4^+CD25^-$ T cells was measured at day 3 after anti-CD3/anti-CD28 activation and addition of prestimulated B cells to the culture. Prestimulated B cells and a 1:2 T cell/B cell ratio were used in all of the experiments. The addition of prestimulated B cells to the coculture induces a significant increase in $CD4^+CD25^-$ T cell apoptosis in the three groups (Figure 1C). Interestingly, no difference was observed in apoptosis levels between cell trace$^+$ and cell trace$^-$ T cells, confirming that the increase in apoptosis was not due to inhibition of T cell proliferation (data not shown). Type I helper T cell (Th1) proinflammatory cytokines (IFN-γ and TNF-α) were analyzed using intracellular staining after 3 days of coculture. IFN-γ T cell production was slightly lower when prestimulated B cells from HVs were added to the culture, but this was due to a slightly higher level of IFN-γ production by $CD4^+CD25^-$ T cells from HVs only (Figure 1D). TNF-α production by T cells from the three groups of patients was unchanged when prestimulated B cells were added to the culture (Figure 1E). Representative pictures of IFN-γ and TNF-α production by T cells are displayed in Figure 1, F and G. Altogether, these data show that B cells from HVs, transplant TOLs, and STAs all inhibit T cell proliferation and induce T cell apoptosis but have no effect on Th1 proinflammatory cytokine production.

B Cell Inhibitory Effect on T Cells Is Dependent to GzmB and Is Contact Dependent

Having previously reported higher production of IL-10 by B cells from tolerant recipients during the differentiation process *in vitro*, as well as B cells having been shown to mainly display regulatory properties through IL-10, we decided to assess the role of IL-10 in our model. We looked at the frequency of IL-10–expressing B cells and the level of IL-10 expression by these B cells after 48 hours of CD40L and ODN stimulation. As Q:5 expected, although the resting B10 level was low, a significant and substantial increase in the frequency of B10 cells was found after activation (Figure 2A). No difference was observed in the frequency of B10 cells and in the relative amount of IL-10 expressed by B cells between the three groups of individuals (Figure 2, B and C). To assess the role of IL-10 in the coculture assay, we blocked its effect using anti–IL-10 antibody. We found that the blockade of IL-10 does not hinder the inhibitory effect of B cells on effector T cell proliferation (Figure 3A). Because other cytokines have been shown to play a role in the function of suppressive B cell populations, TGF-β and GzmB were similarly blocked by adding anti–TGF-β antibody and anti-GzmB peptide to the coculture at day 0. The blockade of TGF-β did not hinder the inhibitory effect of B cells on T cell proliferation (Figure 3B). However, for the three groups of patients, the addition of anti-GzmB peptide to the coculture significantly affects the suppressive effect of B cells on autologous $CD4^+CD25^-$ T cell proliferation (Figure 3C), whereas

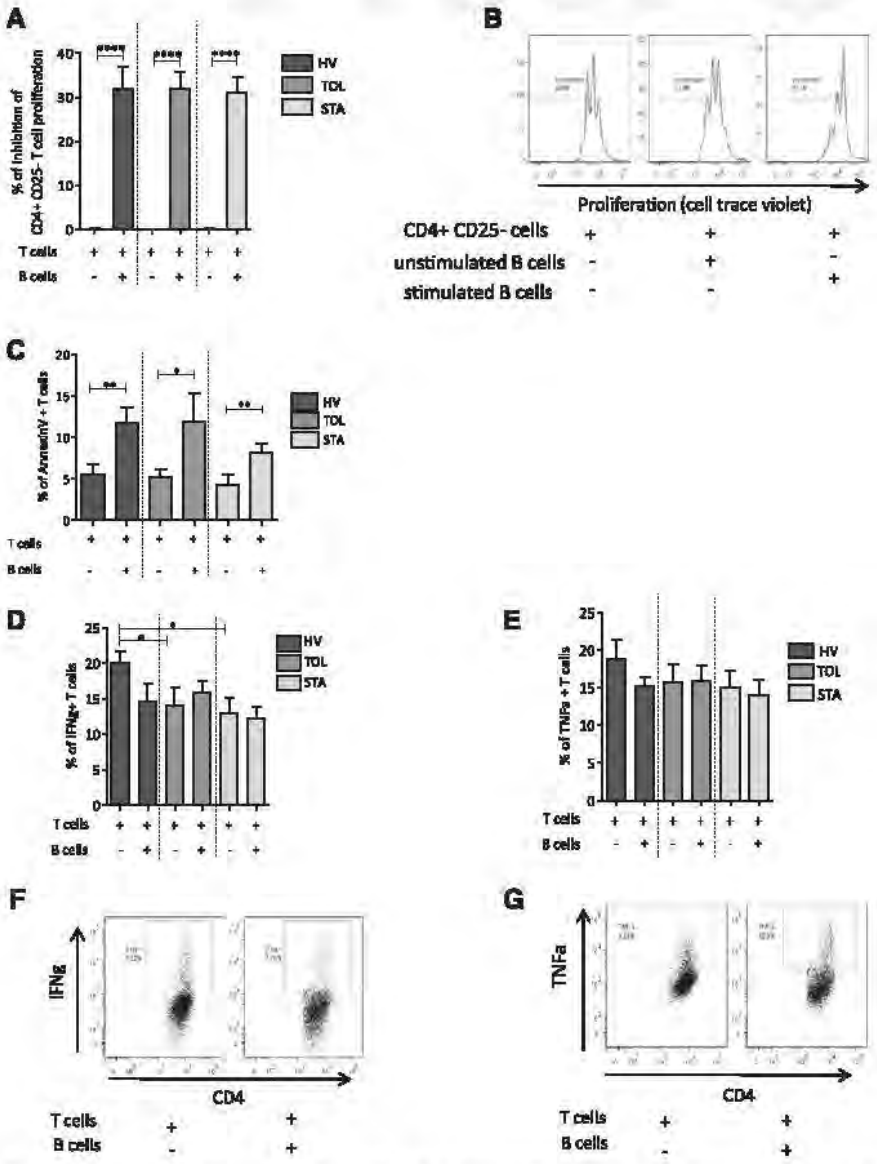

Figure 1. CpG-CD40 prestimulated B cells inhibit $CD4^+CD25^-$ T cell proliferation and induce T cell apoptosis without effect on type Th1 proinflammatory cytokine production. Effector T cell proliferation is followed by CellTrace Violet staining after 3 days activated with anti-CD3/anti-CD28 and cocultured with unstimulated B cells or B cells stimulated with CD40L and ODN 24 hours before the coculture. (A) Proportion of T cell proliferation inhibition when cocultured with or without prestimulated B cells in HVs (n=17), TOLs (n=12), and STAs (n=17) (mean±SEM; ****P<0.001). (B) Representative histograms of proliferation of T cells: alone, with unstimulated B cells, and with stimulated B cells from HVs. Apoptosis of effectors T cells is analyzed by Annexin V staining after 3 days of activation with anti-CD3/anti-CD28 and coculture with B cells prestimulated with CD40L and ODN 24 hours before the coculture. (C) Percentage of Annexin V T cells activated and cocultured with or without stimulated B cells in HVs (n=9), TOLs (n=10), and STAs (n=12) (mean±SEM; *P<0.05; **P<0.01). Secretion of IFN-γ and TNF-α is analyzed by intracellular staining in effector T cells after 3 days of coculture with B cells and anti-CD3/anti-CD28 activation. (D) Percentage of IFN-γ^+ T cells activated and cocultured with or without B cells in HVs (n=17), TOLs (n=12), and STAs (n=14) (mean±SEM; *P<0.05). (E) Percentage of TNF-α^+ T cells activated and cocultured with or without B cells in HVs (n=17), TOLs (n=12), and STAs (n=14) (mean±SEM). (F and G) Representative dot plots of the secretion of IFN-γ and TNF-α by T cells activated and cocultured with or without B cells from HVs.

GzmB inhibitor has no effect on T cell proliferation in the absence of B cells (Figure 3D).

Because the inhibitory functions of B cells involving GzmB have been shown to act through a direct interaction of B cells with their target,[25,26] transwell cocultures were performed to determine whether contact was required by B cells to inhibit T cell proliferation. As shown in Figure 3E, the inhibitory B cell effect disappeared when B and T cells were cultured in transwell, demonstrating that T cell–B cell interaction is necessary for the inhibitory B cell function. Altogether, these data show that B cells inhibit T cell proliferation *via* a GzmB pathway and depend on contact between the B and T cells.

Tolerant Recipients Have a Higher Number of B Cells, Which Act in a Dose-Dependent Manner and Express GzmB

On a cell-by-cell basis, B cells from TOLs added to the coculture have the same ability as B cells from HVs and STAs to regulate autologous effector T cell proliferation in a contact- and GzmB-dependent manner (Figure 3, C and E). We previously reported on a higher number of total B cells in blood from TOLs.[10,12,24] Here we found that the absolute value of GzmB-producing B cells was significantly higher in TOLs compared with HVs and STAs (P<0.05; Figure 4A) and the percentage of $GzmB^+$ B cells is directly correlated with effector $CD4^+CD25^-$ T cell proliferation inhibition (Figure 4B). Altogether, these data show that tolerant recipients have a higher number of peripheral B cells and $GzmB^+$ B cells that are able to inhibit T cell response through a contact- and GzmB-dependent pathway and in a dose-dependent manner.

Resting and Stimulated GzmB-Expressing B Cells Display a $CD5^+$ $CD27^+CD138^+$ Phenotype

The $GzmB^+$ B cell phenotype was analyzed by flow cytometry before and after 3 days of coculture using CD19, CD20, CD138, CD38, CD27, CD24, CD5, CD1d, IgD, IgG, and IgM cell surface markers. Unstimulated $GzmB^+$ B cells express a higher level of CD138, CD27, CD5, and CD38, and fewer IgD markers compared with

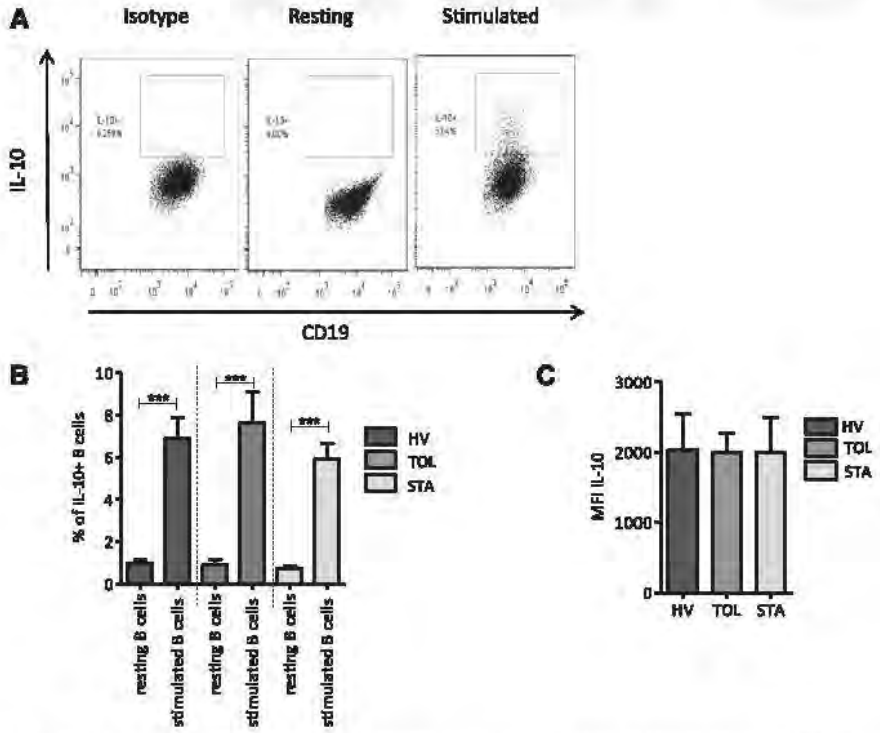

Figure 2. IL-10$^+$ B cells and IL-10 secretion after 48-hour stimulation with CD40L/ODN. IL-10 expression was analyzed on B cells after 48-hour stimulation of PBMCs with CD40L and ODN. (A) Representative dot plot of IL-10 secretion in resting, stimulated B cells, and stimulated B cells staining with isotype control. (B) Percentage of IL-10$^+$ B cells before and after activation in HVs (n=7), TOLs (n=9), and STAs (n=10) (mean±SEM; ***P<0.001). (C) Mean fluorescence intensity from IL-10 stimulated B cells in HVs (n=7), TOLs (n=9), and STAs (n=10).

unstimulated GzmB$^-$ B cells (Figure 4C). After stimulation, GzmB$^+$ B cells have lower CD38 expression compared with GzmB$^-$ B cells (Figure 4D). Resting GzmB$^+$ B cells represented around 2.7%±1.45% of total B cells from TOLs. Three days of activation with CD40L and ODN greatly increases the expression of GzmB (20%±2.5% after activation) (Supplemental Figure 1, A and B). Altogether, these data show that tolerant recipients have a higher number of GzmB-expressing B cells with a CD138$^+$CD27$^+$CD5$^+$CD38$^+$IgD$^-$ phenotype.

B Cell Regulatory Transcriptional Profile Is Not Different in TOLs, HVs, and STAs

We investigated the expression of 60 markers selected either for their participation in the regulation of B cell functions (Supplemental Figure 2, A and B),[26] or their implication in a tolerance-related B signature (Supplemental Figure 2, C and D)[27] in purified and isolated B cells or PBMCs from samples from 32 individuals (10 TOLs, 10 HVs, and 12 STAs). Among these genes, only CD38 and CD1D exhibited a significant difference between STAs and HVs in B cells (Supplemental Figure 2A), whereas more than half were different in PBMCs (Supplemental Figure 2B). The second subset (Supplemental Figure 2, C and D) includes a panel of 35 markers previously linked to tolerance across blood transcriptomic studies and preferentially expressed in B cell subsets for 69% of them.[27] Only two of these markers were different in B cells (EBF1 between TOLs and HVs; PLEKHG1 between STAs and HVs) (Supplemental Figure 2D), whereas all of them were different between TOLs and STAs in PBMCs (Supplemental Figure 2D). These data clearly show that B cells are not intrinsically different between the groups of patients and that, at least for these selected genes, the signature of B cells in blood from tolerant recipients is mainly the result of a higher number of B cells in PBMCs (Supplemental Figure 2).

B Cells from Tolerant Recipients Regulate IL-21–Producing T Cell Levels; IL-21 Production by T Cells Regulates the Number of GZMB$^+$ B cells

GzmB-expressing B cells have been shown to depend on T cell–produced IL-21, and IL-21 is also an important factor in B cell homeostasis. We found that in TOL stimulation with anti-CD3/anti-CD28 of CD4$^+$CD25$^-$ T cells is associated with a lower number of IL-21–producing T cells compared with HVs and STAs (P<0.05). However, when B cells are added to the coculture, IL-21–producing T cells significantly increased in TOLs (P<0.01), whereas no difference, or even decreased IL-21 production, was observed in HVs and STAs (Figure 5, A and B). This correlates with a higher production of IL-21 by T cells stimulated by B cells from TOLs compared with those from HVs and STAs (P<0.05) (Figure 5C). Altogether, these data show that B cells from TOLs regulate the number of IL-21$^+$ T cells and IL-21 production *in vitro*. Finally, to assess the role of IL-21 on GzmB-expressing B cells, we blocked its effect using anti–IL-21 antibody in the coculture assay. Increasing doses of anti–IL-21 (0, 2, 4, 6, and 8 μg/ml) significantly decreased the number of GzmB-expressing B cells (Figure 5D).

DISCUSSION

B cells may have a dual effect, acting as a driver and as a regulator of the immune system.[15–18,28–32] We recently showed that tolerant recipients are characterized by a higher number of circulating B cells,[10,12] a defect in terminal differentiation,[24] and a B cell–transcriptional profile with overexpression of molecules associated with regulation.[9,12] In animal models, that B cells have a role in tolerance is clearly suggested by

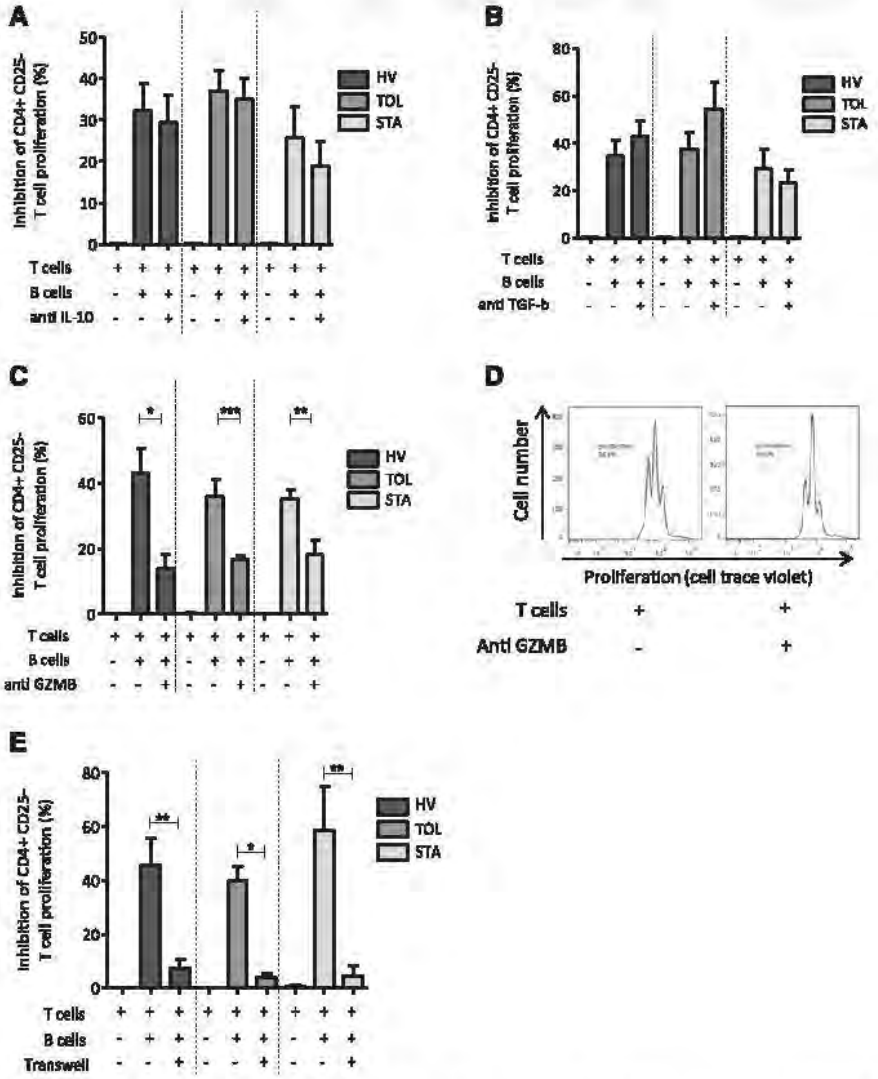

Figure 3. Regulation of effector T cell proliferation by B cells is contact and GzmB dependent. (A–C) T cell proliferation inhibition in HVs (n=12), TOLs (n=10), and STAs (n=7) when T cells are activated with anti-CD3/anti-CD8 and cocultured with B cells with or without anti–IL-10 blocking antibody (A), with or without anti–TGF-β blocking antibody in HVs (n=10), TOLs (n=8), and STAs (n=6) (B), or with or without GzmB inhibitor peptide in HVs (n=9), TOLs (n=9), and STAs (n=14). (D and E) Representative histograms of T cell proliferation with or without GzmB inhibitor peptide (D) and when T and B cells are cocultured in transwell (E) in HVs (n=6), TOLs (n=4), and STAs (n=3) (mean±SEM; *P<0.05; **P<0.01; ***P<0.001).

these rare patients. Haynes *et al.* recently proposed an indirect pathway model in which a decrease in immunosuppression is associated with an increased number of indirect pathway regulatory T cells (Tregs) and of B cells, possibly Bregs, but the mechanisms have not been established.[34] Silva *et al.* showed that peripheral B cells from tolerant recipients maintain the capacity to activate CD40-CD40L signaling, phosphorylate STAT3, and preserve B cell compartment diversity, suggesting a role for these B cells in tolerance.[14] In this article, we explore the potential regulatory role of B cells in such patients. Because of the difficulty in identifying a unique Bregs population with a unique phenotype in humans, we analyzed the *in vitro* suppressive properties of B cells overall. We report a higher number of B cells with dose-dependent suppressive properties in blood from patients with a tolerant kidney graft. The inhibitory effect of B cells is dependent on GzmB and on the interaction of B cells with their T cell targets.

Much evidence suggests that activation is instrumental in Bregs activity.[35] In this article, we show that prestimulated B cells are more efficient at suppressing effector T cell proliferation than nonstimulated B cells, a result in accordance with previous data showing that maturation and B cell activation were important parameters in this process.[28,30–32,36] It may be a little counterintuitive to say that B cells need such activation to regulate the immune system because activated B cells are usually associated with stimulatory activity on other cell types. However, this has been extensively discussed and can be explained as the participation of B cells in a general feedback loop that both induces an immune process and prevents excessive inflammation or unwanted autoaggressive T cell response.[37] Moreover, we clearly show that the inhibitory effect of B cells is dependent on GzmB, the expression of which occurs in resting B cells *in vivo* and is increased in stimulated B cells. Interestingly, as already described for the pro-B10 regulatory cell population, this leads to the conclusion that B cell–mediated regulation is probably inducible and that the effect of B cells is greatly influenced by the nature of the microenvironment.[35,36,38–40]

The expression of IL-10 has been shown to be a frequent characteristic of Bregs.[41–43] Such cells, referred to as B10,[32,44] are involved in the initiation, the onset, and the severity of the accumulation of B cells and formation of germinal centers in tolerant allografts and the ability of B cells to prolong graft survival.[33] However, the role and nature of Bregs in the modulation of immune response in alloimmunity and in tolerance in humans remains unclear. Although tolerant kidney recipients clearly display a strong B-cell signature, with a higher number of B cells, their potential role in establishing and/or maintaining tolerance remains to be determined.

It is tempting to speculate that this increase in B cells with a specific inhibitory profile may be linked to the tolerance of

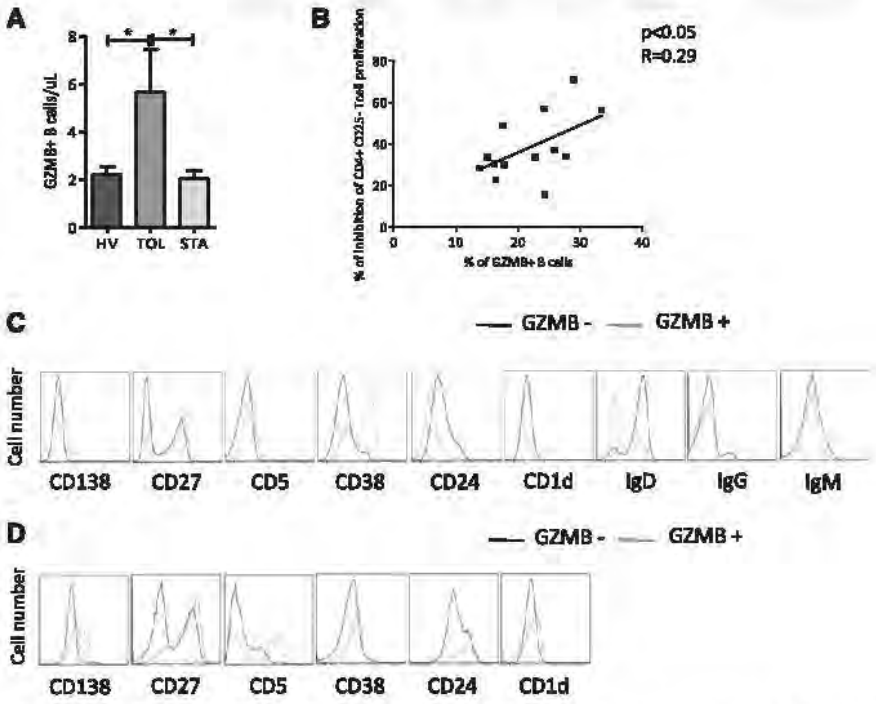

Figure 4. Tolerant recipients have a higher number of B cells expressing GzmB and act in a dose-dependent manner. (A) Number of GZMB$^+$ B cells per microliter of blood from HVs (n=6), TOLs (n=6), and STAs (n=6). (B) Linear regression of percentage of GzmB$^+$ B cells and percentage of inhibition of CD4$^+$CD25$^-$ T cell proliferation (P<0.04). (C) Representative histograms for CD138, CD27, CD5, CD38, CD24, CD1d, IgD, IgG, and IgM expression within the GzmB$^+$ (thin line) and GzmB$^-$ (thick line) in CD19$^+$ B cells before stimulation. (D) Representative histograms for CD38 and CD138 within the GzmB$^+$ (thin line) and GzmB$^-$ (thick line) in CD19$^+$ B cells after stimulation.

various autoimmune diseases, and in transplantation.[45–47] We show here that the suppressive properties of B cells are not dependent on IL-10. Anti–IL-10, used at doses able to efficiently block an IL-10 T cell–dependent response (data not shown), does not diminish the inhibitory effect of B cells on T cell proliferation. These data are in agreement with the work of Deng *et al.*, who reported that anti-CD45RB treatment induces strong and antigen-specific tolerance, which is dependent on the presence of B lymphocytes and is independent from IL-10. In this model, IL-10 clearly counter-regulates tolerance induction, and even exerts a negative effect and causes histologic lesions of rejection.[48] The same observations have been reported in other models.[49] Interestingly, it has been reported that B cells from patients with chronic antibody-mediated rejection have a defect in suppressive properties,[50] in contrast with B cells from STAs, which inhibit T cell proliferation and induce Treg generation through a TGF-β– and IDO–dependent pathway (personal communication, Nouël *et al. Ann Rheum Dis* abstract A8.32, 2014). In our situation, blocking TGF-β has no effect on the suppressive function of B cells, suggesting that TGF-β is not involved in B cell regulation either.

