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« Le travail est indispensable au bonheur de l'homme ; il l'élève, il le console ; et peu importe la nature du travail, pourvu qu'il profite à quelqu'un : faire ce qu'on peut, c'est faire ce qu'on doit. »

Alexandre Dumas Fils.

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Préface

> « Une simple discrimination entre telle entité ou telle autre entité ne suffit pas pour le maintien quotidien ; le système immunitaire doit juger des circonstances qui entourent les antigènes qu'il voit, pour le soi comme pour le non-soi. [...] Le système immunitaire est un système compliqué parce que la régulation de l'inflammation exige un jugement compliqué. » Cohen Irun

Le système immunitaire assure la défense contre les agressions environnementales tout en conservant l'intégrité des tissus de l'hôte. Une infection, qu'elle soit virale, microbienne ou fongique est un processus délétère pour l'hôte. La réponse inflammatoire anti-infectieuse, bien que dirigée directement contre l'agent infectieux grâce au système immunitaire adaptatif, induit une destruction tissulaire importante. Cette inflammation exacerbée est régulée par le système immunitaire qui met en place des mécanismes immunorégulateurs ou inhibiteurs. Ces processus peuvent être intrinsèques ou extrinsèques, agir de manière immédiate ou plus tardivement au travers de mécanismes diversifiés. Cependant, ils partagent tous la capacité de réguler l'intensité et la durée du processus inflammatoire. Certains facteurs environnementaux et génétiques peuvent altérer cette balance immunologique et favoriser l'apparition de certaines pathologies comme l'auto-immunité, dans le cadre d'une reconnaissance inappropriée des antigènes du soi, ou bien l'allergie, dans le cadre d'une reconnaissance inappropriée de peptides environnementaux. La transplantation d'organe est également une situation pathologique ou la problématique de tolérance est importante. Être capable d'induire la tolérance à un organe étranger ou de la rétablir face à un allergène de l'environnement où un antigène du soi nécessite d'abord une analyse et une compréhension approfondies des mécanismes immunologiques de la tolérance. La recherche dans ce domaine est extrêmement riche et dynamique car elle représente un enjeu thérapeutique important. Ainsi depuis mon master et plus fortement pendant ma thèse, j'ai pu participer à des projets traitant d'immunorégulation et de tolérance dans deux situations pathologiques distinctes ; (i) L'étude des mécanismes de la tolérance au greffon rénale avec une analyse des

lymphocytes B et T régulateurs mais aussi (ii) une analyse des lymphocytes B régulateurs dans un modèle d'asthme allergique aux acariens. L'objectif est de comprendre les mécanismes du système immunitaire qui permettent de contrôler une inflammation délétère pour l'hôte. Ce manuscrit traitera essentiellement de la partie B dans l'asthme, vous trouverez néanmoins à la fin de celui-ci l'ensemble des travaux réalisés dans le cadre de la transplantation rénale.

Liste des publications et communications.

Revues

- Chesné J*, **Braza F***, Mahay G, Brouard S, Aronica M, and Magnan A. IL-17 in severe asthma: where do we stand? American Journal of Respiratory and Critical Care Medicine, (Accepted) * These authors contribute equally to this work

- **Braza F**, Chesne J, Castagnet S, Magnan A and Brouard S Regulatory functions of B cells in allergic diseases. Allergy (Accepted) 2014

- J.Chesné, **F.Braza**, A.Magnan T_H17, Neutrophiles et hyperréactivité bronchique. Revue française d'allergologie, Février 2013

- **Braza F**, Soulillou JP, Brouard S.Reconsidering the bio-detection of tolerance in renal transplantation. Chimerism. 2012 Dec 21;4(1).

- Salinas GF, **Braza F**, Brouard S, Tak PP, Baeten D. The role of B lymphocytes in the progression from autoimmunity to autoimmune disease. Clin Immunol. 2013 146: 34-45.

- **Braza F**, Soulillou JP, Brouard S. Gene expression signature in transplantation tolerance. Clin Chim Acta. 2012; 413: 1414-8.

- Danger R, **Braza F** and Brouard S. MicroRNAs, major players in B cells homeostasis and functions. Front Immunol. 2014 Mar 11;5:98

Lettres et Article Originaux

- **Braza F***, Chesne J*, Cheminant MA, Dirou S, Mahay G, Durand M, Lair D, Magnan A and Brouard S. A regulatory $CD9^+$ B cell subset controls HDM-induced allergic airway inflammation (Submitted in JCI). * These authors contribute equally to this work

- Chesne J*, **Braza F***, Mahay G, Chadeuf G, Cheminant MA, Sagan C, Lair D, Brouard S, Loirand G, Sauzeau V and Magnan A. Transcutaneous Sensitization and Bronchial Challenge Induce IL-17A-mediated Neutrophilia and Smooth Muscle Contraction in a House Dust Mite Model of Asthma (In Revision in JACI). * These authors contribute equally to this work

- Chesneau M, Pallier M, **Braza F**, Lacombe G, Le Gallou S, Giral M, Danger R, Guerif P, Michel L, Baron D, Laplaud DA, Degauque N, Soulillou JP, Tarte K and Brouard S. Unique B-cell differentiation profile in tolerant kidney transplanted patients. Am J Transplant. 2014 Jan;14(1):144-55

- **Braza F**, Dugast E, Panov I, Paul C, Vogt K, Pallier A, Degauque N, Meisel C, Guerif P, Volk HD, Giral M, Soulillou JP, Sawitzki B and Brouard S. Central role of $CD45RA^-$ $Foxp3^{hi}$ memory Tregs in clinical kidney transplantation tolerance (Revision in JASN)

Communications orales et posters

- **Poster communication**: "Deficiency of regulatory B cells in a mouse model of asthma" International Severe Asthma Forum, Gothenburg, Sweden, 11-13th October 2012
- **Oral communication**: "Deficiency of regulatory B cells in a mouse model of asthma", World Immune Regulatory Meeting, Davos, Switzerland, 13-16th March
- **Oral communication**: "Unique B-cell differentiation profile in tolerant kidney transplant patients" World Immune Regulatory Meeting, Davos, Switzerland, 13-16th march
- **Oral communication**: “Deficiency of regulatory B cells in a mouse model of asthma” Journées de recherché respiratoire, Montpellier, France, 11-12th October
- **Poster communication**: " Increased Foxp3 TSDR demethylation in circulating CD4+ T cells of tolerant renal transplant patients " The International Basic Science Mentee-Mentor Travel Awards for the 3rd ESOT Basic Science Meeting / 13th TTS Basic Science Symposium (BSM 2013) in Paris, France from November 7-9th, 2013.
- **Oral Communciation**: Central role of CD45RA^{-} Foxp3^{hi} memory Tregs in clinical kidney transplantation tolerance. Nantes Actualités Transplantation 2014. France June 5-6th, 2014.

1. Généralités sur les lymphocytes B.

1.1 Ontogenèse des lymphocytes B

La majorité des cellules B se développe à partir de progéniteurs lymphoïdes au niveau de la moelle osseuse. Les étapes précoces de différenciation impliquent l'interaction des cellules progénitrices avec les cellules stromales de la moelle osseuse mais ne requièrent pas l'antigène. Les étapes suivantes tournent autour de l'expression et du réarrangement d'un récepteur des cellules B (BCR) fonctionnel. Ce processus est finement régulé par des étapes de sélections positive et négative et résulte en la formation de cellules B immatures. Chez la souris les lymphocytes B immatures IgM^+ IgD^- migrent directement dans les tissues lymphoïdes périphériques où ils terminent leur différenciation en cellules folliculaires ou de la zone marginale. Chez l'homme, des cellules B matures naïves co-exprimant l'IgD et l'IgM sont directement générées dans la moelle osseuse et ne dépendent pas d'une étape de maturation dans la rate (van Lochem et al., 2004; Wasserstrom et al., 2008).

Chez la souris, les cellules B sont traditionnellement divisées en trois grands sous-groupes : les cellules B-1, les cellules B de la zone marginale (ZM) et enfin les cellules B folliculaires (FO). Les cellules FO et ZM constituent un ensemble appelé cellules B2. Cette nomenclature permet de les distinguer des cellules B1 qui ont une origine différente et ne dérivent pas des cellules B immatures générées dans la moelle osseuse (Godin et al., 1993; Hardy and Hayakawa, 1991). Ces lymphocytes B-1 sont enrichis dans le péritoine et les cavités pleurales mais on retrouve également ces cellules dans la rate (Baumgarth, 2011). Bien que leur origine ne soit pas encore clairement définie, certains travaux démontrent que leur développement prend place essentiellement dans le foie fœtale, de manière précoce au cours du développement (Godin et al., 1993; Hardy and Hayakawa, 1991). Les B1 sont considérées comme des cellules innées qui produisent la majorité des immunoglobulines « naturelles » de type M et A (IgM et IgA). Étant

données leur polyréactivité et leur capacité à reconnaître un large spectre de motifs bactériens, notamment les antigènes glucidiques, les IgM naturelles agissent comme une première ligne de défense antimicrobienne (Baumgarth, 2011). Les lymphocytes B1 expriment un large spectre de Toll-Like Recepteurs (TLRs) qui modulent leur fonction et leur activation (Rawlings et al., 2012). L'activation par ces TLRs peut ainsi renforcer la réponse humorale mais induit également la production de cytokines leur permettant d'orienter les réponses immunitaires (Rawlings et al., 2012). Ces cellules jouent donc un rôle central à la frontière entre immunité innée et adaptative.

Les lymphocytes B FO et ZM dérivent des cellules immatures. Avant de se différencier dans l'une des deux sous-populations, le lymphocyte B immature subit un contrôle qualité de son BCR. C'est une deuxième étape clef de tolérance au cours de laquelle la majorité des lymphocytes B autoréactifs est supprimée. Au cours de ce processus, les B immatures passent par deux stades de transition distincts. On distingue ainsi les cellules transitionnelles de type 1 (T1) et de type 2 (T2). On les différencie par leur phénotype, leur localisation, et leur fonctionnalité (Chung et al., 2003). En effet les T1 sont localisés dans la zone périarterielle lymphoïde où elles subissent une sélection négative des clones autoréactifs (Su and Rawlings, 2002). Les cellules T1 sont extrêmement sensibles à l'apoptose et ont besoin de la molécule BAFF (« B-cell activating factor »), un facteur de croissance pour survivre et poursuivre leur maturation (Batten et al., 2000). Les lymphocytes T1 migrent ensuite dans la rate ou ils se différencient en cellules T2. Celles-ci acquièrent et expriment progressivement différentes molécules de costimulation, importante dans l'interaction avec les lymphocytes T. De plus, on observe progressivement une maturation du BCR avec la mise en place du signalosome, qui regroupe l'ensemble des molécules de signalisation de la voie du BCR (Btk, Lyn, CD45, BLNK, vav) (Pappu et al., 1999; Xu et al., 2000). Une fois ces étapes de maturation achevées, les B deviennent matures et fonctionnelles. A ce stade le choix entre une différenciation vers un phénotype FO ou ZM dépend de l'intensité du signal BCR et du

clivage de Notch2 (Pillai and Cariappa, 2009). Notch2 est un facteur de transcription qui induit spécifiquement la différenciation en cellules B ZM. Or son activité est inhibée par des signaux intenses transduits par le BCR. Par conséquent les cellules B qui reçoivent des signaux BCR intenses inhibent Notch2 et se différencient en cellules B FO alors que les autres, exposées à des signaux faibles, expriment Notch2 et se différencient en cellules B ZM (Pillai and Cariappa, 2009). On ne sait pas comment la signalisation du BCR peut ainsi moduler la différenciation des sous populations vers tel ou tel lignage B. Néanmoins Il est extrêmement important de voir l'ensemble comme un continuum au cours duquel les cellules immatures, transitionnelles, FO et MZ sont continuellement exposées à des signaux antigéniques assurant bien sur la sélection et l'élimination des clones auto-réactifs mais également la survie, l'activation et la différenciation des lymphocytes B (Pillai and Cariappa, 2009). Les modifications post-transcriptionnelles joueraient un rôle crucial dans le développement des cellules du système immunitaire (Turner et al., 2014). Ainsi plusieurs études ont démontré que l'expression des micro-ARNs régulait la différenciation des cellules B (Danger et al., 2014). Le micro-ARN-185 cible directement le BCR et module l'intensité des signaux transduits influençant ainsi le processus de différenciation des cellules B T2 vers un lignage ZM ou FO (Danger et al., 2014). Sur le plan fonctionnel les cellules B FO et ZM sont extrêmement différentes. Les cellules B ZM sont séquestrées dans les sinus marginaux de la rate où elles sont directement exposées aux antigènes sanguins. Elles expriment d'ailleurs très fortement le CD21, un récepteur du complément, qui permet ainsi de reconnaître les pathogènes recouverts par les molécules du complément et de s'activer de manière non spécifique. Ces cellules n'ont pas besoin de l'aide des lymphocytes T pour se différencier en plasmocytes. Comme les B1, elles sécrètent essentiellement des IgM et expriment également un grand nombre de TLRs permettant de moduler leurs fonctions (Rawlings et al., 2012). Les lymphocytes B FO représentent la majorité des cellules B de la rate (85-90%). Ces cellules, lorsqu'elles rencontrent l'antigène, migrent à la lisière entre follicule B et follicule T où elles entrent dans une réaction de centre germinatif (Victora

and Nussenzweig, 2012). C'est au cours de ce processus que les cellules B de centre germinatif (CG) diversifient leur répertoire BCR. Les cellules recombinent ainsi leur BCR de manière à exprimer une Immunoglobuline de membrane présentant la meilleure affinité pour l'antigène. Pour cela elles introduisent dans la séquence ADN de leur BCR des mutations aléatoires, on parle de mutations hypersomatiques (Victora and Nussenzweig, 2012). De plus, en fonction du type d'antigène et du microenvironnement inflammatoire, la cellule B va produire une Immunoglobuline de classe différente, on parle de commutation de classe. Ainsi une cellule folliculaire B IgD^+ peut exprimer et produire de l'IgM, A, E ou G. Ces processus assurent la diversification du répertoire BCR permettant ainsi une défense antimicrobienne optimale. Lorsque la cellule B exprime un nouveau BCR fonctionnel et suffisamment affin pour l'antigène, les cellules CG se différencient alors en cellules B mémoires ou en plasmocytes. La description de ce processus ne serait pas complète sans souligner l'importance des cellules dendritiques et lymphocytes T folliculaires (CD_{FH} et T_{FH} respectivement) dans les réactions des centres germinatifs (Victora and Nussenzweig, 2012). Les CD_{FH} sécrètent les chimiokines qui séquestrent les cellules B dans le centre germinatif. De plus ces cellules présentent de manière continue des antigènes et permettent ainsi de sélectionner les cellules B les plus affines. Les lymphocytes T_{FH} renforcent la survie des cellules CG en fournissant des signaux de costimulation. Dans cette interaction les voies CD40-CD40L et CD28/B1 sont cruciales (Victora and Nussenzweig, 2012).

1.2. Le lymphocyte B, un acteur clef de l'immunorégulation

Les lymphocytes B ont essentiellement été décrits pour leur capacité à se différencier en plasmocytes produisant des anticorps de différents isotypes (IgG, IgA, IgM, IgD et IgE). Les plasmocytes assurent ainsi une grande partie de l'immunité anti-infectieuse. En effet les anticorps assurent un grand nombre de fonctions : (i) l'opsonisation, processus par lequel l'anticorps recouvre la surface des pathogènes ou antigènes reconnus, favorisant ainsi leur phagocytose (ii) l'activation de la voie du complément et (iii) la dégranulation

de médiateurs pro-inflammatoires par les cellules de l'immunité innée (eosinophiles, mastocytes, basophiles et neutrophiles). Dans un contexte pathologique comme l'auto-immunité, l'allergie ou la transplantation, ces mécanismes deviennent délétères pour l'hôte induisant des lésions tissulaires parfois irréversibles (Salinas et al., 2013). C'est pour cela que le lymphocyte B représente une cible thérapeutique importante notamment en transplantation et en auto-immunité. Le développement du Rituximab, anticorps monoclonal ciblant la molécule CD20 a permis de proposer une thérapeutique efficace pour les patients atteints de ces pathologies. En supprimant l'ensemble cellules B, le Rituximab élimine ainsi les lymphocytes B pathogènes, diminue le taux d'anticorps délétères et contribue à l'amélioration des symptômes (Smith et al., 2006; Tokunaga et al., 2007). D'autres approches existent avec notamment le développement d'anticorps neutralisant la voie Blys/BAFF (Atacicept), empêchant ainsi la maturation des cellules B et la production d'auto-anticorps (Isenberg et al., 2014). Cependant des résultats surprenants ont été obtenus au cours des différents essais cliniques. En effet dans certains cas, l'amélioration clinique observée chez les patients traités au Rituximab précède souvent une réduction du taux d'auto-anticorps (Martin and Chan, 2006). En effet, dans la sclérose en plaque (SEP), la déplétion des lymphocytes B provoque d'abord une forte diminution du nombre de lymphocytes T dans le système nerveux central des patients traités, suggérant que les cellules B promeuvent le développement de cette pathologie en maintenant les réponses T pathogéniques probablement par la présentation antigénique ou la production de cytokines (Bar-Or et al., 2010; Hauser et al., 2008; Kappos et al., 2011; Vallerskog et al., 2007). Egalement, et de manière très intéressante, la déplétion des lymphocytes B chez des patients atteints de colites ulcératives, de psoriasis ou de SEP, entraîne une exacerbation des réponses inflammatoires menant à une aggravation des signes cliniques (Dass et al., 2007; Goetz et al., 2007; Kappos et al., 2014). Ces observations cliniques démontrent clairement la capacité des lymphocytes B à promouvoir ou contrôler les réponses inflammatoires indépendamment de leur fonction humorale. Si l'anticorps n'est pas le seul médiateur des

réponses B nous pouvons nous demander quels autres aspects cruciaux permettent d'assurer les fonctions effectrices du lymphocyte B.

Une des fonctions centrales de cette cellule est sa capacité à orienter les réponses immunitaires en présentant les antigènes aux lymphocytes T mais également en produisant un large spectre de cytokines. En effet la stimulation des lymphocytes B par les molécules de costimulation ou les TLRs induit la sécrétion de plusieurs molécules pro ou anti-inflammatoires jouant ainsi un rôle potentiel dans le cancer, l'auto-immunité, la transplantation et les allergies (Bao and Cao, 2014; Lund and Randall, 2010). Parmi les nombreuses cytokines identifiées, les cellules B sont capables de produire des cytokines pro-inflammatoires comme l'interleukine-2 (IL-2), l'IL-4, l'IL-6, l'IL-17, le TNFα, la lymphotoxine-α (LTα), l'interféron-γ (IFN-γ), mais également des chimiokines comme CXCL13, CCL5, CCL9 et CCL22 leur permettant d'influer sur leur propre migration ainsi que celle des cellules de l'environnement (Bao and Cao, 2014; Lund and Randall, 2010). A contrario les cellules B sont capables d'inhiber l'inflammation notamment au travers du contrôle des réponses T inflammatoires grâce à la production d'IL-10, d'IL-35, de TGF-β, mais également en induisant l'apoptose des cellules T effectrices par l'expression de granzyme-B, de Fas-L ou par la séquestration de métabolites comme l'adénosine via l'expression du couple CD39/CD73 (Bao and Cao, 2014; Chesneau et al., 2013; Lund and Randall, 2010). Dans la suite de ce manuscrit nous synthétiserons donc l'essentiel des données existantes dans le domaine décrivant le rôle des cellules B effectrices et régulatrices. Nous verrons ainsi en quoi celles-ci sont capables de réguler les réponses immunitaires et leur implication en immunopathologie.

2. Les lymphocytes B effecteurs en immunopathologie

2.1. Identification des lymphocytes B effecteurs

En 2000, l'équipe de Lund proposait le concept de B effecteur (Be) (Harris et al., 2000). En effet leurs travaux ont permis d'identifier l'existence de deux grands sous types de cellules B caractérisés par un profil cytokinique distinct. En co-cultivant des cellules T_H1 ou T_H2 en présence de lymphocytes B naïfs ils ont induit respectivement des B effecteurs 1 (Be1), produisant d'importantes quantités d'IFN-γ, d'IL-12, et d'IL-2 et des B effecteurs 2 (Be2), sécrétant de l'IL-2, de l'IL-4, de l'IL-6 et de l'IL-10 (Figure 1). L'identification de ces deux sous-groupes distincts suggérait que les cellules B étaient capables de réguler le développement et les fonctions effectrices des cellules immunitaires. De manière intéressante ils confirment cette hypothèse en démontrant que la capacité des Be1 et Be2 à induire la différenciation des T_H1 et T_H2 passe par la voie du récepteur de l'IFN-γ (Harris et al., 2005a) ou du récepteur de l'IL-4 (Harris et al., 2005b).

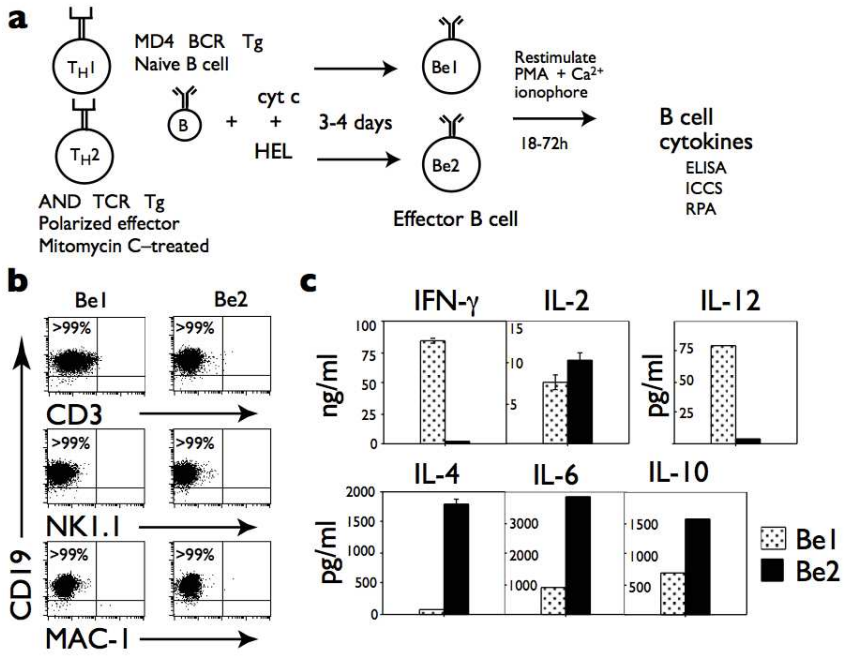

Figure 1: Identification des Be1 et Be2: A, Stratégie expérimentale pour induire les Be1 et Be2; **B,** Pureté des sous-populations leucocytaires triées; **C,** Profil cytokinique des Be1 et Be2.

Le rôle des lymphocytes Be a été étudié *in vivo* dans un modèle d'infection parasitaire par le *Heligmosomoides polygyrus.* Dans ce modèle, les cellules B produisant de l'IL-2 sont cruciales pour le développement des réponses T_H2 et le contrôle de la charge parasitaire (Wojciechowski et al., 2009). En effet des souris chimériques générant des cellules B déficientes pour l'IL-2 contrôlent de manière beaucoup moins efficace l'infection parasitaire. Les cellules T isolées de souris μMT (qui ne développent plus de lymphocytes B) reconstituées avec une moelle osseuse IL-2-/- ne produisent pas d'IL-4 après restimulation *in vitro* confirmant le rôle des cellules B IL-2^+ dans l'induction de réponses T_H2 (Wojciechowski et al., 2009). Bien que ce travail ne soit pas totalement en accord avec les observations initiales, selon lesquelles les cellules Be2 IL-4^+ induisent les T_H2 (Harris et al., 2000), il démontre pour la première fois l'importance des Be *in vivo*.

2.2. Les lymphocytes B effecteurs en auto-immunité

Les essais cliniques impliquant une déplétion des lymphocytes B chez des patients atteints de pathologies auto-immunes, ont démontré la capacité de ces cellules à maintenir des réponses inflammatoires pathologiques indépendamment de la production d'auto-anticorps (Hauser et al., 2008; Kappos et al., 2011; Martin and Chan, 2006; Vallerskog et al., 2007). Depuis, la démonstration de leur rôle dans la promotion de réponses T inflammatoires en pathologie reste faible, bien que celui-ci soit fortement suspecté. Des données initiales obtenues chez des patients lupiques démontrent la forte présence de lymphocytes Be produisant de l'IL-6 (Youinou and Jamin, 2009). Bien qu'aucune donnée expérimentale dans ce travail ne démontre clairement leur rôle dans la régulation des réponses T, la présence de ces cellules suggère néanmoins une implication possible des cellules B dans la différenciation des cellules T_H17. En effet de nombreuse données expérimentales (Acosta-Rodriguez et al., 2007; Burgler et al., 2009; Muranski and Restifo, 2013) et cliniques (Shin, 2013) démontrent l'importance de l'IL-6 dans le processus de différenciation des lymphocytes T_H17. Récemment deux études indépendantes ont démontré l'importance de la production d'IL-6 par les cellules B dans

l'induction de lymphocytes T_H17 et le développement de l'Encéphalite Auto-immune Expérimentale (EAE), un modèle d'étude de la SEP (Barr et al., 2012; Molnarfi et al., 2013). En effet une délétion conditionnelle de l'IL-6 dans les cellules B affaiblit la génération de T_H17 chez les souris, réduisant ainsi l'évolution de la pathologie (Molnarfi et al., 2013) (Figure 2A). *In vitro*, la stimulation des cellules B avec un agoniste du CD40 en combinaison avec un ligand du TLR4 (Lipopolysaccharide, LPS) ou du TLR9 (CpG Oligodeoxynucleotide, ODN) induit fortement la production d'IL-6 (Barr et al., 2012). Bien que l'ensemble des sous-populations B puisse produire de l'IL-6, on note un enrichissement de ces cellules dans la population B ZM. La fréquence de ces cellules augmente progressivement au cours du développement de l'EAE, de plus elles expriment un taux élevé de transcrits codant pour l'IL-6 (Barr et al., 2012). Le rôle de ces B effecteurs dans la pathogénie de la SEP est confirmé par des observations cliniques (Barr et al., 2012). En effet des patients souffrant d'une forme rémittente de SEP possèdent des taux élevés d'IL-6 sérique. Après traitement avec le Rituximab les taux d'IL-6 diminuent significativement, atteignant des niveaux comparables à ceux observés chez des volontaires sains (Figure 2B). Ceci démontre qu'en situation pathologique les cellules B sont une source majeure d'IL-6. De façon intéressante, des cellules mononuclées du sang périphériques issues de patients traités au Rituximab, déplétées en lymphocytes B, produisent moins d'IL-17 après restimulation *in vitro* confirmant ainsi le rôle des Be IL-6 dans le maintien des réponses T_H17 chez des patients atteints de SEP (Barr et al., 2012) (Figure 2B).

Des données similaires concernant le rôle Be IL-6 ont été obtenues dans un modèle de diabète de type 2 lié à l'obésité. Ainsi les cellules B isolées de la rate de souris diabétiques sécrètent d'importantes quantités d'IL-6 par rapport aux souris contrôles après ré-activation *in vitro* à l'aide de différents stimuli (DeFuria et al., 2013). Là encore, la stimulation par les TLRs semble être le moyen le plus efficace d'induire l'IL-6 dans les lymphocytes B. La production d'IL-10 par les cellules B est atténuée dans les

souris obèses suggérant un changement important dans le profil cytokinique des B des souris diabétiques en faveur d'un contexte plus inflammatoire (DeFuria et al., 2013).

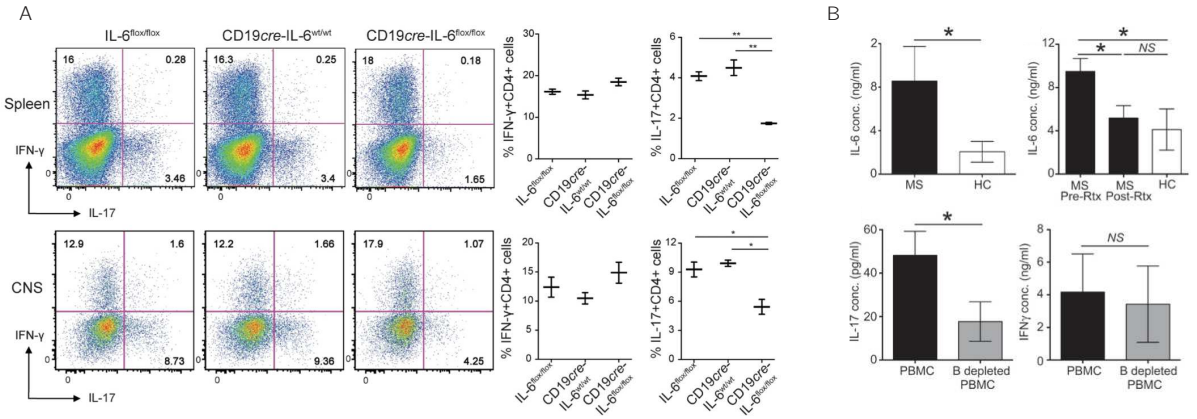

Figure 2 : Rôle des Be IL-6 dans la réponse T_H17 : A, Quantification des cellules T_H17 après restimulation *in vitro* chez des souris EAE; **B,** Panel du haut à gauche : Quantification de l'IL-6 chez des patients atteints d'une sclérose en plaque rémittente traités au Rituximab (MS) comparé à des volontaires sains (HC). Panel du haut à droite : Étude longitudinale de la production d'IL-6 avant et après le traitement au Rituximab. Panel du Bas : Quantification de l'IL-17 et de l'IFN-γ après restimulation *in vitro* des cellules mononuclées du sang de patients avec ou sans déplétion des cellules B.

Les souris obèses μMT développant une insulino-résistance ont des taux sériques diminués d'IL-6 comparées à des souris diabétiques sauvages. De plus les cellules T du tissu adipeux epididymal de souris obèses μMT ont une expression fortement diminuée de l'IL-17A et de l'IL17F, deux cytokines exprimées par les T_H17 (DeFuria et al., 2013). Enfin la co-culture de cellules B et T issues de patients obèses et diabétiques induit une forte production d'IL-17 absente chez les patients obèses non-diabétiques. Cependant les auteurs ne démontrent pas clairement l'implication de l'IL-6 dans ce système. L'ensemble de ces études suggèrent l'idée que les lymphocytes Be sont capables de promouvoir les réponses T inflammatoires en auto-immunité. Dans ce contexte la sous-population Be IL-6 semble jouer un rôle primordial en induisant notamment la différenciation des cellules T_H17 (Figure 3).

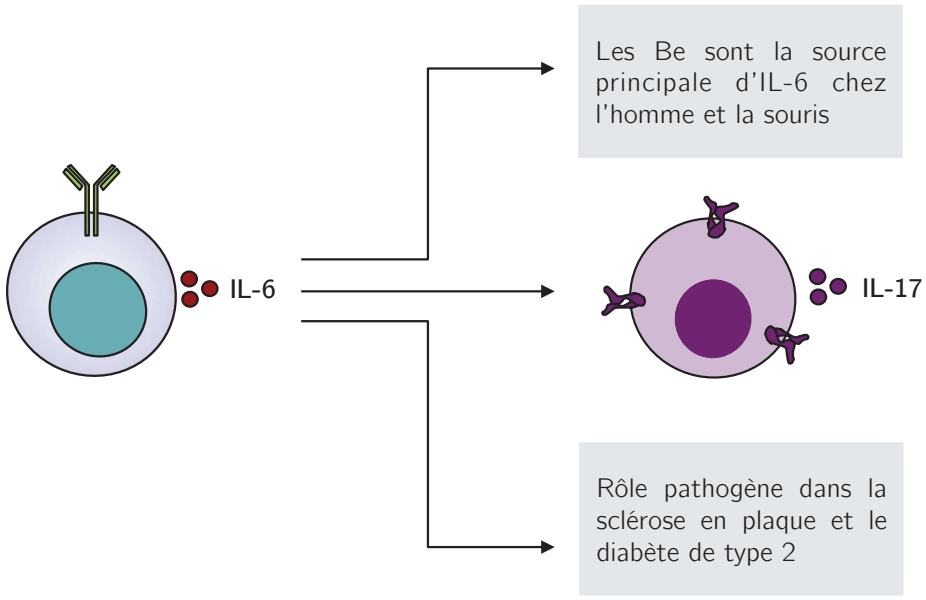

Figure 3 : Rôle des Be IL-6 en Immunopathologie

2.3. Les lymphocytes B effecteurs contribuent à l'immunité anti-infectieuse

De par leur rôle dans la production de cytokines, les Be ont également été décrits comme des acteurs clefs de l'immunité anti-infectieuse. Une étude récente a mis en évidence l'expansion d'une sous-population B FO caractérisée par une forte expression des molécules CD11a et FcγRIII, lors d'infections bactériennes (Listeria monocytogenes, *Escherichia coli)*, virales (vesicular stomatitis virus). Ces cellules B produisent de très hauts niveaux d'IFN-γ et sont capables de prévenir et contrôler l'infection après transfert adoptif (Bao et al., 2014). Le contrôle de l'infection passe par l'activation des macrophages en augmentant leur production de TNF-α et d'oxyde nitrique (Bao et al., 2014). L'investigation des mécanismes cellulaires nécessaires à la génération des Be IFN-γ révèle un rôle central des cellules dendritiques. En effet leur interaction via la voie CD40-CD40L est nécessaire à la production d'IFN-γ par les lymphocytes B (Bao et al., 2014). Dans cette étude les auteurs décrivent ainsi un rôle central des Be IFN-γ dans l'élimination des pathogènes intracellulaires. Récemment une étude très élégante, a démontré l'existence d'une nouvelle population Be capable de contrôler le choc septique induit par le LPS (Rauch et al., 2012). Cette sous-population B produit de fortes concentrations de GM-CSF. Cette découverte est très surprenante. En effet, il était initialement admis que le GM-CSF était exclusivement produit par les cellules non-

hématopoïétiques, les macrophages et dans certains cas les lymphocytes T CD4+ (Hamilton, 2008; Noster et al., 2014). Ici l'injection de LPS provoque une production exclusive de GM-CSF par les cellules B spléniques (Figure 4 A-G). Les données phénotypiques et transcriptomiques classent les Be GM-CSF comme un sous groupe distinct des autres populations B lymphocytaires. Leur phénotype IgM^{hi} CD23^{lo} CD43^{hi} CD93^{+} IgD^{lo} CD21^{lo} CD138^{+} VLA4^{hi} LFA1^{hi} CD284^{+} CD5^{+} les rapproche des plasmocytes CD138^{+} (Rauch et al., 2012).

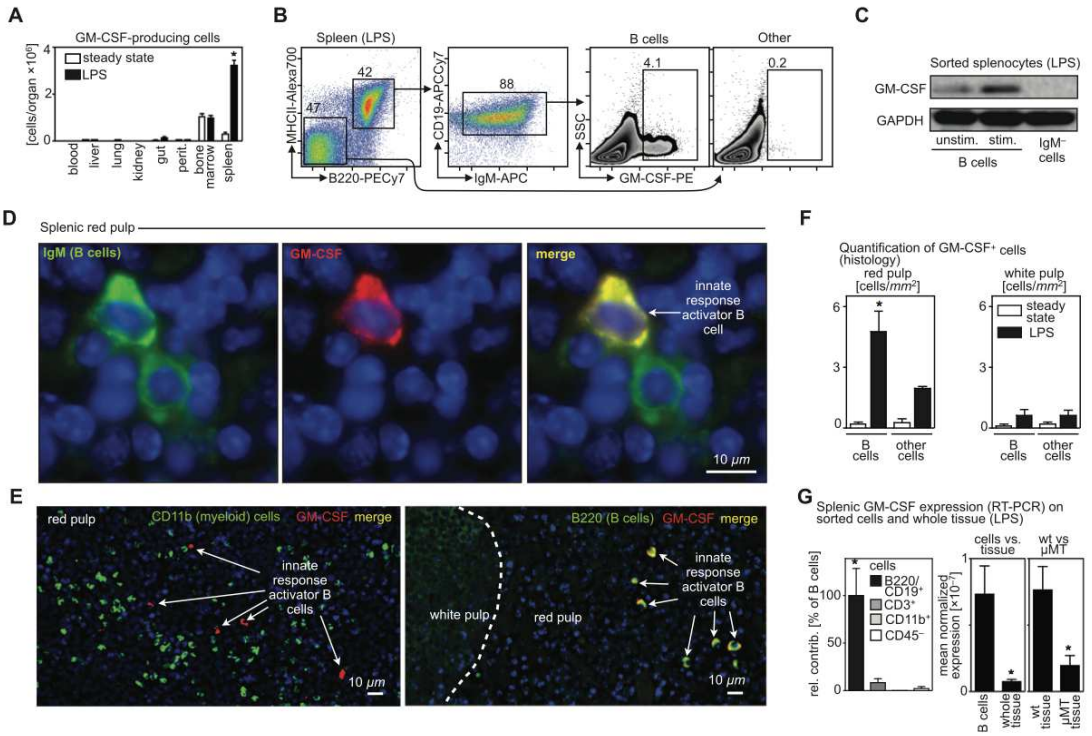

Figure 4 : Identification des cellules B produisant du GM-CSF : A, Quantification des cellules produisant du GM-CSF dans différents tissues à l'état basal et en réponse à plusieurs injections intra-péritonéale de LPS. **B,** Identification des cellules B produisant du GM-CSF dans la rate de souris exposées au LPS. **C,** Analyse par western blot de l'expression de GM-CSF sur des cellules purifiées. **D-F** Colocalisation des cellules IgM^{+} et GM-CSF^{+}. **G,** Expression du GM-CSF dans les splénocytes d'animaux sauvages ou µMT.

Dans le but de caractériser cette population, les auteurs se sont intéressés à leur origine cellulaire. Ils ont d'abord voulu savoir si le progéniteur à l'origine de cette population Be GM-CSF était circulant. En effet, alors que les cellules B1, FO, T1 et T2 sont circulantes, les cellules B ZM et leurs progéniteurs sont exclusivement séquestrés dans la

rate (Arnon et al., 2013). Ainsi à l'aide d'un modèle de parabiose (souris partageant leur système circulatoire sanguin) les auteurs ont pu confirmer le caractère circulant des progéniteurs des Be GM-CSF (Figure 5) (Rauch et al., 2012). Enfin le transfert adoptif des différentes sous-populations B circulantes, dans un hôte murin exposé au LPS, a permis d'identifier les lymphocytes B1a comme population originelle des Be GM-CSF (Figure 5A, B et E).