Interestingly, other regulation pathways have been shown to be involved in B cell regulatory activity. We found that GzmB blockade diminishes the B cell inhibitory effect and restores T cell proliferation. This is associated with a higher number of GzmB-producing B cells in blood from tolerant recipients and B cells that act in a contact- and a dose-dependent manner. GzmB is a 32-kD serine protease mainly known as a component of the cytotoxic granule of T cells and natural killer cells[51,52] but is also produced by other cell types, such as B cells,[53] Tregs,[54] and plasmacytoid dendritic cells.[55] GzmB mediates T cell apoptosis and T cell proliferation suppression, independently of the perforin component.[55] In this work, the fact that a blockade of GzmB with anti-GzmB induces a diminishment of the B cell inhibitory effect indicates that GzmB is functional. Interestingly, we found that B cells not only inhibit T cell proliferation, but also induce their apoptosis. We did not find any involvement of the Fas-FasL pathway in this process (data not shown), but alternative pathways have been described as inducing apoptosis, and GzmB is one of them.[56] These data suggest a pivotal role for GzmB in regulation by B cells in our patients through cell death induction and T cell proliferation inhibition, a mechanism already described for Tregs after polyclonal and antigen-specific activation.[54] Interestingly, we found that B cells had no effect on TNF-α and IFN-γ production. Iwata *et al.* reported that IFN-γ and TNF-α production was dependant from IL-10.[46] This is reinforced by the data from Lemoine *et al.*, who reported that regulation of T cell proliferation was distinct from the differentiation of T cells into Th1 cells that depend on the production of IL-10.[42] These observations are in accordance with ours and particularly reinforce our finding that IL-10 is not involved in B cell regulation in our present situation. To our knowledge, nothing has yet been reported on the effect of GzmB on IFN-γ and/or TNF-α production by B cells.

Several microenvironmental factors have been shown to be instrumental in B cell homeostasis. These factors include molecules such as IL-21.[26,53] We first demonstrated that GzmB-expressing B cells are dependent on IL-21. Increasing doses of anti–IL-21 decrease the number of GzmB-expressing B cells in coculture. Moreover, when B cells are added to the coculture, IL-21–producing T cells increase significantly and there is greater production of IL-21 by T cells from TOLs. IL-21 is a key cytokine for GzmB gene transcription,[26,57] which suggests that the increase in GzmB$^+$ B cells in blood from tolerant recipients may be due to a direct effect of IL-21. These data are reinforced by the effective STAT3 phosphorylation in B cells from tolerant recipients,[14] a key signal for the

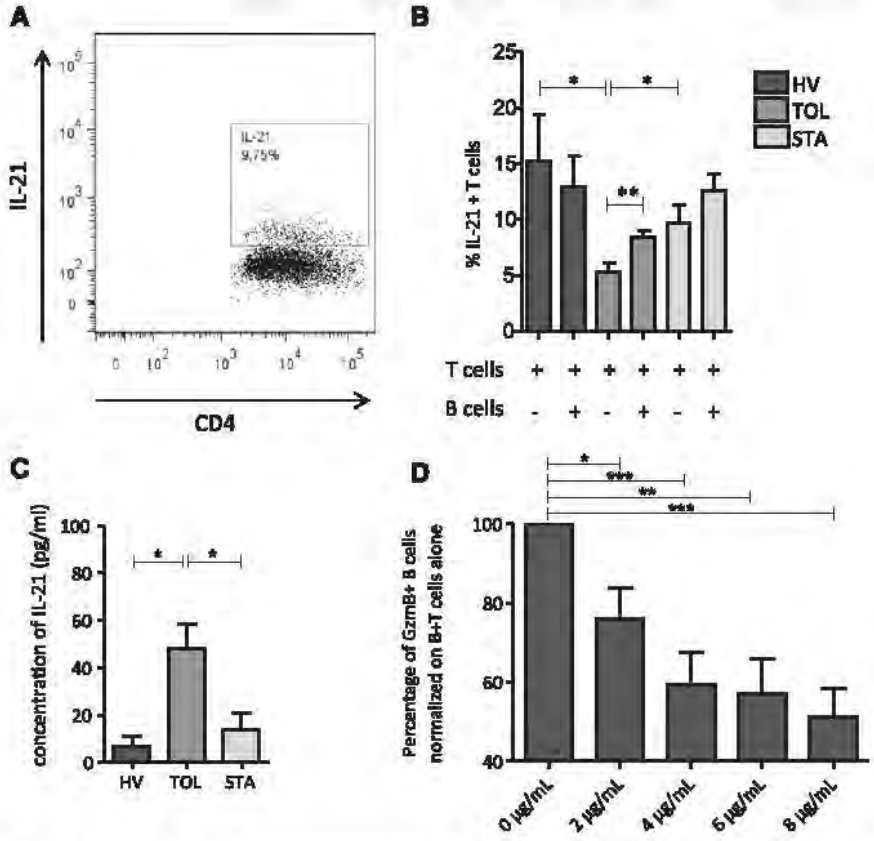

Figure 5. B cells from tolerant recipients regulate IL-21–producing T cells levels and IL-21 production by T cells. (A) Dot plot of the secretion of IL-21 by activated $CD4^+CD25^-$ T cells at day 3 of coculture with stimulated B cells. (B) Percentage of $IL\text{-}21^+$ T cells at day 3 of activation and coculture with or without stimulated B cells in HVs (n=10), TOLs (n=12), and STAs (n=14) (*P<0.05; **P<0.01). (C) Concentration of IL-21 (pg/ml) in supernatants after 3 days of coculture, T cell coculture alone, or with B cells (mean±SEM; *P<0.05). (D) Percentage of $GzmB^+$ B cells normalized on B cells plus T cells alone after increasing doses of IL-21 blocking molecule is added to coculture (0, 2, 4, 6, and 8 µg/ml) (*P<0.05; ***P<0.01).

generation of GzmB activated B cells.[57] Finally, the fact that tolerant recipients have a lower level of $IL\text{-}21^+$ $CD4^+$ T cells, and that B cells from these patients alone (and not HVs and STAs) increase IL-21 production by $CD4^+$ T cells, strongly suggests a negative feedback loop in these patients, increasing excessive B cell activation and inflammation and allowing regulation to take place.

Bregs are able to control the immune response, but an excessive reaction from these cells may also promote tumor cell growth or chronic infection.[26] We hypothesize that this fine-tuning of regulation by B cells and IL-21 production by T cells might be a key factor in tolerance maintenance.

We show that TOLs have more B cells in absolute value with regulatory properties, but on a cell-per-cell basis, their B cells have the same suppressive activity as B cells from STAs and HVs. These results are supported by transcriptional analysis that shows that the B cells are not intrinsically different between the groups of patients and that, at least for these selected genes, the B cell signature in blood from tolerant recipients' results mostly from a higher number of B cells in PBMCs. It is not surprising that the greater suppressive effect in blood of tolerant recipients is due to a higher number of circulating $GzmB^+$ cells, if we consider that clinical outcomes in numerous situations are mainly driven by the quantity of infiltrating and circulating cells, more than their quality.[58] Interestingly, we find that $GzmB^+$ B cells express a specific phenotype with upregulation of CD5, CD27, CD38, and CD138 markers. This is in accordance with our previous data reporting a higher level of memory $CD27^+$ B cells and $CD5^+$ B cells in blood from tolerant recipients.[12] These data are also in accordance with the fact that $CD5^+$ B cells constitutively express GzmB in an IL-21–dependent pathway[59] and that $CD5^+$ B cells express higher levels of IL-21–R than $CD5^-$ B cells.[26] We also found that these cells express CD138 and CD38 and are IgD^-, markers expressed by B cells secreting IL-10 and IL-35 and involved in suppression mechanisms,[60] and also associated with plasma cell maturation.[61] Interestingly, CD138 has also been shown to be associated with GzmB production, regulatory activity, and IL-21 dependence.[57]

In conclusion, we have demonstrated that TOLs have a higher absolute number of $GzmB^+$ B cells with a plasma cell–like phenotype and with dose-dependent suppressive properties through the GzmB pathway. B cells decrease T cell proliferation and induce their apoptosis, two processes that may contribute to a tolerogeneic environment. GzmB-producing B cells are under the control of IL-21. The fact that there is more IL-21 *in vitro* in TOLs after B cell stimulation correlates with a greater number of IL-21–dependent circulating GzmBs and a higher inhibitory effect of B cells, which may act as pro-B10 that need to be activated to display an increased effect. In previous articles, we reported on a decreased number of circulating plasma cells and we showed that B cells from tolerant recipients do not fully differentiate into plasmablasts and are more sensitive to apoptosis.[12,20] Here again, this is in accordance with the fact that *in vitro* unstimulated T cells from TOLs produce less IL-21, a molecule that directly acts on B cell differentiation.[62,63] We hypothesize that these properties may contribute to a favorable tolerogeneic environment and favorable inversion of the B effector–plasma cell/Bregs balance in these patients and their lower antibody production. These data support a role for B cells in patients with operational tolerance. They also raise the question of whether it would be possible to trigger an *ex vivo* or *in*

vivo increase to encourage any potential therapeutic effects, such as counteracting the alloimmune response or even promoting tolerance. These data also question the effectiveness of depleting B cells in order to control antibody-mediated rejection, rather than developing new strategies to maintain the fragile balance between the negative and beneficial effects of B cells in transplantation.

CONCISE METHODS

Patients and HVs

Forty-one patients took part in the study and signed informed consent as follows: (*1*) TOLs with stable kidney graft function (creatinemia<150 μmol/L and proteinuria<1 g/24 h) in the absence of immunosuppression for at least 1 year (n=12) except for 2 who have creatinemia>150 μmol/L±20% but were stable over time, (*2*) STAs with stable kidney graft function (creatinemia<150 μmol/L and proteinuria<1 g/24 h) for at least 3 years under standard immunosuppression (calcineurin inhibitors and corticosteroids) (n=17), and (*3*) HVs without pathologies or infectious episodes in the previous 6 months (n=17) (Table 1).

Cell Culture

Q:9 PBMCs were stimulated at 2×10^6 cells/ml for 48 hours in complete RPMI 1640 (Sigma-Aldrich, St. Louis, MO) containing L-glutamine, penicillin/streptomycin (Life Technologies, Carlsbad, CA), and 10% FCS (Lonza, Verviers, Belgium) in 96-well plates (Nunc, Langenselbold, Germany) at 37°C, 5% CO_2. Stimulation was performed using CpG oligonucleotide (ODN 2006, 10 μg/ml; InvivoGen, San Diego, CA) and recombinant human soluble CD40L (R&D Systems, Minneapolis, MN). PMA (250 ng/ml), ionomycin (1 μg/ml), and brefeldin-A (10 μg/ml) (Sigma-Aldrich) were added for the last 5 hours of the B-cell culture. As a control, PBMCs were cultured in resting conditions for 48 hours.

Analyses of IL-10 Production by Stimulated B Cells

After 48 hours of culture, viability staining was performed using the aqua Live/Dead cell staining kit (Invitrogen/Life Technologies). B lymphocytes were stained with anti–CD19-PC7 (BD Biosciences, San Diego, CA), washed, fixed, and permeabilized using a permeabilization/fixation kit (BD Biosciences). Fcγ receptor inhibitor (eBiosciences, San Diego, CA) was used to avoid nonspecific staining. For detection of intracellular IL-10, staining was performed using anti–IL-10-PE (clone JES3-9D7; BD Biosciences). Appropriate PE-conjugated isotype controls were used for gate setting for IL-10 expression. Unstimulated B cells were stained and used as a control for the gating strategy. Q:10

B Cell Functional Assays: B and T Cell Purification

PBMCs were obtained after Ficoll density centrifugation (Sigma-Aldrich) of fresh blood samples. B cells were purified by negative selection using a Human B Cell Isolation Kit II and an autoMACS PRO Separator, with purity >95% (Miltenyi Biotech, Gladbach, Germany) and stimulated for 24 hours with CD40L (1 μg/ml) and CPG-ODN (10 μg/ml) in a 96-well U-bottom plate at a concentration of 10^6 cells/ml. As a control, a proportion of PBMCs were kept at 4°C in complete medium for 24 hours. B cells were purified using the same technique, without stimulation. $CD4^+CD25^-$ responding T cells were purified by negative selection using successively $CD4^+$ T Cell Isolation Kit II and $CD25^+$ Microbeads II (Miltenyi Biotech), according to the manufacturer's instructions with a purity >95%. Q:11

Coculture Experiments

Coculture assays were performed for 72 hours by adding 1×10^5 autologous prestimulated B cells or unstimulated B cells to 0.5×10^5

Table 1. Summary of clinical and demographic characteristic of patients and HVs

Patient Group	Age (yr)	Sex (F/M)	Donor (LD versus NLV)	HLA Mismatches (n)	Time between Graft and Analysis (mo)	Creatinemia (μmol/L)	Proteinuria (g/24 h)	Time between Immunosuppression Withdrawal and Analysis (yr)
HV (n=17)		8/17						
Median	46.5							
SD	10.8							
Minimum	27.0							
Maximum	61.0							
TOL (n=12)		6/12	4/12					
Median	58.0			3.0	220.6	90.0	0.1	10.5
SD	15.8			1.9	94.9	64.7	0.2	5.5
Minimum	31.4			0.0	71.4	66.0	0.0	1.0
Maximum	85.3			4.0	385.3	280.0	0.6	19.0
STA (n=17)		5/17	1/17					
Median	57.6			4.0	120.4	124.0	0.1	
SD	10.8			1.4	37.6	29.1	0.1	
Minimum	40.4			1.0	66.4	74.0	0.0	
Maximum	74,9			5.0	192.5	175.0	0.2	

F, female; M, male; LD, living donor; NLV, nonliving donor.

$CD4^+CD25^-$ responding T cells, stimulated with anti-CD3 and anti-CD28.2 dynabeads (at a 1:1 ratio of dynabeads/T cells) (Invitrogen, Oslo, Norway). After 72 hours of coculture, brefeldin-A was added at 10 μg/ml for 4 hours. Viability of T and B cells was checked by 4′,6-diamidino-2-phenylindole staining. The proliferation of $CD4^+$ $CD25^-$ responding T cells was measured after staining with CellTrace Violet (Invitrogen). T cell IFN-γ, IL-21, and TNF-α secretion was measured after permeabilization of responding T cells and staining with anti–CD4-PE (BD Biosciences), anti–IFN-γ-APC and anti–TNF-α-FITC (BD Biosciences). For measurement of T cell apoptosis, cells were stained with CD4-APC, CD19-PE-C7, and Annexin V-APV (BD Biosciences). To investigate whether B cells have a dose-dependent effect on T cells, the number of B cells was progressively increased in the coculture (50,000, 100,000, and 200,000), whereas the number of 50,000 T cells remained fixed.

Coculture Experiments Blockade

These coculture assays were performed under the same conditions using transwell polycarbonate inserts (0.4 μm; Corning, Inc.) or using anti–IL-10 (BD Biosciences), anti–TGF-β1 (Abcam, Inc., Cambridge, UK), or anti-GzmB, a peptide that irreversibly inhibits GzmB activity (Ac-IEPD-CHO; BioVision, San Francisco, CA). All antibodies were used at 10 μg/ml concentration.

Microarrays

Purified B cell samples from 32 individuals (10 TOLs, 10 HVs, and 12 STAs) were analyzed with whole-genome Agilent human microarray as previously described[24] according to the manufacturer's instructions (Agilent Technologies). Hybridization signals were normalized using a Lowess procedure.[64] Probe conversion was performed using MADGene[65] and those pertaining to the same gene were averaged. The expression of 60 markers selected either for their participation in the regulation of B cell functions[26] and/or their implication in a tolerance-related B signature[27] was then investigated. Significance of differential expression was evaluated using a one-tailed *t* test and results were compared with those obtained on PBMCs.[27]

$GzmB^+$ B Cell Phenotyping

Anti–human mAbs included the following: CD19-V450 CD38-FITC, CD24-PE, CD5-APC, CD1d-PE, and GzmB-Alexa Fluor 700 from BD Biosciences. CD20-FITC and CD138 PE from Miltenyi Biotech were used for PBMC staining. PBMC suspensions were stained on ice using a predetermined optimal concentration of each antibody for 15–30 minutes. PBMCs were then fixed and permeabilized using a permeabilization/fixation kit (BD Biosciences). For detection of intracellular GzmB, staining was performed using anti–GzmB-A700.

IL-21 Measurement and Inhibition

Coculture assays were performed for 72 hours by adding 1×10^5 autologous prestimulated B cells or unstimulated B cells to 0.5×10^5 $CD4^+CD25^-$ responding T cells stimulated with anti-CD3 and anti-CD28.2 dynabeads (at a 1:1 ratio of dynabeads/T cells) (Invitrogen). Cytokine production was measured in culture supernatants at 72 hours. IL-21 level was determined using BD CBA Human IL-21 FLEX SET as recommended by the manufacturer (BD Biosciences). GzmB production by B cells in B cell/T cell cocultures was measured at day 3 by blocking IL-21 with increasing doses (0, 2, 4, 6, and 8 μg/ml) of anti–IL-21 blocking molecule (recombinant IL-21R Fc chimera) (R&D Systems).

Statistical Analyses

Data are expressed as the means±SEM. Statistical analyses were performed using GraphPad Prism software, and *P* values were calculated by the Mann–Whitney test.

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DISCLOSURES

None.

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Supplemental figures and tables

Supplemental table 1: Detailed clinical data for patients with drug-free long-term graft function (TOL), kidney recipients with stable graft function under standard immunosuppression (STA) and healthy volunteers (HV).

Supplemental Figure 1: B cells GzmB expression before and after activation. (A) Dot plot of secretion of GzmB by unstimulated B cells at day 0 and stimulated B cells at day 3 of coculture with $CD4^+CD25^-$ T cells. (B) Percentage of $GzmB^+$ B cells at day 0 and day 3 in HV (n=6), TOL (n=6) and STA (n=6) (**p<0.01; ***p<0.005). (C) Dot plot of secretion of GzmB by stimulated B cells at day 3 of coculture with $CD4^+CD25^-$ T cells in HV, TOL and STA.

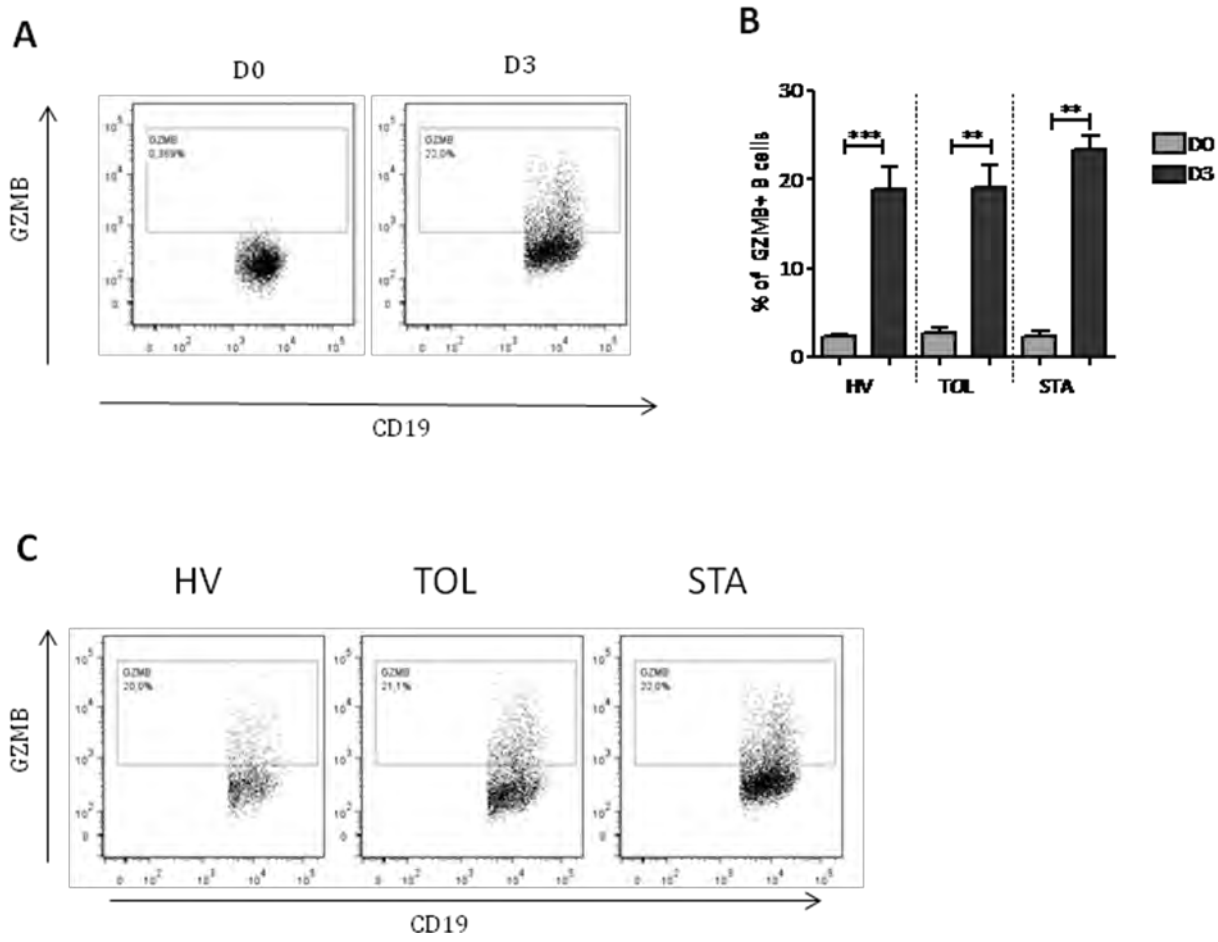
Supplemental Figure 2: Comparative expression of B-cell regulatory molecules (A, B) and tolerance-related markers (C, D) across B-cell (A, C) and PBMC samples (B, D). For each subset (A, B, C, D), gene expression data are represented by a heat map in which each row represents a gene, and each column represents a sample from the Healthy Volunteer (HV), Tolerant (TOL) or Stable patient (STA) group. Each cell in the matrix corresponds to an expression level, with red for over-expression, green for under-expression, and black for gene expression close to the median (see green/red color scale). For each gene, the absolute expression (see purple color scale, Quartile) and the significance of the variations (1: TOL *vs*. STA; 2: TOL *vs*. HV; 3: STA *vs*. HV) between groups (see blue color scale, P-value) are given. Diamond (♦) shows preferential expression in B-cell subsets.

Supplemental figure 3: Absolute number of blood B cells. Number of B cells per µL of blood in HV (n=6), TOL (n=9) and STA (n=6) (* p<0.05).

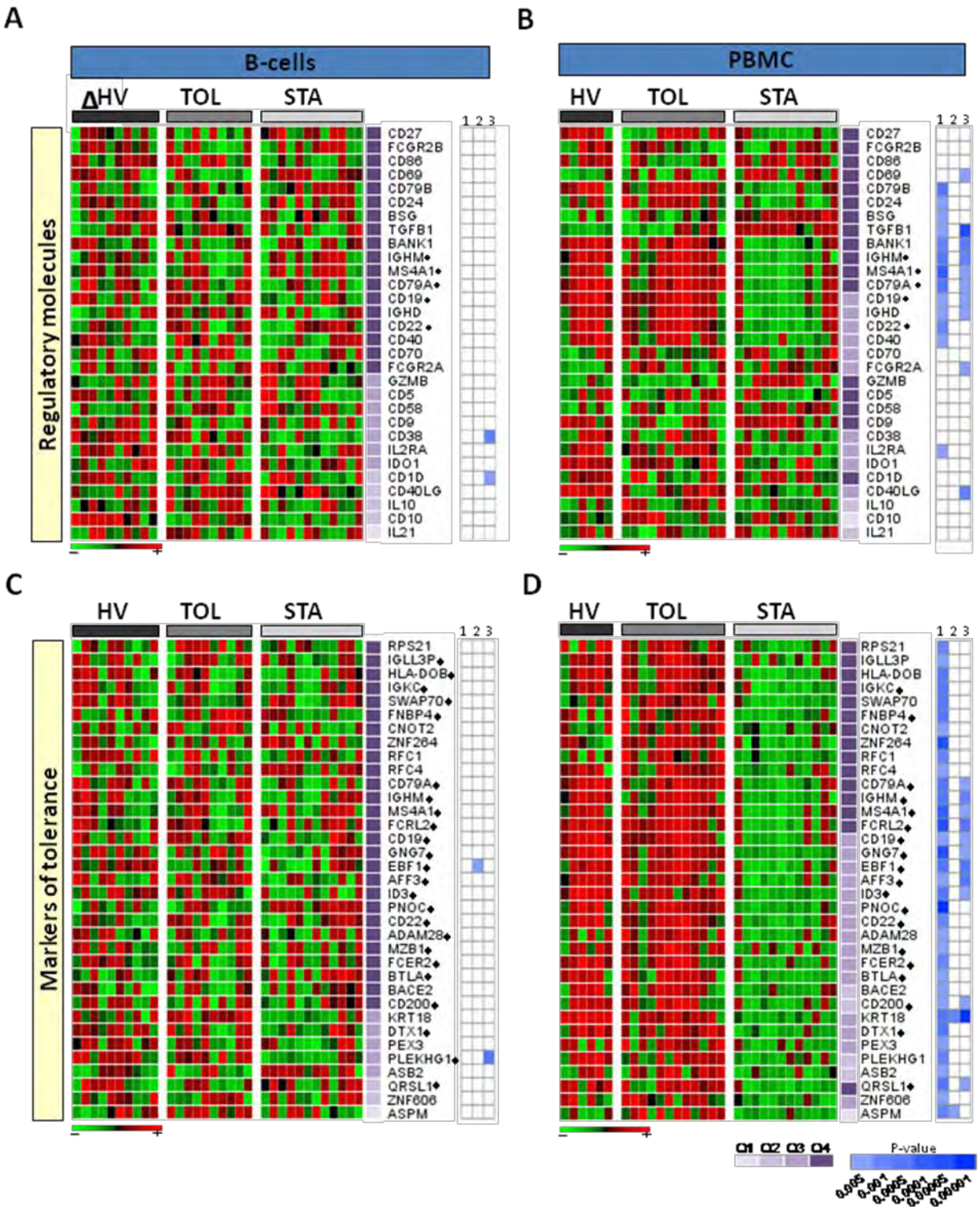
Supplemental table 1

		Age (years)	Gender	Previous episode of acute rejection	Lymphoma status, cancer	Episode of acute infection (CMV)	Time between graft and analysis (months)	Creatinemia (µmol/L)	Proteinuria g/24h	Donor (living vs deceased)	Number of HLA mismatches	Medical treatment	Medical treatment before immunosuppression withdrawal	Time between immunosuppression withdrawal and analysis (years)	Reason of immunosuppression withdrawal	Ab aHLA	Donor specific Abs
TOL		68,9	M	0,0	0,0	1,0	325,2	112,0	0,04	NLD	4,0	0,0	AZA+CNI+CS	13,0	uncompliance	-	-
		58,2	F	2,0	1,0	1,0	244,0	87,0	ND	NLD	3,0	0,0	CC+CNI+CS	19,0	Csa toxicity	+ (CL II)	+ (CL II)
		47,7	M	0,0	0,0	0,0	180,6	103,0	0,04	LD	0,0	0,0	CC+CNI+CS	13,0	uncompliance	-	-
		61,2	F	0,0	0,0	1,0	358,4	71,0	0,09	LD	0,0	0,0	AZA+CNI+CS	12,0	uncompliance	-	-
		59,2	M	0,0	0,0	0,0	197,3	280,0	0,10	LD	0,0	0,0	CNI+CS	13,0	uncompliance	-	-
		49,8	F	0,0	0,0	0,0	136,6	80,0	0,19	NLD	1,0	0,0	MMF+CNI+CS	9,0	uncompliance	+ (CL I - CL II)	-
		85,3	M	0,0	0,0	1,0	246,4	144,0	0,36	NLD	3,0	0,0	CNI+CS	8,0	uncompliance	-	-
		56,8	F	0,0	1,0	1,0	261,9	66,0	0,10	NLD	4,0	0,0	CNI+AZA	4,0	PTLD	+ (CL II)	-
		50,8	M	1,0	0,0	1,0	385,3	79,0	0,08	NLD	4,0	0,0	AZA+CS	18,0	uncompliance	-	-
		44,8	M	0,0	0,0	0,0	168,5	205,0	0,59	NLD	0,0	CC+CNI		5,0	meningio-encephalitis	-	-
		61,0	F	0,0	0,0	1,0	147,8	93,0	0,00	NLD	4,0	0,0	CC+CNI	7,0	uncompliance	-	-
		31,4	F	0,0	0,0	0,0	71,4	75,0	0,00	LD	0,0	0,0	CC+CNI	1,0	uncompliance	+ (CL II)	ND
	Mean	58,0	6F/12	0,0	0,0	1,0	220,6	90,0	0,10	4LD/12	3,0			10,5			
	SD	15,8		0,3	0,4	0,5	94,9	64,7	0,20		1,9			5,5			
	min	31,4		0,0	0,0	0,0	71,4	66,0	0,00		0,0			1,0			
	max	85,3		2,0	1,0	1,0	385,3	280,0	0,59		4,0			19,0			
STA		51,9	F	0,0	0,0	1,0	129,0	81,0	0,10	NLD	5,0	CNI+CS				-	-
		53,7	M	0,0	0,0	0,0	97,0	112,0	0,16	LD	4,0	CS+CC				-	-
		44,1	M	0,0	0,0	0,0	63,4	164,0	0,14	NLD	4,0	CNI+CC				-	-
		60,6	M	0,0	0,0	0,0	98,0	102,0	0,14	NLD	5,0	CNI+CC				-	-
		47,0	M	0,0	0,0	1,0	140,7	93,0	0,16	NLD	1,0	CS+CC				+ (CI II)	-
		54,0	M	0,0	0,0	1,0	192,5	132,0	0,05	NLD	4,0	CNI+CS				+ (CI II)	-
		71,3	M	0,0	0,0	1,0	133,7	175,0	0,14	NLD	5,0	CNI+CC				-	-
		40,4	M	0,0	0,0	0,0	95,5	92,0	0,24	NLD	2,0	CNI+CC				-	-
		52,8	M	1	0,0	0,0	93,1	146,0	0,24	NLD	5,0	CNI+CC+CS				+ (CI II)	-
		44,1	M	0,0	0,0	1,0	142,6	131,0	0,11	NLD	1,0	CNI				-	-
		61,3	M	0,0	0,0	0,0	89,0	133,0	0,20	NLD	4,0	CNI+CC				-	-
		66,3	F	1	0,0	1,0	66,4	74,0	0,04	NLD	5,0	CNI+CS				-	-
		74,9	F	0,0	1	1,0	143,8	88,0	0,18	NLD	4,0	CNI+CC				-	-
		59,8	M	0,0	0,0	0,0	115,9	149,0	0,00	NLD	3,0	CNI+CC				-	-
		53,5	F	0,0	0,0	0,0	109,7	100,0	0,04	NLD	4,0	CNI+CC				-	-
		71,5	M	0,0	0,0	0,0	124,8	117,0	0,04	NLD	5,0	CNI+CC				+ (CI I)	-
		55,4	F	0,0	0,0	1,0	182,9	115,0	0,08	NLD	2,0	CNI+CS				+ (CI II)	-
	Mean	57,7	5F/17	0,0	0,0	0,5	120,4	124,0	0,10	1LD/17	4,0						
	SD	10,8		0,4	0,3	0,5	37,6	29,1	0,10		1,4						
	min	40,4		0,0	0,0	0,0	66,4	74,0	0,00		1,0						
	max	74,9		1,0	1,0	1,0	192,5	175,0	0,24		5,0						

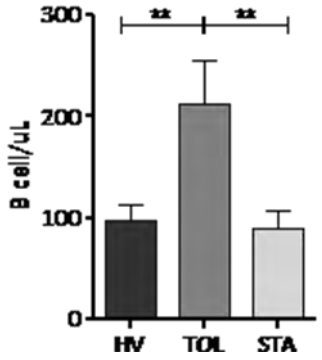
Supplemental figure 1



Supplemental figure 2



Supplemental figure 3



Discusion 2

Les LB peuvent avoir des effets inverses, agir comme inducteurs et comme régulateurs de la réponse immunitaire (Bouaziz et al., 2008; Evans et al., 2007; Fillatreau et al., 2002; Mauri et al., 2003; Yanaba et al., 2008). Nous avons récemment montré que les TOL sont caractérisés par un plus grand nombre de LB circulants, un défaut de la différenciation tardive et un profil transcriptionnel B ainsi qu'une surexpression de molécules associées à la régulation (Brouard et al., 2007; Pallier et al., 2010). Dans les modèles animaux, le fait que les LB ont un rôle dans la tolérance est clairement suggéré par une accumulation de LB et par la formation de centres germinatifs dans les allogreffes tolérées et la capacité des LB à prolonger la survie du greffon (Le Texier et al., 2011). Cependant, le rôle et la nature les LB régulateurs dans la modulation de la réponse immunitaire dans la tolérance et l'allo-immunité chez l'Homme restent flous. Bien que les patients tolérant une greffe rénale montrent clairement une importante signature B, avec une augmentation du nombre de LB, leur rôle potentiel dans l'établissement et le maintien de la tolérance reste à être déterminé.

Il est tentant de penser que cette augmentation de LB avec un profil inhibiteur pourrait être liée à la tolérance de ces rares patients. Le groupe de Haynes a récemment proposé un modèle de voie indirecte dans lequel une diminution de l'immunosuppression est associée à une augmentation du nombre de Treg par voie indirecte et de LB, avec possible Breg, mais les mécanismes n'ont pas été décrits (Haynes et al., 2012). Le groupe de Silva, a également montré que les LB périphériques des TOL maintiennent des capacités d'activation via la signalisation CD40-CD40L, de phosphoryler STAT3 et de préserver une diversité du compartiment B, suggérant un rôle des LB dans la tolérance (Silva et al., 2012). Dans ce papier, nous explorons le potentiel rôle régulateur des LB chez les patients tolérants. En raison de la difficulté à identifier une population unique de LB régulateurs, avec un phénotype unique chez l'Homme, nous avons analysé les propriétés suppressives *in vitro* des LB totaux. Nous montrons un nombre plus important de LB qui possèdent des propriétés suppressives doses dépendantes dans le sang des patients tolérants. L'effet inhibiteur des LB est dépendant du GzmB et de l'interaction des LB avec les LT cible.

Plusieurs preuves suggèrent que l'activation est instrumentale dans la régulation de l'activation des LB. Nous affirmons que les LB stimulés sont plus efficaces comme suppresseurs de la prolifération des LT effecteurs que les LB non stimulés. Un résultat en accord avec les données précédentes montrant que la maturation et l'activation des LB sont des paramètres importants dans ce processus. Et, il est un peu contraire à l'intuition de dire que les LB ont besoin d'une telle activation pour réguler le système immunitaire puisque les LB activés sont souvent associés à une activité stimulatrice sur les autres cellules. Cependant, cela a été longuement discuté et peut-être expliqué comme la participation des LB dans une boucle de régulation qui induit un processus et prévient une inflammation excessive ou une réponse auto-agressive des LT non désirés (Lampropoulou et al., 2012). De plus, nous montrons clairement que l'effet inhibiteur des LB est dépendant du GzmB, l'expression est également visible dans les LB au repos *in vivo* et est augmentée avec la stimulation. De façon intéressante, comme cela a déjà été décrit pour les populations de cellules régulatrices pro-B10, on arrive à la conclusion que la régulation induite par les LB est probablement inductible et que l'effet des LB est fortement influencé par la nature du microenvironnement (Barr et al., 2012; Dang et al., 2014; Fillatreau, 2012; Lampropoulou et al., 2008; Pasare and Medzhitov, 2005).

L'expression d'IL-10 a été montrée comme une caractéristique fréquente des LB régulateurs (Blair et al., 2009; Lemoine et al., 2011; Schmidl et al., 2014). De telles cellules, mentionnées comme B10 (DiLillo et al., 2010; Yanaba et al., 2008), sont impliquées dans l'initiation, le début et la sévérité de différentes maladies auto-immunes et en transplantation (Blair et al., 2010; Iwata et al., 2011; Nouel et al., 2014). Nous montrons ici que les propriétés suppressives des LB ne sont pas dépendantes de l'IL-10. Un inhibiteur de l'IL-10, utilisé à des doses capables d'inhiber efficacement l'action des LB sur la prolifération des LT, ne diminue pas l'effet inhibiteur des LB sur la prolifération des LT. Ces données s'accordent avec le travail de Deng, qui rapporte qu'un traitement anti CD45RB induit une forte tolérance antigène spécifique, qui est dépendante de la présence des LB et indépendante de l'IL-10. Dans ce modèle, L'IL-10 inhibe clairement l'induction de la tolérance et exerce même un effet négatif en provoquant des lésions histologiques et un rejet (Deng et al., 2007). Les mêmes observations ont été rapportées dans d'autres modèles (Singh et al., 2008). De façon intéressante, il a été démontré que les LB de patients avec un rejet chronique induit par des

anticorps présentent un défaut de leurs propriétés suppressives, (Nouel et al., 2014) à l'inverse des LB des patients stables, qui inhibent la prolifération des LT et induisent la génération de LTreg de manière TGF-β et IDO dépendant (personal Communication, annRheum dis abs A8.32, Nouel et al ., 2014). Dans notre cas, le blocage du TGF-β n'a pas d'effet sur la fonction suppressive des LB, suggérant que le TGF-β n'est pas non plus impliqué dans la régulation par les LB.

De façon intéressante, d'autres voies de régulation ont été décrites pour leur implication dans l'activité de LB régulateurs. Nous avons montré que le blocage du GzmB diminue l'effet inhibiteur des LB et restaure la prolifération des LT. Cela est associé à une augmentation du nombre de LB produisant du GzmB dans le sang des patients tolérants et les LB agissent de façon contact et dose dépendants. Le GzmB est une serine protéase de 32-kDa principalement connue comme un composé des granules cytotoxiques des LT et des cellules NK (Ewen et al., 2012; Hoves et al., 2010) mais le GzmB est aussi produit par d'autres types cellulaires, tels que les LB (Jahrsdörfer et al., 2006), les Treg (Gondek et al., 2005) et les cellules dendritiques plasmacytoïdes (Jahrsdörfer et al., 2010). Le GzmB induit l'apoptose des LT et la suppression des LT, par l'intermédiaire de la perforine (Jahrsdörfer et al., 2010). Dans ce travail, le fait que le blocage du GzmB avec un anti-GzmB induit une diminution de l'effet inhibiteur des LB indique que le GzmB est fonctionnel. De façon intéressante, nous montrons que les LB n'inhibent pas seulement la prolifération des LT, mais induisent également leur apoptose. Nous n'avons trouvé aucune implication de la voie Fas-FasL dans le processus (data non montrées), mais des voies alternatives ont été décrites comme induisant l'apoptose et le GzmB fait partie de l'une d'elles (Tretter et al., 2008). Ces données suggèrent un rôle pivot du GzmB dans la régulation par les LB chez nos patients par l'induction de mort cellulaire et l'inhibition de la prolifération des LT, un mécanisme déjà décrit pour les LTreg après activation polyclonale et antigène spécifique (Gondek et al., 2005).

Plusieurs facteurs micro-environnementaux ont été définis comme étant instrumentaux dans l'homéostasie des LB. Ces facteurs incluent des molécules telle que l'IL-21 (Jahrsdörfer et al., 2006; Lindner et al., 2013). Nous démontrons pour la première fois que les LB exprimant GzmB sont dépendantes de l'IL-21. Une augmentation de la dose d'anti-IL-21 diminue le nombre de LB exprimant du GzmB en culture. De plus, quand les LB sont ajoutés à la coculture, les LT produisant de l'IL-21 sont significativement augmentés et il y a une

production plus importante d'IL-21 par les LT des patients tolérants. L'IL-21 est une cytokine clef pour la transcription du gène du GzmB (Lindner et al., 2013; Xu et al., 2014), ce qui suggère que l'augmentation de LB GzmB$^+$ dans le sang des patients tolérants peut être due à un effet direct de l'IL-21. Cette donnée est renforcée par la phosphorylation effective de STAT3 dans les LB des patients tolérants (Silva et al., 2012) un signal clef dans la génération de LB activé sécrétant GZMB (Xu et al., 2014). Finalement, le fait que les patients tolérants ont un plus faible niveau de LT CD4$^+$ IL-21$^+$ et que les LB des TOL augmentent la production d'IL-21 par les LT CD4$^+$ et pas chez les HV et STA, suggère fortement une boucle de régulation chez ces patients, induisant une activation et une inflammation excessive des LB, ce qui permet à la régulation de se mettre en place.

Les LB régulateurs sont capables de contrôler la réponse immunitaire, mais une réaction excessive de ces cellules peut aussi induire une croissance de cellules tumorales et une infection chronique (Lindner et al., 2013). Nous formulons l'hypothèse qu'un bon réglage de la régulation par les LB et une bonne production d'IL-21 par les LT pourraient être un facteur clef dans le maintien de la tolérance.

Nous montrons que les patients tolérants ont plus de LB circulant avec des propriétés régulatrices, mais leurs LB ont la même activité suppressive que les LB des patients STA et HV. Ces résultats sont confortés par des analyses transcriptionnelles qui montrent que les LB ne sont intrinsèquement pas différents entre les groupes de patients et que, au moins pour ces gènes sélectionnés, la signature B dans le sang des patients tolérants résulte principalement d'une augmentation du nombre de LB dans les PBMCs. Il n'est pas surprenant que l'effet suppresseur le plus important dans le sang des patients tolérants est due à un nombre plus élevé de cellules GzmB$^+$ circulantes, si l'on considère que dans de nombreuses situations, le résultat clinique est principalement mené par la quantité de cellules infiltrantes et circulantes, plutôt que par leur qualité (Cherukuri et al., 2014).

En conclusion, nous avons démontré que les patients tolérants ont un plus grand nombre de LB GzmB$^+$ avec un phénotype de type plasmocyte et avec des propriétés suppressives dose-dépendantes via la voie du GzmB. Les LB diminuent la prolifération des LT et induisent leur apoptose, deux processus qui peuvent contribuer à un environnement tolérogène. Les LB qui produisent du GzmB sont sous le contrôle de l'IL-21. Le fait qu'il y ait

plus d'IL-21 chez les TOL après stimulation des LB est en accord avec le plus grand nombre de LB GZMB circulant dépendant de l'IL-21, ainsi qu'un effet inhibiteur plus important des LB. Dans des papiers précédents, nous avons rapporté une diminution du nombre de plasmocytes circulants et nous montrons que les LB des patients tolérants ne se différencient pas complètement en plasmocytes et sont plus sensibles à l'apoptose (Chesneau et al., 2013a; Pallier et al., 2010). Ici encore, cela est en accord avec le fait que les LT non stimulés produisent moins d'IL-21, une molécule qui agit directement sur la différenciation des LB (Berglund et al., 2013; Ding et al., 2013). Nous formulons l'hypothèse que ces propriétés peuvent contribuer à un environnement tolérogènique favorable, favoriser une inversion de la balance cellulaire plasmocytes effecteurs/cellules régulatrices chez ces patients et entraîner une plus faible production d'anticorps. Ces données montrent un rôle des LB chez les patients tolérants et soulèvent également une question : est-il possible d'induire une augmentation *ex vivo* ou *in vivo* de LB pour encourager des effets thérapeutiques potentiels, tel qu'agir contre la réponse alloimmune voire promouvoir la tolérance. Ces données interrogent également sur l'efficacité de la déplétion en LB afin de contrôler le rejet induit par les anticorps, plutôt que le développement des nouvelles stratégies pour maintenir la balance fragile entre les effets bénéfiques et délétères des LB en transplantation.

Conclusions et perspectives

Nous avons montré *in vitro* que les LB des patients tolérants présentent un défaut de leur différenciation tardive en plasmocytes et une sensibilité plus forte à l'apoptose. Ces résultats obtenus *in vitro* sont en accord avec les observations *in vivo*, qui montrent une fréquence moins importante de plasmocytes chez les patients tolérants, comparé aux patients stables.

Dans un deuxième temps, nous avons montré que les LB des patients tolérants ont la capacité de réguler les LT effecteurs en inhibant leur prolifération et en induisant leur apoptose. Cette fonction régulatrice est induite via un contact cellulaire et la sécrétion de GzmB par les LB. De plus, nous avons montré *in vivo*, que les patients tolérants ont plus de LB $GzmB^+$ qui présentent des fonctions régulatrices. Ainsi, les patients tolérants ont un profil lymphocytaire B tolérogène avec un nombre de LB « régulateurs » $GzmB^+$ plus important et moins de plasmocytes, comparé aux patients stables.

L'une des perspectives majeures de ce travail sera d'étudier la voie du CD40.

Il a été démontré que les LB des patients tolérants surexpriment BANK-1 (Pallier et al., 2010). Or, BANK-1 inhibe la voie du CD40 (Aiba et al., 2006), ce qui pourrait laisser penser que chez les patients tolérants qui surexpriment BANK-1 il y aurait un défaut dans la signalisation du CD40, qui a un rôle important dans l'orientation de la réponse B. On sait par exemple, qu'une stimulation IL-21 + BCR avec CD40L induit la différenciation des LB en plasmocytes ou LB mémoires, alors qu'une stimulation IL-21 + BCR sans CD40L oriente la différenciation en LB sécréteurs de GzmB (Hagn et al., 2010, 2012). De plus, en transplantation la voie du CD40 est très importante. En effet, il a été montré que le blocage de la stimulation CD40/CD40L prévient le rejet d'allogreffe dans plusieurs modèles, cependant les tests cliniques utilisant un anti CD154 (anti CD40L) ont été interrompus au vu des effets thromboemboliques de ce traitement, dû à la liaison de l'anti CD154 sur les plaquettes (Kawai et al., 2000; Kirk et al., 2001). Il serait donc intéressant de regarder dans notre modèle s'il y a des inhibiteurs de la voie du CD40 tel que du CD40L soluble. En effet des études montrent que le CD40L soluble supprime les réponses allo-immunes chez la souris. Un taux plus élevé de facteur soluble inhibant la voie CD40/CD40L pourrait expliquer l'apparition de LB régulateurs $GzmB^+$ dans un

microenvironnement inflammatoire (microenvironnement inflammatoire également riche en IL-21). De plus, il a été montré que l'IL -21 induit l'expression de Prdm1 dans les LB via STAT3 et IRF-4. Également connu sous le nom de Blimp1, Prdm1 contrôle la différenciation tardive en plasmocytes. (Ettinger et al., 2005; Kallies et al., 2007; Ozaki et al., 2004). Or, chez les patients tolérants on observe *in vitro* un défaut de l'expression de Prdm1 (Chesneau et al 2013), qui serait associée à la diminution *in vivo* du nombre de plasmocytes chez les patients tolérants. Il faudrait donc également observer l'expression du récepteur à l'IL-21 (IL-21R) sur les LB des patients tolérants (Jin et al., 2004) et doser le GzmB dans leur sérum. Ce travail sera en partie réalisé sur des clones de LB de patients tolérants. Ces patients sont très rares, nous sommes donc restreints au niveau de la quantité de cellules. Nous allons donc établir des lignées de LB immortalisés. Pour cela, je vais utiliser le protocole du groupe d'Hergen Spits, qui a développé une technique de reprogrammation génétique qui permet d'immortaliser des LB humains (Kwakkenbos et al., 2010). Cette technique, que j'ai acquise lors de mon doctorat, utilise un rétrovirus codant BCl-6 et Bcl-xl qui va induire une immortalisation des LB. Ainsi une fois que l'on aura obtenu des lignées de LB de patients tolérants, il sera possible d'étudier la réponse alloimmune des LB des patients tolérants envers les cellules du donneur car nous disposons des cellules de donneurs pour trois patients tolérants.