Parmi les signaux nécessaires à l'induction des B GM-CSF, on retrouve un rôle crucial de la signalisation TLR et Myd88. Des souris délétées pour ces molécules sont incapables d'induire l'expression du GM-CSF dans les lymphocytes B. Ce résultat est logique étant donnée la dépendance de ces cellules aux signaux microbiens. De plus la délétion du récepteur de BAFF, un facteur de croissance des cellules B, inhibe la génération des cellules Be GM-CSF. Ceci reste surprenant car les cellules B1a, dont découlent les Be GM-CSF, peuvent se développer indépendamment de BAFF (Rauch et al., 2012). Cela suggère donc un rôle de cette molécule dans l'induction du GM-CSF dans les lymphocytes B plutôt que dans leur survie. La surexpression de VLA4 et LFA-1 sur ces cellules est en faveur d'un rôle de ces molécules dans leur rétention dans la rate. En effet l'inhibition de l'activité de ces molécules à l'aide d'anticorps neutralisants entraîne la recirculation de ces cellules hors du compartiment splénique (Rauch et al., 2012). Enfin les auteurs démontrent la capacité de ces cellules à diminuer le risque de choc septique après une infection bactérienne par *E. Coli* (Rauch et al., 2012). Mais comment agissent ces cellules *in vivo* ? Comment combattent-elles l'infection ? Il semblerait que ces cellules jouent un rôle indirect en stimulant la production d'anticorps IgM par les cellules B1a (Weber et al., 2014). En effet lors d'une infection pulmonaire on note l'accumulation de cellules B1a et Be GM-CSF dans les voies aériennes. De façon intéressante la coculture de cellules B1a en présence de Be GM-CSF augmente fortement la production d'IgM. Ce mécanisme est dépendant du GM-CSF car des cellules B1a délétées pour le récepteur au GM-CSF ne produisent que très peu d'IgM.

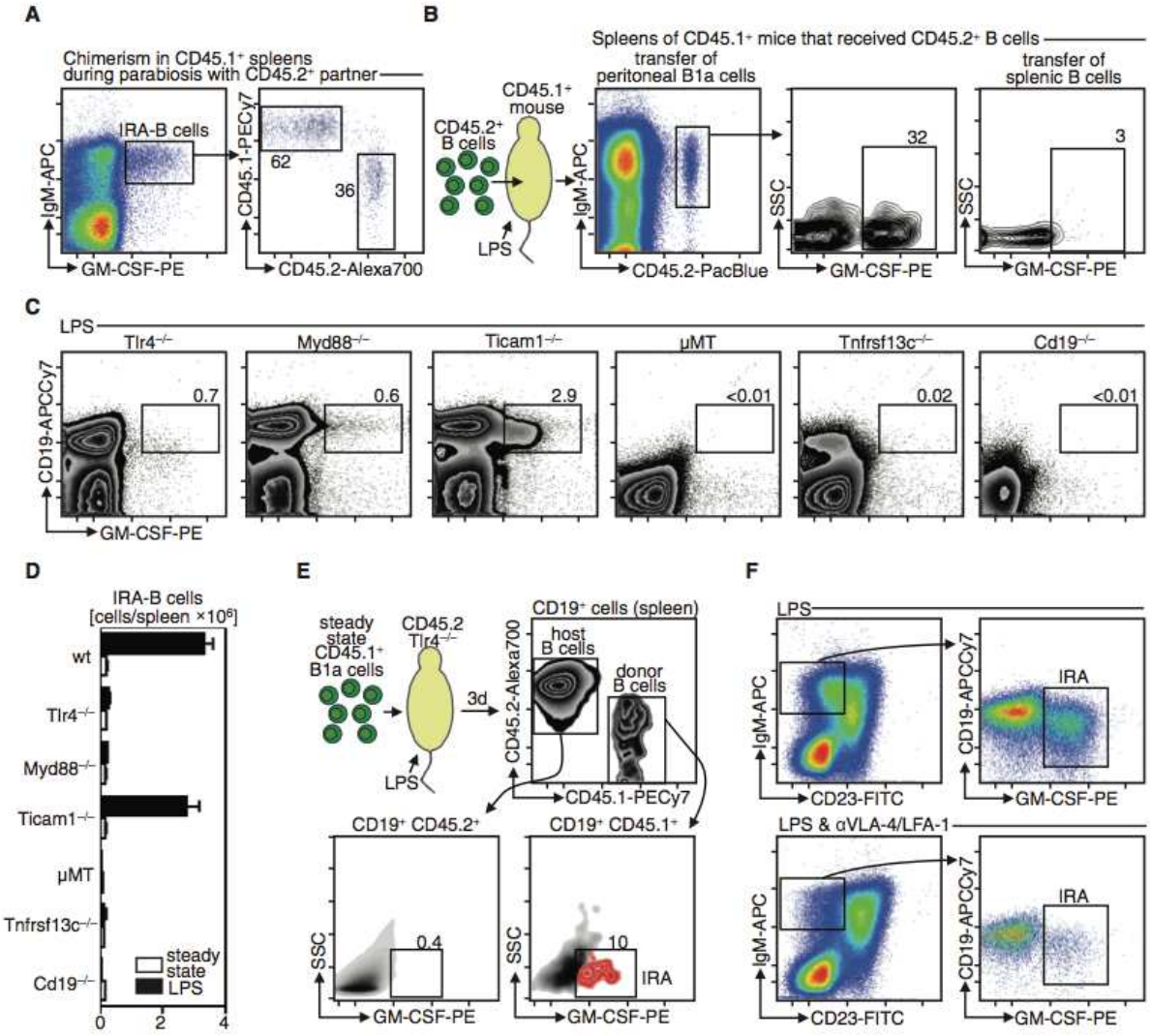

Figure 5 : Les Be GM-CSF se développent à partir des cellules B1a, dépendent fortement des signaux induits par la voie TLR/Myd88 et résident dans les tissues grâce au couple LFA-1/VLA-4 : A, Analyse par cytométrie en flux du chimérisme présent dans les Be GM-CSF dans le système de parabiose. Le chimérisme est suivi à l'aide de l'expression des marqueurs congéniques CD45.1 et CD45.2 **B,** Le transfert adoptif des cellules B1a donne naissance à des cellules Be GM-CSF après exposition au LPS. **C,** Analyse du développement des Be GM-CSF dans des souris $Tlr4^{-/-}$, $Myd88^{-/-}$, $Ticam1^{-/-}$ (gène codant pour TRIF), µMT, $Tnsfrs13c^{-/-}$ (gène codant pour BAFF-R) et $CD19^{-/-}$. **D,** Numération des cellules B GM-CSF dans les souris sauvages et génétiquement modifiées après exposition au LPS. **E,** Transfert de B1a dans un hôte $Tlr4^{-/-}$. **F,** Effet du blocage du couple VLA-4/LFA-1 sur la rétention des cellules B GM-CSF dans la rate.

L'ensemble de ces résultats suggère un rôle important des Be GM-CSF dans l'activation et la maturation des B1a en plasmocytes sécrétant des IgM lors d'infections. En faveur de cette hypothèse, l'absence de cellules Be GM-CSF *in vivo* affaiblit la production

d'IgM antibactérienne et empêche le contrôle l'infection confirmant ainsi leur hypothèse (Weber et al., 2014) (Figure 6).

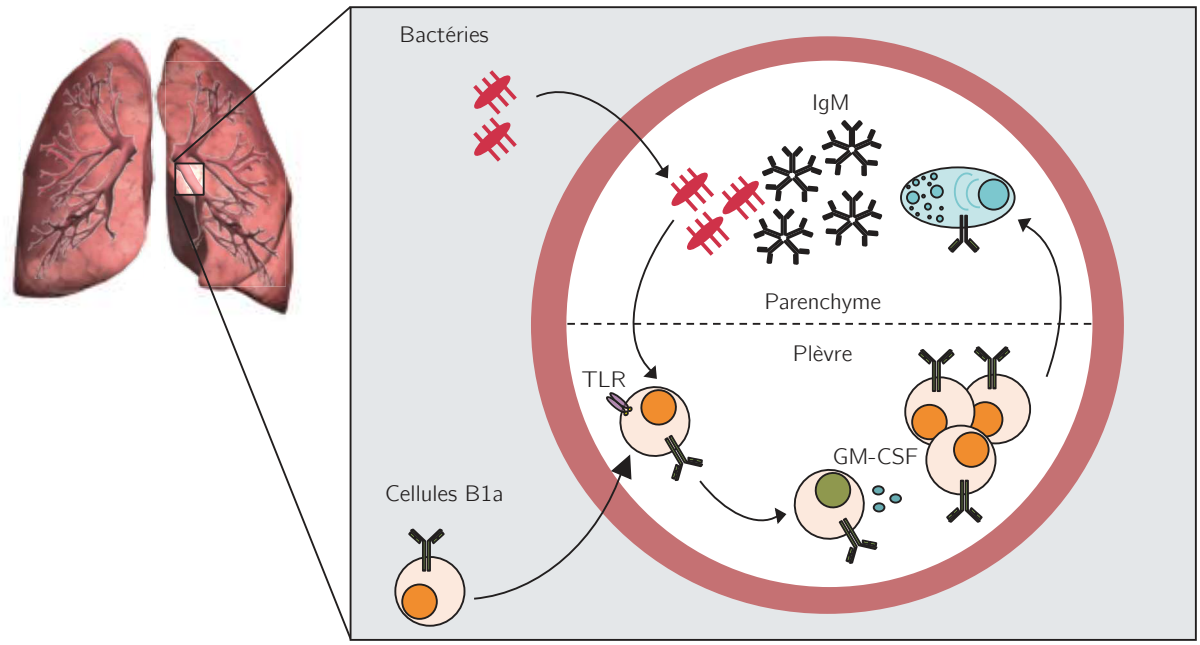

Figure 6 : Modèle décrivant la fonction des cellules B GM-CSF au cours d'une infection pulmonaire. Pendant une pneumonie les cellules B1a de l'espace pleural reconnaissent la bactérie et ces composants grâce aux TLRs. Ceci mène à la génération des cellules Be GM-CSF qui agissent sur les autres cellules B1a induisant ainsi leur différenciation en cellules sécrétrices d'IgM. Ces cellules plasmocytaires migrent alors dans le parenchyme pulmonaire où elles assurent ainsi la défense anti-infectieuse.

2.4. Les lymphocytes B effecteurs peuvent structurer la réponse immunitaire

Derrière la notion « structurer », on sous-entend tout d'abord la capacité des cellules B à influencer la migration d'autres effecteurs du système immunitaire. Le pouvoir chémoattractant des lymphocytes B reste très peu décrit. L'une des plus belles démonstrations a été publiée récemment dans un modèle murin d'infarctus du myocarde. Cette pathologie, caractérisée par une thrombose des artères coronaires, dégrade fortement la fonction cardiaque. Les dommages tissulaires provoquent le relargage de différents composants moléculaires qui vont activer le système immunitaire notamment par l'interaction avec les TLRs. Ces signaux de dangers induisent ainsi la production de

cytokines et chimiokines pro-inflammatoires qui vont recruter des neutrophiles et des monocytes responsables notamment du remodelage cardiaque, une des conséquences altérant la fonction cardiaque. Les auteurs démontrent ainsi qu'une déplétion des lymphocytes B diminue l'inflammation et la gravité de l'infarctus (Zouggari et al., 2013).

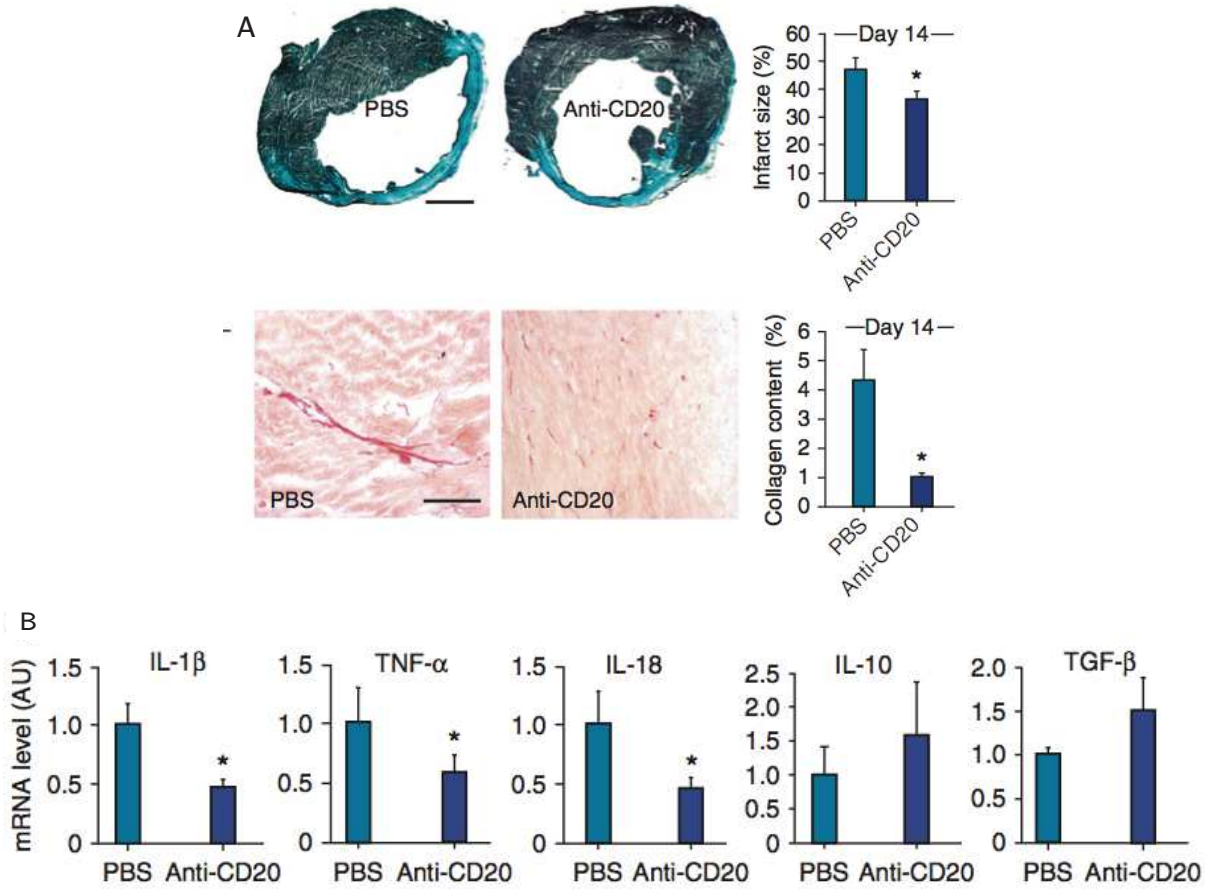

Figure 7 : La déplétion des lymphocytes B réduit l'importance de l'infarctus et limite l'inflammation du myocarde. A, Etude de la fibrose cardiaque par coloration au trichrome de Masson et au rouge Sirius. **B,** Taux d' ARNm des cytokines pro-inflammatoires (IL-1β, TNF-α et IL-18) et des cytokines anti-inflammatoires (IL-10 et TGF-β).

Comme les monocytes sont directement impliqués dans le développement de la fibrose, les auteurs ont quantifié ces cellules au niveau du tissu cardiaque. Ainsi ils ont pu mettre en évidence une diminution significative du nombre de monocytes infiltrant le myocarde suggérant un rôle des cellules B dans leur attraction sur le site inflammatoire. CCL2 et CCL7 sont les deux chimiokines impliquées dans le recrutement des monocytes. Or la

déplétion des lymphocytes B fait chuter le taux de ces deux chimiokines dans le sang des animaux atteints d'infarctus démontrant ainsi l'existence d'une sous-population Be impliquée dans l'attraction des monocytes pro-inflammatoires et donc responsable du développement de la fibrose dans l'infarctus du myocarde (Zouggari et al., 2013).

Toujours dans le cadre de la structuration des réponses immunitaires, il est important de prendre en compte la fonction des lymphocytes B dans la l'organogénèse lymphoïde. La structuration des tissus lymphoïdes dépend essentiellement de la production de lymphotoxine (LT) et de TNF-α. En effet, les souris déficientes pour la lymphotoxine ne développent plus de ganglions lymphatiques structurés et de plaques de Peyer (De Togni et al., 1994). De telles déficiences structurelles altèrent fortement la mise en place de réponses immunes optimales. Malgré cela, ces souris restent immunocompétentes avec la mise en place d'une immunité tissulaire et la formation d'organes lymphoïdes tertiaires (Moyron-Quiroz et al., 2006). La LT est produite par un grand nombre de cellules du système immunitaire (De Togni et al., 1994). Les lymphocytes B en sont une source importante (Sung et al., 1989; Worm and Geha, 1994). De manière intéressante la production de LT par les cellules B assure la formation des follicules lymphoïdes et la génération d'un réseau de CD_{FH} menant ainsi à la structuration des organes lymphoïdes et à la mise en place de réponses immunes optimales (Fu et al., 1998). Une fois mise en place, le maintien de la structure des organes lymphoïdes est orchestré par l'expression de différentes chimiokines parmi lesquelles CXCL13 (Ligand de CXCR5), CCL19 et CCL21 (Ligand de CCR7) jouent un rôle fondamental. La production de LT par les lymphocytes B régule l'expression de CXCL13 (Figure 8B). Ceci permet notamment le positionnement optimal des cellules dendritiques et des lymphocytes T dans les ganglions lymphoïdes (Leon et al., 2012). Dans un modèle d'infection parasitaire avec *Heligmosomoides polygyrus*, la délétion de l'expression de LT dans les B altère le positionnement des DC et des cellules T dans la zone périfolliculaire des ganglions

menant ainsi à un contrôle moindre de la charge parasitaire (Figure 8A) (Leon et al., 2012).

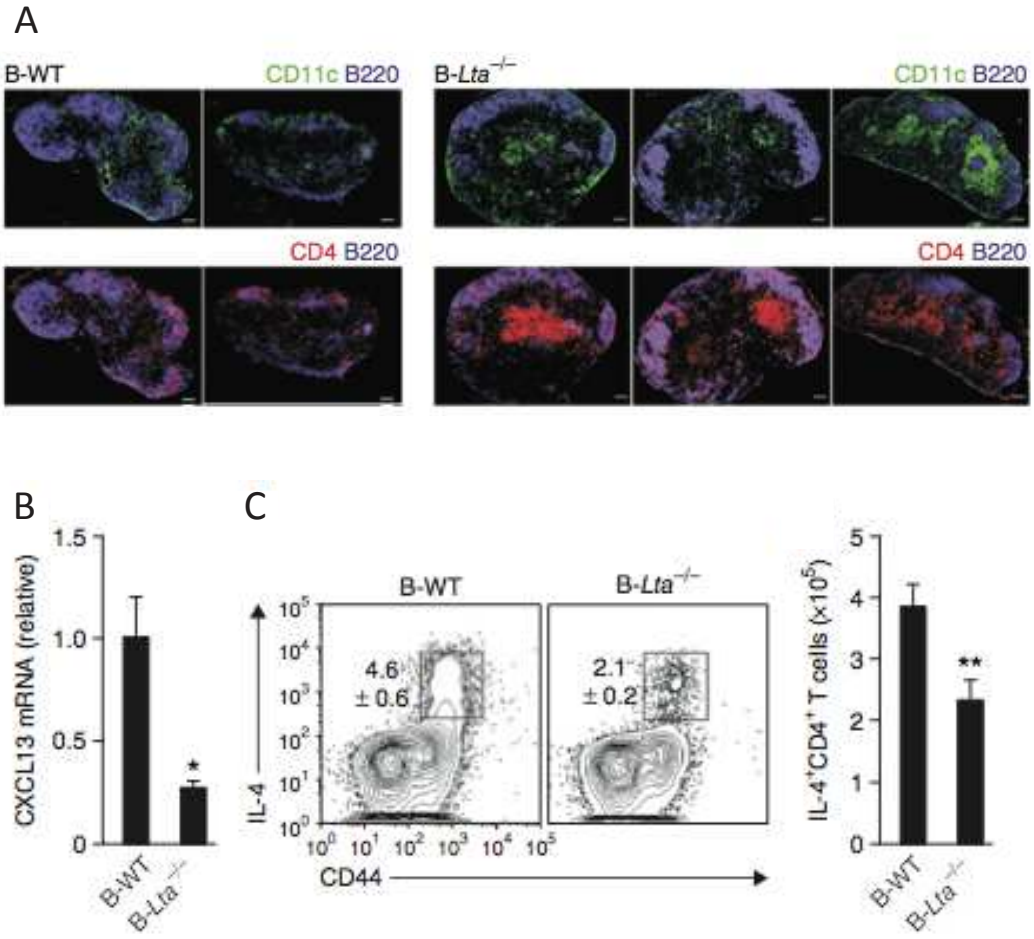

Figure 8 : Les lymphocytes B sécrétant de la lymphotoxine sont essentiels pour le positionnement des cellules T et dendritiques et l'induction des lymphocytes T_H2. A, Coupes d'immunofluorescence avec les marqueurs suivant B220 (pour les cellules B), CD4 (pour les lymphocytes T) et CD11c (pour les cellules dendritiques) dans les ganglions lymphatiques, 8 jours après infection avec *Heligmosomoides polygyrus*. On note un positionnement altéré des populations cellulaires lorsque les cellules B sont déficientes pour la LT. Notez ainsi l'accumulation désorganisée des cellules T et des DC alors qu'on a un repositionnement périfolliculaire optimal chez les souris sauvages. **B,** Expression de CXCL13 dans des souris sauvages et d'autres présentant une délétion de la LT dans les cellules B, 8 jours après infection avec *Heligmosomoides polygyrus*. **C,** Quantification des cellules T produisant de l'IL-4 dans la rate des animaux infectés.

En effet, et de manière surprenante, la production de LT par les lymphocytes est nécessaire à la mise en place d'une réponse T_H2 optimale (Figure 8C). La raison pour

laquelle l'immunité antiparasitaire serait plus efficace dans ce cadre précis n'est pas clairement expliquée dans ce travail. Une des possibilités serait que les cellules B qui captent et présentent les antigènes produisent des cytokines, notamment l'IL-2 (Harris et al., 2005b) et expriment des molécules de costimulation permettant d'orienter la réponse T_H2. Cependant des travaux supplémentaires seraient nécessaires pour élucider la question.

Dans le même registre il semblerait que les cellules B soient cruciales pour la formation d'organes lymphoïdes ectopiques dans les poumons. Les organes lymphoïdes ectopiques sont des structures lymphoïdes tertiaires se formant directement dans les tissus assurant ainsi une immunité locale. Ils se développent lors d'inflammation chronique et dans des conditions similaires aux organes lymphoïdes secondaires (Randall, 2010; Randall and Mebius, 2014). Une des différences fondamentales entre ces deux types de structures est l'absence d'encapsulation suggérant ainsi une interaction avec le milieu et notamment avec les cellules épithéliales (Randall, 2010; Randall and Mebius, 2014). L'étude de ces structures a un intérêt particulier notamment dans les pathologies respiratoires comme l'HyperTension Artérielle Pulmonaire (Perros et al., 2012) (HTAP), la Broncho-Pneumopathie Obstructive Chronique (Bracke et al., 2013; Litsiou et al., 2013) (BPCO) ou dans l'asthme (Baay-Guzman et al., 2012; Randall, 2010) où leur présence est souvent associée à un mauvais pronostic. Le rôle des B dans la formation de ces structures a été décrit dans le cadre de la BPCO (Litsiou et al., 2013). La présence des follicules lymphoïdes tertiaires chez des patients fumeurs est associée à des symptômes plus sévères (Bracke et al., 2013; Litsiou et al., 2013). Ainsi on note chez ces patients de très hauts niveaux de CXCL13 et de LT dans le lavage broncho-alvéolaire (Litsiou et al., 2013). Après digestion de biopsies bronchiques les auteurs ont pu récupérer les cellules et analyser la source de ces molécules. C'est ainsi qu'ils ont identifié le lymphocyte B comme une source majeure de LT mais également de CXCL13 (Litsiou et al., 2013). Ce résultat est assez controversé et à l'inverse des résultats obtenus dans

d'autres types de pathologies pulmonaires. En effet il semblerait que les cellules dendritiques folliculaires, présentes dans les poumons, mais également les cellules épithéliales pulmonaires soient également des sources majeures de cette chimiokine (GeurtsvanKessel et al., 2009; Rangel-Moreno et al., 2007). Quoi qu'il en soit l'ensemble de ces résultats met en avant le rôle des lymphocytes B dans la régulation et l'attraction des cellules immunitaires directement dans les tissus.

En résumé

- Depuis l'identification des Be1 et Be2, les études ont mis en évidence une multitude de sous-populations lymphocytaires B produisant des cytokines pro-inflammatoires et assurant ainsi des fonctions variées en immunopathologie.
- Les Be peuvent ainsi réguler les réponses T en favorisant notamment la différenciation des T_H17 via la sécrétion d'IL-6. Ce mécanisme est fortement suspecté en autoimmunité.
- Les Be participent également à l'immunité infectieuse grâce à (i) la sécrétion d'IFN-γ qui permet l'activation des macrophages mais également (ii) la production de GM-CSF qui promeut fortement la différenciation des B1a en cellules plasmocytaires sécrétant des IgM protectrices.
- Les Be produisent également des chimiokines et des cytokines importantes pour l'organogénèse des tissus lymphoïdes et le recrutement des cellules immunitaires sur le site inflammatoire.

3. Les lymphocytes B régulateurs en immunopathologie

3.1. Identification des lymphocytes B régulateurs

Le concept de « lymphocytes B régulateurs » (Bregs) a émergé il y a 40 ans (Katz et al., 1974). Dans un modèle d'hypersensibilité chez le cochon d'inde, une lymphodéplétion à l'aide de cyclophosphamide augmentait l'inflammation induite par l'hypersensibilité suggérant l'existence d'une population lymphocytaire régulatrice (Katz et al., 1974). De façon intéressante, le transfert de splénocytes d'un animal sensibilisé à un autre permettait de contrôler le développement de la pathologie. Or, le transfert de splénocytes, déplétés en cellules B, ne conférait aucune protection contre l'inflammation, suggérant ainsi pour la première fois l'existence de cellules B capables de réguler d'autres cellules autologues non B. Plus tard les observations démontrant qu'un déficit génétique en lymphocyte B augmentait l'inflammation dans un modèle d'EAE confirmaient le rôle régulateur de ces cellules (Wolf et al., 1996). Ces 10 dernières années, de nombreux travaux ont confirmé qu'une absence de lymphocyte B participait à une inflammation sévère lors du développement de pathologies auto-immunes (Yanaba et al., 2008; Yanaba et al., 2013). Dans les années 90, plusieurs travaux en faveur du rôle des cellules B dans l'induction de la tolérance immunologique ont été publiés (Fuchs and Matzinger, 1992; Lassila et al., 1988). Les travaux de Polly Matzinger démontraient par exemple que les lymphocytes B naïfs étaient incapables d'initier une réponse T (Lassila et al., 1988). De façon intéressante l'injection de ces cellules B naïves pouvait induire la tolérance en transplantation chez le petit animal (Fuchs and Matzinger, 1992). A l'époque elle posait l'hypothèse que les B naïfs étaient plutôt tolérogènes et donc incapable d'activer des lymphocytes T naïfs. Or ce travail montre que les lymphocytes B activés avec du LPS peuvent également induire la tolérance à l'allogreffe. Ce résultat inattendu a probablement été mal interprété. En effet, Polly Matzinger ne se doutait probablement pas qu'elle venait d'identifier l'un des médiateurs

majeurs des lymphocytes B régulateurs (Fillatreau et al., 2002; Fuchs and Matzinger, 1992).

3.2 L'IL-10 est un médiateur clef des fonctions régulatrices assurées par les lymphocytes B régulateurs

3.2.1 Identification des lymphocytes B régulateurs dans les modèles animaux

Initialement, les lymphocytes B régulateurs produisant de l'IL-10 (B10) ont été identifiés dans des modèles animaux de colite ulcérative et d'EAE (Fillatreau et al., 2002; Mizoguchi et al., 2002). Alors que l'EAE régresse spontanément chez des souris sauvages, les souris chimériques n'exprimant plus l'IL-10 dans leurs cellules B développent une EAE beaucoup plus sévère (Fillatreau et al., 2002). Une absence de production d'IL-10 par les lymphocytes B est ainsi associée à une diminution de cellules T_H1 et T_H17, démontrant ainsi le rôle des Bregs dans le contrôle des réponses T inflammatoires *in vivo* et dans le contrôle de l'EAE (Carter et al., 2011; Fillatreau et al., 2002). Les B10 régulent également l'inflammation dans un modèle spontané de colite ulcérative (Mizoguchi et al., 2002; Sattler et al., 2014). La présence des Bregs permet de contrôler la production d'IL-1β diminuant ainsi l'inflammation dans l'organe atteint (Mizoguchi et al., 2002). De récentes données démontrent que l'absence de B10 dans un modèle de colite favorise l'infiltration de granulocytes et une inflammation de type T_H1 dans l'intestin (Sattler et al., 2014). Un rôle protecteur des B10 a également été rapporté dans un modèle d'arthrite induit par le collagène (Evans et al., 2007; Yang et al., 2012). Ces cellules contrôlent le développement de l'arthrite en inhibant l'activation des cellules T_H17 (Yang et al., 2012). Les B10 contrôlent également le développement des pathologies allergiques (Braza et al., 2014). En effet, elles participent à l'inhibition de l'activation des cellules T au niveau de la peau dans des modèles d'hypersensibilités (Jin et al., 2013; Yanaba et al., 2008). Dans le cadre d'allergies respiratoires, un seul travail a démontré que l'infection parasitaire pouvait promouvoir l'émergence de cellules B10 capables de contrôler l'inflammation des voies aériennes et de restaurer la fonction

pulmonaire dans un modèle d'asthme allergique à l'ovalbumine (OVA) (Amu et al., 2010). Enfin des travaux expérimentaux chez la souris ont démontré la capacité des cellules B10 à induire la tolérance en transplantation démontrant ainsi le fort pouvoir régulateur de ces cellules (Chesneau et al., 2013; Ding et al., 2011). À l'inverse, la présence de B10 peut être indésirable. C'est notamment le cas lors du développement d'infections ou de cancers où les Bregs participent aux mécanismes d'échappement infectieux (Das et al., 2012; Neves et al., 2010; Siewe et al., 2013) ou tumoraux (DiLillo et al., 2013; Olkhanud et al., 2011).

3.2.2 Identification des lymphocytes B10 chez l'homme

L'exacerbation de l'inflammation observée après déplétion des lymphocytes B chez des patients atteints de pathologies autoimmunes, renforce l'idée de selon laquelle les cellules B sont capables de contrôler le développement de pathologies inflammatoires (Dass et al., 2007; Goetz et al., 2007; Kappos et al., 2014). Un bel exemple concerne les résultats obtenus lors de l'essai thérapeutique de Rituximab chez des patients atteints de colite ulcérative. En effet la déplétion des B est associée à une perte de l'expression de l'IL-10 dans la muqueuse intestinale et à des atteintes pathologiques beaucoup plus sévères (Goetz et al., 2007). Or chez l'homme, les cellules B produisent également de l'IL-10 après activation *in vitro* suggérant ainsi l'existence d'une population B10. De façon intéressante il semblerait qu'en situation pathologique, la présence des Bregs soit altérée. En effet les cellules B de patients atteints de SEP produisent moins d'IL-10 que des cellules B de volontaires sains suggérant qu'un défaut de B10 pourrait contribuer à la pathogenèse cette maladie (Duddy et al., 2007). De manière intéressante les infections parasitaires sont corrélées à des fréquences réduites de poussées inflammatoires de SEP (Correale et al., 2008). Or les cellules B de patients affectés produisent plus d'IL-10 que les autres suggérant ainsi un rôle des B10 dans le contrôle des poussées (Correale et al., 2008). Le développement de Lupus et de polyarthrite altèrerait la fonction régulatrice des B10 (Blair et al., 2010; Flores-Borja et al., 2013). En effet les Bregs de ces patients sont incapables d'inhiber l'activation des

cellules T et la production de cytokines pro-inflammatoires *in vitro* (Blair et al., 2010; Flores-Borja et al., 2013).

3.2.2 Mécanismes régulateurs assurés par les B10

Les différentes études chez l'animal et l'homme ont pu mettre en avant les fonctions assurées par les B10 (Rosser et al., 2014) (Figure 9). Tout d'abord, elles inhibent directement l'activation et la prolifération des cellules T effectrices. En effet, elles sont capables d'inhiber *in vivo* et *in vitro* la production de cytokines T_H1, T_H2 et T_H17 (Rosser et al., 2014). De manière intéressante, elles sont directement impliquées dans l'induction de cellules T régulatrices (Amu et al., 2010; Carter et al., 2011; Flores-Borja et al., 2013; Matsushita et al., 2010; Olkhanud et al., 2011). Par exemple les lymphocytes B10 induits au niveau de la tumeur participeraient à l'échappement tumoral en induisant des cellules T régulatrices via la production d'IL-10 (Olkhanud et al., 2011). Aussi, l'effet bénéfique observé dans le modèle d'asthme allergique à l'OVA passe par l'induction de cellules T régulatrices dans les voies aériennes (Amu et al., 2010). Également les B10 peuvent influer sur la réponse cellulaire T de manière indirecte en modulant l'activation des autres cellules présentatrices d'antigènes, les CD et les monocytes (Iwata et al., 2011; Lampropoulou et al., 2008; Li et al., 2012). Les B10 régulent la maturation des monocytes et leur production de cytokines pro-inflammatoires, TNF-α, IL-6, IL-12 et IL-23 (Iwata et al., 2011; Lampropoulou et al., 2008; Li et al., 2012). L'ensemble de ces études renforce donc le rôle central des B10 dans le maintien d'une balance immunologique favorable. Or, il est intéressant de souligner qu'un défaut quantitatif ou fonctionnel de ces cellules a été rapporté dans un grand nombre de pathologies autoimmunes, en faisant ainsi une cible thérapeutique privilégiée (Blair et al., 2010; Flores-Borja et al., 2013; Gao et al., 2014; Lemoine et al., 2011; Li et al., 2012; Lin et al., 2014; Zhu et al., 2014). Néanmoins, encore faut-il connaître et maitriser les mécanismes nécessaires à leur développement pour prétendre à une quelconque application ou manipulation thérapeutique (Mauri and Blair, 2014).

3.3 Signaux nécessaires au développement des B10

3.3.1. Rôle des TLRs

Les signaux nécessaires à l'acquisition des propriétés suppressives des B10 ont été partiellement identifiés. De façon surprenante les TLRs, initialement décrits comme des acteurs clefs des processus inflammatoires, ont un rôle critique dans le développement des B10. En effet le rôle des TLRs dans l'induction des Bregs a été initialement décrit par les travaux de Polly Matzinger cités plus hauts. Les expériences *in vitro* confirment le rôle de l'activation des TLRs dans la production d'IL-10 par les lymphocytes B murins et humains (Iwata et al., 2011; Lampropoulou et al., 2008; van de Veen et al., 2013). Enfin des souris qui n'expriment plus le TLR4 à la surface des cellules B, ou la molécule adaptatrice MyD88 (protéine clef dans la signalisation des TLRs) développent une EAE sévère, similaire à celle observée chez des souris déficientes en B10 (Fillatreau et al., 2002). Par ailleurs les cellules B de souriceaux nouveaux nés, sont plus sensibles aux signaux TLRs et produisent ainsi d'importantes quantités d'IL-10. Une absence de signaux TLRs inhibe complètement le développement des B10 démontrant ainsi le rôle central de ces protéines dans la génération des B10 (Yanaba et al., 2009).

3.3.2. Rôle du BCR

Bien que l'activation par les TLRs soit nécessaire et indispensable, elle n'est pas suffisante pour induire les B10 de manière optimale. En effet des souris avec un répertoire BCR limité sont incapables de produire ces cellules (Yanaba et al., 2009) ou de contrôler le développement de l'EAE (Fillatreau et al., 2002). Plusieurs études confirment ainsi l'idée que le BCR contribue à la mise en place des fonctions suppressives des B10. Tout d'abord sur un plan cellulaire, la spécificité antigénique est primordiale (Ding et al., 2011; Matsushita et al., 2010).

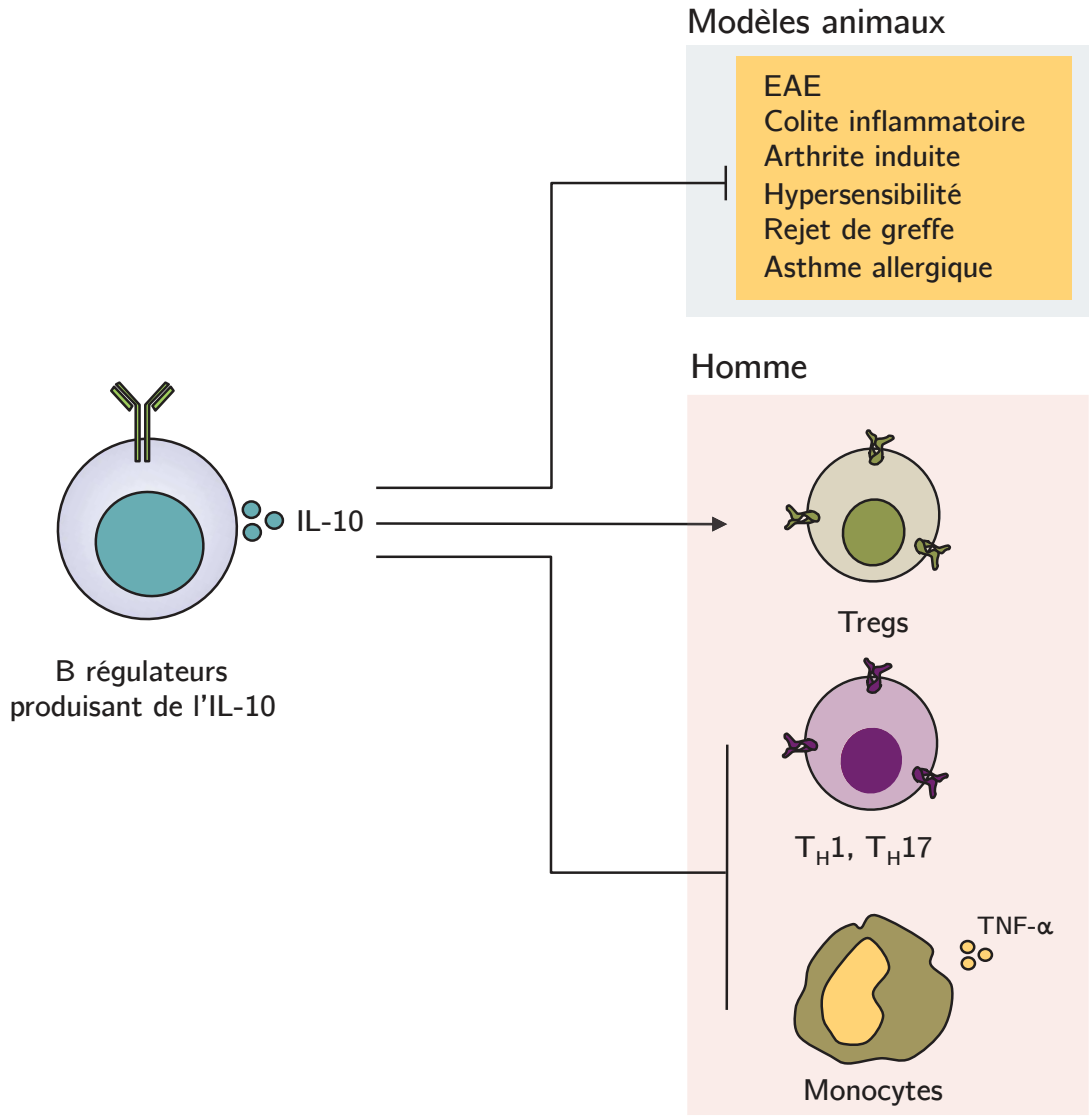

Figure 9 : Rôle des B10 en Immunopathologie

En effet le transfert de B10 de souris naïves ne contrôle par le rejet de greffe chez la souris receveuse. *A contrario*, le transfert de B10 d'une souris préalablement immunisée aux alloantigènes permet d'induire une tolérance à la greffe chez la souris receveuse suggérant ainsi la nécessité d'une activation BCR-spécifique pour assurer leurs fonctions immunosuppressives (Ding et al., 2011). Sur le plan moléculaire, l'activation par les TLRs, suivie d'une activation par le BCR, potentialise fortement la production d'IL-10. Or une délétion de la protéine BLNK dans les lymphocytes B (Jin et al., 2013), ou des molécules STIM-1 et STIM-2, nécessaires à la signalisation calcique induite après

activation du BCR (Matsumoto et al., 2011), affaiblit fortement la génération des B10. Ainsi des souris présentant ce type de défauts génétiques sont incapables de contrôler le développement d'hypersensibilité ou d'EAE (Jin et al., 2013; Matsumoto et al., 2011). Elles présentent en effet une inflammation sévère dans les organes cibles, associée à une altération dans le compartiment des T régulateurs (Jin et al., 2013; Matsumoto et al., 2011). Le fait que l'activation du BCR renforce les fonctions suppressives des B10 nous mène à deux constats : le premier est que le développement de ces cellules semble être séquentiel, avec d'abord une activation via les TLRs qui initie la production d'IL-10, suivi par l'activation spécifique du BCR qui potentialise leurs fonctions suppressives. Nous verrons ensuite que d'autres signaux peuvent intervenir pour amplifier encore la production d'IL-10 ; le deuxième, assez intrigant, est que les Bregs auraient besoin de signaux aussi agressifs pour assurer leur fonction. Or les signaux transduits par le BCR et les TLRs ont plutôt été décrits comme centraux pour les fonctions pro-inflammatoires des B (différenciation en plasmocytes, production d'anticorps ou de cytokine pro-inflammatoires) (Rawlings et al., 2012). Néanmoins ce processus a ses avantages. En effet il pourrait assurer une boucle de rétrocontrôle physiologique permettant de contenir une inflammation sévère en situation pathologique. La présence des B10 ne serait ainsi qu'une pièce, somme toute importante, de la « machine suppressive » du système immunitaire.

3.3.3. Rôle de l'interaction des cellules B avec les cellules T

Des signaux dérivés des lymphocytes T peuvent également induire ou renforcer la production d'IL-10 par les Bregs. Parmi eux, il en existe deux types. Le premier concerne les signaux dépendants de contacts cellulaires. En effet les cellules T peuvent interagir avec les lymphocytes B via différentes voies. Une des voies importantes est la voie du CD40-CD40 ligand. Chez la souris, l'absence de CD40 à la surface des lymphocytes B est associée à une altération des fonctions suppressives des B10 (Fillatreau et al., 2002). De façon similaire, l'injection d'un agoniste du CD40 chez la souris promeut l'expansion de cellules B10 capables d'inhiber le développement de l'arthrite (Mauri et al., 2003). De

même, la surexpression du CD40 chez la souris amplifie de manière significative la production d'IL-10 par les Bregs (Yanaba et al., 2008; Yanaba et al., 2009). Chez l'homme les expériences *in vitro* démontrent également l'importance de l'activation du CD40 dans l'induction de B10 (Blair et al., 2010; Iwata et al., 2011). L'interaction B-T via la voie CD80/86-CD28 optimise la génération de B10. En effet la neutralisation de cette voie affaiblit les fonctions suppressives des cellules B (Blair et al., 2010; Flores-Borja et al., 2013). Le deuxième type de signal, concerne les cytokines produites par les cellules T. En effet bien que de nombreux travaux démontrent l'influence des B10 sur les réponses T, plusieurs études suggèrent également que les réponses lymphocytaires T peuvent avoir un effet sur l'expansion ou l'inhibition des B10. Ainsi, l'IL-21 se positionne comme un facteur de croissance important des B10 (Yoshizaki et al., 2012). Cette cytokine est capable d'induire et de maintenir les B10 *in vitro* mais également *in vivo* (Yoshizaki et al., 2012). Ce résultat est la preuve que l'environnement peut influer sur le développement des Bregs. Néanmoins ces données concernant l'IL-21 restent assez controversées. En effet l'action de l'IL-21 sur les B10 n'est pas retrouvée sur des cellules B issues de souris Balb/c (Holan et al., 2014). L'ensemble du travail a été initialement réalisé sur fond C57B6 suggérant ainsi un effet souche dépendant. De plus, aucun rôle de l'IL-21 sur la génération des B10 humains n'a, à ce jour, été décrit. En effet il semblerait même que cette cytokine n'ait aucun effet sur la production d'IL-10 (van de Veen et al., 2013) mais favoriserait plutôt l'émergence d'un autre type de Bregs, caractérisé par la production de granzyme-B (Lindner et al., 2013) (Chesneau et al., 2014 in revision). D'autres candidats ont été identifiés comme des inducteurs potentiels de la production d'IL-10. Parmi eux, l'IL-12 et l'IFN-γ sont capables de renforcer la sécrétion d'IL-10 par les cellules B (Holan et al., 2014). De façon intéressante, des patients allergiques alimentaires au lait, traités par immunothérapie (ingestion de caséine) ne montrent aucune amélioration clinique, alors que l'ajout d'IFN-γ induit une tolérance à l'allergène. Les patients tolérants au lait sont ainsi caractérisés par une expansion de cellules B10 spécifiques de la caséine démontrant ainsi le rôle de cette

cytokine dans l'induction des B10 chez l'homme (Noh et al., 2012). Parmi les cytokines pro-B10, on retrouve également l'IL-10 et l'IL-35 (Jin et al., 2013; Wang et al., 2014). Ces deux cytokines agissent toutes les deux de la même façon en promouvant l'expansion des B10 mais également des Tregs. Ces observations suggèrent donc qu'il existerait une interaction privilégiée entre Tregs et Bregs, l'un assurant ainsi la fonction de l'autre pour le maintien de la tolérance immunologique. Néanmoins des études sont ici nécessaires pour confirmer le rôle de cette interaction chez l'homme. Mais pour le moment très peu de travaux ont mis en évidence les voies de signalisation impliquées dans les différents processus décrits ci-dessus. Probablement que la décortication des voies de signalisation améliorera notre compréhension concernant le développement des lymphocytes B10.

3.4 Phénotype des B10

2.4.1 Chez la souris

La découverte de nouvelles populations immunitaires régulatrices implique souvent une caractérisation à la fois fonctionnelle et phénotypique. L'identification de marqueurs de surface ou de facteurs de transcription spécifiques d'un sous-groupe facilite grandement l'identification puis la manipulation des cellules. L'exemple des Tregs est assez probant. De l'identification simple avec deux clusters de différenciation, $CD4^+$ $CD25^+$, on arrive aujourd'hui à un phénotype plus complexe et à l'identification de sous-populations Tregs bien distinctes (Miyara et al., 2009; Sanchez Rodriguez et al., 2014; Schmidl et al., 2014; Valmori et al., 2005). Chez la souris, les B régulateurs ont été initialement caractérisés fonctionnellement par leur capacité à réguler l'inflammation via la production d'IL-10 (Fillatreau et al., 2002). Sur le plan phénotypique cette population a d'abord été associée à des cellules B FO qui, sous l'action de signaux spécifiques deviennent régulatrices (Fillatreau et al., 2002; Lampropoulou et al., 2008). Or des souris délétées pour les protéines Gi-α et p110δPI3K qui ont de profondes altérations dans l'homéostasie B (Dalwadi et al., 2003), avec notamment une incapacité à générer

des cellules transitionnelles 2 progénitrices de la ZM (T2-PZM) et ZM développent spontanément des colites inflammatoires suggérant que les B FO ne sont pas les seules cellules à assurer des fonctions régulatrices (Dalwadi et al., 2003). En accord avec ces observations, les travaux de l'équipe de Claudia Mauri démontrent le rôle central des T2-PZM dans le contrôle de l'arthrite induite via la production d'IL-10 (Evans et al., 2007). Une autre théorie suggère que les B10 appartiennent à la sous population B $CD5^{+}$ $CD1d^{hi}$ (Yanaba et al., 2008). Phénotypiquement proche des B1a et ZM, elle semble être une population distincte qui produit des quantités élevées d'IL-10, cependant leur origine cellulaire reste toujours non-élucidée (Yanaba et al., 2008). La molécule TIM-1 a également été décrite comme marqueur des B10 (Ding et al., 2011). Des souris mutées pour cette molécule ont un défaut dans le développement des B10 et présentent des manifestations autoimmunes avec l'âge (Xiao et al., 2012). Cependant des résultats totalement discordants ont été obtenus à partir de souris génétiquement modifiées qui sous le contrôle du promoteur de l'IL-10 expriment une protéine fluorescente. Ceci permet de suivre directement les cellules exprimant l'IL-10. Ces études démontrent que la majorité des cellules B IL-10^{+} est confinée au sein du compartiment plasmocytaire (Madan et al., 2009; Maseda et al., 2012; Neves et al., 2010; Scapini et al., 2011). Cette grande diversité est difficile à expliquer. Il est fort probable ici que le fond génétique des souris, le type de modèle et le microenvironnement influent directement sur le phénotype des B10. En accord avec cette hypothèse la récente identification de B10 dans le tissue adipeux, avec un phénotype inédit mais complexe, renforce l'idée de l'existence de plusieurs sous-populations régulatrices probablement tissus spécifiques (Nishimura et al., 2013). Ceci renforce aussi l'idée que les Bregs ne sont pas des cellules générées de manière naturelle, à l'instar des Tregs, mais sont essentiellement des cellules inductibles.

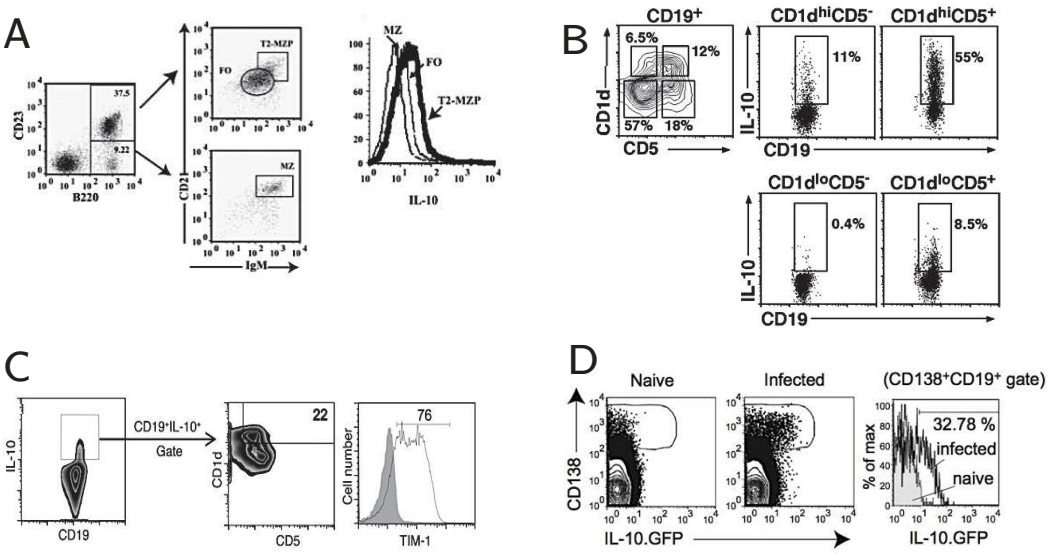

Figure 10 : Phénotypes des B10 chez la souris. A, Dans un modèle d'arthrite dans des souris de type DBA/J, les B10 ont un phénotype de type T2-PZM. **B,** Dans les souris C57B6, les B10 sont enrichis dans une population B caractérisée par l'expression du CD5 et du CD1d. **C,** La molécule TIM-1 a été proposée comme marqueur spécifique des B10. **D,** Dans certains modèles infectieux, les B10 sont enrichis dans les plasmocytes $CD138^+$

3.4.2 Qu'en est-il en biologie humaine ?

Chez l'homme, les cellules B10 ont également été identifiées par leur fonction (Blair et al., 2010; Iwata et al., 2011). Les études phénotypiques ont associé ces cellules aux populations B humaines immatures ($CD19^+$ $CD24^{hi}$ $CD38^{hi}$) (Blair et al., 2010) ou mémoire ($CD19^+$ $CD27^+$) (Iwata et al., 2011) (Figure 11). Récemment une étude transcriptomique par puces à ADN a permis d'identifier de nouveaux marqueurs de surface spécifiques des B10 (van de Veen et al., 2013). Par cette approche les auteurs ont défini un nouveau phénotype pour ces cellules ; $CD19^+$ $CD25^+$ $CD73^-$ $CD71^+$. Ces cellules sont capables d'inhiber la prolifération des cellules T dans un test spécifique de l'antigène (van de Veen et al., 2013). De façon intéressante ces cellules B ont un pourvoir immunosuppresseur beaucoup plus important que les Tregs. En effet dans des conditions expérimentales similaires, l'activité suppressive de ces Bregs est observée à un ratio de 1:25 alors qu'elle n'est observée qu'à partir d'un ratio 1:4 avec des Tregs

(Akdis et al., 2004; van de Veen et al., 2013). Néanmoins aucune information ne concerne leur état de maturation. En effet, on ne sait pas si ces cellules B sont immatures, naïves, mémoires ou plasmocytaires (van de Veen et al., 2013).

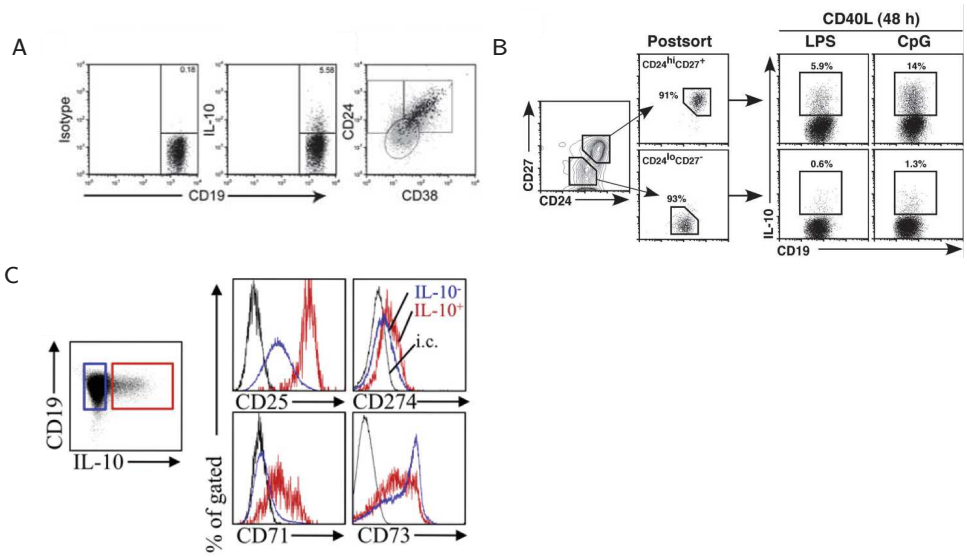

Figure 11 : Phénotypes des B10 chez l'homme. A, Les cellules B immatures sont les premières cellules B10 à avoir été décrites chez l'homme. Elles sont caractérisées par la surexpression du CD24 et CD38. **B,** Les cellules B mémoires $CD27^+$ sont également capables de produire de l'IL-10 et de réguler la maturation des monocytes **C,** Récemment une étude transcriptomique a identifié de nouveaux marqueurs pour identifier les B10.

Récemment une étude très intéressante a démontré qu'au-delà du phénotype, la fonction suppressive dépendait surtout d'un ratio entre la production d'IL-10 et TNF-α (Cherukuri et al., 2014). En effet les auteurs démontrent ici que l'ensemble des sous-populations B est capable de produire de l'IL-10 à des niveaux plus ou moins élevés (Cherukuri et al., 2014). Ceci suggère que n'importe quelle cellule B peut devenir régulatrice, à condition de recevoir les signaux adéquats. Dans leur étude, les auteurs démontrent qu'en plus de sécréter de l'IL-10, les cellules B produisent également du TNF-α qui vient contrebalancer les effets régulateurs de l'IL-10 (Cherukuri et al., 2014). En effet les cellules B sont régulatrices lorsque le ratio IL-10/ TNF-α est à la faveur de la cytokine suppressive. Ainsi malgré le fait que les cellules B naïves et mémoires produisent de l'IL-10, elles n'exercent aucune fonction régulatrice *in vitro* à cause des taux élevés de TNF-α (Cherukuri et al., 2014). L'ensemble de ces données confirme

clairement que le phénotype ne suffit pas à une identification des Bregs. Pour le moment, seules des investigations fonctionnelles permettent de statuer sur le caractère régulateur d'une population B aussi bien chez la souris que chez l'homme.

3.5 Émergence de nouvelles fonctions B régulatrices

Bien que les B10 représentent à ce jour la population Breg majeure, il est important de souligner ici la récente identification de nombreux autres mécanismes régulateurs des lymphocytes B. Tout d'abord la production de TGF-β a été décrite comme une autre fonction immunorégulatrice assurée par ces cellules B (Bao and Cao, 2014). Ainsi l'induction d'une tolérance orale à l'OVA protège les souris du développement de l'asthme via l'expansion d'une population B TGF-β^+ capable d'induire des T régulateurs *in vivo* (Natarajan et al., 2012). Récemment, une sous-population B intestinale caractérisée par l'expression du CD5, du récepteur de la fractalkine (CX3CR1) et de l'intégrine αvβ6 est capable de produire du TGF-β *in vitro* après activation polyclonale (Liu et al., 2013). Le transfert de ces cellules protège les souris du développement d'une allergie alimentaire induite par l'OVA. Les Bregs TGF-β^+ joueraient également un rôle dans le cadre de la tolérance en transplantation rénale (Haynes et al., 2012).

La récente découverte de cellules B régulatrices produisant de l'IL-35 est particulièrement intéressante (Shen et al., 2014; Wang et al., 2014). Des souris n'exprimant plus la sous-unité p35 (une des deux sous-unités de l'IL-35 avec EBI3) développent une EAE sévère (Shen et al., 2014). L'activation par le LPS et le CD40 induit l'expression de cette cytokine par les lymphocytes B (Shen et al., 2014; Wang et al., 2014). L'injection d'IL-35 induit l'expansion des Bregs IL-35^+ *in vivo* permettant de contrôler les réponses inflammatoires dans un modèle d'uvéite autoimmune. L'injection de cellules B cultivées en présence d'IL-35 et issues de (i) souris déficientes en IL-10 ou (ii) déficientes en IL-35, ne permet pas de supprimer l'inflammation *in vivo* (Wang et al., 2014). Ces résultats sont très intéressants car ils suggèrent que les B10 et les Bregs IL-

35 sont intimement liés fonctionnellement (Wang et al., 2014). L'existence de cette population de Bregs IL-35^{+} est confirmée chez l'homme (Wang et al., 2014). Néanmoins, des études complémentaires sont nécessaires pour comprendre leur impact sur le plan immunopathologique.

L'induction de l'apoptose est également une stratégie mise en œuvre par les cellules B régulatrices pour contrôler l'inflammation. Ces cellules B « tueuses » expriment la molécule Fas-L ou produisent du granzyme-B (GrzB) pour stopper l'activation des cellules T. Les premières ont été essentiellement décrites chez la souris. En effet il existe *in vivo* une sous-population B Fas-L^{+} capable d'inhiber l'inflammation bronchique dans un modèle d'allergie à la blatte (Lundy and Boros, 2002). Également des cellules pro-B activées avec du CpG-ODN (Ligand du TLR9) peuvent se différencier en Bregs Fas-L^{+} capables d'inhiber la réponse T inflammatoire dans un modèle de diabète auto-immun (Montandon et al., 2013). Ces cellules migrent dans la rate et le pancréas où elles sont capables d'inhiber de manière spectaculaire la prolifération des T effecteurs. La modulation de cellules pro-B vers des profils régulateurs stables pourrait être une alternative très intéressante dans le cadre de la thérapie cellulaire en auto-immunité (Montandon et al., 2013). Les cellules B produisant du GrzB représentent le deuxième groupe de cellules Bregs inductrices d'apoptose. De façon intéressante l'expression du GrzB semble être un caractère évolutif. En effet les cellules B murines n'expriment pas le GrzB alors que l'on retrouve son expression dans les lymphocytes B humains. Cette population Breg contrôle l'activation des T effecteurs en induisant leur mort cellulaire. Dans notre équipe des travaux rapportent l'augmentation de ces cellules dans le sang de patients tolérant leur greffon rénal sans traitement immunosuppresseur suggérant un rôle de ces cellules dans la tolérance à la greffe (Chesneau et al., 2014 in revision).

En résumé

- Les études chez l'homme et la souris ont permis l'identification d'une population B, produisant de l'IL-10 et capable de contrôler le développement de pathologies inflammatoires.

- Ces cellules appelées B10, sont capables d'inhiber la maturation des cellules présentatrices d'antigènes, d'induire des lymphocytes T régulateurs et de contrôler directement la prolifération et la mise en place de réponses T_H1, T_H2 et T_H17.

- Il n'existe, pour le moment, aucun marqueur ou facteur de transcription capable d'identifier ces cellules de manière précise.

- En plus de la production d'IL-10, les cellules B produisent d'autres médiateurs leur permettant de contrôler l'inflammation. Les cellules B IL-35^+ ou TGF-β^+ assurent des fonctions similaires aux B10.

- La récente découverte de nouvelles cellules Bregs promouvant l'apoptose des cellules T démontre la grande diversité fonctionnelles des cellules Bregs. Une attention particulière est portée aux cellules B produisant du granzyme B qui contrôlent l'activation T et sont augmentées dans le sang de patients tolérant spontanément leur greffon rénal.

4. Qu'est-ce que l'asthme ?

Dans une première partie nous avons pu voir l'ensemble des données scientifiques décrivant le rôle des cellules B dans la régulation des réponses inflammatoires. Dans cette seconde partie nous nous attarderons un peu plus sur le rôle de ces cellules dans le développement de l'asthme allergique. Ainsi dans une première partie, nous nous attarderons sur la complexité de cette pathologie. Puis dans une seconde partie nous décrirons les mécanismes immunopathologiques en jeu dans l'asthme. Ainsi nous verrons comment la cellule B assure les mécanismes physiopathologiques de l'asthme notamment grâce à la production d'IgE. De plus à la lumière des données décrites dans la partie précédente nous nous interrogerons sur le rôle de la cellule B en tant qu'acteur dans la régulation de la réponse immune aux cours de l'asthme allergique.

4.1 Définition et Généralités

L'asthme est une pathologie chronique très commune. Sa prévalence varie dans le monde, néanmoins plus de 5% de la population mondiale souffre actuellement d'asthme (Fanta, 2009). Ce pourcentage peut être plus élevé dans certaines régions, notamment dans les pays industrialisés. L'asthme affecte des individus de tout âge et représente ainsi un sérieux problème de santé public. Cette pathologie affecte la qualité de vie des patients en altérant leur capacité respiratoire. Dans certains cas extrêmes, elle peut causer la mort des patients, notamment ceux avec un asthme non-contrôlé ou mal traité. Sur le plan physiopathologique, l'asthme est caractérisé par un désordre inflammatoire des voies aériennes qui mène à une obstruction bronchique altérant ainsi la fonction respiratoire de l'individu. Des épisodes de dyspnée, d'oppression thoracique (voire de toux) paroxystique, sifflante, récidivante, volontiers nocturne, sont des signes résultant de l'asthme (Fanta, 2009). La pathogenèse de l'asthme est extrêmement complexe et reste parfois incomprise dans certains cas. L'asthme est majoritairement caractérisé par une inflammation de type T_H2-eosinophiles avec la production d'IgE

spécifiques des allergènes. Ces patients sont dits « atopiques » car ils possèdent un fond génétique les prédisposant à répondre de manière incontrôlée aux allergènes de l'environnement. Cependant réduire cette pathologie à la simple cause des allergènes exclurait l'ensemble d'autres types d'asthmes dits « non-allergiques ». Cette première classification clinique met en avant l'existence de sous-groupes de patients distincts. On peut aussi classer les sous-types d'asthme en fonction du type d'inflammation en confirmant l'absence ou la présence d'éosinophiles et/ou de neutrophiles. La fonction respiratoire est également un facteur qui permet de définir des groupes de patients asthmatiques. Le volume expiratoire maximal par seconde (VEMS) est un marqueur important de la fonction respiratoire. Enfin la réponse aux traitements classiques permet de distinguer des groupes avec par exemple des asthmes cortico-sensibles ou cortico-résistants, plus ou moins réversibles aux bronchodilatateurs. Les formes résistantes représentent aujourd'hui les cas les plus sévères. Ce sont des asthmes mal-contrôlés et extrêmement délétères pour l'individu qui nécessitent aujourd'hui le développement de nouvelles thérapies (Wener and Bel, 2013).

Cette première classification de l'asthme reste somme toute assez classique dans son approche et fait preuve d'une limite importante : elle manque énormément de spécificité. Prenons l'exemple de l'inflammation. Classiquement les asthmes à éosinophiles concernent les asthmes allergiques et sont décrits comme des asthmes cortico-sensibles. En réalité l'éosinophilie se retrouve dans d'autres sous-groupes de patients comme l'asthme lié à l'exercice ou encore l'asthme induit par l'aspirine (Wenzel, 2012). De plus, il existe également des cas d'asthme sévère, cortico-résistant, chez des patients éosinophilies (Wenzel, 2012). On se rend compte finalement que la définition précise d'un sous-type d'asthme repose sur la combinaison de plusieurs paramètres cliniques, physiologique et moléculaires. C'est en combinant ces facteurs que l'on arrive petit à petit à appréhender l'extraordinaire hétérogénéité de cette pathologie.

4.2 Phénotype et endotype : comment appréhender l'hétérogénéité de l'asthme ?

4.2.1 Les preuves de l'hétérogénéité de l'asthme

C'est dans les années 90 que les chercheurs ont identifié, à l'aide de modèles animaux, le rôle important de la réponse T_H2 dans l'inflammation et l'hyperréactivité des voies aériennes (Grunig et al., 1998; Wills-Karp et al., 1998; Zhang et al., 1999). Ainsi l'asthme a été longtemps décrit comme allergique, éosinophilique, T_H2 dépendant et cortico-sensible. Cependant les premiers résultats négatifs, obtenus lors d'essais cliniques évaluant l'efficacité de molécules ciblant directement la composante T_H2, suggéraient l'existence d'autres mécanismes pathologiques (Flood-Page et al., 2007; Leckie et al., 2000). En parallèle plusieurs études rapportaient l'existence d'un sous-groupe de patients avec un asthme réfractaire en l'absence d'éosinophiles. De manière intéressante la réponse aux corticoïdes était dépendante du type d'inflammation présente dans les voies aériennes. Ces travaux notent ainsi l'existence d'asthme neutrophilique résistant à la corticothérapie (Green et al., 2002; Wenzel et al., 1999). L'ensemble de ces données suggérait donc l'existence de plusieurs sous-types de patients caractérisés par des caractères cliniques et des réponses aux thérapies distincts. L'asthme serait alors une pathologie unique englobant une multitude de sous-groupes distincts appelés phénotypes.

4.2.2 Du phénotype à l'endotype

Le concept d'hétérogénéité de l'asthme a considérablement évolué ces dernières années, notamment grâce à (i) la mise en place de cohortes de patients permettant des analyses statistiques de « clustering » et (ii) l'émergence de thérapie de plus en plus spécifiques. Ces approches ont permis de confirmer l'existence de plusieurs phénotypes d'asthme (Haldar et al., 2008; Moore et al., 2014; Moore et al., 2010). Un phénotype est ainsi défini par l'ensemble des caractéristiques d'un patient qui résultent de l'interaction entre le fond génétique (par exemple l'atopie) et les facteurs environnementaux. Une étude très intéressante a ainsi classé les patients asthmatiques en fonction de l'intensité des symptômes et de l'inflammation éosinophilique. Ils distinguent ainsi plusieurs phénotypes

d'asthme avec des caractéristiques cliniques bien distinctes (Haldar et al., 2008) (Figure 11). L'identification de ces sous-groupes est un outil très intéressant pour le clinicien qui peut ensuite adapter la conduite thérapeutique en fonction du profil du patient (Haldar et al., 2008).

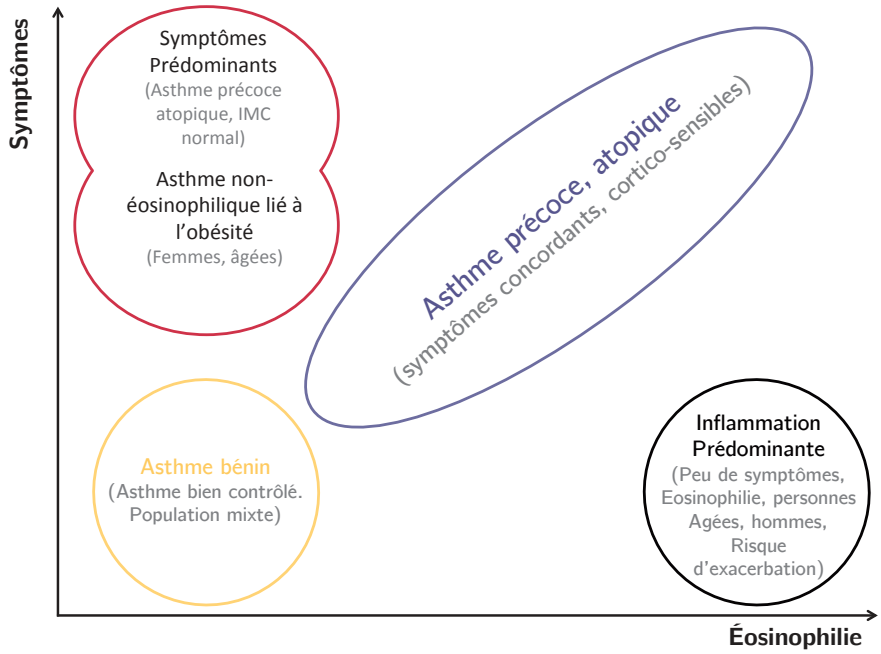

Figure 11 : Phénotypes cliniques de l'asthme. Les groupes ont été constitués selon l'expression relative des symptômes et le degré d'inflammation. Ces paramètres sont des facteurs cliniques pertinents et évoluent avec le type d'asthme.

Aux États Unis, le « Severe Asthma Research Program of the National Heart, Lung, and Blood Institute » a pour but d'identifier et de caractériser les patients avec un asthme sévère et de les comparer aux patients atteints d'asthme bénin à modéré. Ainsi par des approches statistiques similaires, les auteurs ont classé les individus asthmatiques à l'aide de 3 facteurs : la fonction respiratoire (FEV1), la réponse aux bronchodilatateurs (sévérité) et l'âge. Ils identifient ainsi 5 groupes de patients (Moore et al., 2010) (Figure 12). Plus récemment le même groupe a étudié le type d'inflammation retrouvé au sein de différents phénotypes cliniques (Moore et al., 2014). Dans cette étude ils identifient quatre phénotypes cliniques distincts à l'aide de 15 paramètres cliniques préétablis (Figure 12). C'est en étudiant le profil inflammatoire dans chaque groupe qu'ils

remarquent une association intéressante entre sévérité et profil inflammatoire. Ainsi dans les groupes C et D, la présence des neutrophiles est associée à la sévérité de la pathologie. Ces patients sont caractérisés par une fonction pulmonaire altérée et une corti-thérapie importante. De plus une importante corrélation existe entre fonction respiratoire et taux de neutrophiles (Moore et al., 2014; Moore et al., 2010).

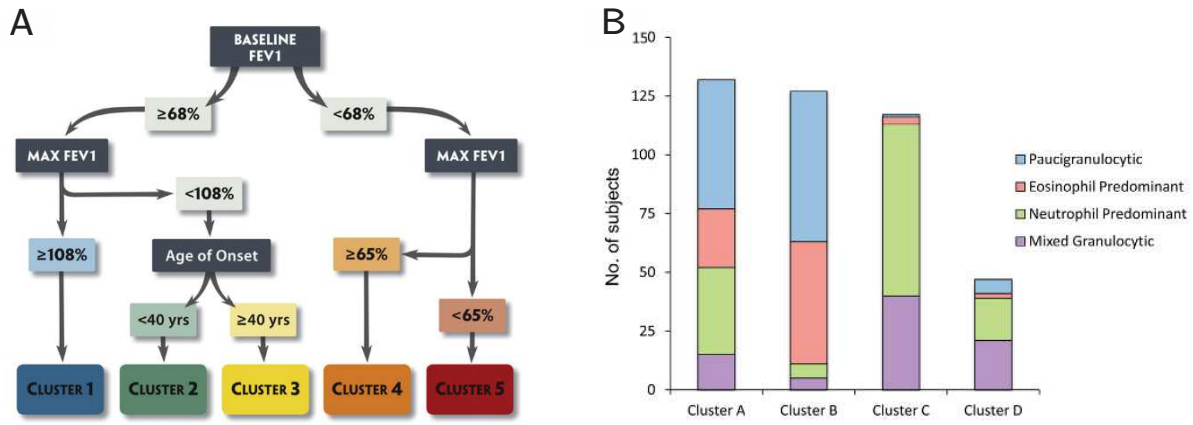

Figure 12 : Phénotypes cliniques de l'asthme sévère. A, Classification des groupes de patients selon les paramètres d'âge et de la fonction respiratoire. Les clusters 4 et 5 correspondent aux asthmes les plus sévères avec la fonction la plus altérée. **B,** Type d'inflammation au sein des différents sous-groupes cliniques. Il est intéressant de noter la forte présence de neutrophile dans les clusters « sévères » C et D.

Bien que ces études aient permis de distinguer différents sous-groupes de patients asthmatiques, elles ne permettent toujours pas l'obtention de sous-groupes homogènes. En effet dans l'étude SARP, les asthmes sévères et réfractaires aux thérapies actuelles sont retrouvés dans l'ensemble des sous-groupes. Bien que l'inflammation neutrophilique prédomine dans les cas d'asthmes sévères, il existe des asthmes éosinophiliques réfractaires. Par conséquent l'objectif aujourd'hui est d'identifier de manière précise de petits sous-groupes de patients. Pour cela plusieurs études se sont intéressées à une caractérisation moléculaire de l'asthme avec pour objectif l'identification de biomarqueurs venant ainsi renforcer la classification clinique. La découverte de marqueurs biologiques permettrait d'orienter l'approche thérapeutique vers une approche clinique beaucoup plus personnalisée. C'est ainsi qu'a commencé récemment, la ruée vers

l'endotype défini par l'identification d'un mécanisme moléculaire particulier menant à un phénotype clinique spécifique.

4.2.3 L'endotype T_H2^{high}

L'observation, selon laquelle seulement une portion des patients asthmatiques a un profil T_H2, a poussé les chercheurs et les cliniciens à orienter leur caractérisation vers des approches plus moléculaires. Ce phénotype moléculaire T_H2 (ou éosinophilique) est présent chez 50% des patients asthmatiques (Wenzel, 2012). Il englobe la majorité (mais pas tous) des patients allergiques asthmatiques ainsi que des patients avec un asthme induits par l'effort physique (Wenzel, 2012). On retrouve dans ce phénotype moléculaire des patients avec un asthme tardif, non-atopique caractérisé par une très forte éosinophilie (Wenzel, 2012). Enfin la sensibilité aux corticostéroïdes est variable avec la présence de patients sensibles et résistants, confirmant ainsi l'hétérogénéité de ce phénotype moléculaire. L'utilisation de biomarqueurs permet de classer et d'identifier avec beaucoup plus de spécificité les phénotypes T_H2. L'éosinophilie sanguine, l'oxyde nitrique exhalé ou les niveaux de périostine peuvent être utilisés pour identifier avec précision ce type de phénotype moléculaire (Wenzel, 2012).