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Enfin, au sein de l'équipe, des analyses épigénétiques ont été réalisées à partir d'études transcriptomiques effectuées sur les LB des différents groupes de patients. De cette étude ressort un enrichissement en protéines de déacétylation ; les histones déacétylases (HDAC). Les HDAC inhibent la transcription de gènes en bloquant l'accès à la machinerie transcriptionnelle au niveau du site de transcription (Yang and Seto, 2003). De façon intéressante, l'expression de gènes spécifiques de la différenciation terminale des LB nécessite le recrutement des HDAC. Dans ces analyses, il ressort notamment une inhibition de CREBBP dans les LB des patients tolérants qui code pour la protéine de liaison CREB (CBP ou CREBP) - protéine nucléaire qui participe en qualité de co-activateur à la régulation des gènes d'expression de l'AMP-cyclique (Chrivia et al., 1993; Hoeffler et al., 1988; Kwok et al., 1994). CBP fait également partie de la famille des histones acétyltransférases (HAT) (Green et al., 2006; Ting and Trowsdale, 2002), CBP joue donc un rôle de coactivateur transcriptionnel et est impliqué dans plusieurs fonctions cellulaires telles que la réparation de l'ADN, la

croissance cellulaire, l'activation et l'apoptose. Ce coactivateur transcriptionnel partage avec son paralogue, la protéine de liaison E1A (P300) - une structure similaire puisque CBP et P300 ont 70% d'homologie. De par sa fonction d'HAT, CBP possède des fonctions très larges du fait de son interaction avec les histones et son implication et est impliqué dans de nombreuses fonctions cellulaires. Chez la souris, une étude montre que CBP et P300 sont impliqués dans l'activité de 36 protéines exprimées par les LB tels que NFkB ou PAX5 (Xu et al., 2006). CBP étant inhibé chez les patients tolérants, il serait intéressant d'analyser l'effet d'un blocage de CBP dans les LB de témoins sains. Or, il a été démontré que le curcumin est un inhibiteur de CBP et P300 (Zhu et al., 2014). Le curcumin est décrit comme étant un agent immunomodulateur pouvant moduler l'activation des LT ; LB ; macrophages ; neutrophiles ; cellules NK (Varalakshmi et al., 2008). En outre, le curcumin a une fonction apoptotique sur les LB car il diminue le taux de BCL-2 - qui est anti-apoptotique - tout en augmentant l'expression de Bax pro-apoptotique (Zhu et al., 2014). Dans le même principe , ICG-001 a été identifié pour son effet inhibiteur sur l'interaction de la β-catenine avec son coactivateur transcriptionnel CBP sans pour autant modifier l'interaction de la β-catenine avec P300 (Arensman et al., 2014). Ainsi , l'un des effets de la molécule ICG-001 via la perturbation de la transcription des Wnt/ β-catenine est la diminution de l'expression de BIRC5 (aka survivin protein) qui est un inhibiteur de l'apoptose entrainant l'activation de caspase 3/7 et induisant l'apoptose (Lazarova et al., 2013a, 2013b; Ma et al., 2005). Les principales méthodes d'analyse de CBP sont l'immunofluorescence et le western blot. Il serait donc intéressant dans un premier temps de regarder par western blot, l'expression de CBP dans les LB des patients tolérants pour ensuite, la comparer avec les patients stables et les témoins sains. Il serait également intéressant de regarder l'expression par western blot de BIRC5 dans les LB. BIRC5 a été décrit comme impliqué à la fois dans l'apoptose (Arensman et al., 2014) et dans la signalisation de CBP, or nous avons montré *in vitro* que les LB des patients tolérants sont plus sensibles à l'apoptose que les patients stables. CBP semble impliqué dans la régulation de plusieurs gènes anti-apoptotiques (P53, c-myc, Bcl-xl…..) (Han et al., 1999). Il faudrait donc regarder l'expression de ces gènes dans les LB des patients tolérants et stables afin de voir si un défaut de CBP chez les tolérants ou à une surexpression de CBP chez les stables pourrait être à l'origine de la plus forte sensibilité à l'apoptose des LB chez les patients tolérants ou une plus faible sensibilité à l'apoptose chez les stable. En effet, l'apoptose LB des patients tolérants étant identique à celle observée chez les témoins sains, la différence révélée entre

TOL et STA serait davantage liée à un défaut d'apoptose chez les STA. Ceci pourrait être expliqué par une résistance associée aux traitements immunosuppresseurs. Ainsi, l'une des hypothèses serait que la tolérance pourrait être liée à une homéostasie des LB conservée. En effet une partie de nos résultats montrent des différences entre STA et HV mais pas entre HV et TOL, (apoptose, phénotype …). Les différences entre STA et TOL seraient en partie expliquées par les traitements IS. Dans ces études, nous sommes restreints au niveau des contrôles, puisque nous ne disposons pas de témoins sains sous IS. Ainsi, dans nos prochaines études, nous utiliserons un groupe supplémentaire de patients qui seront des patients transplantés recevant de faibles doses d'IS (Minimal Immunosuppression) et ayant une fonction stable de leur greffon.

*

En conclusion, les patients tolérants présentent une plus forte proportion des LB ce qui a été la cause principale de cet intérêt aux LB dans la tolérance en transplantation rénale. L'étude de ces LB montre des différences *in vivo*, moins de plasmocytes et plus de LB transitionnels chez les TOL, comparé aux STA et HV. Cependant, l'étude transcriptionnelle des LB ne montre pas de réelles différences entre les TOL et HV ce qui laisserait à penser que la fonction des LB ne serait pas modifiée et que la tolérance serait liée à une homéostasie des LB. Ainsi, chez les patients tolérants, qui ont plus de LB on peut imaginer que du fait de leur nombre plus élevé il y aura moins de LB à être activés donc la fréquence de LB à se différencier en plasmocytes sera moins élevée (Figure 14). Ainsi ce nombre plus élevé de LB chez les patients tolérants pourrait être la base de la tolérance. Sur cette donnée, la question se pose de l'intérêt des traitements immunosuppresseurs visant une déplétion des LB : l'intérêt ne serait-t-il pas de

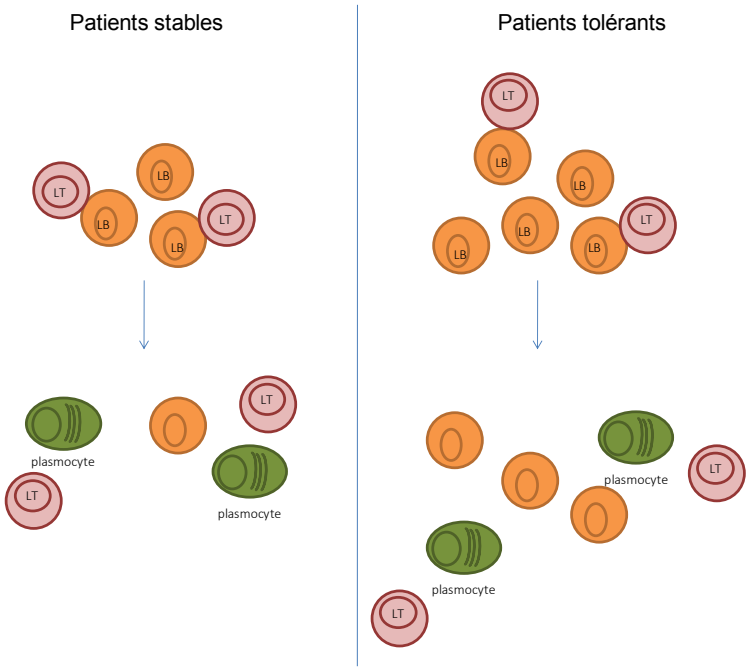

Figure 15 : Schéma représentant l'hypothèse : plus de LB en nombre chez les TOL = moins de plasmocytes et plus de B naïfs en fréquence

trouver un moyen de conserver une balance LB régulateurs / LB effecteurs favorable plutôt que de supprimer les LB ?

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Annexes 1

Lymphocytes B régulateurs et tolérance en transplantation : des modèles animaux à l'Homme.

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Regulatory B cells and tolerance in transplantation: from animal models to human

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Until recently, the role of B cells in transplantation was thought to be restricted to producing antibodies that have been clearly shown to be deleterious in the long-term, but, in fact, B cells are also able to produce cytokine and to present antigen. Their role as regulatory cells in various pathological situations has also been highlighted, and their role in transplantation is beginning to emerge in animal, and also in human, models. This review summarizes the different studies in animals and humans that suggest a B-cell regulatory role in the transplant tolerance mechanisms.

Keywords: transplantation, regulatory B cells, animal, human, treatment, tolerance

INTRODUCTION

Reducing immunosuppressive drug doses is one of the major goals in transplantation. In liver transplantation, up to 20% of recipients could eventually be weaned off immunosuppression (1, 2). Although the kidney is less disposed to successful immunosuppressive drug withdrawal, more than a hundred cases of operationally tolerant (OT) renal transplant patients have been reported (2–5). These are patients who display continued good graft function in the absence of immunosuppressive drugs (6). Understanding the mechanisms involved in these patients would provide invaluable information about the pathways to human transplantation tolerance.

Over recent decades, tremendous progress has been made in the understanding of the biology of regulatory cells and their roles in autoimmunity, infection, cancer, and transplantation. The best-known are the $CD4^+Foxp3^+$ T cells, and their role in transplantation, at least in animal models, has been clearly established (7). Until recently, the role of B cells in transplantation was thought to be restricted to producing antibodies that have been clearly shown to be deleterious in the long-term (8, 9), but B cells are also able to produce cytokine and to present antigen (10, 11). Their role as regulatory cells has also been highlighted in varying pathological situations and their role in transplantation has begun to emerge. The present review will focus on the role of these cells in transplantation, in animal models and in clinic.

REGULATORY B CELLS: PHENOTYPE AND MECHANISMS OF ACTION

While a suppressive role for B cells was already suspected in the mid-70s in a model of delayed hypersensitivity in guinea pigs (12, 13), it is in 1996 that Wolf et al. (14) really pointed toward the existence of "regulatory" B cells. Unlike wild type mice, B-cell deficient mice (μMT) were unable to recover from Experimental Autoimmune Encephalomyelitis (EAE) and this was later attributed to the absence of IL-10-producing B cells. Since this first report, the major role of these cells has been reported in numerous autoimmune disease models. As was the case for regulatory T cells (Treg) in the 1980s, there are, as yet, no validated phenotypic markers of regulatory B cells, and it remains very likely that, as for Treg cells, different regulatory B-cell subsets exist. Two main B-cell populations have been reported in mice. The precursor B cells of the marginal zone (T2-MZP B cells) were described by Evans et al. (15) in a collagen-induced arthritis (CIA) mouse model. These cells produce IL-10, have a $CD19^+CD21^{high}CD23^+CD24^{high}CD93^+$ phenotype, and their adoptive transfer from naïve mice to immunized mice suppresses CIA development in an IL-10 dependent manner. Tedder and colleagues identified another subset of regulatory B cells in mice based on IL-10 expression, called B10 cells. This rare B-cell subset (1–2%) is found predominantly in the spleen $CD1d^{high}CD5^+$ B-cell subset of naïve wild type mice and is defined by its unique capacity to produce IL-10 in response to specific activation signals (16). In a contact hypersensitivity (CHS) model of inflammation, Tedder's group has shown that the B10 cells suppress T-cell dependent inflammation during CHS *in vivo* in an antigen-dependent manner (17). Even if there are significant phenotypic differences between these two B-cell subsets, it cannot yet be excluded that they share a common progenitor. More recently, studies have demonstrated that a combination of IL-21 and B-cell antigen receptor (BCR) stimulation enables B cells to produce and secrete Granzyme B, without the secretion

of perforin. This Granzyme B secretion by B cells may also play a major role in the regulation of autoimmune responses (18). So different subsets of regulatory B cells seem to exist with, most likely, different mechanisms of action.

Concerning the activation of Bregs, several studies demonstrate the major role of CD40 pathway stimulation for Breg IL-10 secretion (19, 20) and also the involvement of Toll Like Receptors (TLRs) (16, 17, 21). Interestingly, Yanaba et al. showed as recently as last year that B10-cell maturation into functional IL-10-secreting effector cells requires IL-21 and CD40-dependent cognate interactions with T cells (22). Some studies have also shown that the regulatory function of B cells was antigen specific in an EAE and in a CHS model (16, 23), and also that these Bregs can differentiate into plasmocytes and plasmablasts secreting polyreactive or antigen-specific antibodies (24). Recently Montandon et al. also described a new population of B cells with regulatory properties in an animal model of type-1 diabetes. These are a hematopoietic progenitor population: innate pro-B cells which protect non-obese diabetic mice against type-1 diabetes. Pro-B cells activated by TLR-9 suppress pathogenic effectors cells by reducing their IL-21 production and by inducing apoptosis via Fas Ligand (25).

Similarly to Tregs, Bregs exert their suppressive properties in different ways: Th1 and Th17 differentiation inhibition (15, 19, 20, 23, 26–28) regulatory T-cell induction (28–30); and also through a direct inhibitory effect on antigen presentation by DC (23). These suppressive mechanisms are summarized in **Figure 1**.

In humans, these regulatory B cells have recently been identified and described. However, their study is still in its infancy and their phenotype needs to be better described. Blair et al. (26) demonstrated that human transitional $CD19^{+}CD38^{hi}CD24^{hi}$ B cells possess regulatory capacities (31). This has also been confirmed in healthy volunteers by Lemoine et al. (32). After CD40 stimulation, these cells suppress the differentiation of T helper 1 cells, partially via the provision of IL-10. Their suppressive capacity is reversed by a blockade with CD80 and CD86 monoclonal antibodies, suggesting a contact-dependent suppressive action. In 2010, the group of Tedder characterized IL-10 competent B cells in humans. They describe a B10 subset defined by its capacity to secrete IL-10 after 5 h of *ex vivo* stimulation, whereas progenitor B10 (B10pro) cells require 48 h of *in vitro* stimulation before they acquire the ability to express IL-10 (33). Both subsets are predominantly found within the memory $CD24^{hi}CD27^{+}$ B-cell subpopulation and are able to negatively regulate monocyte cytokine production through IL-10 dependent pathways during *in vitro* functional assays. In addition, a recent study demonstrated that human B cells can regulate DC maturation and function (34).

AS can be seen from the above, currently the majority of studies looking at Bregs in human autoimmune diseases. However, studies in the area of transplantation have produced a number of arguments pointing to a major implication of B cells in tolerance. The following will focus on the role of Bregs first in animal tolerance models, and then in human.

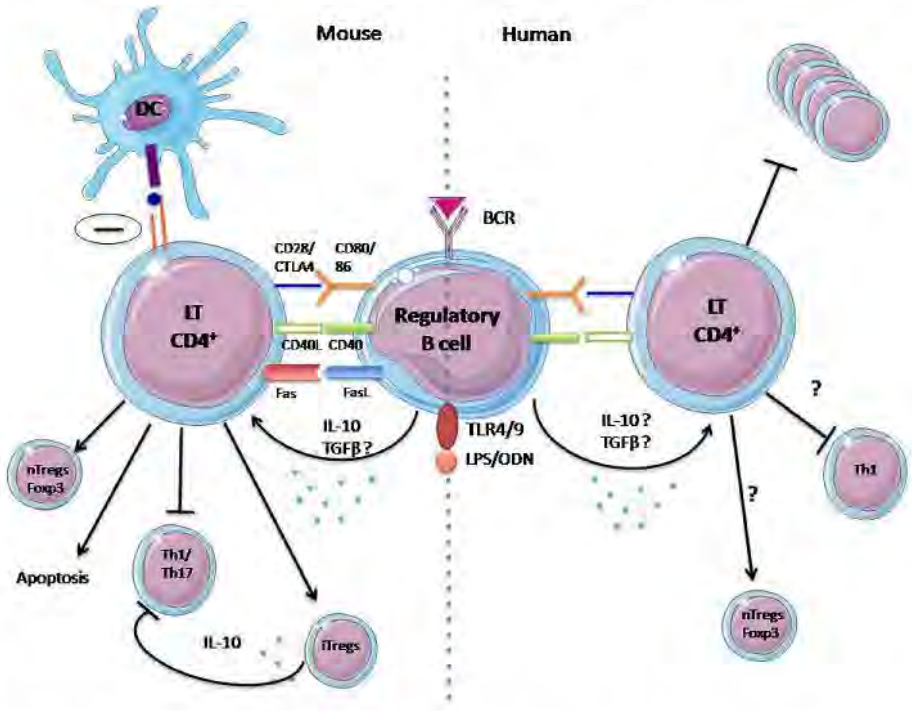

FIGURE 1 | Mechanisms of suppression of regulatory B cells identified in human and animal. In mice, regulatory B-cell suppression is fulfilled by IL-10 secretion, activation of the CD40 pathway, and probably via contact with T lymphocytes. It has numerous effects: (1) inhibition of Th1 and Th17 differentiation, (2) inhibition of antigen presentation by DCs, and (3) induction of natural regulatory T cells. For humans, the mechanisms for the actions of regulatory B cells remain unclear and have yet to be confirm: (1) Probable inhibition of proliferation of $CD4^{+}$ T cells, (2) Possible inhibition of Th1 differentiation, and (3) possible increase of natural regulatory T cells.

PART I: REGULATORY B CELLS IN ANIMAL MODEL OF TRANSPLANTATION

The following provides a review of experimental models demonstrating the implication of B cells as major actors in inducing tolerance (**Table 1**).

The first evidence for a potential role for B cells in allograft tolerance was reported by Parker et al. (35). In a pancreatic islet allograft BALB/c mouse model, survival of C57Bl/6 recipient mice was increased by injection of a large quantity of B cells, in addition to a CD40 ligand (CD40L) blocking antibodies to prevent T-cell/B-cell interaction, 8 days before islet transplantation [from (BALB/C × C57BL/6)F1). Allogenic donor B cells thus permit islet allograft survival when administrated in combination with anti-CD40L (35).

Niimi et al. (36) confirmed the role of B cells in the tolerance induction after blockade of CD40L-CD40 interaction in a cardiac allograft mouse model. They induced tolerance to cardiac allograft in C3H mice by treating the recipients with a donor-specific subset of B cells (resting B cells that are incompetent or non-professional APCs) from C57BL/6 mice and blocking CD40L 14 days prior to graft. Furthermore, induction of tolerance by resting B cells was abrogated in CD40L Knockout mice, confirming that the CD40 pathway plays a critical role in allograft rejection *in vivo* (36).

Yan et al. (37) demonstrated in DA recipients of a kidney allograft from PVG rat, that donor B-cell administration at the time of transplantation induces long-term acceptance more efficiently than donor T cells. However, the mechanism by which B cells induce long-term allograft survival was not elucidated (37). Other studies have also pointed to a role for B cells in inducing tolerance by immunosuppression targeting CD45 (38–40). CD45 is part of family of transmembrane protein tyrosine phosphatases involved in lymphocyte development and activation (51) and serves as a rheostat determining the threshold of antigen stimulation. Deng et al. have shown that short-term administration of anti-CD45-RB antibodies on days 0, 3, 5, and 7 following transplantation efficiently prevents cardiac allograft rejection in both the allogeneic C3H to B6 and the BALB-derived transgenic HA104 [Hemagglutinin (HA) expressing] to TS1 (HA specific TCR) combination (38). In this model, tolerance induced by anti-CD45-RB was lost in B6μMT$^{-/-}$ mice, a model of transgenic mouse lacking B cells and antibodies. Tolerance was restored after B-cell transfer in B6μMT$^{-/-}$ mouse, showing that tolerogenic efficacy of anti-CD45-RB therapy requires host B cells. Long-term survival was not obtained when cardiac allografts were transplanted to B-cell deficient mice reconstituted with splenocytes from knockout mice with a deficiency for the co-stimulatory molecule CD40 or the CD80/CD86 combination. These data show that tolerance induced

Table 1 | Summary table of studies demonstrating the implication of B cells as major actors in tolerance induction in different kinds of experimental animal models.

Reference	Animal model	Modality of tolerance	Implication of B cells
Parker et al. (35)	Mouse pancreatic islet allografts	Treatment with allogenic small lymphocyte or T-depleted small lymphocytes plus blocking antibody to CD40L	Increase survival of recipients treated with T-depleted small lymphocytes plus CD40L
Niimi et al. (36)	Mouse model of cardiac allograft	Resting B cells plus blocking antibody to CD40L	Tolerance induced by B cells involves the CD40 pathway
Yan et al. (37)	Rat model of kidney allograft	I.V. injection of donor B cells at time of transplantation	B cells induce more efficiently long-term acceptance of graft than T cells
Deng et al. (38)	Mouse model of cardiac allograft	Anti-CD45-RB therapy	Anti-CD45-RB is not efficient in transgenic mouse without B cells
Huang et al. (39)	Mouse model of cardiac allograft	Treated with anti-CD45-RB, anti-ICAM, anti-LFA or combination of these agents	Expression of ICAM-1 by B cells and interaction with LFA-1 form a central aspect of transplantation tolerance induced by CD45-RB therapy
Zhao et al. (40)	Mouse model of cardiac allograft	Anti-CD45-RB therapy	IL-10 expressed by B cells inhibits B-cell-mediated tolerance induction in cardiac allograft model
Ding et al. (41)	Mouse model of islet allograft	Anti-TIM-1 therapy	TIM-1 B cells are regulatory and transfer donor-specific long-term graft survival
Le Texier et al. (42)	Rat model of cardiac allograft	Short-term immunosuppression	Accumulation of B cells in PBMC of tolerant recipients and a phenotype of inhibited B cells partially blocked at their IgM to IgG switch and over expressing the inhibitory receptor Fcgr2b
Lee et al. (43)	Mouse model of islet allograft	Anti-CD45-RB and anti-TIM-1 therapy	Combined anti-CD45-RB and anti-TIM-1 treatment induced allograft survival that is B-cell dependent, dependent on B-cell production of IL-10, and is associated with up-regulation of TIM-1 on B cells

by anti-CD45-RB therapy requires host B cells and that tolerance is mediated through the interaction of co-stimulatory molecules on B cells and T cells (38). In the same area of research, Huang et al. have shown that, in a model of cardiac allograft from C3H donors into B6 recipient mice, anti-CD45-RB Ab therapy on days 0, 1, 3, 5, and 7 after transplantation induce tolerance. Splenic B-lymphocytes demonstrate phenotypic alterations including up-regulation of CD54 (intracellular ICAM-1 adhesion molecules) (52). Blockade of ICAM-1/LFA-1 interaction prevents the tolerance induction by anti-CD45-RB and mice deficient in either ICAM-1 or LFA-1 reject their graft even when they are treated with anti-CD45-RB (39). In their study, Zhao et al. investigated the role of IL-10 in an anti-CD45-RB model of mouse cardiac allograft. Surprisingly, in this model, neutralization of IL-10 by treatment with five doses of anti-IL-10 antibody every other day post transplantation improves tolerance induction. The role of B-cell IL-10 production was assessed by transferring IL-10 deficient splenocytes into B-cell deficient recipients in which tolerance could be then induced in a similar manner as in the IL-10 non-deficient splenocytes, confirming that Il-10 expression by B-lymphocytes inhibits B-cell-mediated tolerance induction. Neutralization of IL-10 enhances tolerance induction and improves the long-term outcomes of cardiac allograft (40).

Due to the lack of regulatory B cells markers, certain research focuses on the development of phenotypic markers. T-cell immunoglobulin and mucin domain (TIM) family proteins are potent co-stimulatory molecules in T-cell activation (53). Anti-mouse TIM-1 mAb RMT1-10 given i.p. on day of transplantation at 0.5 mg and on days 2, 4, 6, 8, and 10 after transplantation at 0.25 mg prolongs graft survival in one mice model of cardiac allograft (B6 to BALB/C) (54). Ding et al. (41) showed that anti-TIM-1 (RMT1-10) administered i.p. on days 1, 0, and 5 relative to day of islet (B6) transplantation to Balb/c prolong islet allograft survival. Depletion of B cell (anti-CD20) in recipients prior to transplantation shortened allograft survival compared with B-cell-intact mice, demonstrating that B-lymphocytes are required for prolonged anti-TIM-1-reliant allograft survival. In this model, both islet transplantation and the treatment of the recipient with anti-TIM-1 increased IL-10 and IL-4 expression on B cells. The expression of these two cytokines in TIM-1$^+$ vs. TIM-1$^-$ B cells show that TIM-1$^+$ B cells expressed more IL-4 and IL-10 than other B cells. In this study, TIM-1$^+$ and TIM-1$^-$ B cells from splenic B cells of Balb/c allograft recipients treated with anti-TIM-1 and sacrificed on day 14 were sorted. Transfer of TIM-1$^+$ B cells into untreated JHD recipients of B6 islet prolongs allograft survival whereas TIM-1$^-$ B cells have no effect, demonstrating that TIM-1$^+$ B cells have a regulatory role and are able to transfer donor-specific long-term graft survival properties (41).

Lee et al. studied the effect of anti-CD45-RB treatment in combination with anti-TIM-1 antibodies. This tolerance induction in a mouse islet allograft model of C57BL/6 diabetic recipients and islets from BALB/C demonstrates that this model is dependent on the production of IL-10 by B cells. Transfer of WT B cells prolong graft survival whereas IL-10 deficient B cells do not prolong allograft survival, suggesting that graft survival is dependent on IL-10 production by B cells in this model. Depletion of Treg by anti-CD25 PC61 before transplantation leads to a rejection of allograft suggesting that tolerance induction is also dependent on TIM-1$^+$ regulatory B-cell/Treg interaction (43).