De manière extrêmement intéressante l'utilisation de biomarqueurs permet aujourd'hui d'identifier les patients asthmatiques avec un profil moléculaire distinct et, d'associer ce profil à une thérapie précise : le sous-groupe ainsi identifié est appelé « endotype ». L'exemple le plus frappant concerne la récente identification des endotypes T_H2^{high} et T_H2^{low} (Woodruff et al., 2009). Initialement une étude en 2007 par l'équipe de Prescott Woodruff a identifié une signature biologique dans les brossages bronchiques de patients asthmatiques allergiques. Ainsi la surexpression de 3 protéines (POST ; periostine – CLCA1 ; « Calcium-activated chloride channel regulator 1 » – SERPINB2 ; « Plasminogen activator inhibitor-2 ») permettait de distinguer les patients asthmatique de patients atteins de BPCO et de volontaires sains (Woodruff et al.,

2007). Plus tard les investigateurs ont démontré la possibilité de classer les patients asthmatiques grâce à cette signature biologique. Ainsi les patients sur-exprimant ces trois molécules étaient caractérisés par un phénotype T_H2 très prononcé avec une production très importante d'IL-5 et d'IL-13. Ce groupe fut alors appelé T_H2^{high}. Un deuxième groupe de patients, nommé T_H2^{low} est quant à lui caractérisé par une expression plus faible de la signature biologique corrélée à un phénotype moléculaire T_H2 beaucoup moins prononcé avec notamment des taux d'IL-5 et d'IL-13 beaucoup plus bas (Woodruff et al., 2009) (Figure 13). Sur le plan clinique ces deux groupes de patients sont très différents. En effet les T_H2^{high} sont caractérisés par une éosinophilie plus élevée, de plus haut niveau d'IgE systémiques et un remodelage bronchique plus sévère (Woodruff et al., 2009). Néanmoins ils répondent très bien à la corticothérapie qui améliore de manière significative leur fonction respiratoire. A contrario les T_H2^{low} sont cortico-réfractaires suggérant que ce type de thérapie n'est pas du tout approprié pour ce sous-groupe de patients. Une des critiques majeures de ce travail, est la non-inclusion de patients asthmatiques sévères. En effet seuls des patients avec un asthme bénin à modéré ont été inclus dans cette étude. On ne trouve également aucune information concernant la neutrophilie des patients. Ce paramètre aurait pu donner des informations supplémentaires notamment concernant le groupe T_H2^{low}. Récemment des données obtenues sur des cas sévères, avec une inflammation neutrophilique, démontrent une surexpression de la périostine chez ce type de patients. Néanmoins pour le moment la pertinence de la périostine, en tant que biomarqueurs des T_H2^{high} reste forte avec un usage clinique potentiel avec notamment la possibilité de la doser dans le sérum des patients asthmatiques (Jia et al., 2012).

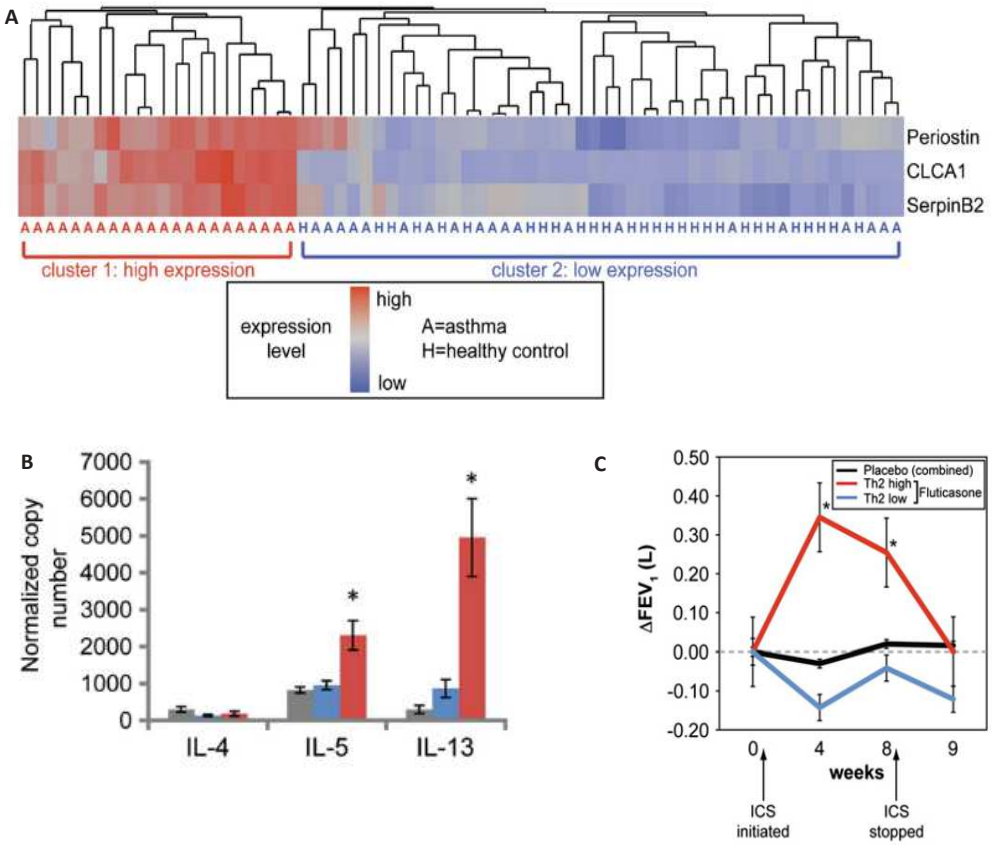

Figure 13 : **Caractérisation de l'endotype T_H2^{high}. A et B,** Identification de trois biomarqueurs permettant de classer les patients asthmatiques selon leur profil T_H2. **C,** Evaluation de la fonction respiratoire après traitement chez les patients $TH2^{low}$ et $TH2^{high}$.

L'utilité clinique de la périostine est illustrée par sa capacité à identifier un sous-groupe exclusif de patients asthmatiques capables de répondre au Lebrikizumab, un anticorps neutralisant l'IL-13 (Corren et al., 2011). Dans cette étude 219 patients avec un asthme mal contrôlé, en dépit d'une thérapie à base de stéroïdes, ont été inclus. Dans un premier temps, les données statistiques obtenues lors de l'étude ne démontraient aucune efficacité thérapeutique de cet anticorps monoclonal sur l'ensemble du groupe traité. Or le classement des patients en fonction de leur phénotype moléculaire T_H2^{high} ou T_H2^{low}, met en avant un effet thérapeutique de la molécule exclusivement dans le groupe $TH2^{high}$ (Corren et al., 2011). D'autres études de ce type démontrent que les patients $TH2^{high}$, lorsqu'ils sont sélectionnés sur la base de biomarqueurs, répondent très bien au Mépolizumab (Anti-IL-5) (Haldar et al., 2009; Pavord et al., 2012) ou au Dupilumab

(Anti IL-4Rα, qui neutralise à la fois le récepteur l'IL-4 et de l'IL-13) (Wenzel et al., 2013). L'ensemble de ces observations démontre la nécessité de « phénotyper » et d' « endotyper » les patients asthmatiques. Ceci permettra clairement de faire évoluer la prise en charge de ces patients vers une démarche médicale personnalisée.

4.2.4 Les asthmes non-T_H2 : un véritable casse-tête clinique et biologique

Les asthmes non-T_H2 représentent une large proportion des patients. Cependant par rapport aux asthmes T_H2, très peu de choses sont connues sur ce groupe de patients. Beaucoup de patients avec un asthme modéré non-atopique appartiennent à cette catégorie (Bisgaard et al., 2009). Ces patients ne répondent pas aux thérapies classiques (Woodruff et al., 2009). Enfin le manque d'efficacité des thérapies T_H2-spécifiques (sur des groupes non-phénotypés) ainsi que l'existence d'autres voies moléculaires capables de promouvoir l'asthme confirment l'existence d'un sous-type d'asthme non-T_H2. L'existence de patients avec une inflammation exclusivement neutrophilique renforce cette hypothèse. De manière intéressante ces patients sont caractérisés par une fonction pulmonaire fortement altérée et une résistance aux corticoïdes (Baines et al., 2010; Baines et al., 2014; Moore et al., 2014). Une donnée surprenante est la forte proportion de patients atopiques avec un asthme neutrophilique (Baines et al., 2010; Baines et al., 2014; Moore et al., 2014). De nombreuses études réalisées par une équipe australienne tente aujourd'hui de caractériser de manière moléculaire les asthmes neutrophiliques. Par des approches à hauts débits (Puce à ADN, protéomique), les investigateurs proposent aujourd'hui l'existence de potentiels biomarqueurs. Parmi les molécules identifiées on retrouve l'IL-6, l'IL-1β, NLRP3 (NOD-like receptor family, pyrin domain containing 3) et CXCR2 (récepteur de l'IL-8 une molécule clef dans l'attraction des neutrophiles). Cependant ces biomarqueurs caractérisent l'ensemble des asthmes neutrophiliques et ne semblent pas liés à un phénotype clinique particulier (Baines et al., 2010; Baines et al., 2014; Moore et al., 2014). Récemment le test d'un antagoniste du CXCR2 chez des patients

neutrophiliques révèle des résultats assez encourageants. En effet la prise de ce médicament induit une diminution de la fréquence des exacerbations avec une amélioration de la qualité de vie des patients (Nair et al., 2012). Néanmoins cette étude reste très préliminaire et ces résultats doivent être confirmés sur une cohorte plus importante.

Ces dernières années l'inflammation T_H17 a fortement attiré l'attention des cliniciens et des scientifiques. En effet ces cellules produisent de l'IL-17 une cytokine qui régule l'attraction des neutrophiles en augmentant l'expression et la production de chimiokines pro-neutrophiles, comme l'IL-8, CXCL1 et CXCL5. Ce lien biologique dans l'asthme est bien démontré dans les modèles animaux. En effet la neutralisation de l'IL-17 dans ces modèles atténue l'hyperréactivité bronchique et diminue l'inflammation neutrophilique (He et al., 2009; He et al., 2007; Wilson et al., 2009). Chez l'homme certaines évidences supportent un rôle de cette cytokine dans l'asthme sévère neutrophilique. En effet de nombreuses études révèlent une corrélation positive entre la présence d'IL-17 et l'infiltrat neutrophilique chez les patients asthmatiques (Bullens et al., 2006; Chesne et al., 2014). Cependant d'autres études cliniques seront nécessaires pour confirmer cette hypothèse chez l'homme (Chesne et al., 2014). De façon intéressante, la neutrophilie coexiste avec l'éosinophilie chez certains patients. Cette inflammation mixte cible les patients les plus sévères (Moore et al., 2014; Nagasaki et al., 2014). Ce type de phénotype suggère l'activation des voies T_H2 et T_H17. La récente identification de cellules produisant à la fois de l'IL-4 et de l'IL-17 dans l'asthme renforce cette hypothèse (Cosmi et al., 2010). De façon intéressante cette sous-population T inflammatoire est augmentée en périphérie et dans les voies aériennes chez les patients caractérisés par une inflammation mixte (Cosmi et al., 2010; Irvin et al., 2014). La présence des cellules T_H2-T_H17 est associée à un asthme sévère, résistant aux corticoïdes et concerne essentiellement des patients atopiques (Irvin et al., 2014). L'ensemble de ces observations démontre bien la complexité des mécanismes

immunopathologiques de l'asthme sévère ainsi que la difficulté à caractériser ces patients non-T_H2. L'évolution dans les approches biologiques et l'indentification de biomarqueurs, via l'emploi de techniques à haut débit, permettra sans doute une meilleur stratification de ces patients et une amélioration de leur prise en charge.

En résumé

- L'asthme est une maladie hétérogène englobant une grande diversité de sous-groupes de patients
- Le phénotype est identifié par l'intégration des caractéristique du patients émergeant de l'interaction entre la génétique (Atopie ?) et l'environnement (Asthme lié au sport ? Allergènes ? Obésité ?)
- La caractérisation moléculaire des phénotypes d'asthme incorpore les caractéristiques biologiques et peut mener à l'identification de biomarqueurs.
- L'endotype est confirmé et défini quand l'inhibition d'une voie moléculaire, préalablement identifiée dans un sous-groupe de patients, mène à une amélioration clinique.
- Les endotypes T_H2 commencent à être identifiés et définis avec l'identification de biomarqueurs et la description de voies moléculaires spécifiques
- Les phénotypes associés à des asthmes non-T_H2 restent très peu compris et difficilement défini: plusieurs suspects sont à l'étude avec notamment un rôle suspecté de l'IL-17

5. Immunologie de l'asthme

Les cellules T $CD4^+$ effectrices sont classées en sous-populations T auxiliaires, appelées T_H1, T_H2, T_H17 (Figure 14) et les récentes T_H9 et T_H22. Chaque sous-population est caractérisée par un programme transcriptionnel distinct et la production de cytokines pro-inflammatoires (Figure 14). Bien qu'il existe une certaine plasticité immunologique, avec par exemple l'existence de cellules T $CD4^+$ partageant un profil à la fois T_H2 et T_H17 (Cosmi et al., 2010; Irvin et al., 2014), la caractérisation des sous populations T auxiliaires reste robuste et fonctionnelle. De nombreuses données scientifiques obtenues chez l'homme et l'animal démontrent le rôle central des T_H2 dans le développement de l'asthme. Elles sont impliquées dans la mise en place d'un grand nombre de processus immunopathologiques menant à l'inflammation des voies aériennes et à la détérioration de l'épithélium bronchique. Ces 5 dernières années ont été riches en découverte avec notamment l'identification d'une nouvelle sous-population immunitaire, les cellules lymphoïdes innées (Walker et al., 2013). C'est un sous-groupe de cellules hétérogènes caractérisé par sa capacité à produire des panels de cytokines distincts proches des cellules T_H1, T_H2 ou T_H17.

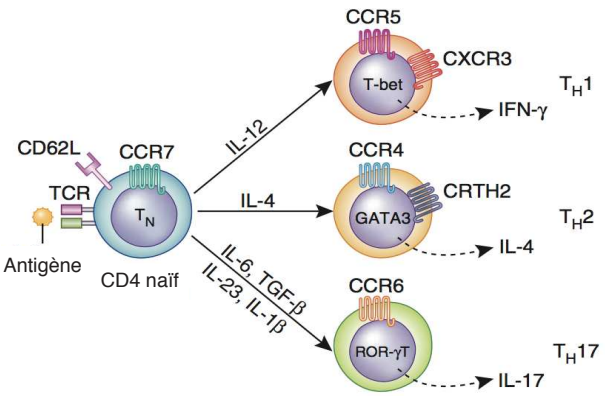

Figure 14 : Caractérisation de l'endotype T_H2^{high}. A et B, Identification de trois biomarqueurs permettant de classer les patients asthmatiques selon leur profil T_H2. **C,** Evaluation de la fonction respiratoire après traitement chez les patients $TH2^{low}$ et $TH2^{high}$.

5.1 La réponse T_H2 : la nouveauté est innée.

Initialement, on pensait que la différenciation des cellules T_H2 dépendait essentiellement de la présence d'IL-4 sécrétée par les cellules T elles-mêmes, les mastocytes, basophiles ou encore les lymphocytes B (Leon et al., 2014). Cependant l'exposition de l'épithélium bronchique aux allergènes, particules polluantes ou virus, induit la production de TSLP, d'IL-25 et d'IL-33 via notamment l'activation des TLRs (Lambrecht and Hammad, 2012). Ces cytokines ont été décrites comme majeures dans la mise en place d'un environnement pro-T_H2. En effet elles agissent directement sur les cellules dendritiques, les cellules sub-épithéliales mais également et surtout induisent l'expansion et l'activation de cellules lymphoïdes innées de type 2 (ILC2). Les ILC2 ont été récemment découvertes et décrites comme des facteurs clés dans l'induction des réponses T_H2 et dans la promotion des réponses allergiques (Walker and McKenzie, 2012). Ces cellules ont été initialement identifiées et décrites comme une source majeure d'IL-4, 5 et 13 (Neill et al., 2010). Étant donné leur profil, la question de leur implication dans les pathologies T_H2 dépendantes s'est rapidement posée. Ainsi de nombreuses études chez l'homme rapportent l'accumulation de ces cellules dans les polypes nasaux de patients atteints de rhinosinusite chronique (Mjosberg et al., 2011), dans la peau de patients atteints de dermatite atopique (Kim et al., 2013). Les données obtenues chez l'animal confirment le rôle de ces cellules dans la mise en place de l'asthme. Cependant la majorité des résultats ont été obtenus à partir de modèles de souris chimériques ou immunodéficientes. En effet pour étudier l'effet spécifique de ces cellules il est nécessaire d'exclure toute influence des cellules T ou B. Néanmoins de nouveaux modèles, avec une déficience génétique menant à une déplétion spécifique des ILC2, se développent (Halim et al., 2014; Oliphant et al., 2014). Les résultats obtenus récemment à l'aide de ces modèles démontrent des choses extrêmement intéressantes. En effet le développement des réponses $CD4^+$ T_H2 est dépendant de la présence des ILC2 (Halim et al., 2014; Oliphant et al., 2014). De plus, ces cellules sont capables

d'interagir avec les cellules T via leur CMH de type II. Cette interaction est importante pour leur propre expansion et l'induction de cellules T_H2 (Oliphant et al., 2014). Cependant, bien que les modèles animaux apportent de nombreuses preuves de leur implication dans l'asthme, aucune donnée en clinique ne permet d'attester leur fonction pathologique chez l'homme.

5.2 Production et rôle de l'IgE dans l'asthme

Une fois présentes, Les cellules T_H2 peuvent interagir directement avec les cellules B via la présentation antigénique et les molécules de costimulation. La production d'IL-4 et d'IL-13 par les cellules T_H2 (et probablement par les ILC2) provoque une commutation de classe isotypique amenant à l'expression d'une nouvelle région constante de l'immunoglobuline (Cε pour l'IgE). Initialement, on pensait que les cellules B IgE+ étaient générées seulement dans les organes lymphoïdes secondaires. Cette vision très simpliste de l'immunologie a beaucoup évolué. En effet on sait aujourd'hui que les réponses tissulaires locales sont centrales en pathologie. Ainsi de nombreuses observations cliniques et biologiques montrent que la sélection clonale, la commutation isotypique et les mutations hypersomatiques peuvent avoir lieu directement dans les tissus respiratoires (Kato et al., 2013). Les mécanismes pathogéniques régulés par l'IgE dépendent de son interaction avec différents récepteurs. Les principaux récepteurs impliqués sont (i) le FcεRI (récepteur de haute affinité exprimé de manière constitutive sur les mastocytes et basophiles et induites à la surface des CD, éosinophiles et neutrophiles et (ii) le CD23 (FcεRII, récepteur de moyenne affinité exprimé sur les cellules épithéliales bronchiques et les cellules B) (Galli and Tsai, 2012). Comment cela se passe-t-il en pratique ? lors d'une première exposition, l'allergène est reconnu par l'épithélium qui produit les cytokines pro-T_H2. L'allergène est également pris en charge par les cellules dendritiques qui vont présenter les peptides aux cellules T et B locales et des ganglions drainants. C'est la première étape pour la mise en place de la réponse T_H2. C'est lors de la réexposition à l'allergène que le processus s'emballe (Figure 15).

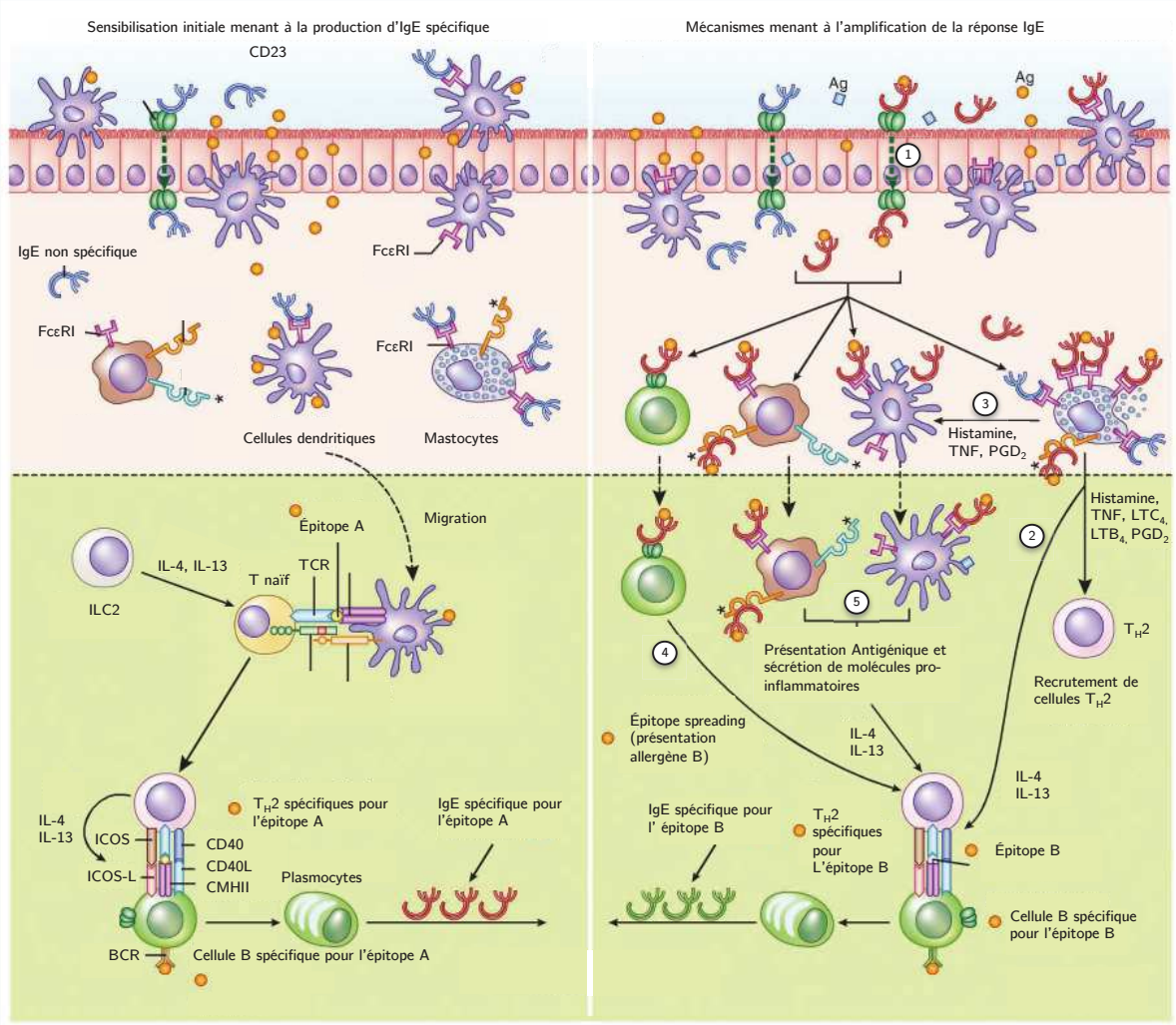

Figure 15 : **Mécanismes de sensibilisation et de production d'IgE. (À gauche)** La première exposition induit une activation des cellules épithéliales et dendritiques menant à la mise en place d'un environnement pro-T_H2. L'allergène est capté et dégradé en épitope par les cellules dendritiques qui migrent vers le ganglion drainant. Les cellules présentatrices activent alors les cellules T vers un profil T_H2 qui migrent vers le site inflammatoire. La voie de reconnaissance de l'allergène par les cellules B n'est pas connue. Il est fort probable que le lymphocyte B puisse reconnaître directement l'allergène non dégradé ou sous forme d'épitopes grâce aux cellules dendritiques. Dans ce contexte pro-T_H2 l'activation du lymphocytes B va favorisait la production d'IgE spécifique de l'allergène (IgE spécifiques pour les épitopes A). **(À droite)** C'est lors d'une nouvelle exposition que la réponse allergique s'emballe. Cette fois ci, les IgE spécifiques reconnaissent directement l'allergène et vont alors activer les cellules innées via l'interaction avec le FcεRI et le CD23. Ceci induit la production de médiateurs lipidiques pro-inflammatoires qui vont exacerber la réponse allergique. En parallèle, on note une expansion des cellules lymphoïde innées et T_H2 qui vont potentialiser la réponse IgE. Par sa fonction de présentation antigénique, la cellule B peut également participer à la promotion des cellules T_H2. Par ailleurs, la réexposition aux allergènes va promouvoir une diversification du répertoire IgE (Epitope spreading) avec la production d'IgE avec une spécificité antigénique différente (IgE spécifiques pour les épitopes B).

Les IgE spécifiques reconnaissent les allergènes et forment des complexes immuns qui interagissent avec le FcεRI à la surface des mastocytes, basophiles et éosinophiles. Cette interaction induit le relargage d'un grand nombre de médiateurs lipidiques pro-inflammatoires comme l'histamine, les leucotriènes et la prostaglandine. Ces molécules vont induire des modifications profondes comme (i) la vasodilation et l'augmentation de la perméabilité vasculaire facilitant ainsi le recrutement d'autres cellules immunitaires, (ii) la bronchoconstriction. La production d'IL-4 et d'IL-13 amplifie le phénomène en promouvant la production d'IgE mais également en augmentant l'expression de CD23. A la surface des cellules épithéliales on observe ainsi un phénomène de transcytose (Figure 15) : les complexes IgE-allergènes sont captés à la surface de l'épithélium par le CD23 puis sont transférés sur le site inflammatoire augmentant ainsi la disponibilité de complexes immuns allergiques. À la surface des cellules B, le CD23 agit comme un récepteur d'endocytose. Le complexe IgE-allergène est alors internalisé et dégradé. Les peptides ainsi générés sont présentés aux lymphocytes T. La présentation antigénique par les B amplifie ainsi la diversification de la réponse T_H2. En effet l'absence de lymphocytes B affaiblit fortement les réponses T_H2 allergiques chez le petit l'animal (Lindell et al., 2008; Linton et al., 2003). Dans quelle mesure les cellules B participent à la promotion des réponses allergiques, via notamment la production de cytokines, reste très mal connue. Il est fort probable que l'inflammation bronchique et l'exposition à l'allergène puissent altérer leur profil cytokinique vers un profil plus inflammatoire, comme observé dans le diabète auto-immun (DeFuria et al., 2013). Cependant ces cellules restent décrites essentiellement pour leur rôle dans la production d'IgE.

5.3 Rôle des cytokines T_H2 dans la physiopathologie de l'asthme

En plus d'avoir un rôle sur la régulation de la réponse immune, les cytokines T_H2 assurent d'autres rôles pathologiques. L'IL-5 est ainsi un facteur clé dans l'attraction et l'activation des éosinophiles (Pavord et al., 2012). Sur le site inflammatoire, les

éosinophiles sont très délétères pour les tissus bronchiques et provoquent des lésions irréversibles. L'IL-13 assure également un grand nombre de mécanismes pathologiques avec des effets directs sur les cellules structurelles. Elle induit l'hyperplasie des cellules caliciformes bronchiques (Erle and Sheppard, 2014; Kuperman et al., 2002). En effet, cette cytokine promeut la production de mucus dans les voies aériennes via l'activation de la voie STAT6 (Erle and Sheppard, 2014; Kuperman et al., 2002). L'hypersécrétion de mucus dans l'asthme altère la qualité de la fonction respiratoire des patients. Aussi, de nombreuse études montrent que cette cytokine joue un rôle central dans le développement de fibrose, processus caractérisé par l'accumulation de composant de la matrice extracellulaire (Wynn and Ramalingam, 2012). En effet l'IL-13 aurait un effet direct, en régulant la prolifération des fibroblastes et des cellules musculaire lisses (Wynn and Ramalingam, 2012), mais aussi indirect via l'induction de TGF-beta (une molécule clé du processus fibrotique) (Wynn and Ramalingam, 2012). Enfin, l'IL-13 régule l'hyperréactivité en induisant un profil transcriptomique particulier dans les poumons, avec la surexpression de gènes impliqués dans la contraction du muscle lisse (Perkins et al., 2011). Des observations *in vitro* ont mis en évidence la capacité de l'IL-13 à augmenter la contraction de trachées de souris (Tliba et al., 2003) et ou de cellules musculaires lisses humaines (Risse et al., 2011). L'IL-13 active ainsi la voie calcique (Tliba et al., 2003) et régule l'expression de petites protéines G de la famille RhoA impliquées dans la réponse contractile du muscle lisse (Kudo et al., 2013).

5.4 L'IL-17 : une cytokine importante dans l'asthme sévère ?

L'hétérogénéité de l'asthme force la constatation suivante : d'autres mécanismes, immunologiques ou non, peuvent être responsables du développement de la pathologie. Ainsi l'IL-17 a été décrite comme une cible potentielle importante dans l'asthme sévère (Chesne et al., 2014). Cette cytokine, majoritairement produite par les cellules T_H17, est augmentée chez des patients caractérisés par un asthme sévère (Al-Ramli et al., 2009; Bullens et al., 2006; Doe et al., 2010). Certaines études cliniques rapportent une

corrélation étroite entre la présence de neutrophiles et les taux d'IL-17 dans l'asthme sévère. Ce lien biologique est très bien illustré dans les modèles animaux ou l'inhibition de la réponse T_H17 (par des approche génétiques ou thérapeutiques avec l'emploi d'anticorps neutralisants) diminue l'inflammation neutrophilique et le développement de l'asthme (He et al., 2009; He et al., 2007; Wilson et al., 2009; Zhao et al., 2013). Par ailleurs l'IL-17 régule directement l'expression des chimiokines pro-neutrophiles assurant ainsi leur attraction sur les sites inflammatoires (Chesné et al., 2014 publication jointe au manuscrit). Ce lien entre IL-17 et neutrophiles est parfaitement illustré chez l'homme dans le cadre d'essais cliniques ciblant la voie IL-17 chez des patients atteints de psoriasis (Papp et al., 2012; Zaba et al., 2009) ou de spondylarthrite ankylosante (Baeten et al., 2013; Baeten and Kuchroo, 2013). En effet l'administration d'anticorps ciblant l'IL-17 ou son récepteur améliore la condition des patients via notamment la diminution de l'infiltrat neutrophilique (Baeten et al., 2013; Papp et al., 2012; Zaba et al., 2009). Une neutropénie est même observée chez certains patients confirmant la forte altération de la voie IL-17 chez les patients traités (Baeten et al., 2013; Papp et al., 2012; Zaba et al., 2009). Néanmoins, le lien IL-17-neutrophiles dans l'asthme ne reste qu'une supposition due au manque de résultats obtenus lors d'essais cliniques sur une population ciblée de patients asthmatiques (Chesne et al., 2014; Wenzel, 2012).

Dans les modèles animaux la population T_H17 est décrite pour induire la cortico-résistance (McKinley et al., 2008; Zhao et al., 2013). En effet le transfert de cellules T_H17 chez la souris immunodéficiente assure la mise en place d'une résistance à la dexaméthasone alors que le transfert de cellules T_H2 n'altère pas la réponse aux stéroïdes (McKinley et al., 2008). Chez l'homme des observations similaires ont été rapportées avec notamment une augmentation des cellules T_H17 dans le cadre de l'asthme sévère chez l'enfant et l'adulte (Gupta et al., 2014; Nanzer et al., 2013). La récente caractérisation moléculaire d'une population T_H17 pathogénique et résistante aux stéroïdes renforce les observations précédentes. Cette sous-population T_H17 est

notamment caractérisée par la surexpression du transporteur ABCB1 (MDR1) (Ramesh et al., 2014). Cette molécule est impliquée dans le développement de multi-résistance aux médicaments et est une cible clé en pharmacologie (Ramesh et al., 2014). Sa surexpression sur les cellules T_H17 renforce le caractère « stéroïde-résistant » de ces cellules. De manière intéressante le traitement par les stéroïdes semble favoriser l'émergence de cette sous-population T_H17 chez l'homme (Ramesh et al., 2014). Ces observations ont des retombées importantes dans le cadre de l'asthme notamment sévère. Il est tout à fait possible que certains patients recevant de très fortes doses de corticoïdes modulent leur profil inflammatoire en favorisant l'émergence de cellules T_H17 aggravant ainsi leur pathologie avec la survenue d'une neutrophilie.

Les mécanismes moléculaires menant à la différenciation en cellules T_H17 sont bien décrits et connus (Muranski and Restifo, 2013). Cependant les facteurs environnementaux ou génétiques impliqués dans le cadre de l'asthme sont très peu connus. Chez le petit animal, la sensibilisation percutanée ou par les voies aériennes favorise la mise en place d'un asthme IL-17 dépendant (He et al., 2009; He et al., 2007; Wilson et al., 2009) avec notamment l'induction de cellules dendritiques ayant un profil pro-T_H17 caractérisé par la sécrétion d'IL-23 (He et al., 2007), une cytokines importante dans le processus de différenciation des T_H17 (Muranski and Restifo, 2013). Également l'exposition à des particules de diesel aggrave la pathologie chez la souris en induisant notamment une très forte expansion des cellules T_H17 (Brandt et al., 2013). La neutralisation d'IL-17 dans ce modèle inhibe l'hyperréactivité bronchique (Brandt et al., 2013). En clinique les enfants asthmatiques atopiques exposés à ces particules ont des taux très élevés de l'IL-17 dans le sang. Les taux d'IL-17 sont associés de manière significative à la fréquence des symptômes et la sévérité de la pathologie (Brandt et al., 2013). Plus récemment une étude très élégante a mis en évidence un potentiel mécanisme moléculaire responsable de l'asthme lié à l'obésité (Kim et al., 2014). En effet des souris obèses ont une hyperréactivité plus élevée et sont caractérisées par des

taux très élevés d'IL-17 dans les voies aériennes. Les auteurs identifient les ILC de type 3 (ILC3) comme source principale d'IL-17 dans leur modèle (Kim et al., 2014). L'expression de NLRP3 et la production d'IL-6 et d'IL-1β sont cruciales pour l'expansion de ces cellules et la mise en place de l'asthme chez les souris obèses. Ce qui est très intéressant ici, c'est que ces trois molécules ont été identifiées comme des biomarqueurs potentiels de l'asthme neutrophilique (Baines et al., 2010; Baines et al., 2014; Wood et al., 2012). De plus les auteurs retrouvent une augmentation du nombre d'ILC3 dans le lavage bronchoalvéolaire des patients atteints d'asthme sévère (Kim et al., 2014). Cette observation a été faite sur une très petite cohorte de patients et mérite d'être confirmée sur un plus grand nombre.

Ces récentes études chez l'animal démontrent une chose : la nécessité de développer des modèles animaux mimant un endotype ou un phénotype moléculaire particulier, notamment les endotypes non-T_H2 (Martin et al., 2014). C'est sur la base de cette hypothèse que nous avons développé au sein de notre équipe un nouveau modèle d'asthme aux acariens caractérisé par une inflammation mixte (Chesné et al., 2014 publication jointe au manuscrit). Dans ce modèle nous démontrons que l'IL-17 joue un rôle central dans l'asthme en régulant l'inflammation neutrophilique et la contraction du muscle lisse bronchique. Notre travail vient ainsi renforcer l'idée d'un rôle de l'IL-17 dans le développement de l'asthme.

5.5 Mécanismes immunorégulateurs dans l'asthme : quel rôle pour les lymphocytes B régulateurs ?

Une des fonctions du tractus pulmonaire est de maintenir la tolérance face l'exposition continue d'antigènes. Plusieurs études expérimentales ont mis en avant le fait que les antigènes entrant par la voie respiratoire sont généralement tolérés induisant parfois une réponse T_H2 résiduelle (Holt et al., 2008). Dans ce processus l'IL-10 est une cytokine cruciale pour la suppression des réponses allergiques dans le tissus pulmonaire (Lloyd

and Hawrylowicz, 2009). De plus bien que cette cytokine ne soit pas primordiale pour contrôler l'inflammation systémique, elle est absolument indispensable pour contenir les réponses inflammatoires dans les muqueuses intestinales et pulmonaires (Rubtsov et al., 2008). Cette tolérance, dépendante de l'IL-10, supporte ainsi l'implication des cellules régulatrices produisant de l'IL-10. Parmi les plus connues et décrites, les lymphocytes Tregs sont décrits comme des acteurs importants dans le contrôle de l'inflammation des voies aériennes au cours de l'asthme (Lloyd and Hawrylowicz, 2009).

Cependant les lymphocytes B sont aussi une source importante d'IL-10 et sont capables de contrôler les réponses inflammatoires. Plusieurs travaux ont ainsi mis en évidence qu'une déficience en B10 contribuait à la mise en place d'une inflammation sévère au cours du développement de pathologies allergiques (Braza et al., 2014). Dans le cadre de l'asthme très peu de choses sont connues. Les données épidémiologiques démontrent que des personnes hôtes de parasites ne développent pas d'asthme (Amu et al., 2010), suggérant que la présence de ces microorganismes induit la mise en place de mécanismes régulateurs capables de contrôler la survenue d'asthme. En accord avec cette hypothèse de récentes études démontrent que l'infection parasitaire induit l'expansion d'une population B10 et empêche la mise en place de l'inflammation des voies aériennes chez la souris. Le transfert des B10 d'une souris infectée à une souris asthmatique réverse complètement le phénotype en inhibant l'inflammation et l'hyperréactivité des voies aériennes (Amu et al., 2010). Chez l'homme les données sont rares. Une seule équipe a récemment démontré que des patients atteints d'un asthme allergique ont une diminution de la fréquence des B10 dans le sang (van der Vlugt et al., 2014). Ces observations reposent sur l'analyse de 12 patients et nécessitent donc d'être confirmées sur une plus grande cohorte. Il aurait également été intéressant d'introduire un paramètre longitudinal à cette étude et de quantifier les Bregs avant, pendant et après l'exacerbation pour déterminer si la pathologie a réellement un impact sur l'homéostasie de ces cellules. Aujourd'hui des stratégies thérapeutiques existent

pour le traitement de pathologies allergiques comme l'immunothérapie spécifique (Pipet et al., 2009). Or différentes études démontrent que l'immunothérapie favorise l'émergence de cellules B régulatrices dans le cadre de l'allergie alimentaire (Noh et al., 2010; Noh et al., 2012) et au venin d'abeille (van de Veen et al., 2013). Cela suggère que la présence des B10 semble un facteur important dans le contrôle des réponses allergiques.

Sur la base de ces observations nous posons l'hypothèse que le développement de l'asthme, menant à une inflammation bronchique et systémique, altère le profil lymphocytaire B. Pour confirmer notre hypothèse nous avons étudié le réponse B dans un modèle d'asthme allergique aux acariens développé au laboratoire (Chesné et al., 2014 publication jointe au manuscrit). Les études phénotypiques et transcriptomiques réalisées dans le cadre de ce travail ont permis de confirmer l'influence de l'inflammation allergique sur le profil lymphocytaire B, en favorisant l'émergence de cellules B inflammatoires au détriment des cellules B régulatrices. Nos investigations supplémentaires se sont focalisées sur la caractérisation de cette population B régulatrice chez la souris. Nous avons ainsi pu identifier de nouveaux marqueurs de surface transposables chez l'homme. L'entièreté de ce travail fait l'objet d'une soumission dans le *Journal of Clinical Investigation*.

En résumé

- L'asthme allergique est caractérisé par une inflammation T_H2. On sait aujourd'hui que la réponse TH2 n'est plus le seul fait des cellules T. En effet les cellules épithéliales, les cellules dendritiques et les cellules lymphoïdes innées participent grandement à la mise en place de l'inflammation des voies aériennes

- La réponse IgE est central dans le cadre de l'asthme. Cet anticorps est le pivot de la réaction inflammatoire.

- L'IL-13 influence l'environnement en altérant le phénotype des cellules épithéliales et en modulant les fonctions contractiles du muscle bronchique.

- L'IL-17 semble être une molécule importante dans les mécanismes physiopathologiques des asthmes non-T_H2. Elle induit une inflammation neutrophilique et peut également moduler les fonctions contractile du muscle lisse

- Plusieurs travaux ont démontré une altération des mécanismes immunorégulateurs dans l'asthme. C'est ans ce contexte que nous nous sommes intéressés à la physiologie des B10 dans l'asthme.

Regulatory functions of B cells in allergic diseases

Braza F, Chesne J, Castagnet S, Magnan A and Brouard S (In press in Allergy)

Abstract

B cells are essentially described for their capacity to produce antibodies ensuring anti-infectious immunity or deleterious responses in the case of autoimmunity or allergy. However abundant data described their ability to restrain inflammation by diverse mechanisms. In allergy some regulatory B cells subsets producing IL-10 have been recently described as potent suppressive cells able to restrain inflammatory responses both in vitro and in vivo by regulatory T cell differentiation or directly inhibiting T cell mediated inflammation. A specific deficit in regulatory B cells participates to more severe allergic inflammation. Induction of allergen tolerance through specific immunotherapy induces a specific expansion of these cells supporting their role in establishment of allergen tolerance. However the regulatory functions carried out by B cells are not exclusively IL-10 dependent. Indeed, other regulatory mechanisms mediated by B cells are (i) the production of TGF-β (ii) the promotion of T cell apoptosis by Fas-Fas ligand or Granzyme-B pathways and (iii) their capacity to produce inhibitory IgG4 and sialylated IgG able to mediate anti-inflammatory mechanisms. This points to Bregs as interesting targets for the development of new therapies to induce allergen tolerance. In this review we highlight advances in the study of regulatory mechanisms mediated by B cells, and outline what is known about their phenotype as well as their suppressive role in allergy from studies in both mice and humans.

Key words: Allergy, B cells, IL-10, Immunotherapy, regulatory B cells

REVIEW ARTICLE

Regulatory functions of B cells in allergic diseases

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Abstract

B cells are essentially described for their capacity to produce antibodies ensuring anti-infectious immunity or deleterious responses in the case of autoimmunity or allergy. However, abundant data described their ability to restrain inflammation by diverse mechanisms. In allergy, some regulatory B-cell subsets producing IL-10 have been recently described as potent suppressive cells able to restrain inflammatory responses both *in vitro* and *in vivo* by regulatory T-cell differentiation or directly inhibiting T-cell-mediated inflammation. A specific deficit in regulatory B cells participates to more severe allergic inflammation. Induction of allergen tolerance through specific immunotherapy induces a specific expansion of these cells supporting their role in establishment of allergen tolerance. However, the regulatory functions carried out by B cells are not exclusively IL-10 dependent. Indeed, other regulatory mechanisms mediated by B cells are (i) the production of TGF-β, (ii) the promotion of T-cell apoptosis by Fas–Fas ligand or granzyme-B pathways, and (iii) their capacity to produce inhibitory IgG4 and sialylated IgG able to mediate anti-inflammatory mechanisms. This points to Bregs as interesting targets for the development of new therapies to induce allergen tolerance. In this review, we highlight advances in the study of regulatory mechanisms mediated by B cells and outline what is known about their phenotype as well as their suppressive role in allergy from studies in both mice and humans.

Allergic diseases encompass many heterogeneous pathologies with distinct clinical manifestations. These pathologies generally result from an uncontrolled inflammatory response to allergens and can lead to a number of disorders, including asthma, allergic rhinoconjunctivitis, anaphylaxis, urticaria, and atopic dermatitis. Mechanisms promoting allergic inflammation in mucosal tissues are characterized by dysregulated type 2 immunity with a dramatic T_H2-driven immunity, elevated allergen-specific IgE, leading to goblet cell hyperplasia and mucus overproduction. One significant cause of the development and persistence of allergic inflammation is an alteration in the immune regulatory processes (1). Regulatory T cells have long been the focus of all attention in the maintenance of allergen tolerance (2, 3), but recently, a new subset of B cells has been identified as regulatory (Bregs), due to their capacity to secrete IL-10 and constrain severe inflammation (4). In humans, B-cell depletion was recently suggested to exacerbate ulcerative colitis, promote psoriasis, or aggravate inflammation in patients with multiple sclerosis (5–7). These observations demonstrated the role of Bregs in the control of T-cell-mediated inflammation *in vivo*. Bregs cells are of special interest in allergic diseases also, as demonstrated by recent reports showing their potential implication in the development of allergen tolerance (8–10). However, the regulatory functions carried out by B cells are not exclusively IL-10 dependent. Indeed, the recent identification of other Bregs expressing TGF-β, granzyme-B, Fas-L (Fig. 1), or producing inhibitory antibodies in allergy cases points to Bregs as interesting targets for the development of new therapies to induce allergen tolerance. In this review, we will synthesize recent data concerning Breg origin, development, and function and also report on recent findings showing how B cells can constrain inflammation, notably in allergic diseases.

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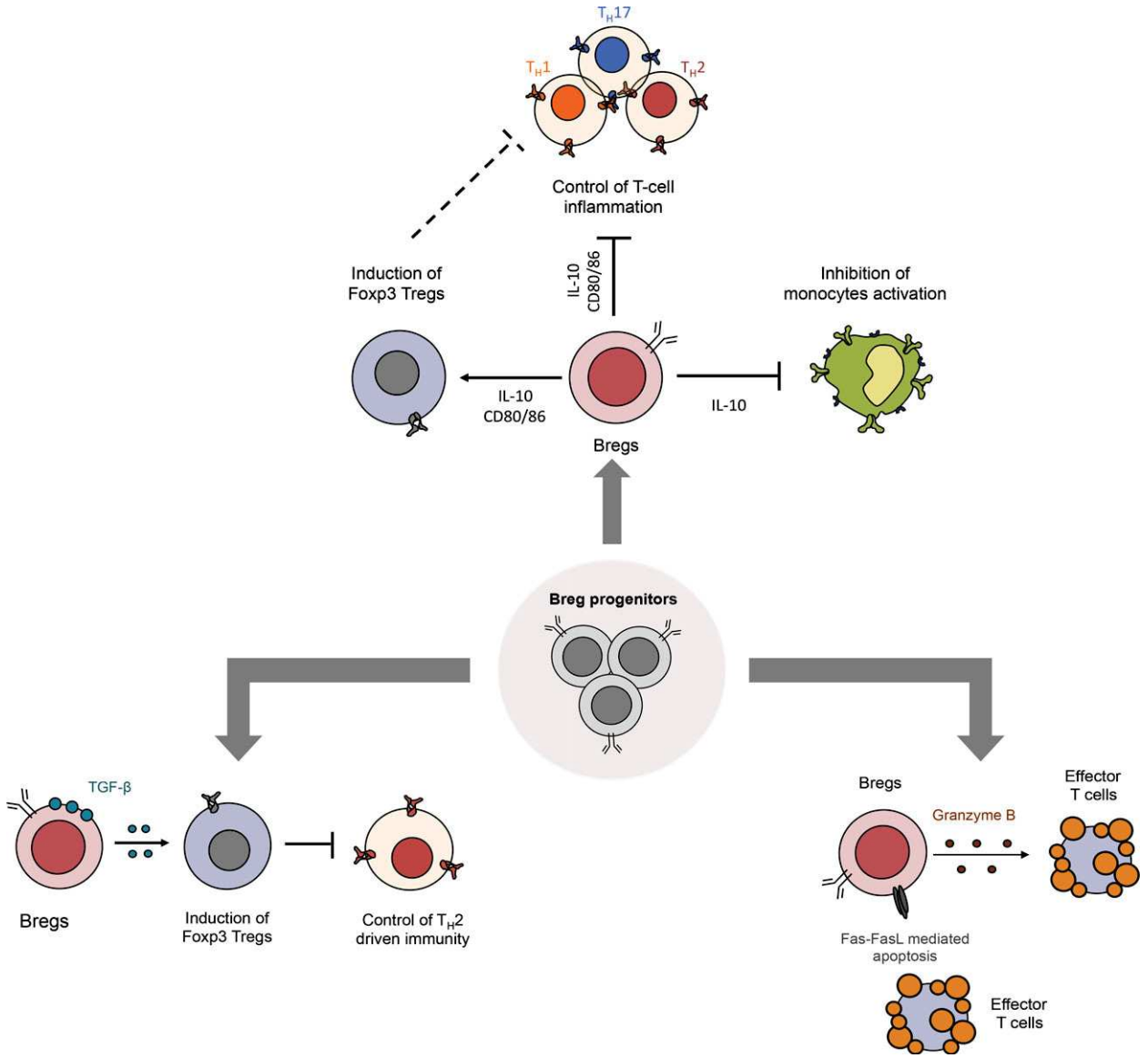

Figure 1 Suppressive functions mediated by Breg subsets in inflammation. Regulatory B cells are distinct B cell subsets exhibiting different functions. These cells probably arise from different progenitors and must be generated under specific instructive signals. Among identified regulatory B cells populations we can find IL-10 producing B cells that are important for the maintenance of Tregs and inhibition of T cell mediated inflammation. A deficit in these cells exacerbates inflammation and induces an alteration in Treg homeostasis. Some reports also demonstrated the ability of IL-10 Bregs to control inflammation by inhibiting monocyte activation and maturation. Reports also described the existence of TGF-β secreting cells controlling deleterious TH2 inflammation essentially through the induction regulatory T cells. Finally the recent identification of killer B cells suggests that Bregs may also constrain T cell responses by inducing their death.

IL-10-producing B cells as central regulators of inflammation and allergic diseases

IL-10$^+$ regulatory B cells in animal models

The first experiments demonstrating the regulatory functions of B cells were performed in a guinea pig model of skin hypersensitivity. Preliminary observations demonstrated that treatment with cyclophosphamide before sensitization enhanced inflammatory responses, suggesting a regulatory role for B cells in this model (11). Adoptively transferred splenocytes from sensitized guinea pig were able to inhibit delayed-type hypersensitivity skin reactions, whereas B-cell-depleted splenocytes were not (11). A later study demonstrated that injection of high doses of sheep erythrocytes in C57BL6 mice induces the expansion of B cells, which, when transferred to naive mice, were able to generate suppressor T

cells (12). More recently, the discovery that B-cell deficiency in mice prevents recovery from EAE or exacerbated autoimmune and inflammatory diseases confirmed the capacity of B cells to control deleterious inflammation (13–16). Consistent with this, chimeric mice lacking endogenous IL-10-producing B cells develop irreversible EAE associated with increased T_H1 inflammation (17). Similarly, B-cell-deficient-TCR alpha KO mice develop more severe colitis lesions than TCR alpha KO mice, indicating that B cells can contribute in part to the suppression of intestinal inflammation (18). Further investigations identified that an IL-10-producing B-cell subset is important in suppressing T_H2-driven inflammation in this model (18). More recently, it was shown that this regulatory subset was also crucial for the suppression of T_H1 and T_H17 immunity and maintenance of regulatory T cells in mice (Fig. 1) (19). A lack of specific transcriptional or surface markers makes the identification of these Bregs difficult and has led to a certain amount of controversy regarding their origin. Animal studies have demonstrated that regulatory functions can be associated with many B-cell subsets (4). Given this great heterogeneity, several models have been proposed to explain Breg origin and development. One hypothesis supposes that Bregs stem from follicular B cells (FO, $CD19^+$ $CD21^{lo}$ $CD23^{hi}$) and are generated following specific instructive signals (17, 20, 21) and that they are able to suppress maturation of monocytes and dendritic cells and so dampen T-cell proliferation (21). However, support for this theory remains modest as KO mice for G protein alpha inhibitory subunit and p110δPI3K, lacking marginal zone B cells (MZ, $CD19^+$ $CD23^{lo}$ $CD21^{hi}$ $CD1d^{hi}$) and transitional 2-marginal zone progenitors B cells (T2-MZP, $CD19^+$ $CD23^+$ $CD21^{hi}$ $CD1d^{hi}$), develop strong colitis. This result suggests that these B-cell subsets are also necessary for immune regulation (22). Consistent with this, work by Mauri's group also identified T2-MZP B cells as the main regulatory B-cell subset in mice (23, 24). Indeed, adoptive transfer of this subset can delay or stop the development of collagen-induced arthritis in mice (24). Another theory suggests that Bregs are derived from the $CD5^+$ $CD1d^{hi}$ B-cell subset, also called B10 cells (15). However, contrasting results have been obtained from IL-10 reporter mice in which IL-10-secreting B cells are confined to plasmablasts and plasma cells *in vivo* (25–27), confirming observations in infectious models (28, 29). A recent publication also suggested that the phenotype of Bregs could be dependent on their tissue localization and probably their microenvironment (30). Indeed, adipose $IL\text{-}10^+$ B cells are $CD1d^{low}$ $CD5^{-/low}$ $CD11b^{low}$ $CD21/CD35^{low}$ $CD23^{low}$ $CD25^+$ $CD69^+$ $CD72^{high}$ $CD185^-$ $CD196^+$ IgM^+ IgD^+, which is distinct from any other known IL-10-producing B cells (30).

Identification of IL-10$^+$ Regulatory B cells in humans

The existence of this subset in human has been primary suggested through studying patients with multiple sclerosis (MS), who exhibit decreased frequency of IL-10-secreting B cells compared with healthy volunteers (31). Interestingly, the partial amelioration of the disease occurring in patients with helminth-infected MS is associated with the proportion and activity of IL-10-producing B cells being restored to control levels (32). Functional Breg defects have also been reported in lupus, rheumatoid arthritis, and patients with immune thrombocytopenia (33–35). In these patients, IL-10-secreting B cells enriched within $CD24^{hi}$ $CD38^{hi}$ transitional B cells have dampened regulatory functions (33) and are unable to suppress T_H17 immunity and induce regulatory T cells *in vitro* (34). However, whereas mainly described within the $CD24^{hi}$ $CD38^{hi}$ immature B-cell subset (33, 34), IL-10 Bregs are also enriched in the $CD27^+$ $CD24^{hi}$ memory B-cell compartment and are the human equivalent of mouse B10 cells (36, 37). Despite these divergent results, expression of CD5 is a common feature of human Bregs (33, 37, 38). Notably, CD5 is directly involved in the secretion of IL-10 and could have a functional role in the suppressive effects of Bregs (39, 40). Recently, the isolation and microarray analysis of induced Bregs in healthy volunteers allowed the identification of new specific markers. In contrast to some previous reports, $IL\text{-}10^+$ Bregs were more enriched in a $CD25^+$ $CD71^+$ $CD73^-$ B-cell population than other described subsets (10). However, no information about their differentiation state (naive, memory, or secreting plasma cells) was given in this study. The fact that these $IL\text{-}10^+$ B cells also produce antibodies could suggest that they belong to a memory or plasma cell subset (10). Recently, an elegant study demonstrated that the regulatory capacity of $IL\text{-}10^+$ Bregs importantly depends on their capacity to also secrete TNF-α (41). Authors demonstrated that the ratio of IL-10/TNF-α expression, a measure of cytokine polarization, might be a better indicator of suppressive function of Bregs than IL-10 expression alone. Then, $CD24^{hi}$ $CD38^{hi}$ transitional B harbored the highest IL-10/TNF-α and so the best suppressive function (41). These data highlight the impossibility of clearly attesting to and identifying the origin and development of Bregs. In addition, the great heterogeneity of phenotypes strongly suggests that B cells must be generated under specific instructive signals rather than arising from a unique progenitor (Fig. 1).

IL-10$^+$ regulatory B cells in allergies

Since the initial experiments on a guinea pig model of hypersensitivity in 1979, very few studies have demonstrated the existence of IL-10-suppressive B cells in allergic disease models. However, observations that CD19−/− mice, which lack of IL-10 B cells, have increased contact hypersensitivity responses have pointed to the role of these cells in regulating skin allergy diseases (42). Consistent with this, a specific deficiency in IL-10-producing B cells dramatically increases allergic skin inflammation in mice with an accumulation of effector T cells and higher production of IFN-γ in the skin (43). Adoptive transfer of Bregs from CHS-mice inhibits skin inflammation and the development of the disease *in vivo* (15, 43). CpG injections reduce inflammation in a late-phase experimental allergic conjunctivitis model (44). Transfer of splenocytes from CpG-treated mice is able to constrain allergen-induced inflammatory responses in recipient mice. Flow cytometry analysis revealed that CpG treatment significantly

increased the proportion of IL-10-producing B cells with a follicular phenotype, able to constrain inflammation and to inhibit eosinophilia (44). Other studies have demonstrated that B cells isolated from helminth-infected mice have a protective function in allergy by controlling fatal anaphylaxis or airway inflammation via IL-10 production (45–48). Immunosuppressive effects are mediated by the induction and maintenance of regulatory T cells independent of TGF-β (45).

Interesting data have come from clinical protocols for specific immunotherapy (SIT). Patients suffering from a food allergy (cow's milk allergy) have a diminution in their peripheral Bregs (49). These cells, called induced Bregs (B_R1), are characterized by the expression of $CD5^+$ and the production of IL-10 (49). Specific *in vitro* restimulation with casein induces a decrease in B_R1 in the allergic group but increases these cells in the tolerant group, suggesting a role for B_R1 in the establishment of cow's milk tolerance. Most interestingly, whereas oral tolerance induced by ingestion of milk alone did not improve clinical outcomes, milk intake associated with IFN-γ injections completely suppressed the disease, probably by increasing B_R1 (9). Patients receiving IFN-γ and milk have significantly higher proportion of B_R1 *ex vivo* and after restimulation with casein *in vitro* (9). In addition, B_R1 are able to suppress the proliferation of $CD4^+$ T cells with a ratio of 1 B cell to 25 PBMCs (7). In comparison, regulatory T cells suppress antigen-specific proliferation in a ratio of 1 suppressor cell to 2 or 4 responder cells in similar experimental conditions (50). Interestingly, in the context of bee-venom allergy, beekeepers who spontaneously develop tolerance toward the allergen and patients undergoing immunotherapy exhibit a higher level of antigen-specific B_R1 in their blood, supporting the view that Bregs are important for the establishment of allergen tolerance (10). The study of $IL\text{-}10^+$ Bregs in patients with asthmatic allergy reveals a significantly lower level of IL-10 regulatory B cells in peripheral blood of $CD24^+$ $CD27^+$ human subjects when compared to healthy controls (51). In addition, $CD24^+$ $CD27^+$ subjects exhibited a nonoptimal induction of IL-10 after stimulation with LPS and allergen resulting in the partial induction of regulatory T cells *in vitro* (51). In contrast, stimulated $CD24^+$ $CD27^+$ of healthy volunteers produce significant levels of IL-10 and induce more regulatory T cells into the system. This suggests that Bregs cells from patients with asthma do not optimally respond to LPS, which may contribute to the increased inflammation found in asthma (51).

Microbes, the hygiene hypothesis, and IL-10$^+$ Bregs: The missing link?

Many instructive signals are known to promote the generation of IL-10-regulatory B cells. BCR signaling is central to the production of IL-10 and generation of effective regulatory B cells. This is shown in mice with B cells deficient for (i) stromal interaction molecules 1 and 2 (STIM-1 and STIM-2), which control calcium signaling downstream BCR, or (ii) B-cell linker protein (BLNK), a molecule involved in the B-cell signalosome downstream the BCR; these mice have impaired IL-10 production and increased inflammation (43, 52). The dialogue with T cells is also important for optimal induction of IL-10 Bregs as CD80/86, CD40, and IL-21 molecules significantly improve generation of Bregs (17, 33, 53–55). The recent role of TIM-1 in the generation of Bregs is of special interest in allergy study as certain polymorphisms of this gene are strongly associated with allergic diseases (56–59). However, no convincing data have yet emerged in human studies. A number of reports have demonstrated that development and generation of IL-10 Bregs require Toll-like receptors (TLRs) and Myd88 signaling in both mice and humans (17, 28, 36, 60). Consistent with this, mice with a B-cell-specific deficiency in both TLR2 and TLR4 or in Myd88 develop a chronic form of EAE, comparable to chimeric mice with IL10-deficient B cells. In contrast, mice with wild-type B cells recover after a short episode of paralysis, strengthening the case for a key role for TLR signaling in the prime regulatory functions of B cells (17). The hygiene hypothesis suggests an early-life farming lifestyle confers protection against asthma, hay fever, and allergic sensitization, arguing that early exposure to microbes could activate a number of regulatory mechanisms during infancy (61). Accordingly, a decrease in parasitic infections is suggested as being responsible for the dramatic increase in the global incidence of allergic diseases, such as asthma (62, 63). Several works in animals and humans have demonstrated the role of TLRs in the protection conferred by parasites and other microbes toward allergy (64–66). It is worth-noticing that in early life, immune cells preferentially produce IL-10 after stimulation with TLR ligands (67). In line with this, a recent work showed that neonate mice exhibit dramatically higher levels of IL-10-regulatory B cells than adult mice (60). Consequently, observations that (i) lower TLR4-induced IL-10 production is significantly associated with the development of atopic dermatitis in children (68), (ii) patients with allergic asthma exhibit attenuated TLR4-induced IL-10 production by B10 cells when compared to healthy individuals (51), and (iii) infection with parasites favors the emergence of Bregs and protects from allergic inflammation (45, 48, 63), all support a potential role for IL-10 Bregs cells in the early control of allergic diseases. We could also postulate that early exposure to pathogens could enhance the generation of Bregs and thus be important for protection against allergy, probably through the maintenance of Tregs (45, 48, 66, 69) (Fig. 2). However, to date, no data have clearly demonstrated this.

New regulatory mechanisms mediated by B cells: a role in allergic diseases?

TGF-β-producing B cells

A number of studies indicate that Bregs, like Tregs, may encompass several subsets that exhibit different immune regulatory mechanisms, including, notably, the production of TGF-β. This multifunctional cytokine carries out many physiological roles in both healthy and pathological processes. TGF-β is involved in both suppression and promotion of inflammation; for example, it ensures the maturation of Tregs and inhibition of effector T cells, and it also participates in the differentiation and priming of Th17 cells. Interestingly,

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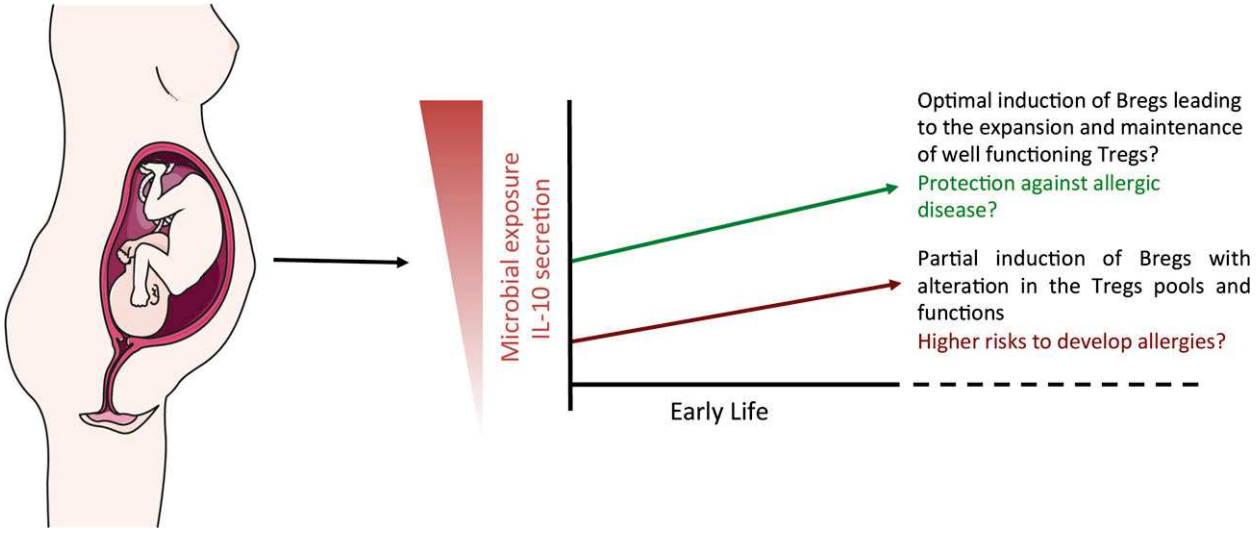

Figure 2 A role for Bregs in hygiene hypothesis and allergy protection? Exposure to parasites and bacteria is associated to a decrease incidence of allergic diseases. Recent evidences suggest an involvement of IL-10 producing regulatory B cells in this process. In early age the immune system preferentially produce IL-10 after stimulation with toll like receptors agonists. There receptors are central in the generation of regulatory B cells that can than promote the induction and maintenance of regulatory T cells necessary for allergy protection.

disruption of TGF-β Receptor signaling predisposes patients to develop allergic pathologies including asthma, food allergy, eczema, and allergic rhinitis (70). In a mouse model of OVA-induced oral tolerance, accumulation of $CD5^+$ TGF-β-producing B cells in lymph nodes suppresses lung inflammation by promoting the expansion of regulatory T cells (Fig. 1) (71). Recently, a subpopulation of intestinal B cells expressing the αvβ6 integrin has been identified in mice. This subset, characterized by the expression of CD5 and CX3CR1, produces high levels of TGF-β. Stimulation of αvβ6 induces the production of TGF-β, suggesting a key role in the regulatory function of B cells. These Bregs are able to suppress T-cell proliferation *in vitro*, induce Treg differentiation, and inhibit food allergy-induced Th2 inflammation in the intestine *in vivo* (72). In line with these results, specific immunotherapy in an OVA-induced food allergy model promotes the emergence of $CD35^+$ Thrombospondin-1 (TSP-1)-producing B cells able to suppress allergic inflammation (73). They control inflammation by inducing regulatory T cells through the production of TGF-β. Interestingly, induction of Tregs is dependent on TSP-1 activity. In addition, it has been demonstrated that this molecule is able to cleave costimulatory molecules at the surface of dendritic cells, dampening global inflammation (73). In humans, despite the identification of a decrease in TGF-β-producing B cells in milk allergy, no convincing data have yet emerged concerning the role of TGF-β-producing Bregs in the context of human allergies.

Fas-L-expressing B cells and granzyme-B-producing B cells: the new killer cells?

The induction of T-cell apoptosis is also one of the mechanisms mediated by B cells to control inflammation. Recently, an elegant study demonstrated that stimulation with TLR-9 ligand favors the emergence of innate pro-B cells that can protect mice from the development of type 1 diabetes (74). Protection was ensured by IFN-γ-mediated upregulation of Fas ligand expression on B cells, which enabled them to kill effector T cells through apoptosis (74). In allergy models, the existence of Killer B cells expressing Fas-L has also been identified as the central regulator of airway inflammation (Fig. 1) (75, 76). In humans, no data have been found so far to demonstrate the role of Fas-L killer B cells in the regulation of T-cell inflammation. However, recent studies shed light on the existence and the role of granzyme-B-producing B cells in the control of autoimmunity and antitumor responses. Production of granzyme-B by human B cells seems to be acquired through evolution (77). B cells preferentially differentiate into granzyme-B-producing B cells in response to IL-21 with a lack of CD40 activation (78). Microarray data obtained in healthy individuals demonstrate that B_R1 also expresses high levels of granzyme-B (10), suggesting a potential role in regulatory functions of B cells. However, for now, no functional data exist in humans permitting the confirmation of the role of these cells in allergic diseases. In addition, the lack of granzyme-B expression in mice B cells limits the investigation of the role of these cells in animal models of allergy and asthma.

Production of inhibitory antibodies: a specific feature of allergen tolerance

Production of inhibitory immunoglobulins by B cells is of special interest in allergy and remains an important mechanism for allergen tolerance. Indeed, what differentiates healthy individuals from those who have allergies is their

capacity to produce IgG, and not IgE, against allergens. Specific allergen immunotherapy (SIT) is an effective clinical approach to reorientating inappropriate immune responses in patients with allergy (79, 80). The emergence of IgG4 is a particular feature of induced allergen tolerance. This immunoglobulin subclass represents 5% of the total IgG pool and exhibits unique structural and functional characteristics (81). A specific amino acid in its Fc fragment leads to weak interaction with Fcy receptors (82). Another unique feature of IgG4 is its half-antibody exchange reaction. Indeed, IgG4 is a dynamic molecule that can easily exchange Fab arms by exchanging a heavy chain and attached light chain with a heavy–light chain pair from another molecule resulting in a bispecific antibody (81, 83). The resulting bispecific but functionally monovalent IgG4 proteins are unable to cross-link antigens limiting their ability to form immune complexes and mediate inflammatory responses (81, 83). Interestingly, many reports demonstrated that IgG4 responses could be promoted by SIT (82, 84–87). For example, the follow-up of patients undergoing sublingual administration of *Phleum pretense* for 2 years reveals a significant switch of the immune response, leading to the amelioration of the clinical state of patients with allergy (87). This is characterized by a modulation of humoral responses with a decrease in allergen-specific IgE and an increase in specific IgG4. In addition, SIT resulted in a decreased production of Th2 cytokines in blood, an increase in IFN-γ-producing cells, and an induction of regulatory T cells (87). Similarly, bee-venom immunotherapy induces the production of allergen-specific IgG4 able to inhibit IgE allergen interactions and favor an increase in regulatory T cells (88). IgE and IgG4 are both induced by IL-4 and IL13. However, in the presence of IL-10, preferential emergence of IgG4 occurs, suggesting a role for Tregs in the induction of IgG4 production. Most interestingly, it has been shown that IgG4 production is confined in B_R1 and that IL-10 dramatically enhances production of IgG4 after *in vitro* stimulation (10). As a result, SIT can lead to sequential induction of allergen tolerance where regulatory T and B_R1 cells are induced and favor the production of IgG4 through IL-10 secretion (Fig. 3). Recent interesting findings showed that the pro-inflammatory and anti-inflammatory functions of IgG are regulated by Fc N-linked glycosylation (89). Whereas desialylated IgG correlates with pro-inflammatory and disease activity in autoimmunity (90), sialylated IgG mediates the immunosuppressive effects observed in intravenous immunoglobulin treatment (89). Recently, the structure of IgG synthetized after a T-dependent stimulation, with and without costimulus signals, was analyzed in a mouse model of allergic asthma: Interestingly, under inflammatory conditions, IgG is predominantly desialylated because of the low expression of 2,6-sialyltransferase by plasma cells. In contrast, under tolerance induction, IgG was more sialylated, able to block antigen-specific T- and B-cell responses, dendritic cell maturation, and allergic airway inflammation

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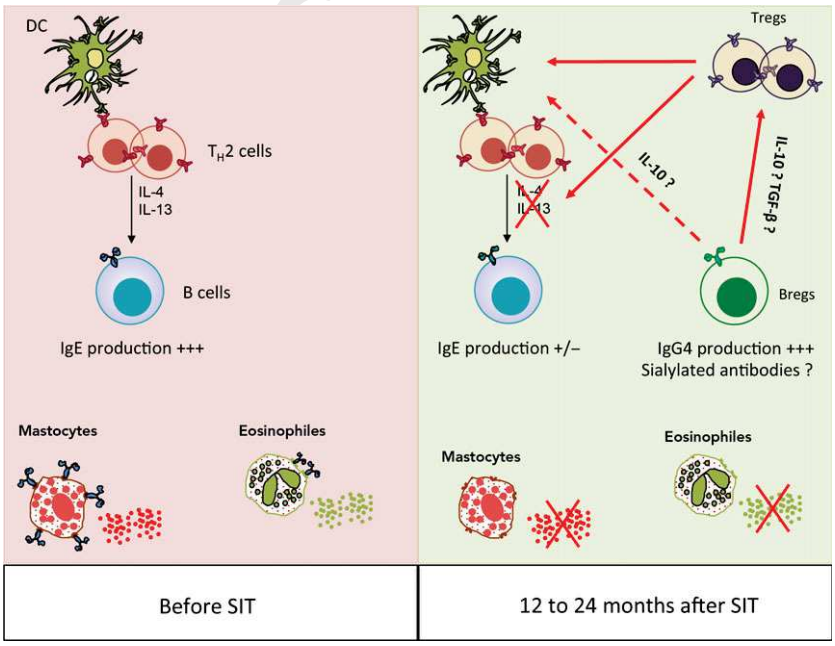

Figure 3 Specific immunotherapy favours the emergence of B cells with regulatory functions. Specific immunotherapy (SIT) induces an important decrease in granulocytes degranulation and a decreased tendency for systemic anaphylaxis. To maintain allergen tolerance allergen-specific Treg cells are then generated leading to suppression of TH2 responses. Also SIT results in the emergence of regulatory B cells producing IL-10 and IgG4. These cells may directly participate to inhibition of TH2 responses and induction of Tregs. Increase production of IgG4 lead to a substantial decrease in the allergen-specific IgE-to-IgG4 ratio. Allergen-specific IgG4 compete with IgE and dampen Fcε-receptor I mediated degranulation of mastocytes, basophils and eosinophils. Global diminution in inflammation may favour the production of sialylated antibodies with anti-inflammatory functions.

(91). Injection of sialylated antibodies in mice inhibits the development of airway inflammation. In addition, successful SIT, in the context of birch pollen allergy, is associated with decreased quantities of allergen-specific IgE, but an increase in allergen-specific IgG (91). Sialylation levels were comparable with antibodies used for intravenous immunoglobulin and IgG in healthy individuals, suggesting that SIT induces the sialylation of antigen-specific IgG and the emergence of inhibitory antibodies (91). Other experimental approaches are being developed to induce inhibitory antibodies. Indeed, a number of studies have focused on the possibility of promoting IgA to the detriment of IgE. It has been suggested that IgA can inhibit the development of allergies (92). Finally, the use of hypoallergenic peptides is of special interest as it leads to the production of inhibitory IgG (93). However, clinical confirmations are needed to validate these approaches.