In a LEW1W/LEW1A heart allograft rat model, administration of LF15-0195, an analog of deoxyspergualin, for 20 days starting at transplantation induces long-term cardiac allograft tolerance (42). The tolerated allograft contains B cells organized in germinal centers with strongly inhibited IgM to IgG switch. The authors report on an accumulation of B cells in the blood of tolerant recipients following cessation of immunosuppressive treatment (at days 30 and 100 after transplantation). Blood B cells from tolerant recipients express a lower ratio of IgG/IgM transcripts and display an over-expression of BANK-1 and FcgR2b compared with B cells from recipients that develop chronic rejection. BANK-1 is an adapter protein involved in B-cell receptor-mediated signaling that negatively regulates CD40-mediated AKT activation (55) and the inhibitory receptor Fcgr2b is a member of the immunoreceptor tyrosine-based activating/inhibitory motif (ITAM/ITIM) family. Colligation of FcgammaRIIb with the BCR results in the abrogation of B-cell activation. Fcgr2b may play a role in the maintenance of peripheral tolerance and its polymorphism is, like that of BANK-1, significantly associated with systemic lupus erythematosus (SLE) (56, 57). This result may reflect an accumulation of inhibitory or inhibited B cells blocked in their switch recombination process. These data suggest that the B cells may not have proceeded to somatic hypermutation and therefore may produce Donor-Specific Antibody (DSA) of low affinity.

Human Alpha-1-Antitrypsin (hAAT) is a clinically available anti-inflammatory circulating glycoprotein known to protect islets from allorejection through the expansion of Tregs and alteration of dendritic cell responses (58, 59). A recent study from Mizrahi et al. demonstrates that B cells could participate toward tolerance in allogeneic transplantation via hAAT. First, they demonstrate that, *in vitro*, hAAT reduces B-cell activation in LPS-stimulated culture by lower expression of activation markers CD40 and CD19 and release of IL-10, and also affects T-cell-dependent B-cell activation by reducing expression of co-stimulatory molecules in B cells (CD80 and CD86) upon stimulation with CD40L. They demonstrated that the presence of IL-10-producing B cells is elevated by hAAT. They also show that B-cell knockout hATT transgenic chimeric mice fail to exhibit the increase in Treg observed in hAAT transgenic mice, suggesting a role for B cells in the induction of Treg by hAAT. This study brings to light the potential regulatory function of B cells in the protection of islet allorejection induced by hAAT.

PART II: REGULATORY B CELLS IN HUMAN TOLERANCE

The following provides a review of studies demonstrating the implication of B cells in kidney transplant operational tolerance (**Table 2**). Despite tolerance now being commonly obtained in animal models, it remains a challenge in clinic. In humans, operational tolerance has been defined as a stable graft function without clinical features of chronic rejection in the absence of any immunosuppressive drugs for more than 1 year (6). A creatinemia and proteinuria below 150 µmol/l and 1 g/24 h respectively have been defined as acceptable thresholds. This definition has been accepted by the two main European and

Table 2 | Summary table of studies demonstrating the implication of B cells in kidney-transplant operational tolerance.

Reference	Groups studied	Implication of B cells
Louis et al. (44)	TOL/STA/CR	TOL display more circulating B cells compared to STA and CR
Brouard et al. (45)	TOL/STA/CR/HV	Transcriptional signature in OT patients. Footprint of 49 genes, several genes implicated B cells, CD79a,b, CD19, CD20
Pallier et al. (46)	TOL/STA/CR/HV	Higher absolute number and frequency of total B cells in blood. Increased expression of BANK-1, CDId, CD5, FCγRIIb in TOL vs. STA
Newell et al. (4)	TOL/STA/HV	B-cell signature in TOL patients, increase of naives B cells and increase of IL-10 expression in TOL vs. STA
Sagoo et al. (5)	TOL/STA/CR/HV	Increase number of B cells and TGF-b producing cells in TOL vs. STA and CR
Danger et al. (47)	TOL/STA	Over-expression of miR142-3p in B cells and increase of TGF-Bl expression in B cells from TOL vs. STA
Silva et al. (48)	TOL/STA/CR/HV	Transitional B cells from TOL preserved ability to activate the CD40/STAT3 signaling pathways in transitional B cells in contrast with CR
Haynes et al. (49)	TOL/STA/CR	Increase of circulating naive B cells in TOL vs. STA and CR. Higher POT score ("probability of being tolerant": score including B-cell parameters and direct pathway T-cell parameters) in TOL vs. STA and CR
Chesneau et al. (50)	TOL/STA/HV	Less plasma cells in TOL vs. STA. *In vitro* their is a default in B-cell differentiation and an increase B-cell sensitivity to apoptosis in late step of differentiation of B cells from TOL vs. STA. Increase of IL-10 expression by activated B cells in TOL vs. STA

American consortia[1,2]. Even if the experiments are performed in the peripheral B cells, there is a certain amount of research indicating a possible involvement of these cells in the mechanisms of tolerance in OT patients. A study, published in 2006, reported that chronic rejection recipients display a significantly lower absolute number of B cells compared to tolerant recipients (44). One year later, we described a specific, sensitive peripheral transcriptional signature in OT patients. Inside the footprint of the 49 genes identified, several genes implicated B cells, such as CD79a, b, CD19, and CD20 (45). Following this study, we reported on an increase in both the absolute number and frequency of total B cells in the blood from 12 tolerant kidney-transplant recipients, due in particular to an expansion of activated and memory B cells. These cells had an inhibitory phenotype, defined by the increased expression of BANK-1 (which negatively modulates CD40-mediated AKT activation), CD1d, and CD5, as well as a decreased proportion of CD32a/CD32b (46). The same year, Newell et al., sponsored by the Immune Tolerance Network (ITN), studied 25 tolerant kidney-transplant recipients. They sought to identify immunity parameters to discriminate tolerant recipients from subjects with stable allograft under immunosuppression, as well as healthy controls (4). This study included the identification by microarray and real-time PCR of 30 genes, over two thirds of which are B-cell specific, that distinguish between tolerant and non-tolerant individuals. Most notably, the tolerant cohort differentially expressed three B-cell genes (IGKV4-1, IGLL1, and IGKV1D-13) that proved to be highly predictive of tolerance in a new set of patients. Moreover, the analysis of the phenotype of peripheral blood B cells revealed an increase in naïve and transitional B cells in the tolerant group. Finally, the stimulated transitional B cells from tolerant recipients produced more IL-10 *in vitro* compared to the non-tolerant group, even if this secretion remained very low. As Il-10 is one of the main features of regulatory B cells, these data suggest that OT patients could present a larger subset of regulatory B cells than the non-tolerant patients (4). Recently Newell et al. showed that IGKV1D-13 appears as the most stable and discriminating gene and propose this single gene as a signature of operational tolerance (Personal communication, ATC, Boston, 2012).

Another multicenter study, including 11 tolerant kidney-transplant recipients conducted by the RISET consortium reported similar results: they also found over-expression of B-cell related genes in OT patients compared to the other groups of patients (stable patients, chronic rejection, and healthy controls) by microarray analysis, and a displayed increase in B cells and NK cells (5). Interestingly, they also reported a relative increase in TGF-β producing cells in OT patients.

Taken together, these findings show that OT patients have a particular blood B-cell phenotype and suggest that B cells may participate in or contribute to the maintenance of long-term graft function in these patients. These three studies were partly validated by a recent study describing a significant increase in circulating naïve B-cell numbers in OT patients compared to stable treated patients or chronically rejecting patients, with a higher POT score ("probability of being tolerant": score including B-cell parameters and direct pathway T-cell parameters (5), in the OT group (49). However, the donor-specific indirect pathway analysis in this study revealed a B-cell independent, IL-10 independent but TGF-b dependent signature in the OT group (49). So, the precise mechanisms by which B cells induce tolerance remain elusive and are not as clear as in animal models. Interestingly, among the specific blood signature of the 49 genes associated with tolerance, 27% of the genes modulated in the blood of OT patients could be regulated by the TGF-b (45). We ourselves recently showed that miRNA 142-3-p is up-regulated in B cells of OT patients with a stable

[1]www.risetfp6.org/
[2]http://www.immunetolerance.org/In

expression over time. This study suggests that a negative feedback loop involving TGF-β signaling and miR-142-3p expression in B cells may contribute to the maintenance of tolerance (47).

A recent study reported a preserved BCR repertoire in OT patients, similar to that in healthy individuals (48). In addition, tolerant patients also displayed a conserved capacity to activate the CD40/STAT3 signaling pathways in transitional B cells, in contrast to patients with chronic rejection. The authors conclude that the B-cell regulatory compartment is preserved in OT patients (48). However, these results need to be confirmed, the numbers of OT patients being low ($n = 5$).

A recent study by Nouël et al. studied B cells in chronic rejection. They demonstrated that B cells from chronic rejection patients are unable to efficiently inhibit autologous T-cell proliferation, as B cells from stable patients or healthy volunteers can. Indeed, B-cell inducing tolerance could be explained by a preserved B-cell compartment (60).

More recently, B cells in operational tolerance in kidney transplantation was also analyzed in the study of Chesneau et al. In this study, we show that B cells from tolerant patients display a lack of plasma cells compared to stable patients that may be due to a default in *in vitro* B-cell differentiation and an increase of B cells sensibility to apoptosis in late step of differentiation of B cells from tolerant patients. Furthermore in this study we show that activated B cells from tolerant patients secrete more IL-10 compared to healthy volunteers and stable patients. This study reinforce the potential regulatory properties of B cells in tolerant patients with an over-expression by B cells of IL-10 after *in vitro* stimulation in tolerant compared to healthy volunteers and stable patients and a default in plasma cell/naives cells balance in tolerant compared to stable patients (50).

Finally, in a model of tolerance induction based on a combined kidney and bone marrow transplantation in humans, Porcheray et al. showed that in three-quarters of tolerant patients, *de novo* antibodies specific to donor antigens and/or C4d deposition in the graft developed. This antibody response coincided with B-cell reconstitution and a high frequency of peripheral transitional B cells (61). The involvement of B cells in the mechanisms of tolerance is not limited to OT patients but also concerns also patients with therapeutic induced tolerance.

CONCLUSION

Interestingly, in contrast to kidney tolerant recipients, in liver transplantation around 20% of patients can be successfully weaned off immunosuppression (62, 63). Contrary to kidney-transplant patients, these liver tolerant patients do not present an increase in the absolute numbers of peripheral blood B-lymphocytes, no modification of the different subsets of B cells and no B-cell transcriptional pattern but a NK cell signature (64). These data suggest that the mechanisms involved in the induction and maintenance of this tolerance process remain as yet undetermined and probably differs depending on the kind of organ transplanted. In kidney transplantation, whether these regulatory B cells are a driving force for tolerance induction, or whether they simply help to stabilize tolerance in the absence of immunosuppression, has not been established. Since the first cases of OT were described, the problem of inadequate comparators has remained unsolved. This paradox is due to the clinical situation of patients who display stable graft function but no longer receive immunosuppression. Healthy volunteers share with these patients the absence of immunosuppression but have not received a transplant, whereas stable patients share graft function stability but are under immunosuppression. This absence of adequate controls remains a difficulty in the comparison immunological parameters.

Nonetheless, all these data lead us to increasing exploration of various therapeutic approaches to inducing tolerance by promoting the development of B cells with regulatory functions. In fact, similarly to Treg, studies suggest that regulatory B cells present varying abilities to suppress immune responses depending on the environment, on the trigger, and probably by using different mechanisms (65). These characteristics will have to be considered and studied, before developing new therapeutic strategies.

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Annexe 2

Biomarqueurs et mécanismes possibles de tolérance opérationnelle chez les patients tolérant une greffe rénale.

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Biomarkers and possible mechanisms of operational tolerance in kidney transplant patients

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Summary: A small number of patients do not reject their graft after weaning from immunosuppressive treatment. Here, we analyze the studies carried out to try to understand the mechanisms involved in this operational transplant tolerance and evaluate the hypotheses proffered on these potential mechanisms.

Keywords: *biomarker, kidney transplantation, operational tolerance*

Introduction

When a disease irreversibly alters kidney function, the treatment of choice is to undergo kidney transplantation. As every single human, aside from identical twins, is genetically different, transplantation is allogenic, and without treatment, a graft will be rejected. Despite dramatic improvements in graft survival during the year posttransplantation, thanks to the discovery of a range of immunosuppressive drugs (1), there are still major problems regarding long-term graft loss (2) and the development of new pathologies related to long-term immunosuppressive drug treatment (3–8).

Which solutions could solve both problems of chronic damage/rejection and immunosuppressive drug toxicity? Currently, research is being conducted to find less harmful, more efficient treatments and/or to minimize immunosuppressive drug treatment to decrease their negative effect on the patient. Ideally, the absolute goal in kidney transplantation is to reach a state of fully functional graft in the absence of immunosuppressive treatment, a situation known as 'tolerance' (9).

Tolerance

The concept of tolerance emerged in the middle of the previous century with Billingham, Brent, and Medawar (10),

who showed that it is possible to induce skin allograft tolerance in mice by inoculating foreign homologous tissue cells during the fetal period. Two different types, or phases, of tolerance, spontaneous tolerance and induced tolerance, are described in the literature. They are independent and probably involve different mechanisms. Numerous studies have been performed on animal models in which induction protocols for obtaining tolerance (11, 12) have been described (13), but unfortunately many of these models are not transposable to human. All tolerance induction protocols are based on two natural phenomena known as central and peripheral tolerance. These protocols include the induction of mixed allogenic hematopoietic chimerism, used to induce tolerance via central tolerance mechanisms (14, 15) and the blockade of costimulation signals, using peripheral tolerance mechanisms (16–18).

As the name suggests, in contrast to induced tolerance, 'spontaneous' tolerance does not follow a tolerance induction protocol and is usually observed by chance when transplanted recipients no longer taking their immunosuppressive drugs do not reject their graft. Spontaneously tolerant patients stop their immunosuppressive treatment for two major, distinct reasons; non-compliance and the occurrence of deleterious side-effects of the immunosuppressive drugs (8, 19, 20). Despite weaning off immunosuppressive drugs, these patients conserve good graft function and resistance against infection (21). The term 'operational' is often used to characterize these patients, since their tolerance status is only defined by the graft function, given that no biopsy can be performed to confirm normal histology (22, 23). 'Spontaneous operational tolerance', while more frequently observed in liver transplants (24–26), is also observed in some rare cases of kidney transplant. This review focuses on such 'operationally tolerant' kidney transplant patients.

About 100 cases of kidney operational tolerance have been described worldwide since the 1970s (27–31). The majority of these patients had immunosuppressive treatment involving azathioprine and corticosteroids, and the reason for weaning was mainly non-compliance. There is no differentiation between these patients as to whether they received a kidney from a deceased or living donor, and the number of HLA incompatibilities is at the same level as in other transplant recipients (23, 27–36). This tolerance process is interesting for several reasons. First, deciphering its mechanisms would allow a better understanding of graft survival in transplantation. Secondly, this tolerance appears as the ultimate solution for graft rejection/loss. Identifying tolerance biomarkers would thus be of interest for detecting patients among a large cohort of patients under immunosuppression, who could benefit from an improved regulation of their treatment and better control of any secondary effects.

Tolerance biomarkers

According to the Biomarker Definitions Working Group, a biomarker (Biological marker) is 'a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention' (37). A biomarker can be in various forms such as protein, sugar, lipid, gene, single nucleotide polymorphism. In renal transplantation, two biomarkers are routinely used: creatinine and proteinuria levels. These markers give essential information on kidney function and allow the medical staff to adapt the patient's treatment. Other biomarkers are more indicative of a precise pathological process, such as the C4d complement molecule (38, 39), but none of the commonly used biomarkers of graft rejection, merely reflecting the functional status of the kidney in the moment. However, many studies are now focusing on finding predictive rather than diagnostic biomarkers in order to adapt patient treatment more rapidly and to intervene in graft rejection episodes more quickly (40). Many different technological processes are being used to define these new biomarkers, including transcriptomic (32, 41), proteomic (42), genomic (43), epigenetic (44), peptidomics (45), metabolomics (43), enzyme-linked immunosorbent assay (ELISA) (46), electrophoresis (42), flow cytometry (47) and immunohistochemistry (48).

In the search for new biomarkers and more specifically tolerance biomarkers, there are two major dilemmas. The first concerns the origins of the samples, and the second is the control population. Regarding the origin of the samples, peripheral blood, graft biopsy (48), and urine have all been used for analysis (34). In kidney transplantation, the graft biopsy is recognized as the 'gold standard' for rejection diagnosis (39). However, in most cases, operationally tolerant patients refuse biopsy and it is ethically questionable to perform a biopsy of a fully functional graft. Cellular infiltrates have been reported to be very low in tolerant kidney graft (48) and would therefore probably not yield a great deal of information. Analyzing the peripheral blood has the advantage of being less invasive and less expensive than biopsy, which makes it the main method used to analyze gene profiles. Unfortunately, this probably does not always

reflect what is happening in the graft (49). In kidney transplantation, analyzing urine may have several advantages: its collection is non-invasive and inexpensive and urine is in direct contact with the grafted organ, which could give relevant information on kidney function (50, 51).

The other significant dilemma is choosing the right control population, which is not easy for tolerant patients. On one hand, these patients have been grafted and have good graft function, so we might suppose that stable patients under immunosuppressant treatment would be the best control, but the absence of immunosuppressive drugs in the tolerant patients could influence the results. On the other hand, absence of treatment makes tolerant patients similar to healthy individuals, but we cannot ignore the absence of transplantation in the latter group. Comparison of tolerant patients with patients undergoing chronic transplant rejection has also been used, but these patients are clinically very different. It cannot be excluded that some tolerant patients could present functionally undetectable subclinical graft lesions. The absence of systematic kidney biopsies for these tolerant patients prevents any conclusions from being drawn. Faced with the lack of the perfect control population, the use of multiple controls may be the best alternative.

In the last 10 years, a number of studies have been conducted to identify new, robust, tolerance biomarkers, and two major consortia have been involved in the discovery of such biomarkers: the Immune Tolerance Network in the USA (http://www.immunetolerance.org/In) and the Indices of Tolerance in Europe (http://www.risetfp6.org/). The existence of such consortia is essential, as operationally tolerant patients are rare (as previously stated, less than 200 known in the world), and so it is the only way to develop multicenter studies and to have access to larger cohorts of patients. Despite an effort to coordinate such studies, the studies that report on operationally tolerant kidney patients are very heterogeneous, either due to the techniques and controls used or to the various clinical profiles of the tolerant patients (22, 23, 32–36, 48, 52–63) (*Table 1*). Moreover, the cohorts studied are small, which prevent a statistical approach. Finally, the lack of biopsies is a problem in these patients, as some indications of graft deterioration may not be detected (64).

Potential mechanisms involved in operational tolerance

Depending on the status of the patient, biological differences can be observed. These differences are probably due to the different mechanisms involved following kidney transplantation. The mechanisms involved in the human tolerance process are complex and difficult to decipher. Besides differing and depending on patient history and great inter-individual variability (22), these mechanisms are also different according to the type of organ grafted (59).

The role of regulatory T cells in human tolerance

Different sub-groups of regulatory T cells (Tregs) have been described in transplantation: naturally occurring (nTregs) and induced regulatory T cells (iTregs). In addition to iTregs, Th3 cells are induced by transforming growth factor-β (TGF-β) and Tr1 cells by interleukin-10 (IL-10) (65, 66). In most cases, Treg cells express the transcription factor Foxp3, with the exception of IL-10 induced Tregs (Tr1) (67). Miyara *et al.* (68) describe distinct Treg subpopulations that depend on CD45RA expression and the levels of Foxp3, $CD45RA^{+}FoxP3^{lo}$ resting Tregs, $CD45RA^{-}FoxP3^{hi}$ activated Tregs, and $CD45RA^{-}FoxP3^{lo}$ non-suppressive T cells. The proportion of these Treg cells is altered in immunological diseases like systemic lupus erythematosus and in aging (68), and several studies have shown their role in tolerance induction in animal transplantation models (69, 70).

The presence and the role of regulatory T cells in tolerance in human remain debatable (51, 71). Regarding the frequency of regulatory T cells in these patients, they have been shown to be at the same levels in tolerant patients, stable patients, and healthy controls, whereas the levels are lower in blood from chronic rejection cases (54, 58). These data suggest that chronic rejection may be linked to a lack of regulation (47, 54) and also that these regulatory cells may have migrated from the blood of operationally tolerant recipients in another compartment. The latter alternative is supported by Becker *et al.* (48), who reported on cellular infiltrates with a greater level of $Foxp3^{+}$ regulatory T cells in biopsies from 4 operationally tolerant kidney recipients compared to patients with interstitial rejection, borderline changes or long-term allograft function without rejection (48). They also reported on an association between the $Foxp3^{+}$ Tregs cells and the HLA-DR molecule, which is characteristic of Tregs with strong immunosuppressive capacities (72).

It is thus clear that some T cells with a Treg cell phenotype may be seen in operationally tolerant recipients, but what is their role? Using the DTH test, it has been demonstrated that T-helper 1 (Th1) and Th2 T cells induce activation of macrophages and B cells via pro-inflammatory

Table 1. Majors information from studies including operationally tolerant kidney allograft cohorts

Number of tolerant patients	Donor status	HLA incompatibility	Cause of discontinuation	Period of tolerance (years)	Tissues analyzed	Technical methods used	Control groups	Biomarkers/majors information	References
3	1D and 2L	2–0–5	Uncompliance	5-27-8	Blood	DTH	STA/AR	Weak donor-reactive DTH response in TOL and TGF-β or IL10 dependant.	Vanbuskirk *et al.* (52)
5	ND	3 or ND	Uncompliance-CNItox- PTLD	3–12	Blood	TcLandscape and qPCR	LD/HI/CR/STA	Altered CDR3-LD in TOL CD8+T cells, abnormal Vβ/HPRT transcript ratios and lack of cytokines transcript accumulation in TOL	Brouard *et al.* (53)
10	ND	0–4	Uncompliance—initial renal degradation—PTLD	1–12	ND	ND	ND	Operational tolerance = stable graft function at a good or acceptable level for several month or years in the absence of immunosuppressive treatment	Roussey-kesler *et al.* (23)
8	7D and 1L	0–4	Uncompliance-CNItox- PTLD	0.5–17	Blood	FC/qPCR/ELISA/Proliferation test	LD/HI/CR/STA	Higher level of CD4+CD25+regulatory T cells in TOL versus CR. Higher level of B cells in TOL versus CR and STA. Tolerant profile similar to HI	Louis *et al.* (54)
6	ND	ND	Uncompliance-lymphoma	2–12	Blood	FC/Proliferation test/RT-PCR and Cytotoxicity-Associated Degranulation Assay	CR/HI/STA	CD8+ cells pattern similar between TOL and HI but different between TOL and CR	Baeten *et al.* (55)
5 + 12	D and L	2–4	CNI tox/PTLD/uncompliance	8.8 ± 4.9	Blood	Microarray analysis/RT-PCR	CR/HI/MIS/AR	Set of 49 genes differentially expressed by TOL many of which are related to TGF-β. Three clusters: 1: reduced immune activation; 2: downregulation of signal transduction genes and RNA binding genes and 3: upregulation of cell-cycle regulator genes/underexpression of costimulatory genes/immune quiescence and ignorance of donor Ag.	Brouard *et al.* (32)
8	1L and 7D	0–4	CNI tox/PTLD/uncompliance	1.6–17.2	Blood	Microarray analysis/RT-PCR	CR/HI	223 differentially expressed genes (12 functional groups among the downregulated genes). Genes implicated in immune response and host defense	Braud *et al.* (56)
8	1L and 7D	0–4	CNI tox/PTLD/uncompliance	ND	Blood	RT-PCR/FC	STA/CR/RF/HI	Higher level of MyD88 and TLR4 mRNA in CR versus Tolerant PBMC. Higher number of $TLR4^+$cells in CR versus TOL. Tolerant profile similar to HI	Braudeau *et al.* (57)
5	4L and 1D	0–4	Uncompliance	1.5–8	Blood	RT-PCR/FC	LD/STA/CR/HI	Higher level of $CD4^+CD25^+$regulatory T cells in Tolerant versus CR. Tolerant profile similar to HI. Decreased in the activation of the IL4/STAT6 pathway in monocytes. High expression of IL4Rα in PBMC	Moraes-Vieria *et al.* (58)
12	1L and 11D	0–4	CNI tox/PTLD/uncompliance	3–21	Blood	RT-PCR/FC/Microarray/Multiplex screening test	STA/CR/HI	High number of B cells with activated-memory phenotype and expressing costimulatory molecules. Upregulation of B-cells associated genes related to cell-cycle, proliferation, development and maturation. B cells have an inhibitory profile (decreased in CD32a/CD32b ratio, accumulation of BANK1, higher number of $CD5^+$ B cells and $CD1d^+$ B cells). Transcriptional profile favoring B-cell survival	Pallier *et al.* (35)
35	ND	ND	ND	ND	Blood	FC/Luminex/ELISpot/RT-PCR/MicroArray	HI/STA low prednisone/STA withoutCNI/STA with CNI/CR	Increase in B and NK cells, altered T/B cells ratio, altered expression of B cells genes in TOL, altered ratio of transitional and memory B cells, increased number of TGF-β-producing B cells	Sagoo *et al.* (36)
25	18L and 5D (2 data missing)	0.83 ± 1.59 and 0.67 ± 1.15	Medical reason or uncompliance	1–32	Blood Urine	RT-PCR/FC/Microarray/Massarray	STA/HI	Increased expression of B cells related genes in TOL compared to STA (principally B cells differentiation genes). Increased number of B cells in TOL versus STA and HI. Increased in IL-10 secreting B cells in TOL (need to be confirmed)	Newell *et al.* (34)
12	ND	0-5	Uncompliance	2–13	Blood	Microarray/FC/RT-PCR	STA/CR/HV	Clear difference of gene expression profile between liver and kidney tolerance. Liver TOL linked to $CD56^+$ lymphocyte and liver non-TOL linked to $CD19^+$ and	Lozano *et al.* (59)