Conclusion

B cells have been extensively described for their deleterious role in immunopathology. Notably, they are the source of IgE, responsible for many inflammatory effects observed in allergies. Although there are many arguments painting B cells as detrimental cells, they are also able to control and dampen allergic inflammation through varying mechanisms such as the production of immunosuppressive cytokines and inhibitory antibodies, notably IL-10 and IgG4 (10), respectively. B cells therefore represent an important target for the development of new treatments. However, many factors have to be taken into account, in particular concerning their identification. First of all, no transcription or surface marker allows the perfect identification of any regulatory B-cell subset. In addition, an overall understanding of their differentiation and induction has not yet emerged. In contrast to regulatory T cells, the signals necessary for the generation of different regulatory B-cell subsets are not well understood and seem extremely heterogeneous. Regulatory B cells should probably be considered as plastic antigen-presenting cells able to regulate immune responses under specific conditions rather than a natural suppressive subset, as regulatory T cells. One other important question to elucidate is how chronic inflammation in allergic settings alters the functions of Bregs. Although defects in terms of numbers and functions have been recently reported in humans, we cannot state that allergies arise from a specific defect in regulatory mechanisms mediated by B cells. One possibility will be to investigate in what extent chronic inflammation alters the tolerance balance and how Bregs are modified under these settings. Understanding these mechanisms will permit to identify new therapeutic targets to restore immunological tolerance balance.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Soyer OU, Akdis M, Ring J, Behrendt H, Crameri R, Lauener R et al. Mechanisms of peripheral tolerance to allergens. *Allergy* 2013;**68**:161–170.
2. Larché M. Regulatory T cells in allergy and asthma. *Chest* 2007;**132**:1007–1014.
3. Taylor A, Verhagen J, Akdis CA, Akdis M. T regulatory cells in allergy and health: a question of allergen specificity and balance. *Int Arch Allergy Immunol* 2004;**135**:73–82.
4. Mauri C, Bosma A. Immune regulatory function of B cells. *Annu Rev Immunol* 2012;**30**:221–241.
5. Dass S, Vital EM, Emery P. Development of psoriasis after B cell depletion with rituximab. *Arthritis Rheum* 2007;**56**:2715–2718.
6. Goetz M, Atreya R, Ghalibafian M, Galle PR, Neurath MF. Exacerbation of ulcerative colitis after rituximab salvage therapy. *Inflamm Bowel Dis* 2007;**13**:1365–1368.
7. Hartung HP, Kieseier BC. Atacicept: targeting B cells in multiple sclerosis. *Ther Adv Neurol Disord* 2010;**3**:205–216.
8. Lee SJ, Noh G, Lee JH. In vitro induction of allergen-specific interleukin-10-producing regulatory B cell responses by interferon-gamma in non-immunoglobulin E-mediated milk allergy. *Allergy Asthma Immunol Res* 2013;**5**:48–54.
9. Noh J, Noh G, Lee SJ, Lee JH, Kim A, Kim HS et al. Tolerogenic effects of interferon-gamma with induction of allergen-specific interleukin-10-producing regulatory B cell (Br 1) changes in non-IgE-mediated food allergy. *Cell Immunol* 2012;**273**:140–149.
10. van de Veen W, Stanic B, Yaman G, Wawrzyniak M, Sollner S, Akdis DG et al. IgG4 production is confined to human IL-10-producing regulatory B cells that suppress antigen-specific immune responses. *J Allergy Clin Immunol* 2013;**131**:1204–1212.
11. Katz SI, Parker D, Turk JL. B-cell suppression of delayed hypersensitivity reactions. *Nature* 1974;**251**:550–551.
12. Shimamura T, Habu S, Hashimoto K, Sasaki S. Feedback suppression of the immune response in vivo. III. Lyt-1+ B cells are suppressor-inducer cells. *Cell Immunol* 1984;**83**:221–224.
13. Matsushita T, Horikawa M, Iwata Y, Tedder TF. Regulatory B cells (B10 cells) and regulatory T cells have independent roles in controlling experimental autoimmune encephalomyelitis initiation and late-phase immunopathogenesis. *J Immunol* 2010;**185**:2240–2252.
14. Wolf SD, Dittel BN, Hardardottir F, Janeway CA Jr. Experimental autoimmune encephalomyelitis induction in genetically B cell-deficient mice. *J Exp Med* 1996;**184**:2271–2278.
15. Yanaba K, Bouaziz JD, Haas KM, Poe JC, Fujimoto M, Tedder TF. A regulatory B cell

subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses. *Immunity* 2008;**28**:639–650.
16. Yanaba K, Kamata M, Ishiura N, Shibata S, Asano Y, Tada Y et al. Regulatory B cells suppress imiquimod-induced, psoriasis-like skin inflammation. *J Leukoc Biol* 2013;**94**:563–573.
17. Fillatreau S, Sweenie CH, McGeachy MJ, Gray D, Anderton SM. B cells regulate autoimmunity by provision of IL-10. *Nat Immunol* 2002;**3**:944–950.
18. Mizoguchi A, Mizoguchi E, Takedatsu H, Blumberg RS, Bhan AK. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. *Immunity* 2002;**16**:219–230.
19. Carter NA, Vasconcellos R, Rosser EC, Tulone C, Munoz-Suano A, Kamanaka M et al. Mice lacking endogenous IL-10-producing regulatory B cells develop exacerbated disease and present with an increased frequency of Th1/Th17 but a decrease in regulatory T cells. *J Immunol* 2011;**186**:5569–5579.
20. Lampropoulou V, Calderon-Gomez E, Roch T, Neves P, Shen P, Stervbo U et al. Suppressive functions of activated B cells in autoimmune diseases reveal the dual roles of Toll-like receptors in immunity. *Immunol Rev* 2010;**233**:146–161.
21. Lampropoulou V, Hoehlig K, Roch T, Neves P, Calderon Gomez E, Sweenie CH et al. TLR-activated B cells suppress T cell-mediated autoimmunity. *J Immunol* 2008;**180**:4763–4773.
22. Dalwadi H, Wei B, Schrage M, Spicher K, Su TT, Birnbaumer L et al. B cell developmental requirement for the G alpha i2 gene. *J Immunol* 2003;**170**:1707–1715.
23. Blair PA, Chavez-Rueda KA, Evans JG, Shlomchik MJ, Eddaoudi A, Isenberg DA et al. Selective targeting of B cells with agonistic anti-CD40 is an efficacious strategy for the generation of induced regulatory T2-like B cells and for the suppression of lupus in MRL/lpr mice. *J Immunol* 2009;**182**:3492–3502.
24. Evans JG, Chavez-Rueda KA, Eddaoudi A, Meyer-Bahlburg A, Rawlings DJ, Ehrenstein MR et al. Novel suppressive function of transitional 2 B cells in experimental arthritis. *J Immunol* 2007;**178**:7868–7878.
25. Madan R, Demircik F, Surianarayanan S, Allen JL, Divanovic S, Trompette A et al. Nonredundant roles for B cell-derived IL-10 in immune counter-regulation. *J Immunol* 2009;**183**:2312–2320.
26. Maseda D, Smith SH, DiLillo DJ, Bryant JM, Candando KM, Weaver CT et al. Regulatory B10 cells differentiate into antibody-secreting cells after transient IL-10 production in vivo. *J Immunol* 2012;**188**:1036–1048.
27. Scapini P, Lamagna C, Hu Y, Lee K, Tang Q, DeFranco AL et al. B cell-derived IL-10 suppresses inflammatory disease in Lyn-deficient mice. *Proc Natl Acad Sci USA* 2011;**108**:E823–E832.
28. Neves P, Lampropoulou V, Calderon-Gomez E, Roch T, Stervbo U, Shen P et al. Signaling via the MyD88 adaptor protein in B cells suppresses protective immunity during Salmonella typhimurium infection. *Immunity* 2010;**33**:777–790.
29. Shen P, Roch T, Lampropoulou V, O'Connor RA, Stervbo U, Hilgenberg E et al. IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases. *Nature* 2014;**507**:366–370.
30. Nishimura S, Manabe I, Takaki S, Nagasaki M, Otsu M, Yamashita H et al. Adipose Natural Regulatory B Cells Negatively Control Adipose Tissue Inflammation. *Cell Metab* 2013;**???**:???–???.
31. Duddy M, Niino M, Adatia F, Hebert S, Freedman M, Atkins H et al. Distinct effector cytokine profiles of memory and naive human B cell subsets and implication in multiple sclerosis. *J Immunol* 2007;**178**:6092–6099.
32. Correale J, Farez M, Razzitte G. Helminth infections associated with multiple sclerosis induce regulatory B cells. *Ann Neurol* 2008;**64**:187–199.
33. Blair PA, Norena LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR et al. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. *Immunity* 2010;**32**:129–140.
34. Flores-Borja F, Bosma A, Ng D, Reddy V, Ehrenstein MR, Isenberg DA et al. CD19+ CD24hiCD38hi B cells maintain regulatory T cells while limiting TH1 and TH17 differentiation. *Sci Transl Med* 2013;**5**:173ra123.
35. Li X, Zhong H, Bao W, Boulad N, Evangelista J, Haider MA et al. Defective regulatory B-cell compartment in patients with immune thrombocytopenia. *Blood* 2012;**120**:3318–3325.
36. Bouaziz JD, Calbo S, Maho-Vaillant M, Saussine A, Bagot M, Bensussan A et al. IL-10 produced by activated human B cells regulates CD4(+) T-cell activation in vitro. *Eur J Immunol* 2010;**40**:2686–2691.
37. Iwata Y, Matsushita T, Horikawa M, Dilillo DJ, Yanaba K, Venturi GM et al. Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. *Blood* 2011;**117**:530–541.
38. Lemoine S, Morva A, Youinou P, Jamin C. Human T cells induce their own regulation through activation of B cells. *J Autoimmun* 2011;**36**:228–238.
39. DiLillo DJ, Weinberg JB, Yoshizaki A, Horikawa M, Bryant JM, Iwata Y et al. Chronic lymphocytic leukemia and regulatory B cells share IL-10 competence and immunosuppressive function. *Leukemia* 2013;**27**:170–182.
40. Gary-Gouy H, Harriague J, Bismuth G, Platzer C, Schmitt C, Dalloul AH. Human CD5 promotes B-cell survival through stimulation of autocrine IL-10 production. *Blood* 2002;**100**:4537–4543.
41. Cherukuri A, Rothstein DM, Clark B, Carter CR, Davison A, Hernandez-Fuentes M et al. Immunologic Human Renal Allograft Injury Associates with an Altered IL-10/TNF-alpha Expression Ratio in Regulatory B Cells. *J Am Soc Nephrol* 2014;**???**:???–???.
42. Watanabe R, Ishiura N, Nakashima H, Kuwano Y, Okochi H, Tamaki K et al. Regulatory B cells (B10 cells) have a suppressive role in murine lupus: CD19 and B10 cell deficiency exacerbates systemic autoimmunity. *J Immunol* 2010;**184**:4801–4809.
43. Jin G, Hamaguchi Y, Matsushita T, Hasegawa M, Le Huu D, Ishiura N et al. B-cell linker protein expression contributes to controlling allergic and autoimmune diseases by mediating IL-10 production in regulatory B cells. *J Allergy Clin Immunol* 2013;**131**:1674–1682.
44. Miyazaki D, Kuo CH, Tominaga T, Inoue Y, Ono SJ. Regulatory function of CpG-activated B cells in late-phase experimental allergic conjunctivitis. *Invest Ophthalmol Vis Sci* 2009;**50**:1626–1635.
45. Amu S, Saunders SP, Kronenberg M, Mangan NE, Atzberger A, Fallon PG. Regulatory B cells prevent and reverse allergic airway inflammation via FoxP3-positive T regulatory cells in a murine model. *J Allergy Clin Immunol* 2010;**125**:1114–1124.
46. Mangan NE, Fallon RE, Smith P, van Rooijen N, McKenzie AN, Fallon PG. Helminth infection protects mice from anaphylaxis via IL-10-producing B cells. *J Immunol* 2004;**173**:6346–6356.
47. Mangan NE, van Rooijen N, McKenzie AN, Fallon PG. Helminth-modified pulmonary immune response protects mice from allergen-induced airway hyperresponsiveness. *J Immunol* 2006;**176**:138–147.
48. van der Vlugt LE, Labuda LA, Ozir-Fazalalikhan A, Lievers E, Gloudemans AK, Liu KY et al. Schistosomes induce regulatory features in human and mouse CD1d(hi) B cells: inhibition of allergic inflammation by IL-10 and regulatory T cells. *PLoS ONE* 2012;**7**:e30883.
49. Noh J, Lee JH, Noh G, Bang SY, Kim HS, Choi WS et al. Characterisation of allergen-specific responses of IL-10-producing regula-

tory B cells (Br 1) in Cow Milk Allergy. *Cell Immunol* 2010;**264**:143–149.
50. Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiannidis C, Crameri R et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med* 2004;**199**:1567–1575.
51. van der Vlugt LE, Mlejnek E, Ozir-Fazalalikhan A, Janssen Bonas M, Dijksman TR, Labuda LA et al. CD24(hi)CD27(+) B cells from patients with allergic asthma have impaired regulatory activity in response to lipopolysaccharide. *Clin Exp Allergy* 2014;**44**:517–528.
52. Matsumoto M, Fujii Y, Baba A, Hikida M, Kurosaki T, Baba Y. The calcium sensors STIM1 and STIM2 control B cell regulatory function through interleukin-10 production. *Immunity* 2011;**34**:703–714.
53. Mann M, Maresz K, Shriver L, Tan Y, Dittel B. B cell regulation of CD4+ CD25+ T regulatory cells and IL-10 via B7 is essential for recovery from experimental autoimmune encephalomyelitis. *J Immunol* 2007;**178**:3447–3456.
54. Mauri C, Gray D, Mushtaq N, Londei M. Prevention of arthritis by interleukin 10-producing B cells. *J Exp Med* 2003;**197**:489–501.
55. Yoshizaki A, Miyagaki T, DiLillo DJ, Matsushita T, Horikawa M, Kountikov EI et al. Regulatory B cells control T-cell autoimmunity through IL-21-dependent cognate interactions. *Nature* 2012;**491**:264–268.
56. Ding Q, Yeung M, Camirand G, Zeng Q, Akiba H, Yagita H et al. Regulatory B cells are identified by expression of TIM-1 and can be induced through TIM-1 ligation to promote tolerance in mice. *J Clin Invest* 2011;**121**:3645–3656.
57. Lee KM, Kim JI, Stott R, Soohoo J, O'Connor MR, Yeh H et al. Anti-CD45RB/anti-TIM-1-induced tolerance requires regulatory B cells. *Am J Transplant* 2012;**12**:2072–2078.
58. McIntire JJ, Umetsu SE, Akbari O, Potter M, Kuchroo VK, Barsh GS et al. Identification of Tapr (an airway hyperreactivity regulatory locus) and the linked Tim gene family. *Nat Immunol* 2001;**2**:1109–1116.
59. Xiao S, Brooks CR, Zhu C, Wu C, Sweere JM, Petecka S et al. Defect in regulatory B-cell function and development of systemic autoimmunity in T-cell Ig mucin 1 (Tim-1) mucin domain-mutant mice. *Proc Natl Acad Sci USA* 2012;**109**:12105–12110.
60. Yanaba K, Bouaziz JD, Matsushita T, Tsubata T, Tedder TF. The development and function of regulatory B cells expressing IL-10 (B10 cells) requires antigen receptor diversity and TLR signals. *J Immunol* 2009;**182**:7459–7472.
61. Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med* 2002;**347**:869–877.
62. Yazdanbakhsh M, Kremsner PG, van Ree R. Allergy, parasites, and the hygiene hypothesis. *Science* 2002;**296**:490–494.
63. Fallon PG, Mangan NE. Suppression of TH2-type allergic reactions by helminth infection. *Nat Rev Immunol* 2007;**7**:220–230.
64. Lauener RP, Birchler T, Adamski J, Braun-Fahrländer C, Bufe A, Herz U et al. Expression of CD14 and Toll-like receptor 2 in farmers' and nonfarmers' children. *Lancet* 2002;**360**:465–466.
65. Ege MJ, Bieli C, Frei R, van Strien RT, Riedler J, Ublagger E et al. Prenatal farm exposure is related to the expression of receptors of the innate immunity and to atopic sensitization in school-age children. *J Allergy Clin Immunol* 2006;**117**:817–823.
66. von Mutius E, Vercelli D. Farm living: effects on childhood asthma and allergy. *Nat Rev Immunol* 2010;**10**:861–868.
67. Kollmann TR, Levy O, Montgomery RR, Goriely S. Innate immune function by Toll-like receptors: distinct responses in newborns and the elderly. *Immunity* 2012;**37**:771–783.
68. Belderbos ME, Knol EF, Houben ML, van Bleek GM, Wilbrink B, Kimpen JL et al. Low neonatal Toll-like receptor 4-mediated interleukin-10 production is associated with subsequent atopic dermatitis. *Clin Exp Allergy* 2012;**42**:66–75.
69. Lluis A, Depner M, Gaugler B, Saas P, Casaca VI, Raedler D et al. Increased regulatory T-cell numbers are associated with farm milk exposure and lower atopic sensitization and asthma in childhood. *J Allergy Clin Immunol* 2014;**133**:551–559.
70. Frischmeyer-Guerrerio PA, Guerrerio AL, Oswald G, Chichester K, Myers L, Halushka MK et al. TGFbeta receptor mutations impose a strong predisposition for human allergic disease. *Sci Transl Med* 2013;**5**:195ra194.
71. Natarajan P, Singh A, McNamara JT, Secor ER Jr, Guernsey LA, Thrall RS et al. Regulatory B cells from hilar lymph nodes of tolerant mice in a murine model of allergic airway disease are CD5+, express TGF-beta, and co-localize with CD4+ Foxp3+ T cells. *Mucosal Immunol* 2012;**5**:691–701.
72. Liu ZQ, Wu Y, Song JP, Liu X, Liu Z, Zheng PY et al. Tolerogenic CX3CR1+ B cells suppress food allergy-induced intestinal inflammation in mice. *Allergy* 2013;**68**:1241–1248.
73. Zhang HP, Wu Y, Liu J, Jiang J, Geng XR, Yang G et al. TSP1-producing B cells show immune regulatory property and suppress allergy-related mucosal inflammation. *Sci Rep* 2013;**3**:3345.
74. Montandon R, Korniotis S, Layseca-Espinosa E, Gras C, Megret J, Ezine S et al. Innate pro-B-cell progenitors protect against type 1 diabetes by regulating autoimmune effector T cells. *Proc Natl Acad Sci USA* 2013;**110**:E2199–E2208.
75. Lundy SK, Boros DL. Fas ligand-expressing B-1a lymphocytes mediate CD4(+)-T-cell apoptosis during schistosomal infection: induction by interleukin 4 (IL-4) and IL-10. *Infect Immun* 2002;**70**:812–819.
76. Klinker MW, Reed TJ, Fox DA, Lundy SK. Interleukin-5 supports the expansion of fas ligand-expressing killer B cells that induce antigen-specific apoptosis of CD4(+) T cells and secrete interleukin-10. *PLoS ONE* 2013;**8**:e70131.
77. Hagn M, Belz GT, Kallies A, Sutton VR, Thia KY, Tarlinton DM et al. Activated mouse B cells lack expression of granzyme B. *J Immunol* 2012;**188**:3886–3892.
78. Hagn M, Sontheimer K, Dahlke K, Brueggemann S, Kaltenmeier C, Beyer T et al. Human B cells differentiate into granzyme B-secreting cytotoxic B lymphocytes upon incomplete T-cell help. *Immunol Cell Biol* 2012;**90**:457–467.
79. Pipet A, Botturi K, Pinot D, Vervloet D, Magnan A. Allergen-specific immunotherapy in allergic rhinitis and asthma. Mechanisms and proof of efficacy. *Respir Med* 2009;**103**:800–812.
80. Akdis M, Akdis CA. Mechanisms of allergen-specific immunotherapy: multiple suppressor factors at work in immune tolerance to allergens. *J Allergy Clin Immunol* 2014;**133**:621–631.
81. Aalberse RC, Stapel SO, Schuurman J, Rispens T. Immunoglobulin G4: an odd antibody. *Clin Exp Allergy* 2009;**39**:469–477.
82. Canfield SM, Morrison SL. The binding affinity of human IgG for its high affinity Fc receptor is determined by multiple amino acids in the CH2 domain and is modulated by the hinge region. *J Exp Med* 1991;**???**:???–???.
83. van der Neut Kolfschoten M, Schuurman J, Losen M, Bleeker WK, Martinez-Martinez P, Vermeulen E et al. Anti-inflammatory activity of human IgG4 antibodies by dynamic Fab arm exchange. *Science* 2007;**317**:1554–1557.
84. Bohle B, Kinaciyan T, Gerstmayr M, Radakovics A, Jahn-Schmid B, Ebner C. Sublingual immunotherapy induces IL-10-producing T regulatory cells, allergen-specific T-cell tolerance, and immune deviation. *J Allergy Clin Immunol* 2007;**120**:707–713.
85. Piconi S, Trabattoni D, Rainone V, Borgonovo L, Passerini S, Rizzardini G et al. Immunological effects of sublingual immu-

notherapy: clinical efficacy is associated with modulation of programmed cell death ligand 1, IL-10, and IgG4. *J Immunol* 2010;**185**:7723–7730.

86. Vickery BP, Lin J, Kulis M, Fu Z, Steele PH, Jones SM et al. Peanut oral immunotherapy modifies IgE and IgG4 responses to major peanut allergens. *J Allergy Clin Immunol* 2013;**131**:128–134.
87. Suarez-Fueyo A, Ramos T, Galan A, Jimeno L, Wurtzen PA, Marin A et al. Grass tablet sublingual immunotherapy downregulates the TH2 cytokine response followed by regulatory T-cell generation. *J Allergy Clin Immunol* 2014;**133**:130–138.
88. Meiler F, Zumkehr J, Klunker S, Ruckert B, Akdis CA, Akdis M. In vivo switch to IL-10-secreting T regulatory cells in high dose allergen exposure. *J Exp Med* 2008;**205**:2887–2898.
89. Kaneko Y, Nimmerjahn F, Ravetch JV. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science* 2006;**313**:670–673.
90. Parekh RB, Dwek RA, Sutton BJ, Fernandes DL, Leung A, Stanworth D et al. Association of rheumatoid arthritis and primary osteoarthritis with changes in the glycosylation pattern of total serum IgG. *Nature* 1985;**316**:452–457.
91. Oefner CM, Winkler A, Hess C, Lorenz AK, Holecska V, Huxdorf M et al. Tolerance induction with T cell-dependent protein antigens induces regulatory sialylated IgGs. *J Allergy Clin Immunol* 2012;**129**:1647–1655.
92. Gloudemans AK, Lambrecht BN, Smits HH. Potential of immunoglobulin A to prevent allergic asthma. *Clin Dev Immunol* 2013;**2013**:542091.
93. Chen KW, Focke-Tejkl M, Blatt K, Kneidinger M, Gieras A, Dall'Antonia F et al. Carrier-bound nonallergenic Der p 2 peptides induce IgG antibodies blocking allergen-induced basophil activation in allergic patients. *Allergy* 2012;**67**:609–621.

A regulatory CD9$^+$ B cell subset controls HDM-induced allergic airway inflammation

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Abstract

In allergic diseases, evidence that an increase in regulatory B cells is necessary for allergen tolerance suggests that development of allergic asthma could be associated with a defect in the regulatory B-cell compartment. In this study we showed that induction of allergic asthma alters the homeostasis of IL-10$^+$ regulatory B cells and favors the production of inflammatory cytokines by B cells. A deepen transcriptomic and phenotypic characterization of regulatory B cells revealed that they were enriched in a CD9$^+$ B cell subset. The adoptive transfer of CD9$^+$ B cells normalizes airway inflammation and lung function in mice by inhibiting T_H2 and T_H17 driven inflammation in an IL-10 dependent manner restoring a favorable immunological balance in lung tissues. This finding demonstrates the central role of IL-10 producing B cells in the control of lung inflammation and airway hyperresponsiveness and strengthens the possibility to develop orientated regulatory B cell therapies in allergic asthma.

A regulatory CD9^{+} B cell subset controls HDM-induced allergic airway inflammation

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Key words: Regulatory B cells, IL-10, allergic asthma, airway inflammation

Abbreviations used:

BAL: Bronchoalveolar lavage

Bregs: Regulatory B cells

HDM: House dust mite

LPS: Lipopolysaccharide

MZ: Marginal zone

PMA: Phorbol 12-myristate 13-acetate

PIM: PMA + ionomycin + monensin

Abstract (163/200)

In allergic diseases, evidence that an increase in regulatory B cells (Bregs) is necessary for allergen tolerance suggests that the development of allergic asthma could be associated with a defect in the Breg compartment. In this study, we showed that the induction of allergic asthma alters the homeostasis of IL-10^{+} Bregs and favors the production of inflammatory cytokines by B cells. Deeper transcriptomic and phenotypic analysis of Bregs revealed that they were enriched in a CD9^{+} B cell subset. This marker was sufficient to identify Bregs in mice as well as humans, where this molecule was expressed in CD24^{hi} CD38^{hi} Bregs. In mice, the adoptive transfer of CD9^{+} B cells normalized airway inflammation and lung function by inhibiting T_H2- and T_H17-driven inflammation in an IL-10-dependent manner, restoring a favorable immunological balance in lung tissues. This finding demonstrates the central role of IL-10-producing B cells in the control of lung inflammation and airway hyperresponsiveness and strengthens the potential for Breg-targeted therapies in allergic asthma.

Introduction

Allergic asthma is a chronic inflammatory disease characterized by airway hyperresponsiveness and deregulated inflammation in response to allergens. This pathology is controlled by $CD4^+$ T helper (T_H) lymphocytes, which cause cellular infiltration in the lungs, overproduction of mucus and airway constriction. In general, B cells have been described in asthma as deleterious cells secreting allergen-specific IgE under pro-T_H2 inflammatory conditions. IgE interacts with Fcε receptor I at the surface of basophils and mast cells, promoting the release of pro-inflammatory mediators such as chemokines, prostaglandins and leukotrienes, which leads to airway inflammation and airway narrowing (1).

Beyond their antibody secretion capacities, B cells can also present antigens, produce cytokines and regulate T cell-mediated immune responses (2). Indeed, B cells can secrete both pro-inflammatory and inhibitory cytokines, the balance of which influences the immune response (2, 3). Recently, a subpopulation of regulatory B cells (Bregs) that produces large amounts of IL-10 was identified in both mice and humans (4, 5). These cells are able to suppress inflammation by constraining T_H1 and T_H17 responses and inducing regulatory T cells (Tregs) (4, 5). Accordingly, functional impairment of the IL-10-producing B cell subset is associated with exacerbated persistent autoimmunity and dermal allergic inflammation (6-10). In allergic asthma models, infection with parasites protects mice from the development of lung inflammation and airway hyperresponsiveness through the generation of IL-10-producing B cells (11, 12). Notably, elevated levels of allergen-specific, IL-10-producing Bregs have been found

after immunotherapies for food and bee venom allergies, suggesting that increased proportions of Bregs are a specific feature of induced tolerance toward allergens (13, 14). However, to what extent the development of allergic asthma influences the homeostasis of Bregs has not yet been clearly addressed. To investigate this, we took advantage of an acute model of house dust mite (HDM)-induced allergic asthma.

Herein, we show that B cells from allergic mice preferentially secrete a pro-inflammatory cytokine profile that counters the effects of the anti-inflammatory cytokine IL-10. Consistently, the frequency of IL-10$^+$ Bregs was decreased in the spleen and lungs of asthmatic mice. Microarray and cytometry analysis further demonstrated that Bregs were enriched in a CD9$^+$ B cell subset that was decreased in asthmatic mice, and adoptive transfer of these CD9$^+$ B cells abrogated asthma in an IL-10-dependent manner.

88 **Materials and Methods (1,572 words)**

89 ***Mice*** – Six- to eight-week-old wild-type and IL-10$^{-/-}$ BALB/c mice were purchased from
90 Charles River Laboratories (Ecully, Fr). Allergic asthma was induced in the mice using a
91 total HDM extract *(Dermatophagoïdes farinae)* provided by Stallergenes. The mice were
92 sensitized on days 0, 7, 14 and 21 by cutaneous application of 500 µg of HDM extract in
93 20 µL of dimethylsulfoxide (DMSO, Sigma-Aldrich) on the ears and challenged
94 intranasally with 250 µg of HDM in 40 µL of sterile PBS on days 27 and 34. Analyses
95 were performed 1 day after the last HDM challenge. Lung function was analyzed by
96 unrestrained, single-chamber barometric plethysmography (EMKA) to determine the
97 enhanced pause (Penh) in response to progressive methacholine concentrations (0, 5,
98 10, 20, 40 mg/mL). The Regional Ethical Committee for Animal Experiments of the Pays
99 de la Loire approved all animal protocols.
100
101 ***Der f1-Specific IgE*** – To measure HDM-specific IgE in the bronchoalveolar lavage
102 (BAL) fluid and serum, wells were coated with purified natural Der f1 protein (Indoor
103 Biotechnologies) overnight. After saturation of the wells with 1% BSA for 12 h, BAL or
104 diluted serum was added overnight. The plates were washed, and horseradish
105 peroxidase (HRP)-conjugated anti-mouse IgE (MCA 419P; 1:2,000; AbD serotec^{TM}) was
106 added for 5 h, followed by incubation with TMB substrate at room temperature for 20
107 min. ELISA stop solution was added, and the absorbance was measured at 405 nm with
108 Victor TMX3 (PerkinElmer, France). All assays were conducted using duplicate
109 samples.

Flow cytometry – BAL cells were stained for multiparameter flow cytometric analysis using an LSR II cytometer (BD Bioscience). Ly6G-PercP.Cy5.5 (1A8), CD8-APC-H7 (53-6.7) (BD Biosciences), CD3-APC (145.2C11), CD19-PeCy7 (1D3), F4/80-FITC (BM8) (eBioscience), CCR3-PE (83101, R&D systems), and DAPI were used to identify BAL-infiltrating cells, as previously described (Amu et al., 2010). Briefly, stained cells were acquired on a BD LSR™ II (BD Biosciences) and analyzed using BD FACSDiva™ software (BD Biosciences). Dead cells were excluded using DAPI. After gating on live cells, polymorphonuclear neutrophils ($Ly6G^{hi}$ F4/80- cells), macrophages (large, Ly6g-, autofluorescent, $F4/80^{+}$ mononuclear cells), and eosinophils (F4/80-, Ly6G-, CCR3+ cells) were identified. Lymphocytes were identified as follows: forward scatter $(FSC)^{lo}$, side scatter $(SSC)^{lo}$, F4/80-, Ly6G- and CCR3-. T ($CD19^{-}$ $CD3^{+}$) and B ($CD19^{+}$ $CD3^{-}$) cells were also identified.

B cell immunophenotyping in the spleen and lungs was performed by multicolor staining with the following antibodies: CD19-PeCy7 (1D3), IgM-APC or PE (II/41), IgD-eFluor®450 or APC (11-26c), GL7-A488 (GL-7), CD9-FITC (KMC8), CD70-PerCP-eFluor®710 (FR70), Ctla4-APC (UC10-4B9), CD73-efluor®450 (TY/11.8), CD5-APC (53-7.3), CD1d A488 (1B1), CD23-FITC (B3B4), CD21-efluor®450 (4E3) (all from eBioscience), CD95-Bv421® (Jo2), and PD-1-Bv421® (J43) (BD Biosciences). To identify Tregs, cells were stained with CD3-APC (145-2C11), CD4-FITC (OKT4), CD25-PE (PC61), Foxp3-PECy7 (FJK-16s). Intranuclear staining of Foxp3 was performed as recommended by the manufacturer (eBioscience). In all staining experiments, Fc receptors (FcR) were blocked with anti-mouse FcR monoclonal antibody (mAb) (93;

eBioscience, USA).

To investigate the T helper responses primed in the lung mucosa, 8 x 10^5 lung cells were transferred to a 96-well round-bottom plate and then stimulated for 5 h with a mixture containing phorbol 12-myristate 13-acetate (PMA) (50 ng/mL) and ionomycin (500 ng/mL; Sigma-Aldrich) together with either monensin (PMA + ionomycin + monensin; PIM) for T_H17 analysis (2 mg/mL; BD Biosciences) or brefeldin A for T_H2 assessment (1 mg/mL; BD Biosciences). For cytokine detection, the FcRs were blocked with mouse CD16/CD32 mAbs (eBioscience, USA). Prior to surface-specific staining, the cells were stained with Fixable Viability Dye 450 (BD Biosciences) to exclude dead cells. The following antibodies were used for surface staining: CD3 PeCy7 (145-2C11) and CD8 APC-H7 (53-6.7) (BD Biosciences). The cells were then fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences) and stained intracellularly with anti-IL-4 (11B11; eBioscience), -IL-13 (eBio13A; eBioscience), and -IL-17A antibodies (TC11-18H10; BD Biosciences).

Peripheral blood samples were obtained from the Etablissement Français du Sang Pays de la Loire (Nantes, France) upon informed consent and approval by the Institutional Review Board. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient sedimentation using LSM 1077 lymphocyte separation medium (PAA Laboratories). Staining of human B cells was performed in the PBMC population with the following antibodies: CD19-Bv510® (SJ25C1), CD27-APC (M-T271), CD24-FITC (ML5), CD38 APC-H7 (HB7), and CD9 PercPCy5.5 (M-L13) (all from BD Biosciences).

Immunohistochemistry – Lungs were fixed in 4% paraformaldehyde overnight before

paraffin embedding. Five-micrometer thick, formalin-fixed, paraffin-embedded lung sections were stained with hematoxylin-eosin (H&E) or periodic acid schiff (PAS) or probed with a primary goat anti-B220 (RA3-6B2) antibody (BD Biosciences). The primary antibody was detected with a biotinylated donkey anti-goat antibody (A11057) and revealed with streptavidin conjugated to HRP.

RNA isolation and real-time quantitative PCR (RT-PCR) – B cells from the lungs were sorted using fluorescence-activated cell sorting (FACS) and then disrupted and homogenized in TRIzol® reagent (Invitrogen). Then, RNA was extracted by phenol-chloroform separation. The total quantity of RNA was determined using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). cDNA was synthesized from 2 µg of RNA using the MMLV Transcriptase according to the manufacturer's instructions. RT-PCR was performed on a ViiA7 Fast Real-Time PCR System using commercially available primers (Applied Biosystems). A pool of cDNA from naive mice and the housekeeping gene HPRT were used to normalize RNA expression with the $2^{(-\Delta\Delta ct)}$ technique.

Analysis of IL-10 production – Splenocytes and lung cells were stimulated with lipopolysaccharide (LPS) (10 µg/mL, Sigma), PMA (50 ng/ml; Sigma), ionomycin (500 ng/ml; Sigma) and monensin (eBioscience; 2 mM) for 5 h (LPS+PIM). In some experiments, splenic B cell subsets were purified using the Pan B Cell Isolation Kit (Miltenyi Biotech) or the FACS ARIA III (BD Biosciences) and then incubated for two

days with a CD40 agonist (BD Biosciences, 2 µg/mL), Tim-1 (BioXcell, 10 µg/mL), IL-21 (eBiosciences, 100 ng/mL) or BAFF (R&D system, 20 ng/mL) and activated with LPS + PIM for the last 5 h. For IL-10 detection, the FcRs were blocked with mouse FcR mAb (93; eBiosciences). Dead cells were excluded using a yellow LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen-Molecular Probes) before cell surface staining. The stained cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's recommendations and then stained with an anti IL-10-PE (JES5-16E3, Biolegend) for 45 min in the dark at 4°C. Unstimulated cells stained for IL-10 were used as negative controls. Secreted IL-10 was quantified using Flowcytomix® (eBioscience) or ELISA according to the manufacturer's instructions. In some experiments, purified splenic B cells and sorted $CD9^+$ or $CD9^-$ B cells were activated and cultured in 96-well round-bottom tissue culture plates (4 x 10^5 cells per well), and then the IL-10 levels in the supernatants were quantified using the IL-10 Quantikine ELISA kit (R&D systems) according to the manufacturer's protocol. All assays were conducted using duplicate samples.

Whole-mouse genome microarray analysis – Purified splenic B cells were activated using a CD40 agonist for 48 h, and then LPS + PIM was added during the final 5 h. IL-10-secreting splenic B cells were identified using an IL-10 secretion detection kit (Miltenyi Biotec) with subsequent staining for CD19 expression before cell sorting. Five thousand IL-10^+ $CD19^+$ cells and 5,000 IL-10^- $CD19^+$ cells were purified using a FACSAria III cell sorter (BD biosciences) with "single-cell purity" and were lysed in

198 SuperAmp Lysis Buffer (Miltenyi Biotec) according to the manufacturer's instructions
199 and stored at -80°C. Gene expression analysis using the Agilent platform (Agilent
200 Technologies, Palo Alto, USA) was performed at the Miltenyi Biotec gene array facility
201 (Bergisch Gladbach, Germany). RNA samples were prepared and hybridized on an
202 Agilent Whole Mouse Genome Oligo Microarray 8x60K (Agilent Technologies). Data
203 extraction of the fluorescence signals without background subtraction was performed
204 using Feature Extraction software v10.7.1.1 (Agilent Technologies). Raw microarray
205 data were deposited in the Gene Expression Omnibus (GEO) database (accession
206 number: GSE57772). Preprocessing of the microarray data included normalization using
207 the Lowess algorithm and incorporation of the Rosetta error model (Rosetta
208 Inpharmatics LLC, Seattle, USA). Expression differences for individual reporters
209 between the test and control groups were identified using the single-group t-test
210 followed by the multiple testing correction (Benjamini & Hochberg) for the log10 ratio
211 data and effect size (fold change). The most differentially expressed genes between the
212 control and HDM-treated mice (p-value ≤ 0.01) are listed in Table 1 in the supplemental
213 data.
214
215 ***Cytokine quantification*** – BAL cytokines and chemokines were quantified using
216 Flowcytomix® technology (eBiosciences) with the mouse T_H1/T_H2 10 plex kit according
217 to the manufacturer's instructions. All assays were conducted using duplicate samples.
218 Data analysis was performed using the Flowcytomix Pro Software version 3.0.
219 Quantification of CXCL13 in the BAL of mice was performed using the mouse CXCL13

Quantitkine ELISA kit according to the manufacturer's instructions (R&D systems).

Proliferation assays – To investigate the suppressive function on B cells, splenic B cells were purified by negative selection (B cell isolation Kit II, Miltenyi) and stimulated with agonistic CD40 mAb for 48 h and LPS for 5 h. $CD4^+CD25^-$ T cells were FACS-sorted and labeled with CellTrace blue dye according to the manufacturer's instructions (Invitrogen). Dye-labeled $CD4^+CD25^-$ T cells (5 x 10^5/mL) were cultured alone or with CD40/LPS-stimulated B cells (5 x 10^5/mL). In some conditions, IL-10 signaling was neutralized with 10 µg/mL of purified anti-IL-10 antibody (Clone, fournisseur). Proliferation of T cells was analyzed with FlowJo software using the proliferation index module.

Adoptive transfer – Spleen cells from asthmatic WT or IL-$10^{-/-}$ mice were stained for CD19 and CD9. Fluorescence minus one was used as a negative control for CD9 expression. $CD9^+$ and $CD9^-$ B cells were sorted using a FACSAria III (BD Bioscience). HDM-treated mice received 5 x 10^5 sorted cells intraveneously (i.v.) one day before the second challenge.

Statistical analysis – Comparisons of experimental values between two groups were analyzed using the Mann Whitney test. The non-parametric Kruskal–Wallis test with Dunn's post-test was used for comparisons between more than two groups. Enhanced pause (Penh) and lung resistance results were analyzed using a two-way analysis of

variance (ANOVA). All statistic analyses were performed in GraphPad Prism v6.

Results

Exposure to allergen elicits attraction of B cells to the airways – Exposure to total HDM extract (Figure 1A) induced airway hyperresponsiveness (Figure 1B) associated with severe inflammation, as characterized by the presence of mucus-secreting cells, eosino-neutrophilic influx, production of systemic Der f1-specific IgE and T_H2-T_H17 cytokine secretion (Figure 1, C-E). Notably, we found an increased expression of the B cell chemoattractant CXCL13 in the lungs and a higher level of CXCL13 in the BAL of asthmatic mice 24 h after the last challenge (Figure 2A). The expression of this pro-B cell chemokine was associated with a significant increase in B cells in the BAL of HDM-treated mice (Figure 2, B and C). We also detected significant perivascular and peribronchial B cell infiltrate in the lung tissues from mice exposed to the allergen (Figure 2D). The formation of these B cell follicles is of importance and suggests a potent local B cell response toward the allergen. As expected, our cytometry analysis confirmed the increased frequency and absolute values of B cells in the lung mucosa after allergen inhalation (Figure 2, E and F). Notably, germinal center B cells ($CD19^+$ Fas^+ $PD1^+$) and total switched memory B cells ($CD19^+$ IgM^- IgD^-) were significantly increased in the lungs of HDM mice (Figure 2, G-J), which was associated with the production of Der f1-specific IgE in BAL fluid, thus confirming the local B cell response toward the allergen (Figure 2, K). Collectively, these data showed that mature and activated B cells accumulate in the airways of mice after exposure to HDM.

Asthma influences the cytokine profile of B cells – Various cytokine-producing B

cells capable of regulating the immune response have been recently described (2). To more precisely define the cytokine profile of B cells in asthma, we assessed the mRNA levels of a panel of cytokines, including IFN-y, IL-2, IL-4, IL-6, IL-10 and TGF-β, in purified *ex-vivo* spleen (Figure 3A) and lung (Figure 3B) B cells obtained from control and HDM-treated mice. We detected a significant increase in IL-4 and IL-6 mRNA levels in B cells from both the spleen and lungs of asthmatic mice (Figure 3, A and B), whereas the expression of IL-10 was significantly decreased in the lung B cells from HDM mice (Figure 3B). This suggests that in asthma, B cells preferentially express inflammatory cytokines, which counteract the effects of suppressive molecules.

Asthmatic mice display altered IL-10$^+$ Breg homeostasis – The decreased expression of IL-10 in the B cells of asthmatic mice prompted us to examine whether allergic asthma affects the homeostasis of Bregs. Spleen and lung cells were isolated and stimulated for 5 h with LPS and PIM, followed by staining for cytoplasmic IL-10 expression (15). IL-10-secreting B cells represented 1.6% (+/-0.22) and 3.14% (+/-0.4) of total B cells in the spleen and lung of control mice, respectively, whereas only 0.97% (+/-0.05, $p<0.01$) and 2.1% (+/-0.3, $p<0.01$) were observed in the spleen and lung of asthmatic mice (Figure 3, C-F). We confirmed these observations in the lungs, where stimulated B cells from asthmatic mice produced lower levels of IL-10 than control mice (Figure 3G). Similarly, we found increased levels of IL-10-producing B cells among the splenocytes of control mice after 4 days of *in vitro* activation with total HDM extract (Figure 3H). Altogether, these results indicate altered homeostasis of IL-10-secreting B

cells in the context of allergic asthma.