Table 1. (*Continued*)

Number of tolerant patients	Donor status	HLA incompatibility	Cause of discontinuation	Period of tolerance (years)	Tissues analyzed	Technical methods used	Control groups	Biomarkers/majors information	References
11	ND	ND	ND	ND	Blood	Microarrays	STA/CR/HI	$CD4^+$ lymphocytes, kidney TOL linked to $CD19^+$ lymphocytes and kidney STA linked to $CD14^+$ and $CD56^+$ lymphocytes. 35 genes differentially expressed in kidney TOL 24 of which expressed by B cells Lower expression of PARVG gene in TOL versus STA and CR. 2 SNPs for this gene	Danger *et al.* (60)
5	4L and 1D	0-4	Uncompliance	1.5–8	Blood	RT-PCR/FC	LD/STA/CR/HI	Higher expression of GATA3 (Th2 transcription factor) in TOL versus STA/CR/LD. TOL have higher percentage of regulatory type of gene expression and lower inflammatory type gene expression compared to other groups. Higher expression of TGFB1 gene and its receptor in TOL versus STA and LD	Morales-Viena *et al.* (61)
5	4L and 1D	0-4	Uncompliance	1.5–8	Blood	FC/BCR repertoire analysis (immunoscope)	LD/STA/CR/HI	Decreased number of B cells in CR and lower BCR diversity for IgM. Overrepresentation of the 16-aa CDR3 length in VH3 Ig family and the 5-aa CDR3 length in the CH1 Ig family. Higher capacity to generate pSTAT3$^+$Breg in TOL versus CR	Silva *et al.* (62)
15	12L and 3D	0-4	ND	1.8–14.9	Blood	TLDA/Taqman miRNA assays/ microarray	STA/HI	4 miRNA overexpressed and 4 under expressed in TOL versus STA. miR142-3p is over expressed in TOL and downregulated after PHA/IL2 stimulation of PBMCs. Higher expression of the miR142-3p in B cells from TOL versus STA but same level than HI. Over-expression of miR142-3p in B cells from TOL affects the TGF-β pathway. TGF-β 1 expression is increased in B cells of TOL. Negative feedback loop between TGF-β and miR142-3p in B cells	Danger *et al.* (63)
27	ND	0-5	CNI tox/PTLD/ uncompliance/ cancer		Blood Biopsy	Multiplex screening/ LCT/ELISA/ Luminex/IHC	STA/REJ	REJ group who stop the IS are younger than TOL group. Higher proportion of HLA-matching in TOL. Less induction therapy in TOL. Loss of tolerance in more frequent in presensitized patients	Brouard *et al.* (22)
4	ND	ND	ND	ND	Biopsy	IHC	IR/BC/NR	Presence of cellular infiltrate in TOL but similar to other groups. Higher level of $Foxp3^+$cells among infiltrating cells in TOL $Foxp3^+$ cells in cellular agglomerates often associated with HLA-DR in TOL. Lower expression of PI3K and c-rel in TOL versus IR and BC	Becker *et al.* (48)
11	ND	ND	ND	ND	Blood	DTH/FC	TWIN/LD/ STA//CR	Lower indirect pathway Teff response and higher regulatory response in TOL. Importance of Th3- TGFβ in the regulatory response. Higher number of naïve B cells in TOL. No effect of B cells on the indirect pathway	Haynes *et al.* (33)
9	3L and 6D	0-4	ND	9.54–28.65	Blood	Immunophenotyping/ apoptosis and proliferation test/ qPCR/microarray/ cytokines and Ig measurement	STA/HI	Different B-cell subset frequencies between STA and TOL. TOL B cells maturate activate and produce IgM and IgG *in vitro*. Defective expression of molecules involved in later stages of differentiation in B cells from TOL. Tolerant B cells have normal proliferation and higher IL-10 production *in vitro*. B cells from TOL show higher cell death susceptibility than STA. TOL B cells downregulate B-cell differentiation genes and anti-apoptotic genes	Chesneau *et al.* AJT (2013, in press)

STA, stable with normal regiment of immunosuppression; AR, acute rejection; LD, low dose of immunosuppression in stable patient; CR, chronic rejection; MIS, minimal immunosuppression in stable patient; HI, healthy individual; CNI, calcineurine inhibitor; Rej, rejection; IR, interstitial rejection; BC, borderline changes; NR, long-term allograft function without rejection; TOL, tolerant; D, deceased donor; L, living donor; PTLD, posttransplant lymphoproliferative disorder; ND, not determined; RT-PCR, real time-polymerase chain reaction; qPCR, quantitative polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; IHC, immunohistochemistry; BCR, B-cell receptor; TLDA, TaqMan Low Density Array; DTH, delayed-type hypersensitivity; Ag, antigen; PBMC, peripheral blood mononuclear cell; TLR4, Toll-like receptor 4; FC, flow cytometry.

secreted cytokines [tumor necrosis factor (TNF) and interferon-γ (IFN-γ)] (73–75). In tolerant patients, Haynes's (33) and VanBuskirk's (52) groups both reported on a decreased DTH response specific to the donor and dependent at least partially on TGF-β in blood from operationally tolerant patients. They reported on the presence of regulatory T cells in the blood from these patients, rather than Th3 regulatory cells induced by TGF-β, as they observed a latent indirect T-effector response after the blockade of TGF-β for four of five tolerant patients (33). The possible involvement of TGF-β signaling in operational tolerance was first reported by our group, who show that 30% of the genes differentially expressed in blood from operationally tolerant patients are modulated by TGF-β (32). These data have been confirmed in other studies (33, 36, 52, 61). Interestingly, while TGF-β modulates gene expression in blood from operationally tolerant recipients, its expression is not different in tolerant patients compared to other transplant recipients (32, 61), suggesting the existence of a feedback loop and fine-tuned regulation in these patients. This was demonstrated by Danger *et al.* (63), who reported on the regulation of TGF-β by mir142-3p in blood from operationally tolerant recipients and inversely (63). Finally, Moraes-Vieira *et al.* (61) reported on an increase in the mRNA and protein level of GATA3, a major transcription factor of Th2 cells, in blood from operationally tolerant recipients, which may lead to a pro-tolerogenic systemic environment (61).

Very little information is available on the $CD8^+$ T-cell compartment. However, it has been shown that $CD8^+$ T cells from tolerant recipients do not accumulate IL-2, IL-8, IL-13, TGF-β or IFN-γ cytokines, suggesting their hyporesponsiveness (53), whereas patients with chronic rejection exhibit a higher level of peripheral $CD8^+CD28^-$ T cells with cytotoxic properties and impairment of their apoptosis process (55). No data are available regarding the possible involvement of $CD8^+$ Tregs cells in these patients, this having been principally described in murine models where the cells exert a protective role on the graft and generate long-term allograft acceptance (76, 77).

How can these different studies and the possible role of Treg in the tolerance process in human be reconciled? One possible scheme would be that Th3 TGF-β-dependent Tregs recruited in the graft may lead to inhibition of the local inflammatory response in operationally tolerant patients, a reduced anti-donor DTH response and an increase in TGF-β-dependent genes in the blood, leading to a pro-tolerogenic Th2 environment, concomitant with the absence of or a hyporesponsive $CD8^+$ cytotoxic T-cell response.

The role of B cells in human tolerance

After the initial identification of a set of genes associated with operational tolerance (32), transcriptional analysis shows a B-cell signature in blood from tolerant patients compared to stable patients receiving immunosuppressive therapy (32, 36). Newell *et al.* (34) confirm these data and show that this B-cell signature is also present in the urine of these patients. This B-cell signature is thus reproducible in the different cohorts of operationally tolerant patients around the word (32, 34, 36). Brouard *et al.* report that this B-cell signature is associated with an increase in the absolute number and frequency of B cells in the blood of these patients (35, 54) and with an inhibitory phenotype, such as an over-expression of FCγRIIb, a receptor transducing inhibitory signal (78) and BANK-1, which negatively modulates B-cell CD40-mediated AKT activation (35, 79) compared to stable patients or patients with chronic rejection. These data were reproduced in a rat model of long-term cardiac allograft tolerance induced by short-term immunosuppression (80). In addition, Newell *et al.* (34) show that this increase in B cells may be in part explained by an increase in circulating transitional B cells, which secrete more IL-10 after *in vitro* polyclonal stimulation, in the blood from these patients. These data, which suggest that a 'regulatory' role of B cells may be involved in operationally tolerant patients, were confirmed by Silva *et al.* (62), who reported on a preserved functional B-cell compartment in blood from operational tolerant patients and healthy volunteers with normal capacity to phosphorylate signal transducer and activator of transcription 3 (STAT3) after activation, compared to patients with chronic rejection. Finally, these data are reinforced by recent findings from Chesneau *et al.* (manuscript in press), who report on a lack of plasma cells in blood from these tolerant patients, compared to stable patients, which may be due to a fault in B-cell differentiation and an increase in B-cell sensibility to apoptosis in a late stage of differentiation in B cells from tolerant patients.

These data on B cells, with a possible increase in B-cell populations with regulatory properties, and a decrease in plasma cells producing deleterious antibodies, in these tolerant patients, are very encouraging and have been reproduced in varying studies in different cohorts of tolerant patients, but to date no functional data allow us to

specify the role of B cells in the process. If confirmed by further studies, reconciling this possible role of B cells with a concomitant role for T cells will not be difficult due to their well-documented interaction and the strong link between cellular and humoral immunity (81, 82). Interestingly, some data could indicate common regulation mechanisms for these T and B cells, such as the expression of mir142-3p or TGF-β, and that both play a role in regulatory cell function (83).

Innate immune cell involvement in transplant tolerance

While extensive evidence suggests the presence of and, possibly, a role for adaptive immunity with T and B lymphocytes in operational tolerance, what about that of innate immune cells? These cells are principally involved in a response against pathogens, but there is now evidence of their involvement in graft rejection (84).

Several studies report a NK cell profile in the blood of liver graft tolerant patients (59, 85, 86). This NK signature was only reported in blood from kidney transplant recipients in a study from Sagoo *et al.* (36) and compared to healthy volunteers in the study from Newel (34). These conflicting results may be due to different immunosuppressive regimens and the presence or absence of donor-specific antibodies, which have been clearly shown to influence NK cell phenotype and frequency (87) or to the heterogeneity of the cohorts of patients. Braudeau *et al.* (57) reported on a decrease in TLR4 and Myd88 mRNA levels in blood from tolerant patients associated with lower levels of $TLR4^+$ monocyte cells (57). These results were confirmed by Sagoo *et al.* (36), who showed a decrease in expression of two monocyte-related genes, TLR5 and SLC8A1, in blood from tolerant patients compared to patients with chronic rejection. TLR4 and TLR5, the

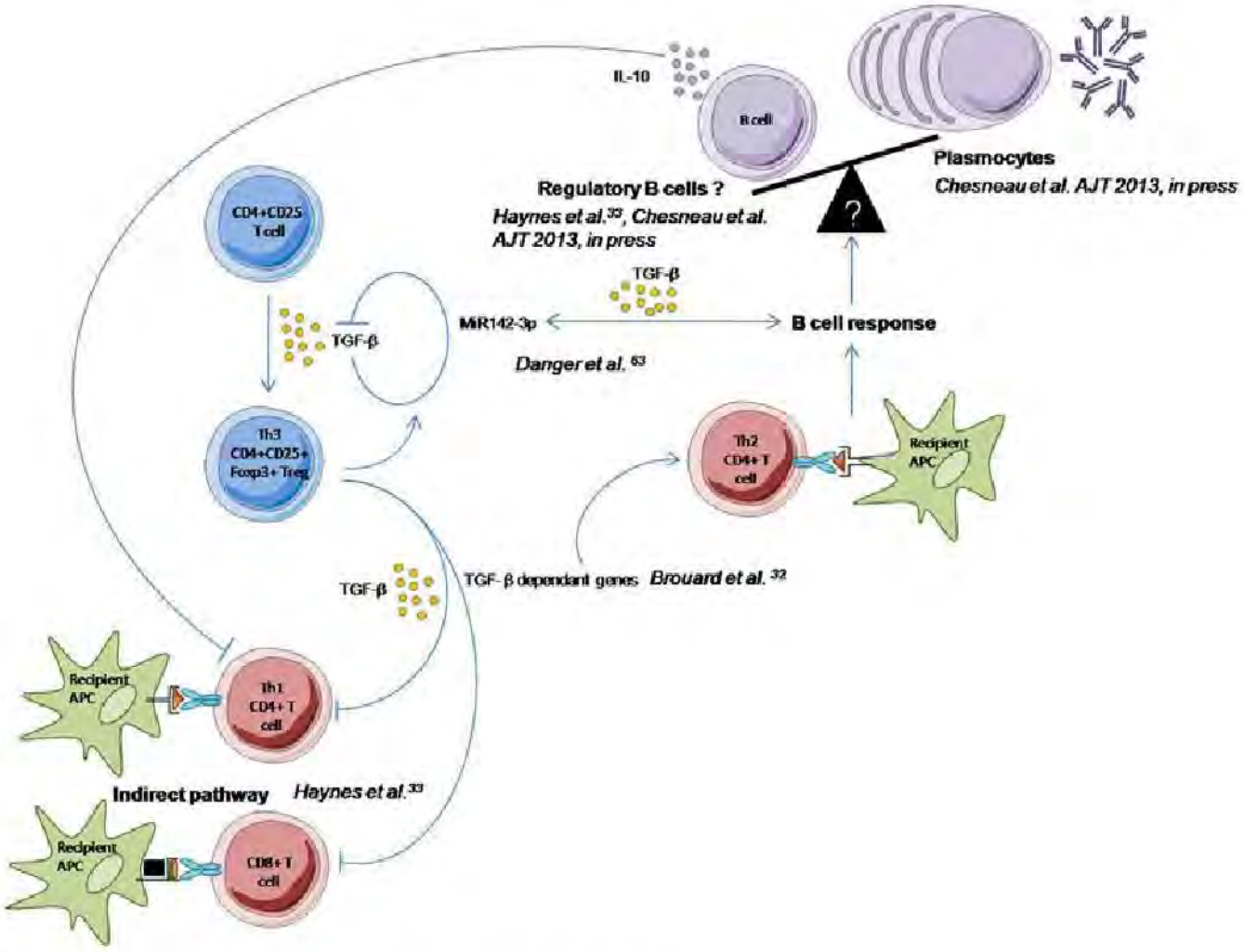

Fig. 1. Hypothesis on the T- and B-cell involvement in a tolerant patient.

receptors of LPS and flagellin, recruit the adapter protein MyD88, which leads to the activation of NF-κB, which in turn induces cytokine secretion and an inflammatory response (88). The absence of the adapter protein MyD88 induces kidney allograft tolerance in a fully MHC-mismatched allograft mouse model (89). These data, which suggest an absence or reduction in the TLR-MyD88 signaling pathway in blood from operationally tolerant recipients, are consistent with a decrease in SLC8A1 mRNA, a gene coding for a protein that controls the Ca^{2+} signal and induces the production of TNF-α, a strong pro-inflammatory cytokine (36). Altogether, the data suggest that a lower level of these molecules in tolerant patients may participate toward a pro-tolerogenic environment that is absent in patients with chronic rejection.

Tolerance profile: specific or a highly controlled physiological cell homeostasis?

Animal models give a lot of information about and show possible research paths toward tolerance, but such studies are not always relevant to human cases. Studies in non-human primate models are informative, and some new immunosuppressive drugs have been shown to be very promising (90). The immune processes occurring during operational tolerance following a kidney allograft are very complex and involve different cell types. Nevertheless, it is noteworthy that although operationally tolerant recipients (both adaptive and innate immunity) differ from patients with chronic rejection on large number of points, they have striking similarities to healthy volunteers, with the same $CD4^+CD25^+$ Treg, NK, $CD8^+$, and B-cell levels (34–36, 54, 55, 58, 62) and also conserved signaling pathways such as STAT3, GATA3, mir142-3p, TGF-β, and TLR4/MYD88 (36, 57, 61–63), all suggesting that this tolerance may be due to a highly controlled physiological cell homeostasis, such as control of indirect Th17 and Th1 responses (33), conserved B-cell activation (62), and an anti-inflammatory environment (32) that, if disturbed, may lead to chronic rejection. The similarity between the tolerance and 'normal' profiles also raises the question of a specific tolerance signature and an absence-of-treatment profile. Indeed, some minimally immunosuppressed patients, under corticosteroids, show a similar transcriptional profile (32) and display an indirect T-cell response close to that of tolerant patients (33). However, several reports also suggest strong differences between operationally tolerant recipients and healthy volunteers: the transcriptional blood profile of operationally tolerant recipients is not shared by healthy individuals (32), B cells from tolerant patients express specific molecules with inhibitory properties, such as BANK1, FCγRIIb (35), and B cells from operationally tolerant patients do not fully terminally differentiate *in vitro*, whereas B cells from healthy volunteers do (Chesneau *et al.*, in press). Haynes's group suggest that B cells could play a role in the maintenance of tolerance but not in its induction and that their development, like that of Tregs, may be due to the progressive weaning off immunosuppression, which may allow regulatory populations to emerge. Perhaps there are also some specific treatments which help this maintenance and/or induction phenomenon (91–94). Finally, histological analysis of a graft would give significant information on the patient's status. This seems difficult to envisage in patients with a well-functioning graft and no clear need for biopsy, but would probably be the only way to ascertain the role of B cells in tolerance, as suggested by results in animal models (80), where the isolation and injection of B cells with the same characteristics as human B cells from tolerant patients (35) transfers tolerance to naive untreated rats.

Conclusion: the tolerance profile, cause or consequence?

At this stage, it remains to be established whether Tregs and/or regulatory B cells are a cause or an effect of tolerance. *Fig. 1* suggests a hypothesis on T- and B-cell involvement in tolerant patients. Currently, a major challenge is understanding what makes a patient move to a tolerant profile or a rejection profile. However, this still needs clearly establishing, as no evidence of overregulation of Tregs or B-regulatory cells has been established in these patients, and the different data available clearly show that the balance between regulatory and effective cells may be fundamental in such a delicate process. This process has been shown to be metastable and fragile over time, demonstrated by a non-negligible proportion of patients who lose their graft for immunological reasons or simply due to physiological age defects (22, 64). No studies have yet been performed on patients who lose their tolerance over time, and such a study would very likely be informative.

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Annexe 3

Fréquence et fonction des lymphocytes B régulateurs non modifiés chez des patients avec une sclérose en plaque.

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Unaltered regulatory B-cell frequency and function in patients with multiple sclerosis

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KEYWORDS:
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Abstract Multiple sclerosis (MS) is a chronic disease of the central nervous system (CNS) typically characterized by the recruitment of T cells into the CNS. However, certain subsets of B cells have been shown to negatively regulate autoimmune diseases and some data support a prominent role for B cells in MS physiopathology. For B cells in MS patients we analyzed subset frequency, cytokine secretion ability and suppressive properties. No differences in the frequencies of the B-cell subsets or in their ability to secrete cytokines were observed between MS and healthy volunteers (HV). Prestimulated B cells from MS patients also inhibited $CD4^+CD25^-$ T cell proliferation with a similar efficiency as B cells from HV. Altogether, our data show that, in our MS patient cohort, regulatory B cells have conserved frequency and function.

Abbreviations: MS, multiple sclerosis; CNS, central nervous system; CSF, cerebrospinal fluid; EAE, experimental autoimmune encephalomyelitis; HV, healthy volunteers; RR-MS, relapsing-remitting multiple sclerosis; PP-MS, primary-progressive multiple sclerosis; SP-MS, secondary-progressive multiple sclerosis; BFA, Brefeldin-A

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1. Introduction

Multiple sclerosis (MS) is classically thought to be initiated by autoreactive T cells recognizing peptides on myelin sheath proteins [1,2]. However, there is no compelling evidence that the frequency of autoreactive cells is higher in the periphery in MS vs. healthy volunteers and the hypothesis remains controversial [3–20]. Currently, some lines of enquiry suggest a prominent role for B cells in MS physiopathology. While the intrathecal synthesis of immunoglobulins in the CSF of sufferers is a well-known feature of MS, Serafini et al. identified the additional presence of B-cell follicles in the brain meninges of patients [21,22]. Furthermore, peripheral B-cell depletion using anti-CD20 monoclonal antibodies results in a significant improvement in the clinical and radiological inflammatory criteria of MS patients [23,24] that has been associated with the homeostasis of B cells after depletion, favoring a repopulation by B cells with regulatory properties [25].

It has recently emerged that specific subsets of B cells may negatively regulate disease symptoms in mouse models of inflammation, cancer, infection and autoimmunity [26]. In experimental autoimmune encephalomyelitis (EAE), the animal model of MS, B cell μMT-deficient mice (B cells deficient in the μ-chain transmembrane region) have higher EAE severity during the late phase of the disease compared to wild-type mice and do not fully recover [27]. Adoptively transferred wild-type B cells normalize EAE severity in μMT mice, suggesting the existence of a regulatory B-cell sub-population [28]. While the hallmark of these B-cell subsets is the secretion of IL-10, they also have been shown to exert their suppressive properties by other mechanisms: via the secretion of TGFβ [29], granzyme B [30] and/or by contact-dependent mechanisms [31,32].

In humans, some regulatory B cells have recently been identified. However, the study of these cells is still in its infancy and their phenotype and mechanisms of their action need more comprehensive description. In MS, several studies have reported a decreased secretion of IL-10 by B cells [25,33,34], but these results remain contested [35] and the function of these cells has never fully been explored. The aim of our study is to characterize the frequency and functional properties of regulatory B cells (Bregs) in the blood of MS patients by comparison with healthy volunteers (HV). We show that cytokine secretion following a 48 h stimulation of B cells was the same for our MS patients and HV. Moreover, these prestimulated B cells from MS patients were able to inhibit $CD4^+CD25^-$ responding T cell proliferation in vitro in the same manner as B cells from HV. We show that this suppressive B-cell activity is independent of IL-10 and TGFβ but dependent on contact between the T and B cells. Altogether, our data do not suggest that a lack of peripheral B-cell regulation contributes to the physiopathology of MS, at least in our cohort of patients.

2. Patients, materials and methods

2.1. Patients and healthy volunteers

The 63 patients included in the study suffered from MS defined by the revised MacDonald criteria 2005, as indicated in Table 1 [36]. Different forms of MS were included: relapsing-remitting (RR-MS, $n = 47$), primary progressive (PP-MS, $n = 10$) and secondary progressive MS (SP-MS, $n = 11$). Fourteen patients were suffering relapses at the time of sampling. The mean age was 43.1 ± 1.5 years (range: 18–73 years). All patients had been without treatment for at least 3 (immunomodulatory treatment) or 6 months (immunosuppressant drugs). Some of the patients, being recently diagnosed, had not yet been treated, some were refusing treatment, and others had low disease activity and did not wish to be treated.

Sixty-one healthy volunteers were studied in parallel as controls. The mean age, 39 ± 1.7 years (range: 18–68 years), was not significantly different from that of the MS patients. Patients and HV were recruited after obtaining informed consent. The University Hospital Ethical Committee and the Committee for the Protection of Patients from Biological Risks approved the study.

2.2. Human cell isolation and phenotype analysis

Patient and HV blood samples (100 ml) were always collected at the same time and used for parallel experiments. Fresh PBMCs were separated on a Ficoll (Eurobio®) gradient layer. 10^7 PBMCs were stained with anti-CD19-PC7 (clone SJ25C1), anti-CD24-PE (clone ML5), anti-CD38-FITC (clone HIT2), anti-CD5 APC (clone UCHT2), anti-CD27-alexa700 (clone M-T271) and anti-CD10-APC (clone HI10a) (BD Biosciences, San Jose, USA). Viability staining was performed using the live/dead cell aqua staining kit (Life Technologies, Invitrogen, Carlsbad, USA). Cells were analyzed using a LSRII flow cytometer (BD Immunocytometry Systems) and data were analyzed using FlowJo Version 9.0.1 (TreeStar).

2.3. Cell culture

B cells were purified using the B-cell isolation Kit II, Human (Miltenyi, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Purity was higher than 94%. B cells were then seeded in complete RPMI 1640 in 96-well U-bottom plates at a final concentration of 10^6 cells/ml. Stimulation using CD40 ligand (1 μg/ml, RD Systems, Minneapolis, USA) and CpG oligonucleotide (ODN 2006, 10 μg/ml) was performed for 48 h at 37 °C, 5% CO_2 PMA (250 ng/ml). Ionomycin (1 μg/ml) and Brefeldin-A (10 μg/ml) (Sigma-Aldrich, St Louis, USA) were added for the last 5 h of culture. Culture supernatants were removed and stored after 48 h to wells in which BFA had not been added. Control B cells were cultured for 48 h without stimulation.

2.4. Analyses of B-cell cytokine production and phenotype of IL-10^+ B cells

After the 48 h of culture, viability staining was first performed using the live/dead cell staining kit aqua (Invitrogen, Life Technologies). B lymphocytes were then stained with anti-CD19-PC7, anti-CD24 FITC (clone ML5), anti-CD27-alexa700, anti-CD38 PC5 (clone HIT2), and anti-CD5-APC (BD Biosciences). Cells were then washed, fixed, and permeabilized using the permeabilization/fixation kit (BD Biosciences). Fc gamma receptor inhibitor (Ebiosciences, San Diego, USA) was used to avoid non-specific staining. The different IL-10, IL-2,

t1.1 **Table 1** Description of the different cohorts of MS patients included in the study.

t1.2
t1.3

	n/Form of the disease	Sex ratio	Disease duration (years, mean ± SEM)	EDSS (mean ± SEM)	ARR (mean ± SEM)	Time since last relapse (months, mean ± SEM)
t1.4 Phenotype analysis	9 PP	3 M/6 F	3.8 ± 1.6	4.8 ± 0.6	0	No
t1.5	6 SP	1 M/5 F	9.3 ± 2.2	4.6 ± 0.8	0.7 ± 0.2	13.3 ± 5.8
t1.6	34 RR	8 M/26 F	6.5 ± 0.9	2.3 ± 0.3	0.9 ± 0.1	16.3 ± 4
t1.7 Cytokine production (ProB10)	16 RR/4 SP/3 PP	6 M/17 F	6.9 ± 1.2	2.2 ± 0.4	0.9 ± 0.2	18.3 ± 5.7
t1.8 Coculture part	8 RR/4 SP	1 M/ 11 F	13.1 ± 3.1	3.5 ± 0.7	0.7 ± 0.1	17.8 ± 6.2

t1.9 PP: primary progressive MS patients.
t1.10 SP: secondary progressive MS patients.
t1.11 RR: relapsing-remitting MS patients.
t1.12 M: male; F: female.
t1.13 ARR: annualized relapse rate.

IL-6, IFNγ, and TNFα cytokines were measured in the culture supernatants using the Th1/Th2 human kit cytometric bead assays (CBA Human Th1/Th2 Cytokine Kit II, BD Biosciences) according to the manufacturer's instructions. For detection of intracellular IL-10, staining was performed using anti-IL-10-APC (clone JES3-9D7) or anti-IL-10-PE (clone JES3-9D7). Unstimulated B cells were also stained and used as a control for the gating strategy. Appropriate PE or APC-conjugated isotype controls were used in both cases. All antibodies were purchased from BD Biosciences except for IL-10 APC (Biolegend).

2.5. B-cell functional assays

In order to investigate the regulatory function of the B cells, coculture experiments were performed with prestimulated B cells and responding T cells using fresh blood samples from 11 HV and 10 MS patients. PBMCs were split into two batches. One batch was used to purify B cells using the B-cell isolation Kit II Human. The B cells were then stimulated for 24 h with CD40L (1 μg/ml) and CpG ODN (10 μg/ml) in a 96-well U-bottom plate at a concentration of 10^6 cells/ml. A 24 h stimuation was performed because the B cells continue to be stimulated by T cells by the CD40L pathway, but also because, during the prestimulation of the B cells, the PBMCs were kept at 4° in order to perform the sorting of $CD4^+CD25^-$ responding T cells at 24 h. These $CD4^+CD25^-$ responding T cells and unstimulated B cells were purified using specific MACS separation kits ($CD4^+$ T cell human Isolation Kit, $CD25^+$ microbeads II, B-cell Isolation Kit II, Miltenyi). For the analysis of responding T-cell proliferation, Cell Trace violet (Invitrogen, Life Technologies) was added to the T lymphocytes before coculture. Coculture assays were performed using 1×10^5 prestimulated or unstimulated B cells and 0.5×10^5 $CD4^+CD25^-$ responding T cells stimulated with anti-CD3 and anti-CD28.2 dynabeads (Invitrogen) for 72 h. The best stimulation conditions were tested for T lymphocytes using different ratios of dynabeads. BFA was added for the last 4 h at 10 μg/ml. Viability of T and B lymphocytes was checked in half of the samples by DAPI staining. Then, the cells were stained with anti-CD4-PE (clone, BD Biosciences), permeabilized and stained with anti-IFNγ-APC (clone B27) and anti-TNFα-FITC (clone 6401.1111, BD Biosciences). Coculture assays were performed in parallel using transwell polycarbonate inserts (0.4 μm, Corning Incorporated, USA) and blocking experiments were performed using anti-IL-10 (clone JES3-9D7), BD Biosciences) and anti-TGFβ 1 (Abcam, Cambridge, UK) at 10 μg/ml.

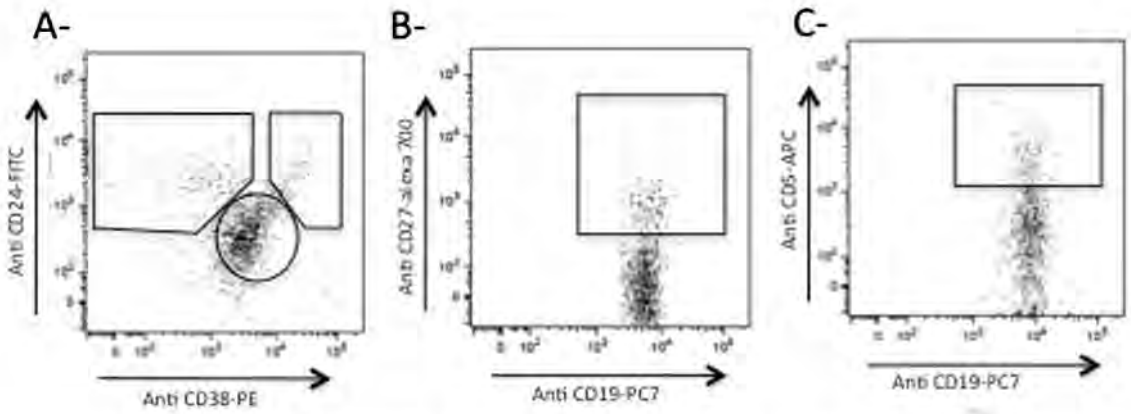
2.6. Statistics

All values are expressed as mean ± SEM. Analysis of significance was performed using Mann–Whitney (MW) or Wilcoxon paired tests with Prism software (Graphpad, La Jolia, USA). *P*-values below 0.05 were considered as statistically significant.

3. Results

3.1. Frequencies of the B cell sub-populations in MS patients

We studied the frequencies of the different B cell subsets in 44 HV (mean age = 38.6 ± 1.9 years) and 49 MS patients (mean age = 43 ± 1.7 years) (NS, MW test) (Table 1). B cells were gated using CD19 staining on the lymphocyte morphology (FSC-A, SSC-A) after exclusion of dead cells. The association of CD38 and CD24 was used to distinguish $CD24^{hi}CD38^{hi}$ immature/transitional B cells, $CD24^{dim}CD38^{dim}$ mature naive B cells, and $CD24^{hi}CD38^-$ memory cells [37], which are also reported as being $CD19^+CD27^+$ cells. The use of CD10 enabled us to analyze the frequency of the $CD24^{hi}CD38^{hi}CD10^+$ transitional T1 B cells. Finally, we looked at CD5 expression by B cells, CD5 being a marker for spleen Bregs in mice [38]. All gating strategies are represented in Fig. 1. No significant difference was found for the frequencies of $CD24^{hi}CD38^{hi}$ immature/transitional B cells (6.4 ± 0.6% in MS, 6 ± 0.5% in HV, NS), $CD24^{hi}CD38^{hi}CD10^+$ T1 cells (2.4 ± 1.6% in MS, 2.3 ± 1.3% in HV, NS) (Fig. 2A, D), $CD24^{dim}CD38^{dim}$ mature naive B cells (57.2 ± 2.7% in MS, 53.6 ± 2.4% in HV, NS) (Fig. 2B), $CD24^{hi}CD38^-$ B cells (26 ± 2.8 in MS, 29.2 ± 2.3 in HV, NS) (Fig. 2C), $CD27^+$ memory B cells (24.7 ± 2.6% in MS, 27.3 ± 2.1% in HV, NS) and $CD5^+$ B cells (12.5 ± 1.1% in MS, 15.8 ± 1.3% in HV, NS) (Fig. 2F, E) between MS and HV. A sub-analysis was performed for each different form of MS and no difference was detected (Supplementary Table 1).



Q4 **Fig. 1** Representation of the gating strategies for the frequencies of the different subsets of B cells. A—Staining of B cells with anti-CD38-PE and anti CD24-FITC allowed to differentiate transitional/immature B cells $CD24^{hi}CD38^{hi}$, $CD24d^{im}CD38^{dim}$ mature naive B cells and $CD24^{hi}CD38^{-}$ memory B cells. B—Staining of B cells with anti CD27-alexa 700 allowed to distinguish the $CD27^{+}$ memory B cells. C—Staining of B cells with anti CD5-APC allowed to identify the $CD5^{+}$ B cells.

3.2. Inhibition of autologous $CD4^{+}CD25^{-}$ T cell proliferation by CD40L/ODN prestimulated B cells

As stimulation of B cells by CD40L and ODN has been shown to result in increased frequency of IL-10 producing B cells in various models of auto-immune diseases in animals and human [12], we developed a coculture assay to compare the suppressive properties of B cells in 12 MS patients (8 RR, 4 SP MS patients, mean age = 47 ± 3.3 years) vs. 15 HV (mean age = 44.5 ± 4.1 years, NS, MW test). When CD40L/ODN prestimulated B cells from HV were added to the responding $CD4^{+}CD25^{-}$ T cells at a ratio of 2:1, we observed a significant decrease in $CD4^{+}CD25^{-}$ T-cell proliferation (38.5 ± 3.5% LT cell trace low) after anti-CD3-CD28 stimulation compared to the $CD4^{+}CD25^{-}$ T cells alone (72.4 ± 2.4% of LT cell trace low, $p < 0.001$) with a mean inhibition of 47%. Interestingly, the inhibition of $CD4^{+}CD25^{-}$ T-cell proliferation was also observed when the HV B cells added to the culture were not prestimulated, but with a lower efficiency (inhibition of 31.3 ± 4.7%, $p < 0.001$; Fig. 3A). $CD4^{+}CD25^{-}$ T-cell proliferation from MS patients ($n = 12$) was significantly decreased when their autologous prestimulated B cells were added (63.9 ± 4.9% LT cell trace low vs. 36.9 ± 4%, $p < 0.001$) with a mean inhibition of 42% (Fig. 3B). A sub-analysis with the different groups of patients (SPMS and RRMS) was performed without significant differences in the suppressive properties of B cells (Supplementary Fig. 1). Moreover, no correlation was observed between the duration of disease and the inhibition of proliferation by B cells (Supplementary Fig. 3).

Different B/T cell ratios (1:1, 2:1 and 4:1) were tested with a maximum suppression obtained with a ratio of 2 B cells for 1 T cell (data not shown). Altogether these data show that B cells (prestimulated or not) from MS patients are able to inhibit the proliferation of $CD4^{+}CD25^{-}$ T cells in the same manner as B cells from HV.

3.3. Prestimulated B cells do not alter the secretion of IFN-γ and TNF-α by $CD4^{+}CD25^{-}$ T cells

Using similar experimental settings, we analyzed the effect of B cells on IFNγ and TNFα secretion by $CD4^{+}CD25^{-}$ T cells after stimulation. In the absence of B cells, 13.3 ± 2% and 10 ± 1.4% of $CD4^{+}CD25^{-}$ T cells from HV secrete IFNγ and TNFα respectively after anti-CD3-CD28 stimulation. When autologous prestimulated B cells were added to the culture (ratio 2:1), the percentage of $CD4^{+}CD25^{-}$ T cells secreting either IFNγ or TNFα remained stable (13.05 ± 2.3% of IFN-$\gamma^{+}CD4^{+}CD25^{-}$ and 11.2 ± 1.8% of TNF-$\alpha^{+}CD4^{+}CD25^{-}$ in HV (Fig. 4A)). Similar results were observed for MS patients with 12.1 ± 1.8% IFN-$\gamma^{+}CD4^{+}CD25^{-}$ T cells and 8.2 ± 1.25% TNF-$\alpha^{+}CD4^{+}CD25^{-}$ T cells after stimulation before addition of prestimulated B cells. When autologous prestimulated B cells were added to the culture, the mean frequency of $CD4^{+}CD25^{-}$ T cells from MS patients secreting IFN-γ^{+} or TNF-α^{+} was 11.3 ± 1.1% and 7.4 ± 0.6% respectively, (NS) (Fig. 4B). Altogether, these data show that, while prestimulated B cells decrease the proliferation of autologous $CD4^{+}CD25^{-}$T cells, they have no effect on their production of TNF-α and IFN-γ, either in MS or in HV.

3.4. The inhibition of responding $CD4^{+}CD25^{-}$ T-cell proliferation by prestimulated B cells is contact-dependent

Experiments using blocking anti-IL-10 and anti-TGFβ antibodies and coculture using transwell inserts were performed to characterize the suppression mechanisms of $CD4^{+}CD25^{-}$ responding T-cell proliferation by prestimulated B cells in HV and MS patients. First, we found that the inhibition of $CD4^{+}CD25^{-}$ T-cell proliferation by prestimulated B cells could be abrogated when the cells were physically separated by transwell inserts. In the presence of transwells, the proliferation of responding T cells (63 ± 2.6% of cell trace low in HV and 69.2 ± 7.3% in MS) was similar to that observed in the control setting (72 ± 2.4% and 64 ± 4.9% cell trace low in HV and MS patients respectively; Fig. 3A, B). In contrast, the addition of blocking anti-IL-10 or anti-TGFβ antibodies had no effect on the ability of prestimulated B cells to inhibit responding $CD4^{+}CD25^{-}$ T-cell proliferation (Fig. 3C), both in MS and HV (40.6 ± 5.5% and 42 ± 8.3% LT cell trace low with anti-IL-10 mAb, and 31.6 ± 6.5% and 31.7 ± 5.7% with anti-TGFβ mAb for HV and MS respectively, as compared to 38.5 ± 3.5% and 36.8 ± 4% in HV and MS respectively without

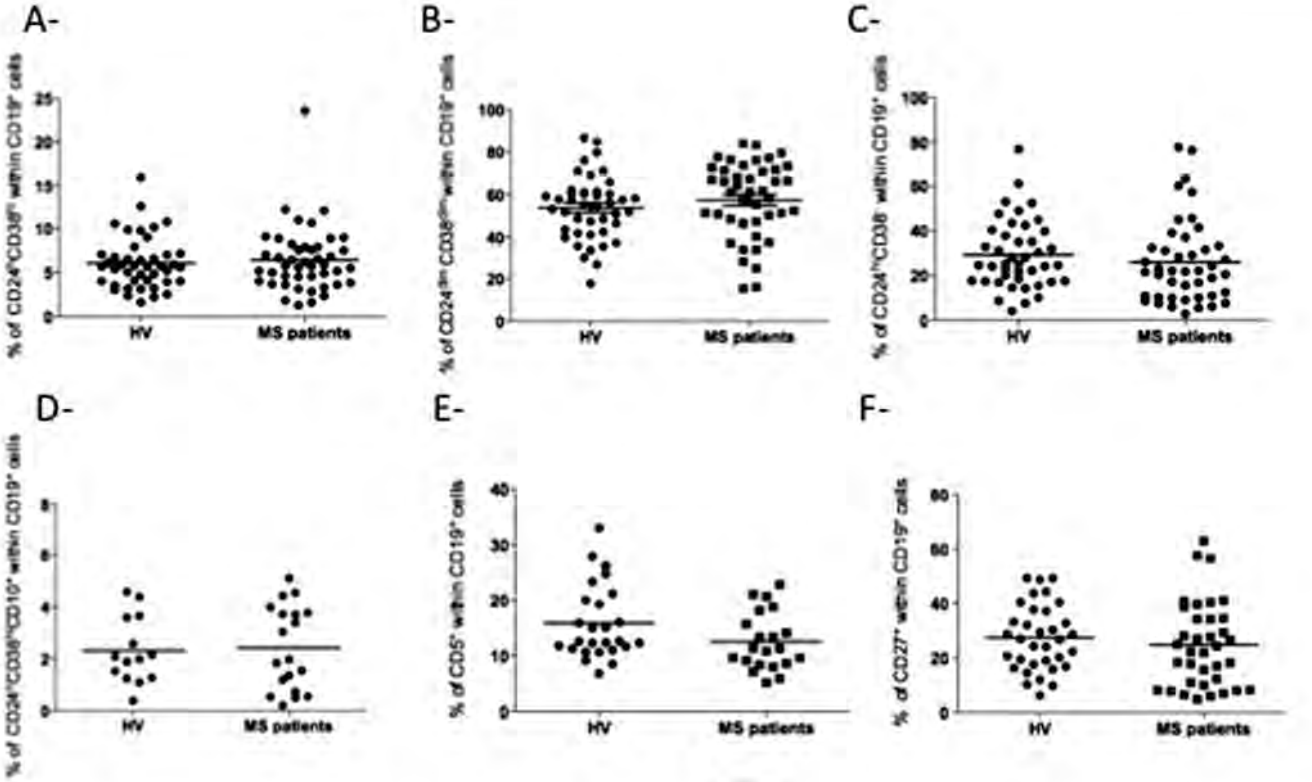


Fig. 2 Frequencies of the different subsets of B cells in HV and MS patients. The frequency of the various B cell subsets was analyzed in the blood of HV and MS patients and is expressed as a percentage of each B cell subset within the B cells. A–D. $CD24^{hi}CD38^{hi}$ transitional (A) and $CD24^{hi}CD38^{hi}CD10^{+}$ transitional T1 (D). B. $CD24^{dim}CD38^{dim}$ mature naive B cells. C–F. $CD24^{hi}CD38^{-}$ memory B cells (C) and $CD27^{+}$ B cells (F). E. $CD5^{+}$ B cells. All the tests performed are MW tests (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

any blocking antibody). Altogether these data show that the regulatory effect of prestimulated B cells on the proliferation of autologous $CD4^{+}CD25^{-}$ T cells requires contact between B and T cells and is not mediated by IL-10 and/or TGFβ cytokines. No significant difference was observed between HV and MS patients, suggesting similar mechanisms for B cells in the two groups.

3.5. Cytokine secretion and frequency of IL-10^{+} B cells in MS

We analyzed, using cytometry, the frequency of IL-10^{+} B cells in MS patients and HV after 48 h of stimulation with CD40L and ODN (with PIB for the last 5 h), a setting able to generate IL-10^{+} B cells in mice and human. The frequency of IL-10^{+} B cells did not differ between MS patients and HV (6.2 ± 0.7% in HV, 6 ± 0.8% in MS patients, $p = 0.94$, MW test) (Fig. 5A). A sub-analysis of the different sub-groups of MS patients was performed without revealing significant differences (Supplementary Fig. 2). Moreover, no correlation was observed between disease duration and the frequency of IL-10^{+} B cells (Supplementary Fig. 3).

In addition, IL-10^{+} B cells produce the same amount of IL-10, the measurements being taken from the culture supernatants of the 48 h stimulated B cells from MS patients ($n = 8$ relapsing form in a remission period) and HV ($n = 11$) (Fig. 5B) (2787 ± 603 pg/ml in HV, compared to 3357 ± 770 pg/ml in MS patients, NS, MW test). Similarly, when measuring the quantity of a number of other cytokines (IL-2, IL-6, IFNγ, TNFα) in the culture supernatants from the 48-hour stimulated B cells using a Th1/Th2 human kit cytometric bead assays, we found no difference between patients with MS and HV (Supplementary Table 2) even if B cells of MS patients tend to secrete more TNFα than HV ($p = 0.1$, MW test). Altogether, these data suggest that cytokine secretion by B cells is not different between our cohort of MS patients and HV and particularly no difference is observed in IL-10^{+} B cells regarding their ability to secrete IL-10 after stimulation.

3.6. Phenotypic characterization of IL-10^{+} B cells

In order to characterize the phenotype of IL-10^{+} B cells, different combinations of cell staining were performed after the 48 h of stimulation: anti-CD24 and anti-CD27 for the memory phenotype ($CD24^{+}CD27^{+}$), anti-CD24 and anti-CD38 for the different subsets of B cells (immature/transitional: $CD24^{hi}CD38^{hi}$, mature naive: $CD24^{dim}CD38^{dim}$, and memory: $CD24^{hi}CD38^{-}$ B cells) and anti-CD5.

In HV, IL-10^{+} B cells were mainly $CD24^{+}CD27^{+}$/$CD24^{hi}CD38^{-}$ memory cells and $CD24^{hi}CD38^{hi}$ immature/transitional B cells. Indeed, 54.7 ± 4.8% of IL-10^{+} B cells were $CD24^{+}CD27^{+}$ memory cells compared to only 36.4 ± 4.1% of the total B cell population ($p < 0.0001$; Fig. 6A). Similarly, 44.5 ± 4.5% of IL-10^{+} B cells displayed a $CD24^{hi}CD38^{-}$ memory phenotype compared to 34.8 ± 4.3% of the total B cell population ($p < 0.05$, Fig. 6C) and 8.9 ± 1.1% of IL-10^{+} B cells displayed a $CD24^{hi}CD38^{hi}$

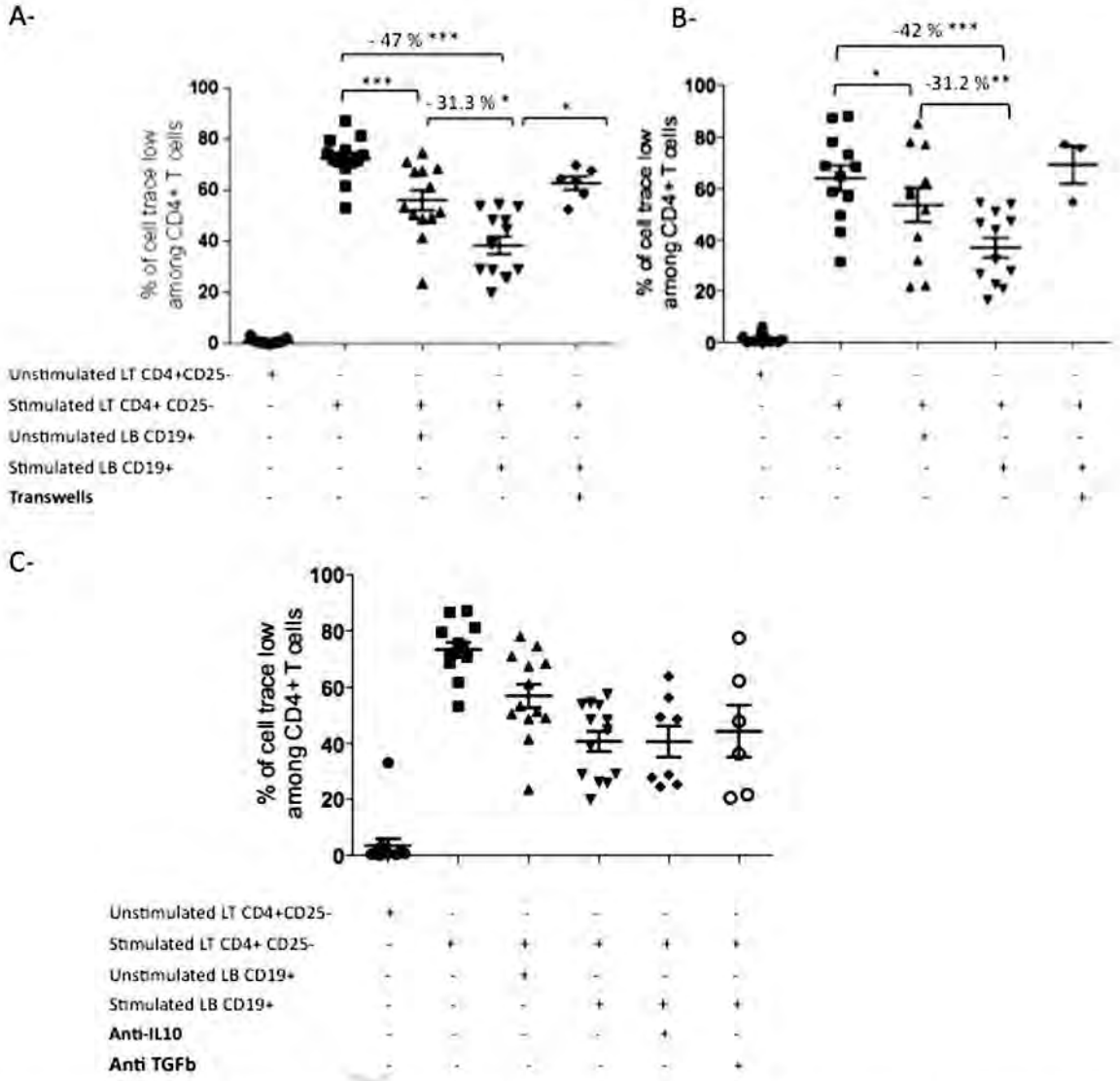


Fig. 3 Prestimulated B cells are able to inhibit $CD4^+CD25^-$ T-cell proliferation. Coculture assays in HV (n = 15) and MS patients (n = 15) were performed by culturing prestimulated or unstimulated B cells with autologous $CD4^+CD25^-$ T cells at a ratio of 2:1 for 3 days in the presence or absence of anti-CD3 and anti-CD28 coated beads (stimulated LT or unstimulated LT). Regulation of $CD4^+CD25^-$ T cell proliferation in HV (A, C), and MS (B) was analyzed by flow cytometry. Blocking anti-IL-10 and anti-TGFβ antibodies were added for some experiments (C). All the tests performed are Wilcoxon paired tests (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

transitional B cell phenotype compared to only 3.7 ± 0.5% for the total B cell population ($p < 0.001$; Fig. 6C). These results were confirmed when we analyzed IL-10 secretion by the $CD24^+CD27^+$ and $CD24^-CD27^-$ B cell subsets after 48 h stimulation. Similarly to the $CD24^+CD27^+$ memory IL-10^+ B cell phenotype, we found a significantly higher IL-10 secretion by $CD24^+CD27^+$ memory cells compared to the $CD24^-CD27^-$ naive B cells (12.6 ± 1.4% vs. 5.6 ± 0.8% respectively, $p < 0.001$) (Fig. 6B). Finally we found that a greater number of IL-10^+ B cells expressed CD5 compared to the total B cell population (15.7 ± 2.3% vs. 10.9 ± 1.6% in the total B cell population, $p < 0.01$) (Fig. 6E). Interestingly, the same analysis performed in MS patients did not reveal any difference with HV (Fig. 6A, B, D, E). Altogether, these data show that memory and transitional/immature B cells preferentially contain the majority of IL-10^+ B cells. No significant difference was observed between MS and HV in the frequency and the phenotype of the B cell subsets, including the IL-10^+ subset. These data comfort the absence of any defect in the regulatory B cell phenotype and function in the blood of MS patients.