B cells from asthmatic mice have altered regulatory capacities – We next evaluated whether the regulatory capacities of IL-10$^+$ B cells were modified in asthmatic mice. To test this, CD19$^+$ B cells were isolated from the spleens of HDM and control mice, pre-activated to induce IL-10 and co-cultured in the presence of activated CD4$^+$ CD25$^-$ effector T cells. In contrast to other recent data (16-18), pre-stimulation with a CD40 agonist was found to optimally induce IL-10 production in purified splenic B cells from naïve Balb/c mice (Figure 4, A-C). Stimulation of the CD40 pathway for two days induced a similar quantity of IL-10 and equivalent levels of IL-10$^+$ B cells compared to splenic B cells obtained from HDM-treated and control mice (Figure 4, D and E). In a proliferation assay, CD40-activated B cells from control mice dampened the proliferation of T cells in an IL-10-dependent manner (Figure 4, F and G). Notably, this reduced T cell proliferation was significantly weaker with activated B cells from asthmatic mice (Figure 4, F and G), which may have been the result of a functional defect of Bregs in the context of asthma or the capacity of activated B cells from HDM mice to secrete significant levels of pro-inflammatory cytokines upon activation, thus counterbalancing the suppressive effect of IL-10 (19, 20). To examine the latter possibility, purified splenic B cells from control and HDM mice were activated as described above, and then cytokine secretion was quantified. CD40-activated B cells from asthmatic mice secreted significantly higher levels of pro-inflammatory cytokines, such as IL-2, IL-4, IL-6 and TNF-α, which can counterbalance the effect of IL-10 and thus sustain T cell activation

and proliferation (19, 20) (Figure 4H). Accordingly, the ratios for IL-10/TNF-α and IL-10/IL-6 were significantly lower in HDM mice, demonstrating an altered balance in cytokine secretion by B cells during asthma (Figure 4I).

To summarize, we confirm here the preferential production of inflammatory cytokines by B cells in asthmatic mice after *in vitro* activation. Notably, *ex vivo* quantification of Bregs in the spleen and lungs revealed that the development of asthma was associated with altered homeostasis of this cell subset, which most likely contributed to the more severe airway inflammation.

Transcriptomic analysis highlights new specific surface markers for IL-10-secreting B cells – Next, we sought to better define the phenotype and profile of Bregs. Indeed, no specific markers have yet been identified to precisely discriminate these cells. Thus, we analyzed the transcriptomic profile of sorted IL-10^+ B cells and IL-10^- B cells from the spleens of asthmatic and control mice using whole-genome microarray expression analysis (Figure 5 A; Table E1). We identified 3,420 and 5,374 differentially expressed probes in IL-10^+ and IL-10^- B cells in control and HDM mice, respectively (Fold >0.3; $p<0.01$, Table E1). Similarly, 954 common probes were differentially expressed in both control and HDM mice (Supplemental Table 1).

To investigate the Breg phenotype, we focused on the expression of surface markers that were differentially expressed in IL-10^- and IL-10^+ cells in both control and HDM mice (Figure 5B). Notably, classical markers described for Bregs, including CD5, CD1d, CD23, CD21, CD43, IgM and TIM-1, were not differentially expressed between IL-10^-

and IL-10$^+$ B cells in our microarray experiments (Supplemental Table 1). Instead, surface proteins with higher expression in IL-10$^+$ B cells included CD80 (1.04-fold in control mice, p=1.3*10^{-21}; 1.23-fold in HDM mice, p=0.00004), CD70 (0.81-fold in control mice, p=0.001; 1.45-fold in HDM mice, p=5 x 10^{-9}), Nt5e (coding for CD73; 1.46-fold in control mice, p=5.7 x 10^{-9}; 1.32-fold in HDM mice, p=1.4 x 10^{-15}), CD9 (1.24-fold in control mice, p=7.9 x 10^{-8}; 1.09-fold HDM mice, p=6.9 x 10^{-11}), Pdcd1 (coding for PD-1; 1.38-fold in control mice, p=1.6 x 10^{-7}; 1.22-fold in HDM mice, p=4.8 x 10^{-11}) and Ctla4 (1.38-fold in control mice, p=4.5 x 10^{-7}; 1.49-fold HDM mice, p=2.8 x 10^{-9}). Surface markers with significantly lower expression in IL-10$^+$ B cells included Pdpn (coding for podoplanin; 1.12-fold in control mice, p=0.00004; 0.8-fold in HDM mice, p=0.005), Itga10 (coding for Integrin-α10; 0.74-fold in control mice, p=0.00004; -0.97-fold in HDM mice, p=0.0005), and Itgb3 (coding for Integrin-β3; 0.67-fold in control mice, p=0.003; 1.27-fold in HDM mice, p=0.0005).

Differential expression of surface CD9, CD70, CD73, CD80, but not PD-1 and CTLA-4, was confirmed by flow cytometry analysis (Figure 5, C-D), further supporting the identification of new markers to phenotype IL-10$^+$ Bregs.

CD9 is a specific marker for mouse and human Bregs – To phenotype Bregs, we focused on the CD9, CD73 and CD70 molecules. Cytometry analysis using these newly identified surface markers revealed that the combination of CD9, CD73 and CD70 expression identified subsets that were highly enriched in IL-10-producing B cells (Figure 5E). Other B cell markers that have been linked to Breg cells in previous reports

374 (21, 22) were analyzed as well (Figure 5F), and we found an equivalent enrichment in
375 IL-10^{+} B cells in the CD5^{+} CD1d^{hi} and T2-MZP populations (Figure 5 F and G). However,
376 a higher enrichment in IL-10 production was found in splenic CD9-expressing B cells
377 (Figure 5G), suggesting that CD9 alone was sufficient to identify a highly enriched, IL-
378 10-secreting B cell subset (Figure 5G). Because these newly identified markers could
379 be induced by our activation protocol, we stimulated the B cells with LPS-PIM for 5 h in
380 order to identify spontaneous Bregs only (15). CD9 expression was unchanged and was
381 still differentially expressed between the IL-10^{+} and IL-10^{-} B cells after activation (Figure
382 6, A and B). In addition, significant production of IL-10 was found after two days of *in*
383 *vitro* activation of sorted splenic CD9^{+} B cells, further confirming the reliability of this
384 marker for identifying Bregs (Figure C and D). In the lungs, the phenotype of Bregs was
385 similar, with higher expression of CD9, CD73 and CD70 at the surface of IL-10-
386 secreting B cells (Figure 6 E and F). Notably, the *ex vivo* frequency of CD9^{+} B cells in
387 the lungs of the mice was lower after HDM inhalation, suggesting that a defect in Bregs
388 could arise from a deficiency of this subset in the context of HDM-induced asthma
389 (Figure 6 G and H).
390 Finally, in humans, expression of CD9 was dramatically increased at the surface of
391 CD24^{hi} CD38^{hi} immature B cells (Figure 6, I and J). Indeed, the CD9-expressing B cells
392 were all immature CD24^{hi} CD38^{hi} B cells (Figure 6K); these B cells have been described
393 as an important Breg subset that can control T cell inflammation (19, 23, 24).
394 Collectively, these data point to CD9 as a potent marker to purify both mouse and
395 human Bregs.

Injection of $CD9^+$ B cells inhibits allergic asthma by restoring the lung immunological balance – Finally, we explored the ability of $CD9^+$ B cells to regulate airway inflammation and asthma exacerbation *in vivo*. A total of 5 x 10^5 splenic $CD9^+$ or $CD9^-$ B cells were isolated by cell sorting and transferred into mice 24 h before the second challenge (Figure 7A). Lung function and allergic airway inflammation were analyzed, and we found that the recipients of splenic $CD9^+$ B cells from HDM mice were protected from HDM-induced asthma, with lower bronchial hyperreactivity (Figure 7B) and dampened cellular infiltration (Figure 7C), including significantly lower levels of neutrophils and eosinophils (Figure 7, D-G). In contrast, $CD9^-$ B cells from the spleens of HDM mice did not influence the course of HDM-induced allergic airway inflammation, with comparable levels of airway hyperresponsiveness (Figure 7B) and cellular infiltration (Figure 7C-G) than HDM mice treated with PBS (Figure 7, C-E). Similarly, transfer of $CD9^+$ B cells from IL-$10^{-/-}$ allergic animals was not protective, confirming the IL-10-dependent regulatory mechanism *in vivo* (Figure 7, C-G).

Next, we investigated to what extent $CD9^+$ B cells influenced T cell responses in the airways. Figures 8 A-C show that injection of $CD9^+$ Bregs significantly reduced the production of IL-4, IL-13 and IL-17 by $CD4^+$ T cells in the lung tissues. Additionally, the levels of inflammatory cytokines were significantly reduced in the BAL after the second challenge in $CD9^+$ recipient allergic mice (Figure 8D).

Given the intimate link between Tregs and Bregs (6, 11), we investigated whether injection of $CD9^+$ Bregs promoted the expansion of Tregs in the lung mucosa. As shown in Figure 8 E and F, adoptive transfer of $CD9^+$ Bregs did not affect the proportion of lung

418 CD25$^+$ Foxp3$^+$ Tregs. However, consistent with our findings, we found a decreased
419 frequency of CD25$^+$ Foxp3$^-$ effector T cells (Teff) after injection of CD9$^+$ B cells, resulting
420 in a significantly higher Treg/Teff cell ratio (Figure 8 G and H). These data confirm that
421 CD9$^+$ Breg cells prevent the development of asthma by inhibiting allergic airway
422 inflammation via IL-10-dependent mechanisms. Finally, by increasing the Treg/Teff
423 ratio, injection of CD9$^+$ Bregs is likely to contribute to immunological tolerance in allergic
424 asthma.
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Discussion

The delicate balance between allergen-induced inflammation and tolerance is crucial for the management of exacerbated inflammatory responses in allergic diseases (25). Tregs have been extensively described as key protective cells against the development of these pathologies (25). However, a major role for IL-10-producing Bregs has also been recently reported in the control of exacerbated inflammation in different animal models as well as humans (4), and uncontrolled inflammation can arise from a Breg deficit (6, 8, 23, 24, 26). Notably, allergen immunotherapy utilizing bee venom (14) or casein (13) was shown to promote the generation of IL-10$^+$ Bregs, thus ensuring the establishment of allergen tolerance. Here, our results demonstrate that an IL-10-producing B cell subset, able to control T cell activation *in vitro*, was less prevalent in both the spleen and lungs after allergen exposure. IL-10 expression in lung B cells was also lower in asthmatic mice, confirming that development of this disease alters the homeostasis of IL-10$^+$ regulatory B cells. Similar results have recently been reported in the context of allergy, in which patients displayed altered Breg functions and a lower frequency of IL-10-secreting B cells when compared to healthy volunteers or allergen-tolerant patients (12, 13).

In the present work, we found that exposure to HDM preferentially induced the expression of inflammatory cytokines by B cells. In particular, we detected elevated IL-4 and IL-6 mRNA levels in the lung and spleen B cells of allergic mice. Furthermore, IL-6, IL-4, IL-2 and TNF-α production was significantly increased following CD40 stimulation of B cells from allergic mice. These findings fit perfectly with data obtained in

462 experimental autoimmune encephalitis (EAE) and diabetes mouse models,
463 demonstrating a key role for inflammatory B cells in the development of the pathology
464 and maintenance of T cell-mediated inflammation (27-30). The ability of B cells to
465 preferentially produce inflammatory cytokines under pathogenic conditions suggests
466 that pathogenic B cells and Breg responses might be generated with different kinetics
467 and under specific conditions (27, 29, 30), which illustrates how pathogenic and
468 regulatory activities may often overlap and be dependent on a delicate balance between
469 pro- and anti-inflammatory B cells (29, 30). Consistent with this, a recent report
470 demonstrated that the regulatory capacities of IL-10^{+} Bregs depends greatly on their
471 capacity to also produce inflammatory cytokines (19). In our experiments, the ratio of
472 cytokines produced was shifted towards inflammatory cytokines, confirming the altered
473 balance between inflammatory B cells and Bregs in the context of asthma.
474 Our work also proposes new markers to identify Bregs in mice. Indeed, despite recent
475 attempts to phenotype Bregs, definitive characterization still remains elusive (4). No
476 specific markers or transcriptional factors permit the definitive characterization of these
477 cells, in mice or in humans. In mice, Bregs have been associated with many
478 phenotypes, including innate pro-B-cell progenitor B cells (31), CD5^{+} CD1d^{hi} B cells
479 (22), TIM-1-expressing B cells (16), B1 B cells (32), transitional-II marginal zone
480 progenitor B cells (21) and plasma cells (33, 34). Our cytometric analysis revealed that
481 these cells were equally enriched in CD5^{+} CD1d^{+} B10 cells and CD19^{+} CD23^{-} CD21^{hi}
482 IgM^{hi} T2-MZP B cells, the most documented Bregs, indicating that this subset is not
483 restricted to a single B cell population. Furthermore, microarray and flow cytometry

analysis allowed us to identify new surface markers that were significantly and differentially expressed on IL-10^{+} B cells. In particular, we found increased expression of CD9, CD73, and CD70 in Bregs from both control and asthmatic mice. CD70 is a co-stimulatory molecule that binds to CD27, although no studies have described a role for this molecule in Breg function. CD73 is a cell surface enzyme highly expressed on Tregs that suppresses T cell-mediated immune responses by producing extracellular adenosine (35). Lack of CD73 is thus associated with increased inflammation and potent anti-tumor activity (35). The recent identification of CD73-expressing B cells able to regulate T cell-mediated responses in humans is of special interest and confirms that IL-10 secretion cannot be the only regulatory mechanisms mediated by induced Bregs (36). However, more in-depth investigations must be performed to determine if adenosine works in cooperation with IL-10 in immune suppression and the maintenance of tolerance in allergic settings.

In our study, the expression of CD73 and CD70 was upregulated by *in vitro* stimulation, whereas expression of CD9 was constitutive, stable and sufficient to identify and purify the majority of IL-10-secreting B cells in mouse spleens. Notably, CD9 was upregulated at the surface of human CD24^{hi} CD38^{hi} immature B cells, a Breg subset described for its capacity to inhibit T cell-mediated inflammation and induce Tregs *in vitro* (19, 23, 24). This supports the idea that CD9 may be used to identify Bregs in humans. CD9 is a tetraspanin molecule involved in the enhancement and maintenance of IL-10 secretion in murine and human antigen-presenting cells (37, 38), most likely by enhancing calcium signaling (39), an important pathway for Breg generation (9). CD9 knockout

mice do not display abnormalities in their B cell compartments (40). This marker is expressed at the surface of B1 and MZ B cells and can be induced in plasma cells for a long time post-activation (41). In the lungs, IL-10-producing B cells also overexpress CD9, supporting its relevance in the identification of Bregs. Notably, exposure to allergen significantly decreased the proportion of this subset, suggesting that the lower proportions of IL-10-producing B cells observed in asthmatic mice could arise from a lack of $CD9^+$ B cells in the airways.

The existence of $CD9^+$ $IL\text{-}10^+$ B cells has already been demonstrated in mice (42); however, no data regarding their regulatory capacities have yet been reported. Herein, we establish a role for $CD9^+$ Bregs in the regulation of allergic asthma by demonstrating that injection of $CD9^+$ B cells altered the course of HDM-induced airway inflammation and bronchial hyperreactivity, thus confirming a therapeutic effect of Breg injection in our allergic asthma model. The $CD9^+$ Breg-induced inhibition of T_H2 and T_H17 cells was also associated with a major drop in neutrophil and eosinophil counts, and these results are in accordance with previously published data in humans and mice demonstrating that IL-10-producing Bregs can inhibit T_H17- (17, 18, 24) and T_H2-mediated inflammation (11). The ability of Bregs to inhibit T cell IL-17 production is of particular interest given the suspected role of pathogenic T_H17 cells in severe asthma (43). Administration of splenic $CD9^+$ B cells from $IL\text{-}10^{-/-}$ mice did not regulate the asthmatic response mediated by HDM, demonstrating the IL-10-dependent mechanism of $CD9^+$ Bregs *in vivo*. As IL-10 is absolutely required to constrain allergic inflammation at mucosal surfaces such as the gut or lungs (44), our results support an important role for

528 Bregs in this pathology.

529 IL-10-producing Bregs can mediate many immune regulatory mechanisms (5, 45). For
530 example, several studies have demonstrated that IL-10-producing B cells are important
531 for the generation or maintenance of Foxp3 Tregs (6, 11). In our study, the injection of
532 Bregs did not influence Tregs in the lungs. Instead, the Bregs controlled T cell
533 activation, restoring a favorable immunological balance and most likely leading to less
534 severe inflammation. Similar findings have also supported the idea that IL-10^{+} Bregs are
535 not necessary for the induction of Tregs (30, 46). Other Breg subsets, expressing TGF-
536 β, have been described as central Treg inducers, notably in allergic diseases (45). Our
537 results suggest that Bregs directly influence the activation and maturation of local
538 antigen-presenting cells, leading to partial T cell activation. Indeed, several studies have
539 demonstrated the role of IL-10 in the downregulated expression of MHC-II and
540 costimulatory molecules at the surface of antigen-presenting cells (47). Moreover, Bregs
541 can influence the ability of dendritic cells to present antigen to T cells (48). In addition,
542 IL-10 from B cells can inhibit the production of IL-6 and IL-23 by dendritic cells *in vitro*,
543 which might explain how Bregs can reduce T_H17 responses *in vivo* (20). Nevertheless,
544 how Bregs regulate T_H2 responses is less clear. Indeed, T_H2 cells might be induced
545 independently of dendritic cells via basophil-dependent or innate lymphoid cell-
546 dependent mechanisms (49). IL-10-producing B cells might therefore regulate T_H2
547 immunity by acting on other immune components besides dendritic cells.

548 In conclusion, our data show how the development of asthma modulates the profile of B
549 cells, with a notable alteration of the Breg pool. The ability of Bregs to normalize lung

function and airway inflammation points to these cells as an interesting target in allergic asthma. Thus, the development of new therapies intending to restore the Breg pool are encouraged for the treatment of allergic asthma.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Dullaers M, De Bruyne R, Ramadani F, Gould HJ, Gevaert P, and Lambrecht BN. The who, where, and when of IgE in allergic airway disease. *J Allergy Clin Immunol.* 2012;129(3):635-45.
2. Lund FE, and Randall TD. Effector and regulatory B cells: modulators of CD4+ T cell immunity. *Nat Rev Immunol.* 2010;10(4):236-47.
3. Bao Y, and Cao X. The immune potential and immunopathology of cytokine-producing B cell subsets: A comprehensive review. *J Autoimmun.* 2014.
4. Mauri C, and Bosma A. Immune regulatory function of B cells. *Annu Rev Immunol.* 2012;30(221-41.
5. Candando KM, Lykken JM, and Tedder TF. B10 cell regulation of health and disease. *Immunol Rev.* 2014;259(1):259-72.
6. Carter NA, Vasconcellos R, Rosser EC, Tulone C, Munoz-Suano A, Kamanaka M, Ehrenstein MR, Flavell RA, and Mauri C. Mice lacking endogenous IL-10-producing regulatory B cells develop exacerbated disease and present with an increased frequency of Th1/Th17 but a decrease in regulatory T cells. *J Immunol.* 2011;186(10):5569-79.
7. Fillatreau S, Sweenie CH, McGeachy MJ, Gray D, and Anderton SM. B cells regulate autoimmunity by provision of IL-10. *Nat Immunol.* 2002;3(10):944-50.
8. Jin G, Hamaguchi Y, Matsushita T, Hasegawa M, Le Huu D, Ishiura N, Naka K, Hirao A, Takehara K, and Fujimoto M. B-cell linker protein expression contributes to controlling allergic and autoimmune diseases by mediating IL-10 production in regulatory B cells. *J Allergy Clin Immunol.* 2013;131(6):1674-82.
9. Matsumoto M, Fujii Y, Baba A, Hikida M, Kurosaki T, and Baba Y. The calcium sensors STIM1 and STIM2 control B cell regulatory function through interleukin-10 production. *Immunity.* 2011;34(5):703-14.
10. Mizoguchi A, Mizoguchi E, Takedatsu H, Blumberg RS, and Bhan AK. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. *Immunity.* 2002;16(2):219-30.
11. Amu S, Saunders SP, Kronenberg M, Mangan NE, Atzberger A, and Fallon PG. Regulatory B cells prevent and reverse allergic airway inflammation via FoxP3-positive T regulatory cells in a murine model. *J Allergy Clin Immunol.* 2010;125(5):1114-24 e8.
12. van der Vlugt LE, Labuda LA, Ozir-Fazalalikhan A, Lievers E, Gloudemans AK, Liu KY, Barr TA, Sparwasser T, Boon L, Ngoa UA, et al. Schistosomes induce regulatory features in human and mouse CD1d(hi) B cells: inhibition of allergic inflammation by IL-10 and regulatory T cells. *PLoS One.* 2012;7(2):e30883.
13. Noh J, Lee JH, Noh G, Bang SY, Kim HS, Choi WS, Cho S, and Lee SS. Characterisation of allergen-specific responses of IL-10-producing regulatory B cells (Br1) in Cow Milk Allergy. *Cell Immunol.* 2010;264(2):143-9.
14. van de Veen W, Stanic B, Yaman G, Wawrzyniak M, Sollner S, Akdis DG, Ruckert B, Akdis CA, and Akdis M. IgG4 production is confined to human IL-10-producing

regulatory B cells that suppress antigen-specific immune responses. *J Allergy Clin Immunol.* 2013;131(4):1204-12.
15. Matsushita T, and Tedder TF. Identifying regulatory B cells (B10 cells) that produce IL-10 in mice. *Methods Mol Biol.* 2011;677(99-111.
16. Ding Q, Yeung M, Camirand G, Zeng Q, Akiba H, Yagita H, Chalasani G, Sayegh MH, Najafian N, and Rothstein DM. Regulatory B cells are identified by expression of TIM-1 and can be induced through TIM-1 ligation to promote tolerance in mice. *J Clin Invest.* 2011;121(9):3645-56.
17. Yang M, Deng J, Liu Y, Ko KH, Wang X, Jiao Z, Wang S, Hua Z, Sun L, Srivastava G, et al. IL-10-producing regulatory B10 cells ameliorate collagen-induced arthritis via suppressing Th17 cell generation. *Am J Pathol.* 2012;180(6):2375-85.
18. Yoshizaki A, Miyagaki T, DiLillo DJ, Matsushita T, Horikawa M, Kountikov EI, Spolski R, Poe JC, Leonard WJ, and Tedder TF. Regulatory B cells control T-cell autoimmunity through IL-21-dependent cognate interactions. *Nature.* 2012;491(7423):264-8.
19. Cherukuri A, Rothstein DM, Clark B, Carter CR, Davison A, Hernandez-Fuentes M, Hewitt E, Salama AD, and Baker RJ. Immunologic Human Renal Allograft Injury Associates with an Altered IL-10/TNF-alpha Expression Ratio in Regulatory B Cells. *J Am Soc Nephrol.* 2014.
20. Lampropoulou V, Hoehlig K, Roch T, Neves P, Calderon Gomez E, Sweenie CH, Hao Y, Freitas AA, Steinhoff U, Anderton SM, et al. TLR-activated B cells suppress T cell-mediated autoimmunity. *J Immunol.* 2008;180(7):4763-73.
21. Evans JG, Chavez-Rueda KA, Eddaoudi A, Meyer-Bahlburg A, Rawlings DJ, Ehrenstein MR, and Mauri C. Novel suppressive function of transitional 2 B cells in experimental arthritis. *J Immunol.* 2007;178(12):7868-78.
22. Yanaba K, Bouaziz JD, Haas KM, Poe JC, Fujimoto M, and Tedder TF. A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses. *Immunity.* 2008;28(5):639-50.
23. Blair PA, Norena LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, and Mauri C. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. *Immunity.* 2010;32(1):129-40.
24. Flores-Borja F, Bosma A, Ng D, Reddy V, Ehrenstein MR, Isenberg DA, and Mauri C. CD19+CD24 hiCD38 hi B cells maintain regulatory T cells while limiting TH1 and TH17 differentiation. *Sci Transl Med.* 2013;5(173):173ra23.
25. Akdis M, and Akdis CA. Mechanisms of allergen-specific immunotherapy: multiple suppressor factors at work in immune tolerance to allergens. *J Allergy Clin Immunol.* 2014;133(3):621-31.
26. Xiao S, Brooks CR, Zhu C, Wu C, Sweere JM, Petecka S, Yeste A, Quintana FJ, Ichimura T, Sobel RA, et al. Defect in regulatory B-cell function and development of systemic autoimmunity in T-cell Ig mucin 1 (Tim-1) mucin domain-mutant mice. *Proc Natl Acad Sci U S A.* 2012;109(30):12105-10.
27. Barr TA, Shen P, Brown S, Lampropoulou V, Roch T, Lawrie S, Fan B, O'Connor RA, Anderton SM, Bar-Or A, et al. B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells. *J Exp Med.* 2012;209(5):1001-10.
28. Molnarfi N, Schulze-Topphoff U, Weber MS, Patarroyo JC, Prod'homme T, Varrin-

Doyer M, Shetty A, Linington C, Slavin AJ, Hidalgo J, et al. MHC class II-dependent B cell APC function is required for induction of CNS autoimmunity independent of myelin-specific antibodies. *J Exp Med.* 2013;210(13):2921-37.

29. DeFuria J, Belkina AC, Jagannathan-Bogdan M, Snyder-Cappione J, Carr JD, Nersesova YR, Markham D, Strissel KJ, Watkins AA, Zhu M, et al. B cells promote inflammation in obesity and type 2 diabetes through regulation of T-cell function and an inflammatory cytokine profile. *Proc Natl Acad Sci U S A.* 2013;110(13):5133-8.
30. Matsushita T, Yanaba K, Bouaziz JD, Fujimoto M, and Tedder TF. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. *J Clin Invest.* 2008;118(10):3420-30.
31. Montandon R, Korniotis S, Layseca-Espinosa E, Gras C, Megret J, Ezine S, Dy M, and Zavala F. Innate pro-B-cell progenitors protect against type 1 diabetes by regulating autoimmune effector T cells. *Proc Natl Acad Sci U S A.* 2013;110(24):E2199-208.
32. Maseda D, Candando KM, Smith SH, Kalampokis I, Weaver CT, Plevy SE, Poe JC, and Tedder TF. Peritoneal cavity regulatory B cells (B10 cells) modulate IFN-gamma+CD4+ T cell numbers during colitis development in mice. *J Immunol.* 2013;191(5):2780-95.
33. Neves P, Lampropoulou V, Calderon-Gomez E, Roch T, Stervbo U, Shen P, Kuhl AA, Loddenkemper C, Haury M, Nedospasov SA, et al. Signaling via the MyD88 adaptor protein in B cells suppresses protective immunity during Salmonella typhimurium infection. *Immunity.* 2010;33(5):777-90.
34. Shen P, Roch T, Lampropoulou V, O'Connor RA, Stervbo U, Hilgenberg E, Ries S, Dang VD, Jaimes Y, Daridon C, et al. IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases. *Nature.* 2014;507(7492):366-70.
35. Antonioli L, Pacher P, Vizi ES, and Hasko G. CD39 and CD73 in immunity and inflammation. *Trends Mol Med.* 2013;19(6):355-67.
36. Saze Z, Schuler PJ, Hong CS, Cheng D, Jackson EK, and Whiteside TL. Adenosine production by human B cells and B cell-mediated suppression of activated T cells. *Blood.* 2013;122(1):9-18.
37. Ha CT, Waterhouse R, Wessells J, Wu JA, and Dveksler GS. Binding of pregnancy-specific glycoprotein 17 to CD9 on macrophages induces secretion of IL-10, IL-6, PGE2, and TGF-beta1. *J Leukoc Biol.* 2005;77(6):948-57.
38. Peng WM, Yu CF, Kolanus W, Mazzocca A, Bieber T, Kraft S, and Novak N. Tetraspanins CD9 and CD81 are molecular partners of trimeric FcvarepsilonRI on human antigen-presenting cells. *Allergy.* 2011;66(5):605-11.
39. Halova I, Draberova L, Bambouskova M, Machyna M, Stegurova L, Smrz D, and Draber P. Cross-talk between tetraspanin CD9 and transmembrane adaptor protein non-T cell activation linker (NTAL) in mast cell activation and chemotaxis. *J Biol Chem.* 2013;288(14):9801-14.
40. Cariappa A, Shoham T, Liu H, Levy S, Boucheix C, and Pillai S. The CD9 tetraspanin is not required for the development of peripheral B cells or for humoral immunity. *The Journal of ....* 2005.
41. Won W-JJ, and Kearney JF. CD9 is a unique marker for marginal zone B cells, B1 cells, and plasma cells in mice. *Journal of immunology (Baltimore, Md: 1950).*

2002;168(11):5605-11.
42. Ostrowski M, Vermeulen M, Zabal O, Zamorano PI, Sadir AM, Geffner JR, and Lopez OJ. The early protective thymus-independent antibody response to foot-and-mouth disease virus is mediated by splenic CD9+ B lymphocytes. *J Virol.* 2007;81(17):9357-67.
43. Chesne J, Braza F, Mahay G, Brouard S, Aronica M, and Magnan A. IL-17 in Severe Asthma: Where Do We Stand? *Am J Respir Crit Care Med.* 2014.
44. Lloyd CM, and Hawrylowicz CM. Regulatory T cells in asthma. *Immunity.* 2009;31(3):438-49.
45. Braza F, Chesne J, Castagnet S, Magnan A, and Brouard S. Regulatory functions of B cells in allergic diseases. *Allergy.* 2014.
46. Hoehlig K, Shen P, Lampropoulou V, Roch T, Malissen B, O'Connor R, Ries S, Hilgenberg E, Anderton SM, and Fillatreau S. Activation of CD4(+) Foxp3(+) regulatory T cells proceeds normally in the absence of B cells during EAE. *Eur J Immunol.* 2012;42(5):1164-73.
47. O'Garra A, Barrat FJ, Castro AG, Vicari A, and Hawrylowicz C. Strategies for use of IL-10 or its antagonists in human disease. *Immunol Rev.* 2008;223(1):114-31.
48. Matsushita T, Horikawa M, Iwata Y, and Tedder TF. Regulatory B cells (B10 cells) and regulatory T cells have independent roles in controlling experimental autoimmune encephalomyelitis initiation and late-phase immunopathogenesis. *J Immunol.* 2010;185(4):2240-52.
49. Paul WE, and Zhu J. How are T(H)2-type immune responses initiated and amplified? *Nat Rev Immunol.* 2010;10(4):225-35.

753 **Figure Legends**
754
755 **Figure 1: Exposure to HDM induces mixed inflammation in the lungs of mice.** (**A**),
756 Schematic representation of the HDM antigen–allergic model. (**B**), Penh and airway
757 resistance values at day 35 after challenge. (**C**), Representative lung sections of control
758 and asthmatic mice at day 35 stained with H&E or PAS. (**D**), BAL levels of eosinophils
759 and neutrophils. (**E**) BAL levels of systemic Der f1-specific IgE, IL-4, IL-13 and IL-17 in
760 control and asthmatic mice. ***p<0 .001 and **p< 0.01.
761
762 **Figure 2: HDM promotes the infiltration and maturation of B cells.** (**A**), Expression
763 of CXCL13 in lung tissues and protein levels of CXCL13 in the BAL of mice. (**B and C**),
764 quantification of B cells in the BAL. (**D**) Representative B cell staining in the lung tissue
765 (Red $B220^+$ B cells). (**E and F**), Quantification of $CD19^+$ IgM^+ mature B cells and $CD19^+$
766 IgM^- IgD^- in lung tissue. (**G and H**) Quantification of memory B cells and (**I and J**) $CD19^+$
767 Fas^+ $GL7^+$ germinal center B cells in the lung tissue. (**K)**, Levels of Der f1-specific IgE in
768 the BAL. ***p<0 .001, and **p< 0.01.
769
770 **Figure 3: HDM-induced asthma alters the homesotasis of Bregs.** The mRNA levels
771 of cytokines were analyzed using RT-PCR in purified B cells from the spleen (**A**) and
772 lungs (**B**) of control and allergic mice. (**C-F),** $IL\text{-}10^+$ B cell frequencies in the spleen and
773 lungs of mice. Splenocytes were cultured with LPS + PIM for 5 h, stained with CD19
774 mAb, permeabilized, and stained with IL-10 mAb for flow cytometric analysis.
775 Representative results demonstrate the frequency of IL-10–producing cells within the
776 indicated gates among total $CD19^+$ B cells in the spleen (**C-D**) or lungs (**E-F**). (**G**), IL-10
777 protein levels in the supernatant of purified lung B cells activated with LPS + PIM for 5 h.
778 (**H**), Splenocytes from control and HDM-treated mice were stimulated for 4 days with a
779 total extract of HDM. At the end of the culture period, the splenocytes were restimulated
780 with PMA, and the $IL\text{-}10^+$ B cell frequency was analyzed by flow cytometry. ***p<0.001,
781 **p<0.01 and *p<0.05.
782
783 **Figure 4: Impaired regulatory functions of B cells in the context of allergic**
784 **asthma.** (**A-B**), Purified splenic B cells from naïve Balb/c mice were cultured with LPS +
785 PIM for 5 h or were cultured with agonistic CD40 mAb, BAFF, anti-TIM-1 or IL-21 for 48
786 h with LPS + PIM added during the final 5 h of culture. The $IL\text{-}10^+$ B cell frequencies
787 were determined by flow cytometry. (**C**), IL-10 protein levels in the supernatant of
788 purified splenic B cells from naïve Balb/c mice activated with LPS + PIM for 5 h or
789 cultured with agonistic CD40 mAb, BAFF, anti-TIM-1 or IL-21 for 48 h. (**D-E**) Purified
790 splenic B cells from control and HDM mice were cultured with agonistic CD40 mAb and
791 restimulated for the final 5 h of culture. The frequency of $IL\text{-}10^+$ B cells was analyzed by
792 flow cytometry, (**D**) and IL-10 protein was quantified in the supernatant by ELISA (**E**). (**F-**
793 **G**), CD3/CD28-activated, CellTrace-labeled $CD4^+$ $CD25^-$ T cells were cocultured with
794 CD40 pre-activated B cells in the presence or absence of blocking IL-10 antibody (n=5-
795 8 mice in each group). (**H**), Quantification of IL-2, TNF-α, IL-6 and IFN-γ in the
796 supernatants of B cells isolated from the spleens of control and HDM mice, which were

treated with agonistic CD40 mAb for 48 h with LPS + PI added for the final 5 h of culture. (**I**) The IL-10/TNF-α and IL-10/IL-6 ratios in control and HDM mice ***p<0.001, **p<0.01 and *p<0.05.

Figure 5: Characterization of IL-10$^+$ and IL-10$^-$ B cells. (**A**), Representative sorting of IL-10$^+$ and IL-10$^-$ B cells. (**B**), Heatmap showing upregulated and downregulated genes coding for surface proteins between IL-10$^+$ and IL-10$^-$ B cells. (**C**), Representative staining of CD9, CD70, CD73, CD80, CTLA4 and PD-1 at the surface of IL-10$^+$ and IL-10$^-$ B cells. (**D**), Differential protein expression of CD9, CD70, CD73 and CD80 at the surface of IL-10$^+$ B cells of control and HDM mice. (**E-F**), Purified B cells from the spleens of control and HDM mice were activated for two days with CD40 mAb agonist and LPS + PIM during the last 5 h. Then, IL-10-secreting B cells were quantified in the CD9$^+$, CD5$^+$ CD1d$^+$ (B10), CD23$^-$ CD21^{hi} IgM^{hi} (T2-MZP) and newly identified CD9$^+$ B cell subsets. **p<0.01.

Figure 6: CD9 is a specific marker of Bregs. (**A-B**) Protein expression in spleen IL-10$^+$ B cells (relative to IL-10$^-$ cells) after 5 h of activation with LPS + PIM. (**C**), Representative sorting of spleen CD9$^+$ and CD9$^-$ B cells. (**D**), Sorted CD9$^+$ and CD9$^-$ B cells were stimulated for 48 h with LPS + PIM for the last 5 h, and then the IL-10 levels were measured in the supernatants by ELISA. (**E-F**), Total lung cells were isolated from naïve Balb/c mice and stimulated for 5 h with LPS + PIM, and then the expression of CD9, CD70 and CD73 was quantified at the surface of IL-10$^+$ B cells. (**G**), Representative staining for CD9 in lung B cells from control and HDM mice. (**H**), Frequency of CD9+ B cells in the lungs of control and HDM mice. (**I-J**), Expression of CD9 in human memory, naïve and immature B cells. (**K**), Phenotype of human CD9-expressing B cells. **p<0.01.

Figure 7: CD9$^+$ B cells control asthma exacerbation. (**A**), Schematic representation of the adoptive transfer experiments. The experiments were performed in 5 groups, including control mice and asthmatic mice injected with CD9$^+$ cells, CD9$^-$ cells, CD9$^+$ IL-10$^{-/-}$ cells and PBS. (**B**), Penh values at day 35 after the adoptive transfer of B cells. (**C**), Total BAL cell counts. (**D**), Representative flow cytometry staining of BAL neutrophils. (**E**), Total neutrophil BAL cell counts. (**F**), Representative flow cytometry staining of the BAL eosinophils. (**G**), Total eosinophil BAL cell counts.

Figure 8: Adoptive transfer of CD9$^+$ B cells influences airway inflammatory T cell responses. (**A-B**), Representative intracellular staining for lung T cells producing IL-17, IFN-γ, IL-4 and IL-13 after *ex vivo* restimulation with PIM (5 h). (**C**), Quantification of lung IL-17, IFN-γ, IL-4, and IL-13 producing T cells (n=5-10 mice per group). (**D**) IFN-γ, IL-17, IL-4 and IL-5 levels in the BAL (n=5-10 mice per group). (**E**), Representative staining for CD25$^+$ Foxp3$^-$ effector T cells and CD25$^+$ Foxp3$^+$ regulatory T cells. Frequency of regulatory T cells and effector T cells (**F-G**) and the regulatory T cell:effector T cell ratio (**H**) in the lungs of control mice and asthmatic mice injected with CD9$^+$ cells, CD9$^-$ cells or CD9$^+$ IL-10$^{-/-}$ cells. **p<0.01, *p<0.05 and ns, non significant.