4. Discussion

Regulatory mechanisms in MS have been extensively explored, especially those involving natural regulatory T cells (nTregs) [39]. In this study, we looked at the role of B cells and, to our knowledge, this is the first analysis to report on their suppressive capacity in patients with MS. We report on the ability of B cells from MS patients to significantly inhibit the proliferation of autologous activated $CD4^+CD25^-$ T cells.

While IL-10 secretion appears to be involved in the regulatory effects of B cells in several animal models of

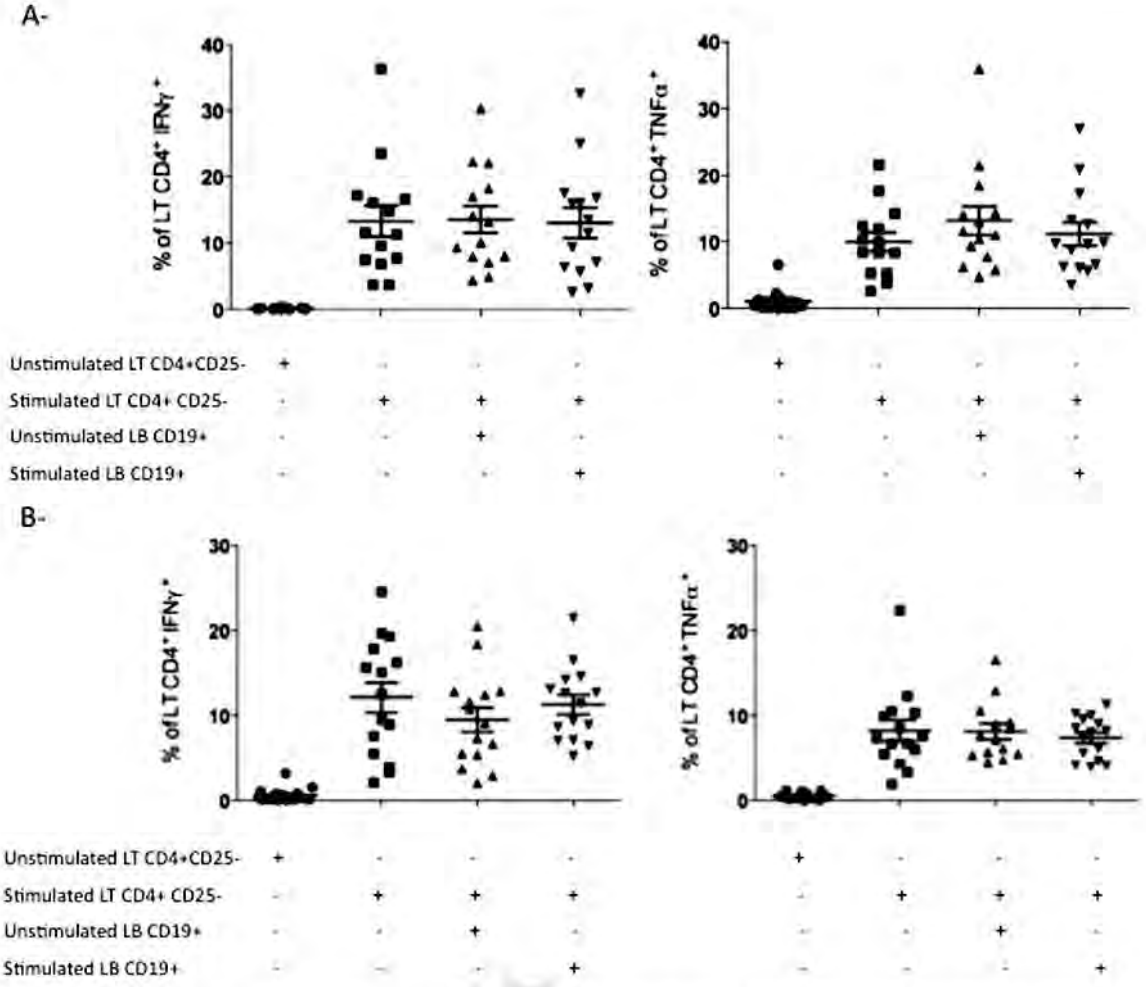


Fig. 4 Prestimulated B cells are not able to inhibit IFN-γ and TNF-α production. Coculture assays in HV (n = 15) and MS patients (n = 15) were performed by culturing prestimulated or unstimulated B cells with autologous $CD4^+CD25^-$ T cells at a ratio of 2:1 for 3 days in the presence or absence of anti-CD3 and anti-CD28 coated beads (stimulated LT or unstimulated LT). Regulation of $CD4^+CD25^-$ T cell secretion of TNF-α and IFN-γ in HV (A) and MS (B) was analyzed by flow cytometry. All the tests performed are Wilcoxon paired tests (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

autoimmune diseases [28,38,40–42], the role of other cytokines, such as TGFβ, has also been discussed [29]. Moreover, contact between regulatory B cells and T cells has also been shown to be required for B cell suppressive properties to be activated in EAE or colitis [31,32]. In the present study, we show that the inhibition of $CD4^+$ T cell proliferation by B cells in MS patients is contact-dependent but is not dependent on IL-10 and/or TGFβ secretion. The absence of a role for IL-10 in particular is supported by the fact that, in our study, untreated patients with MS display the same frequency of $IL\text{-}10^+$ B cell and secrete the same level of IL-10 (measured by CBA) as healthy volunteers.

The studies reporting on differential IL-10 secretion by B cells from MS patients compared to HV are contradictory. Two groups reported in the literature that B cells from MS patients exhibit a decrease in IL-10 production and an increase in the secretion of pro-inflammatory cytokines such as TNFα and LTα [25,34,43], whereas another group made contrary findings, with a significantly higher frequency of $IL\text{-}10^+$ B cells in patients with MS [35]. These discrepancies may be explained by the fact that the protocols used in these studies differ greatly in terms of time of culture and the kind of stimulations, both of which having been shown to impact greatly on cytokine secretion by B cells [11,17]. Moreover, two of these reports included treated MS patients [34,35] and Knippenberg's group reported a significant decrease in IL-10 secretion by B cells in treated MS patients, associated with a significant decrease in memory $CD27^+$ B cells in MS patients compared to HV. As the authors stated, the decrease in memory $CD27^+$ B cells in their study was probably due to the treatment by interferon [44]. Interestingly, Iwata et al. showed that IL-10 secretion was mainly carried out by memory B cells, which could explain the decreased IL-10 secretion in Knippenberg's study. In our study, we chose to include only untreated MS patients to avoid alteration of the B-cell phenotype or cytokine secretion due to the treatment. We chose to stimulate the cells using a paradigm using CD40L and ODN, described by Iwata et al., as inducing the greatest secretion of IL-10 in human [35]. It is therefore difficult to compare our results to other studies in MS that used other kinds of stimulation, which partly explains these discrepancies. Moreover, no patients

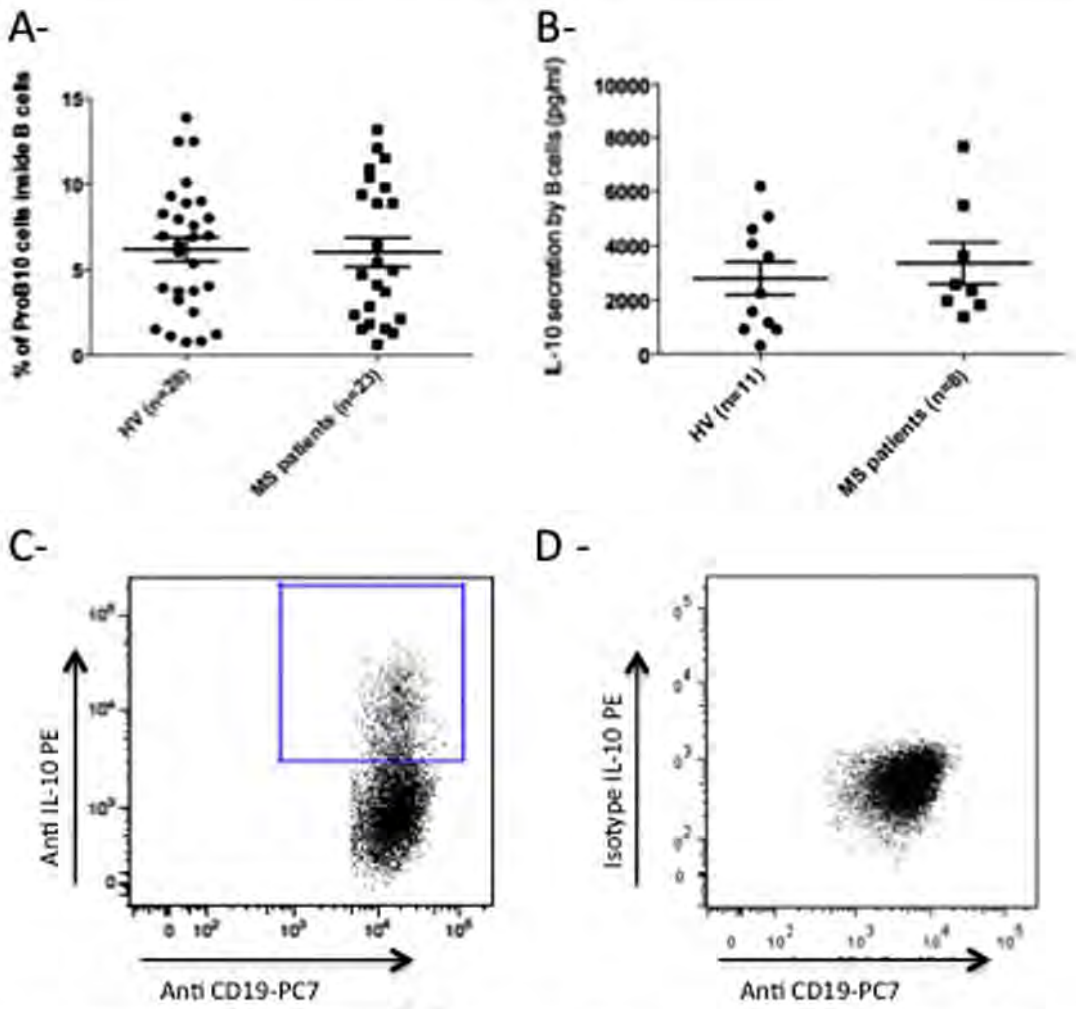


Fig. 5 B cells from HV and MS patients exhibit similar IL-10 production. A—Identification of the B10pro cells in HV and MS patients after 48 h of stimulation with CD40L, CpG ODN and PIB for the last 5 h. B—Measurement of IL-10 in the 48 h culture supernatants of B cells after stimulation by CD40L, CpG ODN, and PI for the last 5 h by cytometric beads assay. C—Representative experiment of IL-10 secretion by flow cytometry in one HV. B cells were stimulated for 48 h with CD40L and CpG ODN and PIB for the last 5 h. IL-10 secretion was studied using anti-IL-10-PE and anti-CD19-PC7. D—Representative staining with isotype-PE in one HV. B cells were stimulated for 48 h with CD40L and CpG ODN and PIB for the last 5 h. Unspecific staining was checked using isotype-PE. All the tests performed are MW tests (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

going through relapse were included in this part of the study and so we cannot exclude there being a change in cytokine secretion by B cells during a relapse period of the disease. However, the B cells of our MS patients do tend to secrete more TNFα compared to HV which concords with descriptions in several studies [25,43].

We also analyzed and compared the phenotype of IL-10$^+$ B cells in HV and MS patients. We provide new data on the different markers expressed by IL-10$^+$ B cells. Particularly, we found a significant enrichment of IL-10$^+$ B cells within the CD24^{hi}CD27$^+$memory B cell compartment, but we also observed that IL-10$^+$ B cells are not restricted to this compartment and are present within the different subsets of B cells, such as transitional/immature B cells expressing the CD38 marker. In contrast with Blair et al. [45], we did not find an exclusive CD19$^+$CD38^{hi}CD24^{hi} immature/transitional B cell phenotype in IL-10$^+$ B cells. These results are in accordance with those of Bouaziz and colleagues and Iwata and colleagues [35,46]. However, we cannot exclude the possibility that, as in mice models, different regulatory B cell populations may coexist, and that the different stimulation protocols used recruit different kinds of suppressive B cells.

We did not find any inhibition by autologous prestimulated B cells of Th1 cytokine secretion by CD4$^+$CD25$^-$ cells in vitro. These data also contrast with the results from Blair et al., who reported on the inhibition of TNFα secretion by B cells from HV in an IL-10 dependent manner and on the inhibition of TNFα and IFNγ secretion in a CD80/86 dependent manner [45].

In addition, the contact-dependent regulatory mechanism of B cells that we report on in this study is in line with a number of animal studies. Mann et al. demonstrated in EAE that B cells regulate CD4$^+$CD25$^+$ Tregs via B7 [31]. In a similar way, in a model of chronic colitis, Mizoguchi et al. showed that the regulatory effects of B cells required the presence of functional costimulatory molecules: CD40 and CD86 [32]. As in our study, Lemoine et al. show that the inhibition of CD4$^+$ T cell proliferation was contact-dependent [47].

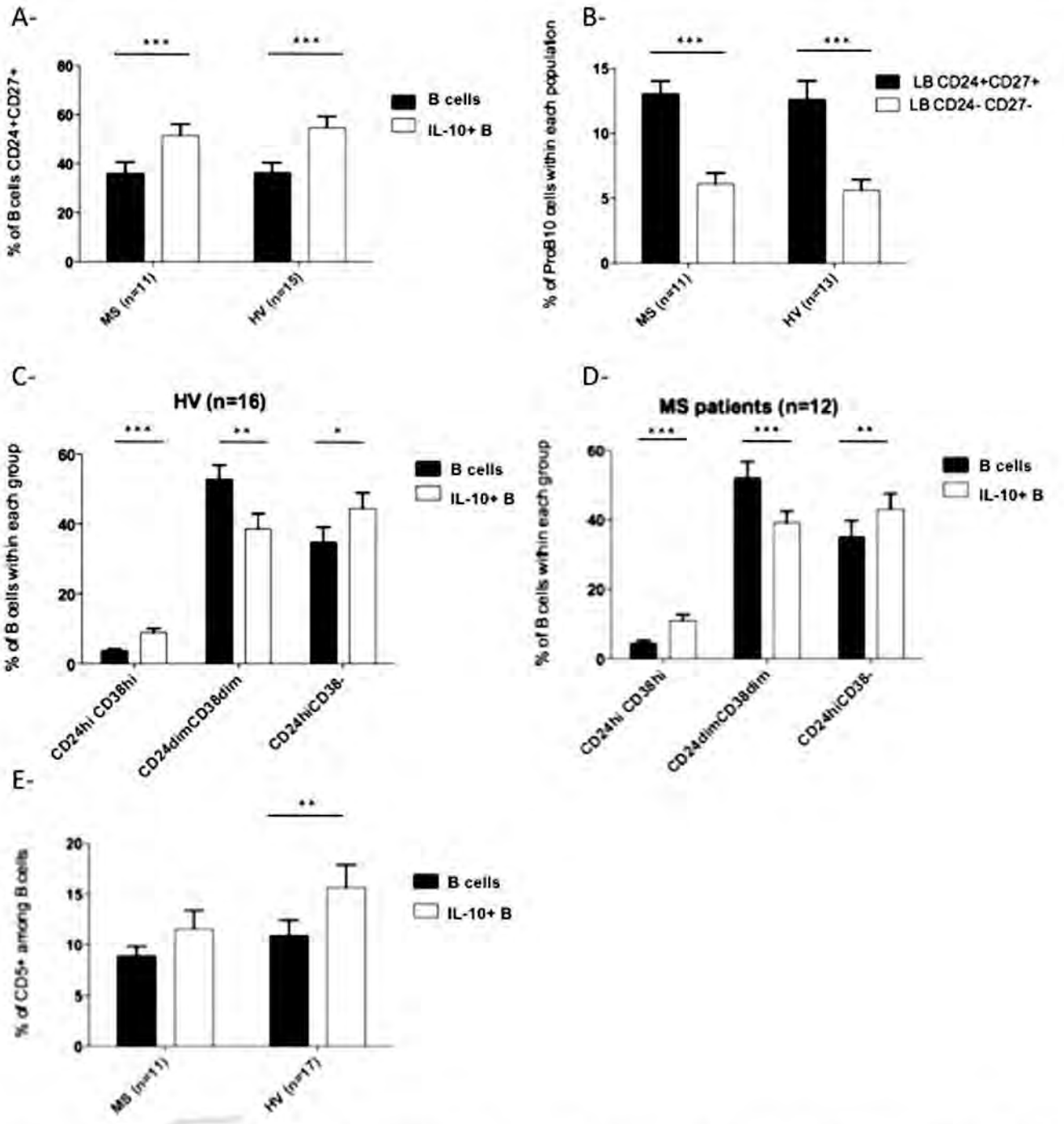


Fig. 6 Analysis of the phenotype of B10 cells in HV and MS patients by flow cytometry. A—Expression of CD24 and CD27 by B10 and B cells. B—IL-10 secretion by $CD24^{+}CD27^{+}$ memory cells compared to the $CD24^{-}CD27^{-}$ cells. C–D—Phenotype of B10 using the CD24/CD38 combination. E—CD5 expression by B10 cells. All the tests performed are Wilcoxon paired tests (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Interestingly, we also observed a significant decrease in $CD4^{+}$ T cell proliferation when adding autologous unstimulated B cells from MS patients and HV, even though the level of inhibition was lower than when the B cells were presimulated. These data fit with previous reports from Niimi et al. that show that, in a mouse model of cardiac allograft tolerance, resting B cells that are incompetent or nonprofessional APCs may induce tolerance [48,49].

Additionally, in our cohort of MS patients, B cells (stimulated by CD40L and TLR9 ligand) present regulatory properties and are equally efficient in the periphery compared to healthy volunteers. Examining the peripheral compartment may be limiting and we cannot exclude the possibility that regulatory B cells are circulating but are defective in the context of an inflammatory CNS. As shown for nTregs in EAE, regulatory B cells could be overridden in an inflammatory context [50].

However, obtaining access to these central regulatory B cells is difficult, due the rarity of these cells in the CSF, the limited accessibility of cerebral material and a lack of specific staining for these Breg cells. It is also possible that, as for nTregs that are rare or undetectable in MS brain lesions [51,52], regulatory B cells may have difficulty accessing the CNS and would not be able to regulate autoreactive T cells inside the brain. To our knowledge no studies have yet explored the presence of such B cells inside the cerebral parenchyma in MS patients. Finally, the presence of lymphoid follicles in the meninges of some patients [21,22] and the antigen specificity of CSF Igs reported in several studies [53,54], suggest an Ag-specific proliferation of B cells in the CNS of MS patients. A defect of an Ag-specific regulatory B cell subset in patients with MS that we could not detect using a polyclonal stimulation is entirely possible.

In conclusion, we have reported on a normal B cell frequency and phenotype in the blood of patients with MS. We show that B cells from MS are able to inhibit autologous $CD4^+CD25^-$ responding T-cell proliferation in vitro in a contact-dependent manner and to the same extent as B cells from HV, suggesting that, in this case, a lack of peripheral regulation by B cells does not contribute to the physiopathology of the disease. Further studies are necessary to better characterize these B cells in a specific inflammatory context.

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Authorship contribution

LM, MC and PM performed the experiments and wrote the manuscript.

AG, MS and AEN performed some experiments.

FL, MJF and SW participated to the inclusion of MS patients and HV.

DAL, JPS and SB designed the project and corrected the manuscript.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Thèse de Doctorat

Mélanie CHESNEAU

Analyse de la différenciation et de la régulation des lymphocytes B chez des patients tolérants un greffon rénal

Analysis of differentiation and regulation of B cells in tolerant patient kidney transplant

Résumé

La transplantation rénale est le traitement de choix de l'insuffisance rénale terminale. Cependant elle nécessite l'administration d'un traitement immunosuppresseur au long cours afin de pallier au rejet du greffon. Malgré les avancées importantes réalisées dans la recherche sur les traitements immunosuppresseurs, ces traitements induisent de nombreux effets secondaires (cancers, néphrotoxicités, infections…). Une tolérance opérationnelle a été décrite chez quelques patients rares. Ces patients présentent une fonction stable de leur greffon après arrêt de leur traitement immunosuppresseur. Les études réalisées sur ces patients tolérant une greffe rénale ont mis en évidence un profil transcriptionnel spécifique des lymphocytes B (LB). Cependant, le rôle joué par les LB dans cette tolérance n'a pas encore été décrite. Cette thèse porte donc sur l'étude de cette population de LB chez les patients tolérants afin d'essayer de mieux comprendre les mécanismes impliqués dans la tolérance opérationnelle. Dans un premier temps l'analyse de la capacité de différenciation des LB en plasmocytes (cellules sécrétrices d'anticorps néfastes en transplantation) a permis de mettre en évidence *in vitro* chez les patients tolérants un défaut dans la capacité de différenciation tardive des LB en plasmocytes. Dans un deuxième temps, l'analyse de la fonction régulatrice des LB montre une fonction régulatrice chez les patients tolérants non modifiés. L'étude de cette population de LB régulant *in-vitro* la prolifération des LT effecteurs a permis de mettre une évidence un phénotype proche des plasmocytes et une fonction dépendante du contact LT/LB et de la sécrétion de Granzyme B.

Mots clés

Lymphocytes B, tolérance, transplantation rénale, régulation, différenciation

Abstract

Kidney transplant is the treatment of choice of kidney terminal failure. However, transplantation requires administration of long term immunosuppression to avoid rejection. Despite progress in research in immunosuppression, these treatments induced side effects (cancer, nephrotoxicity, infections…). Operational tolerance has been described in rare patients. These patients present a stable graft function after immunosuppression withdrawal. Studies on these patients reveal a whole blood transcriptional B cell signature. However, the role of B cells in tolerance was not described yet. This thesis studies B cell population in tolerant patients, to try to better understand a part of the mechanisms involved in operational tolerance. At first, analysis *in vitro* of B cell differentiation abilities into plasma cells (antibody secreting cells with damaging effect in transplantation) allows to highlight a defect in late stage of plasma cell differentiation in B cells from tolerant patients. And secondly, analysis of regulatory function of B cells shows unmodified regulatory function of B cells in tolerant patients compared to healthy donor and stable patients. Study of this B cells that inhibit effector T cell proliferation *in vitro* allowed the characterization of plasma cell like phenotype that exert regulatory properties in contact and Granzyme B dependant manner.

Key Words

B cells, tolerance, kidney transplantation, regulation, differentiation

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