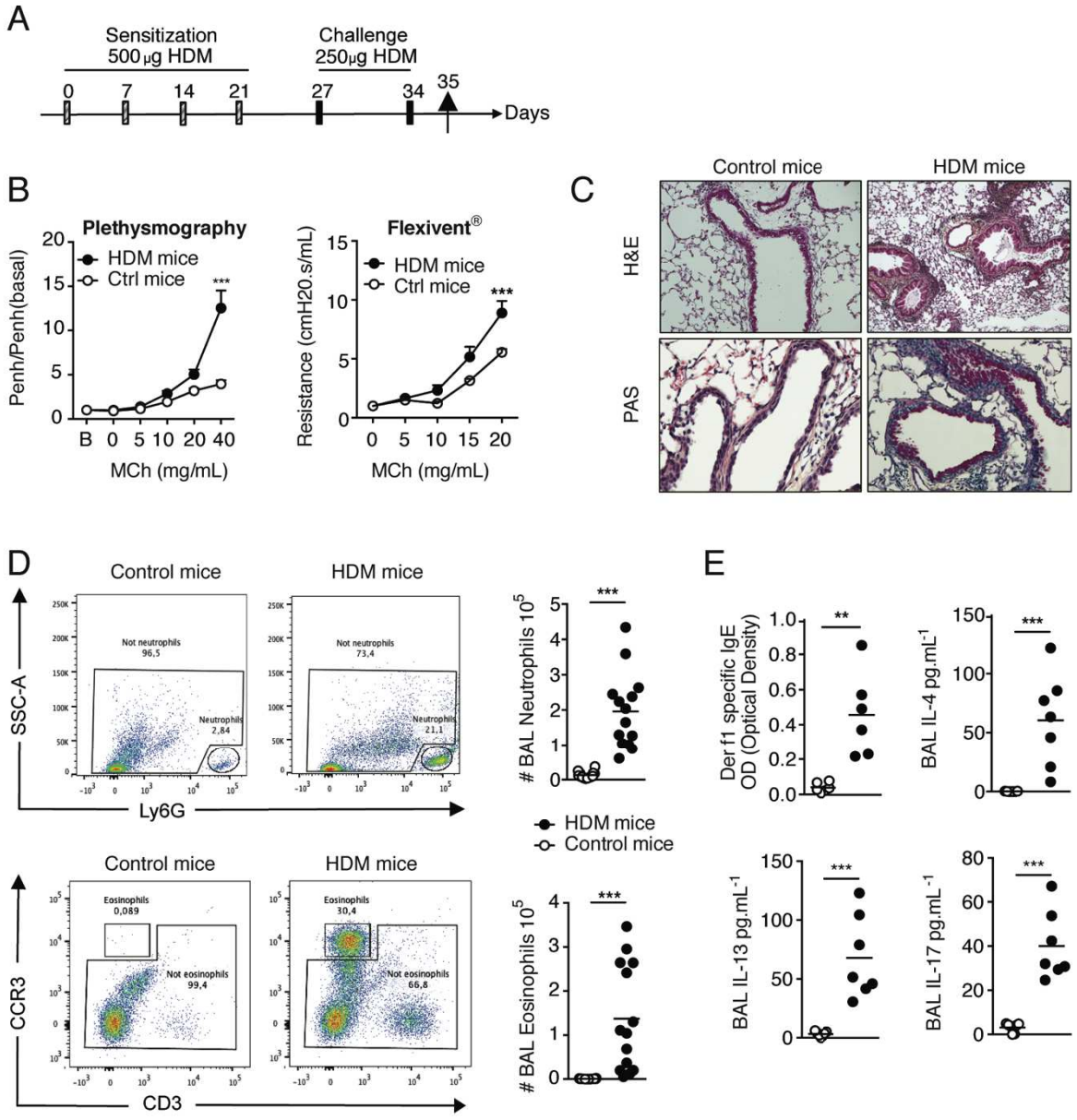
A
Sensitization
500 µg HDM
Challenge
250µg HDM
0
7
14
21
27
34
35
Days
B
Plethysmography
HDM mice
Ctrl mice
***
Penh/Penh(basal)
MCh (mg/mL)
Flexivent®
Resistance (cmH20.s/mL)
C
Control mice
HDM mice
H&E
PAS
D
Control mice
HDM mice
SSC-A
Ly6G
Not neutrophils
96,5
Neutrophils
2,84
Not neutrophils
73,4
Neutrophils
21,1
BAL Neutrophils 10^5
CCR3
CD3
Eosinophils
0,089
Not eosinophils
99,4
Eosinophils
30,4
Not eosinophils
66,8
BAL Eosinophils 10^5
E
Der f1 specific IgE
OD (Optical Density)
BAL IL-4 pg.mL-1
BAL IL-13 pg.mL-1
BAL IL-17 pg.mL-1

Figure 1

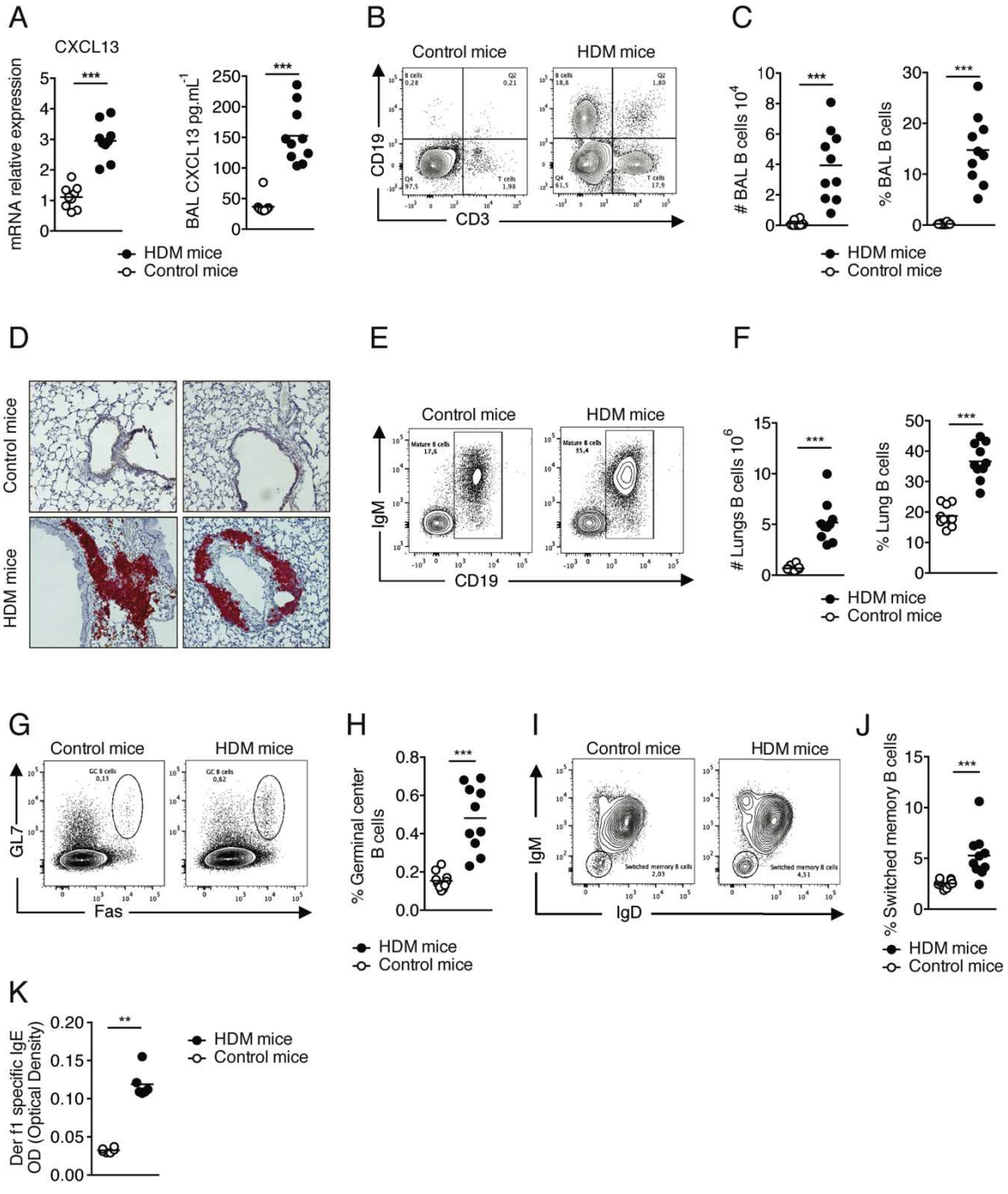
A
CXCL13
mRNA relative expression
BAL CXCL13 pg.mL-1
***
HDM mice
Control mice
B
Control mice
HDM mice
CD19
CD3
C
BAL B cells 10^4
% BAL B cells
D
Control mice
HDM mice
E
IgM
CD19
F
Lungs B cells 10^6
% Lung B cells
G
GL7
Fas
H
% Germinal center B cells
I
IgM
IgD
J
% Switched memory B cells
K
Der f1 specific IgE OD (Optical Density)
**

Figure 2

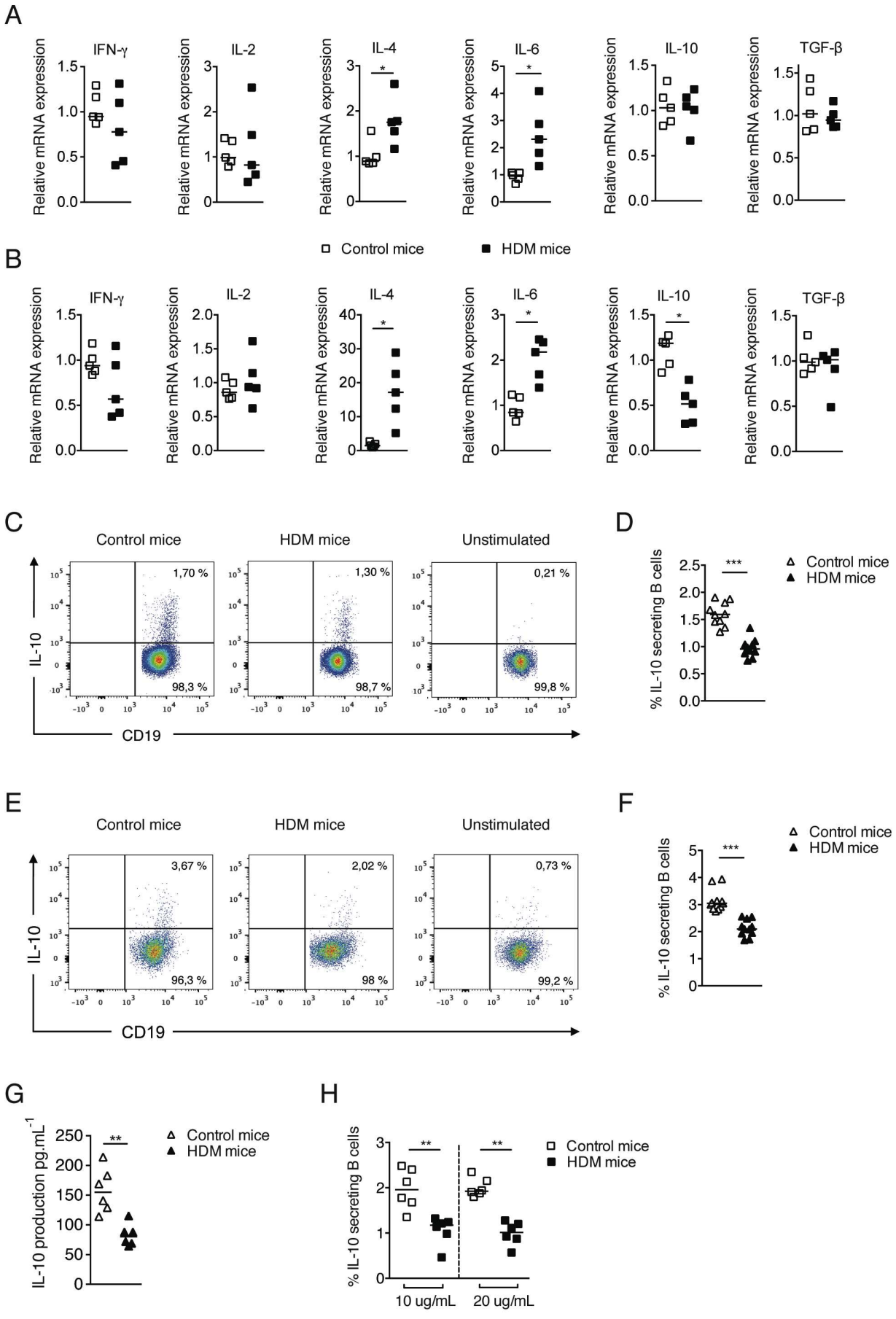
A
IFN-γ
IL-2
IL-4
IL-6
IL-10
TGF-β
Relative mRNA expression
Control mice
HDM mice
B
C
Control mice
HDM mice
Unstimulated
1,70 %
98,3 %
1,30 %
98,7 %
0,21 %
99,8 %
IL-10
CD19
D
% IL-10 secreting B cells
E
3,67 %
96,3 %
2,02 %
98 %
0,73 %
99,2 %
F
G
IL-10 production pg.mL-1
H
10 ug/mL
20 ug/mL

Figure 3

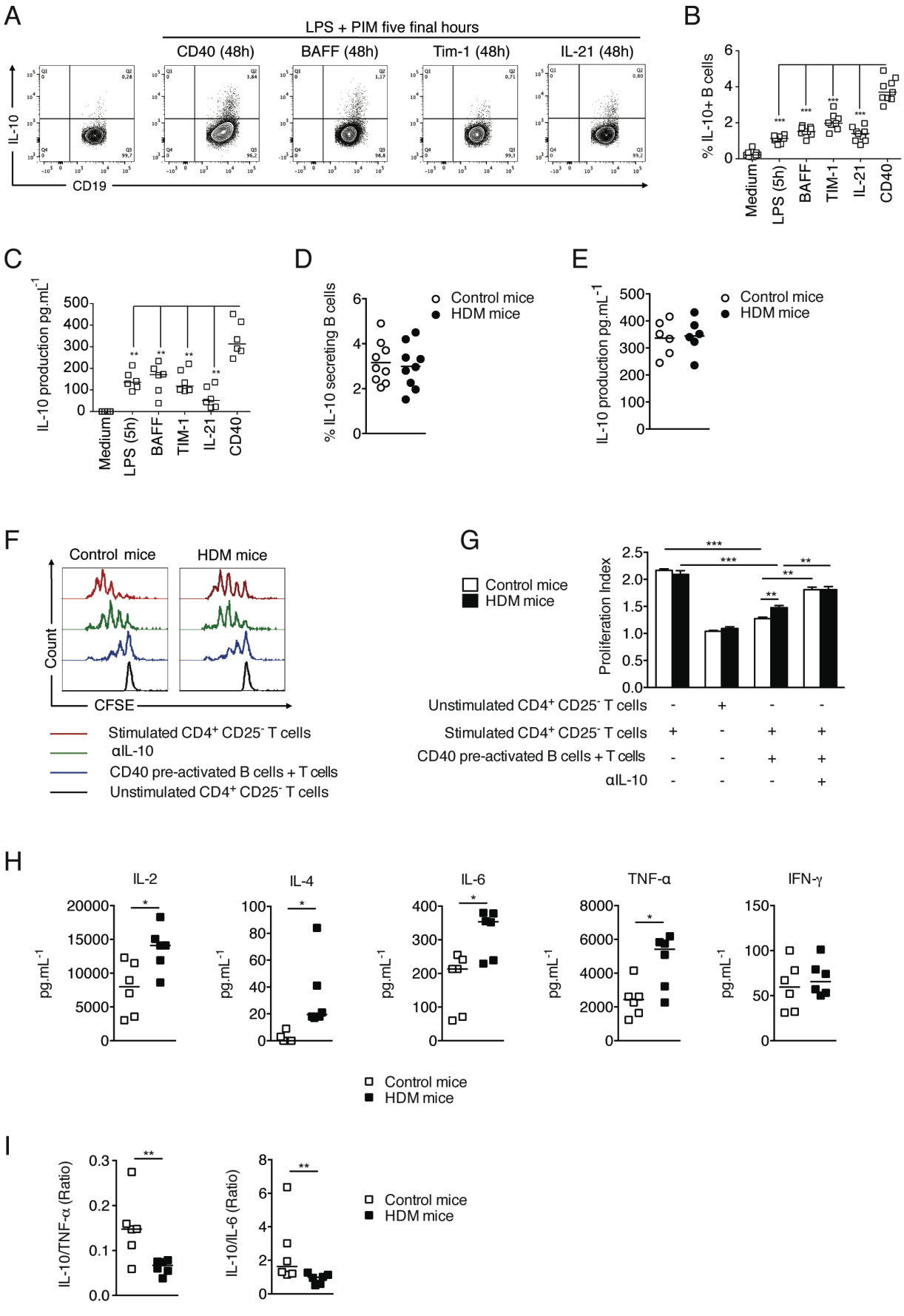
A
LPS + PIM five final hours
CD40 (48h)
BAFF (48h)
Tim-1 (48h)
IL-21 (48h)
IL-10
CD19
B
% IL-10+ B cells
Medium
LPS (5h)
BAFF
TIM-1
IL-21
CD40
C
IL-10 production pg.mL-1
Medium
LPS (5h)
BAFF
TIM-1
IL-21
CD40
D
% IL-10 secreting B cells
Control mice
HDM mice
E
IL-10 production pg.mL-1
Control mice
HDM mice
F
Control mice
HDM mice
Count
CFSE
Stimulated CD4+ CD25- T cells
αIL-10
CD40 pre-activated B cells + T cells
Unstimulated CD4+ CD25- T cells
G
Control mice
HDM mice
Proliferation Index
Unstimulated CD4+ CD25- T cells
Stimulated CD4+ CD25- T cells
CD40 pre-activated B cells + T cells
αIL-10
H
IL-2
IL-4
IL-6
TNF-α
IFN-γ
pg.mL-1
Control mice
HDM mice
I
IL-10/TNF-α (Ratio)
IL-10/IL-6 (Ratio)
Control mice
HDM mice

Figure 4

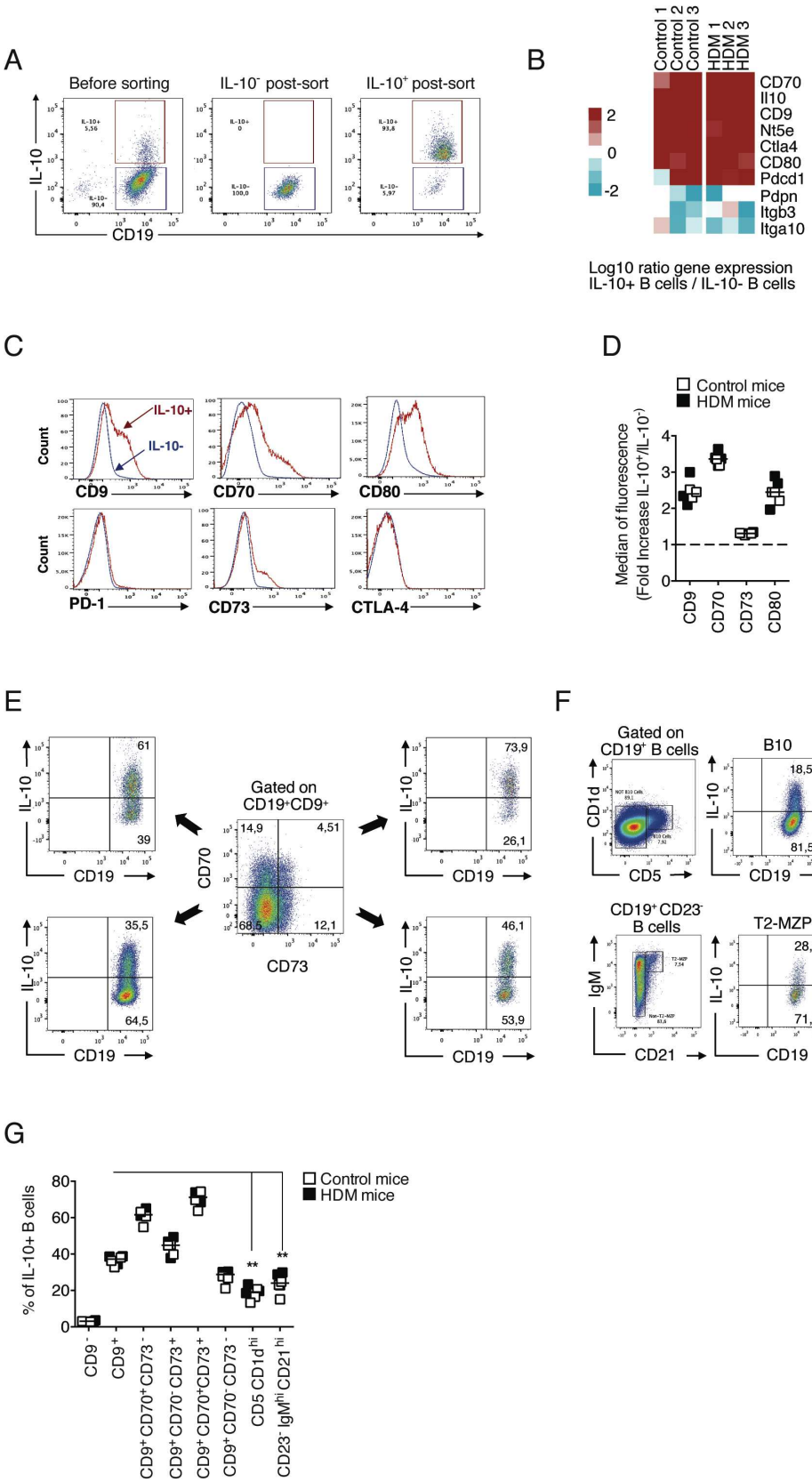
A
Before sorting
IL-10⁻ post-sort
IL-10⁺ post-sort
IL-10
CD19
B
Control 1
Control 2
Control 3
HDM 1
HDM 2
HDM 3
2
0
-2
CD70
Il10
CD9
Nt5e
Ctla4
CD80
Pdcd1
Pdpn
Itgb3
Itga10
Log10 ratio gene expression
IL-10+ B cells / IL-10- B cells
C
IL-10+
IL-10-
Count
CD9
CD70
CD80
PD-1
CD73
CTLA-4
D
Control mice
HDM mice
Median of fluorescence
(Fold Increase IL-10⁺/IL-10⁻)
CD9
CD70
CD73
CD80
E
Gated on
CD19⁺CD9⁺
CD70
CD73
IL-10
CD19
61
39
14,9
4,51
68,5
12,1
73,9
26,1
35,5
64,5
46,1
53,9
F
Gated on
CD19⁺ B cells
CD1d
CD5
B10
Not B10
18,5
81,5
3,71
96,4
CD19⁺CD23⁻
B cells
IgM
CD21
T2-MZP
Not T2-MZP
28,8
71,2
18
82
G
Control mice
HDM mice
% of IL-10+ B cells
CD9⁻
CD9⁺
CD9⁺CD70⁺CD73⁻
CD9⁺CD70⁻CD73⁺
CD9⁺CD70⁺CD73⁺
CD9⁺CD70⁻CD73⁻
CD5 CD1d^hi
CD23⁻ IgM^hi CD21^hi

Figure 5

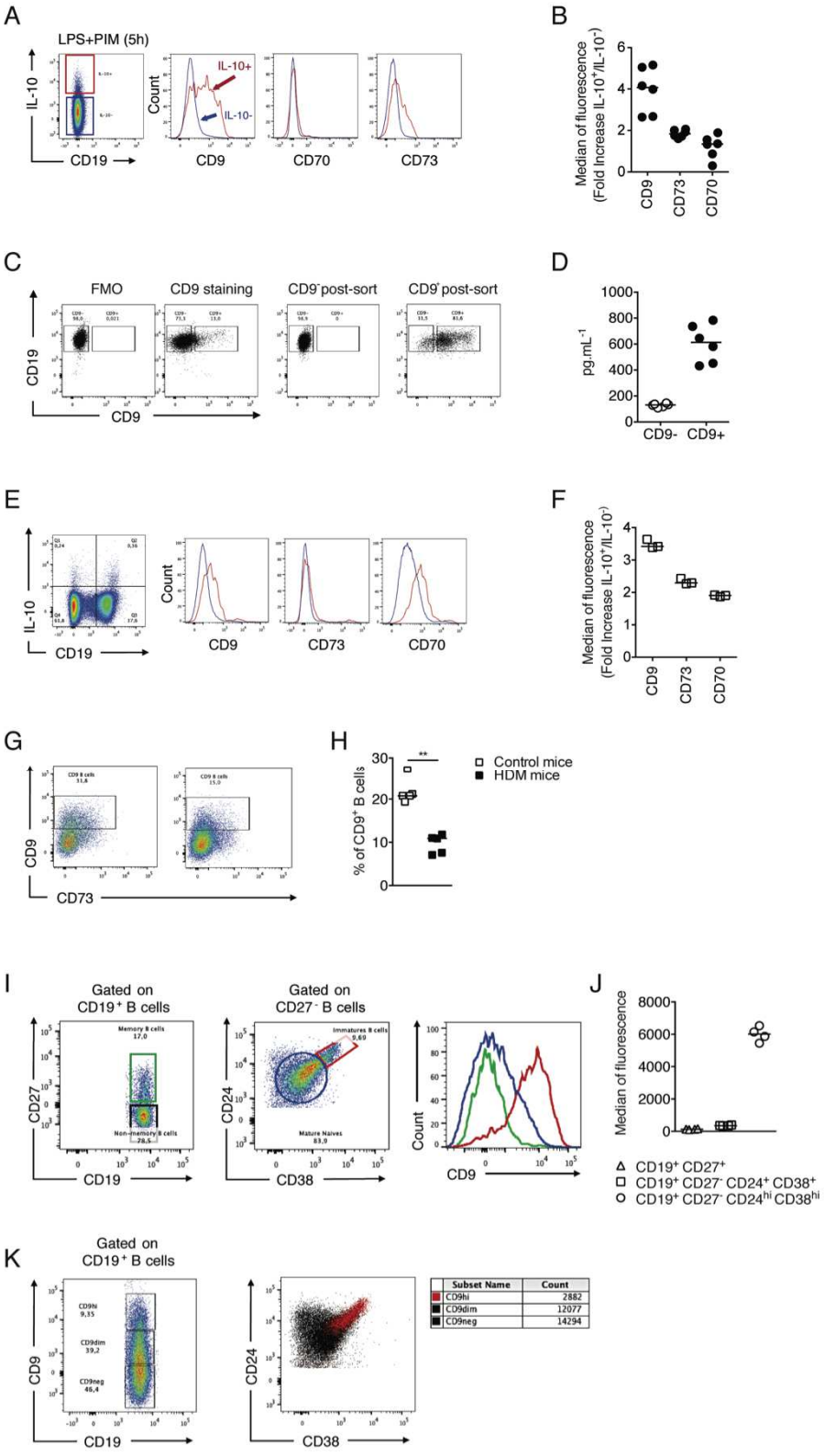
A
LPS+PIM (5h)
IL-10
CD19
Count
IL-10+
IL-10-
CD9
CD70
CD73
B
Median of fluorescence (Fold Increase IL-10+/IL-10-)
CD9
CD73
CD70
C
FMO
CD9 staining
CD9- post-sort
CD9+ post-sort
CD19
CD9
D
pg.mL-1
CD9-
CD9+
E
IL-10
CD19
Count
CD9
CD73
CD70
F
Median of fluorescence (Fold Increase IL-10+/IL-10-)
CD9
CD73
CD70
G
CD9
CD73
H
% of CD9+ B cells
Control mice
HDM mice
I
Gated on CD19+ B cells
Memory B cells 17,0
Non-memory B cells 29,5
CD27
CD19
Gated on CD27- B cells
Immatures B cells 9,69
Mature Naives 83,9
CD24
CD38
Count
CD9
J
Median of fluorescence
CD19+ CD27+
CD19+ CD27- CD24+ CD38+
CD19+ CD27- CD24hi CD38hi
K
Gated on CD19+ B cells
CD9hi 9,35
CD9dim 39,2
CD9neg 46,4
CD9
CD19
CD24
CD38
Subset Name
Count
CD9hi 2882
CD9dim 12077
CD9neg 14294

Figure 6

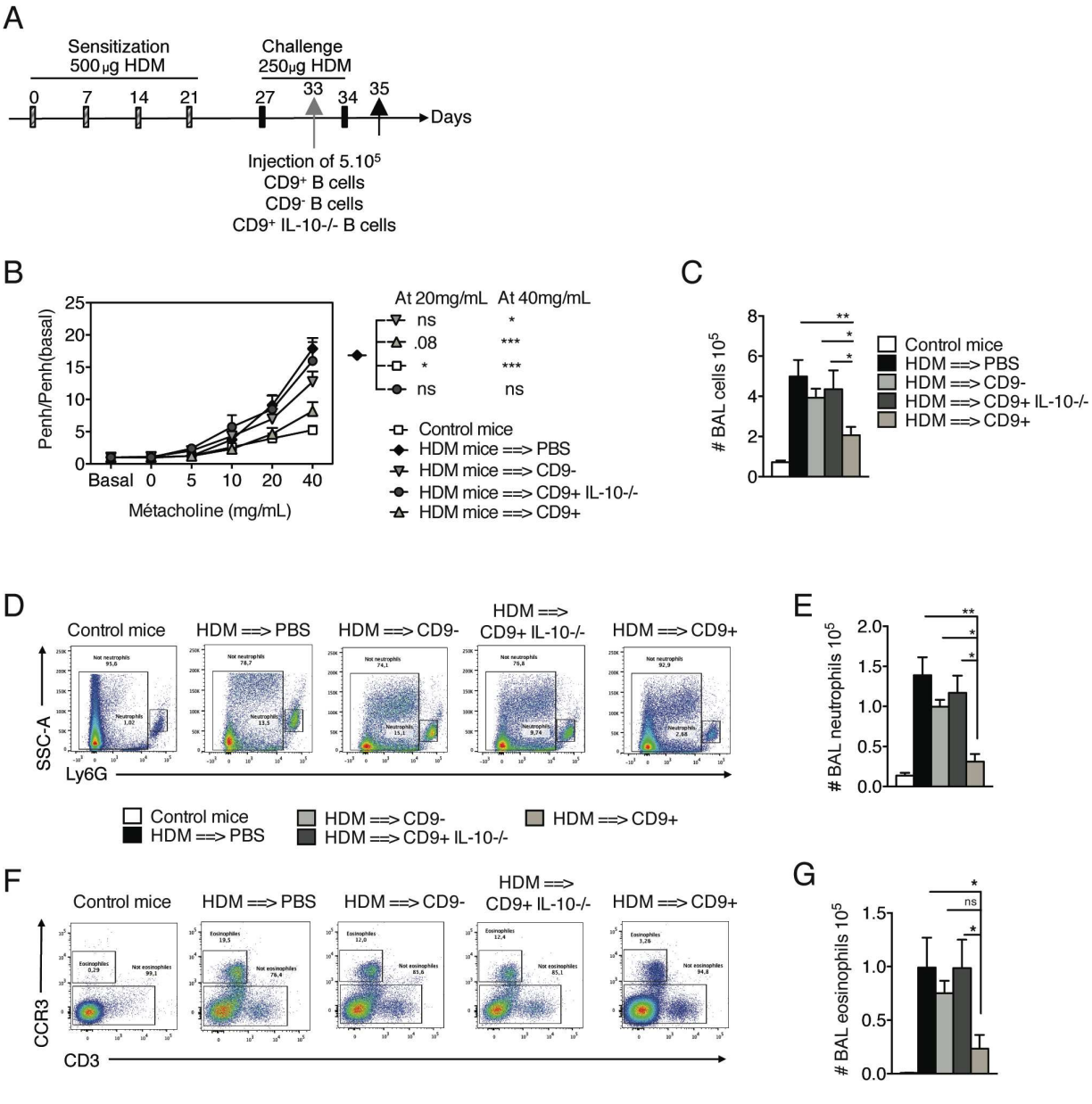
A
Sensitization
500 µg HDM
Challenge
250µg HDM
0
7
14
21
27
33
34
35
Days
Injection of 5.10⁵
CD9⁺ B cells
CD9⁻ B cells
CD9⁺ IL-10-/- B cells
B
Penh/Penh(basal)
Basal 0 5 10 20 40
Métacholine (mg/mL)
At 20mg/mL At 40mg/mL
ns *
.08 ***
* ***
ns ns
Control mice
HDM mice ==> PBS
HDM mice ==> CD9-
HDM mice ==> CD9+ IL-10-/-
HDM mice ==> CD9+
C
BAL cells 10⁵
Control mice
HDM ==> PBS
HDM ==> CD9-
HDM ==> CD9+ IL-10-/-
HDM ==> CD9+
D
Control mice
HDM ==> PBS
HDM ==> CD9-
HDM ==> CD9+ IL-10-/-
HDM ==> CD9+
SSC-A
Ly6G
Control mice
HDM ==> PBS
HDM ==> CD9-
HDM ==> CD9+ IL-10-/-
HDM ==> CD9+
E
BAL neutrophils 10⁵
F
Control mice
HDM ==> PBS
HDM ==> CD9-
HDM ==> CD9+ IL-10-/-
HDM ==> CD9+
CCR3
CD3
G
BAL eosinophils 10⁵
ns

Figure 7

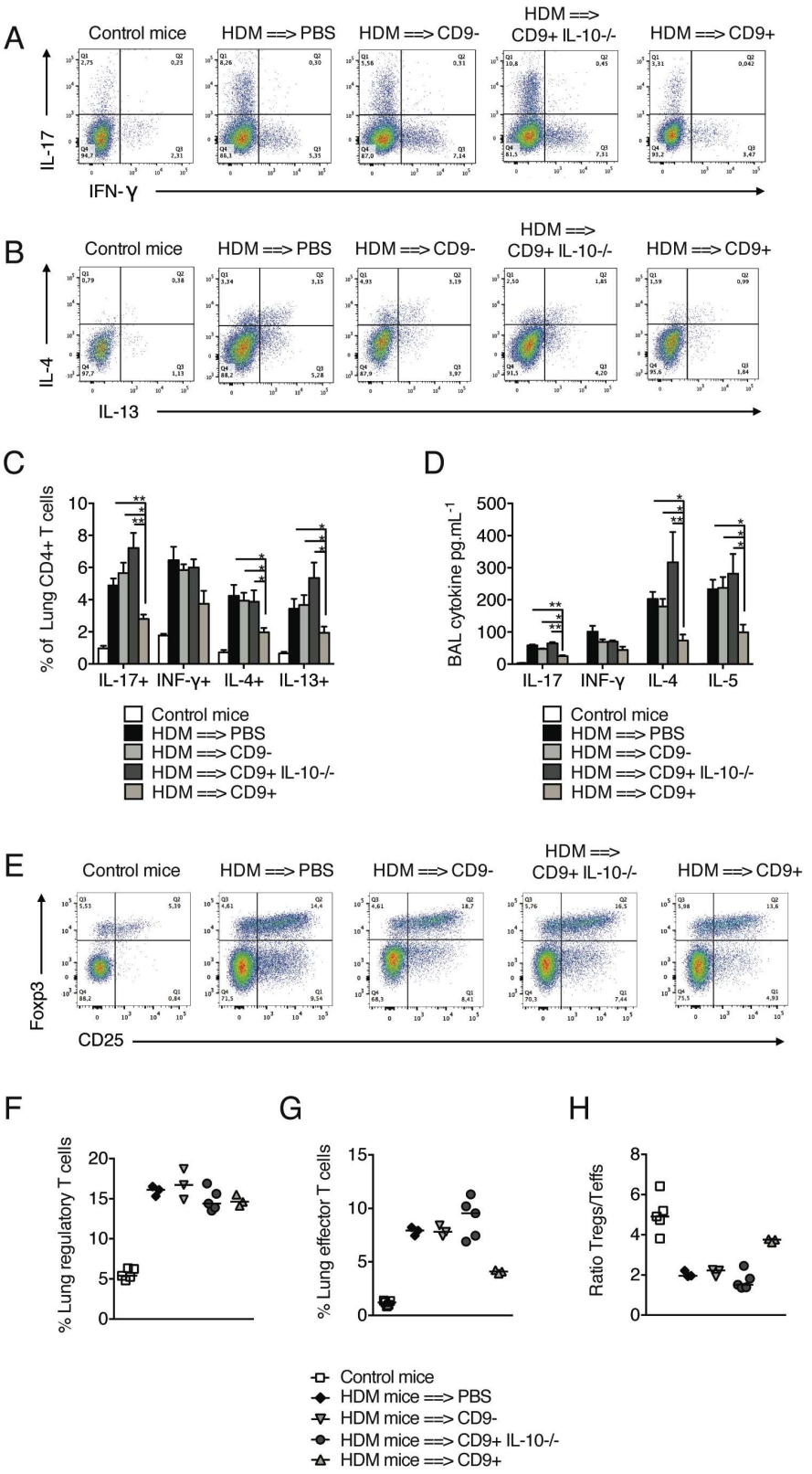
A
Control mice
HDM ==> PBS
HDM ==> CD9-
HDM ==> CD9+ IL-10-/-
HDM ==> CD9+
IL-17
IFN-γ
B
IL-4
IL-13
C
% of Lung CD4+ T cells
IL-17+
INF-γ+
IL-4+
IL-13+
D
BAL cytokine pg.mL-1
IL-17
INF-γ
IL-4
IL-5
E
Foxp3
CD25
F
% Lung regulatory T cells
G
% Lung effector T cells
H
Ratio Tregs/Teffs
Control mice
HDM mice ==> PBS
HDM mice ==> CD9-
HDM mice ==> CD9+ IL-10-/-
HDM mice ==> CD9+

Figure 8

Conclusions et perspectives

Les premières données générées dans notre modèle démontrent ainsi une accumulation des cellules B dans les voies aériennes associée à une production locale d'IgE anti-Der f1, le peptide immunodominant de l'extrait total d'acariens utilisé dans ce modèle. Ceci démontre la mise en place d'une réponse B locale et renforce ainsi l'idée d'une immunologie tissulaire. Ce constat est vif dans le cadre de l'immunité infectieuse où la réponse tissulaire est essentielle et suffisante pour combattre les infections respiratoires (GeurtsvanKessel et al., 2009; Halle et al., 2009; Moyron-Quiroz et al., 2006). Dans le cadre de l'asthme, la présence et le rôle de ces organes lymphoïdes restent controversés (Randall, 2010). Dans notre modèle on observe bien une accumulation de B dans les tissus pulmonaires. Néanmoins, la maturation des follicules n'est pas complète chez les souris asthmatiques. En effet, le réseau de cellules dendritiques est diffus et non structuré (Figure 16). Il est fort probable que la mise en place d'un modèle chronique permettrait d'étudier de manière plus approfondie la maturation locale des cellules B. Or des données préliminaires montrent que l'exposition à un 3ème challenge exacerbe l'infiltrat B et structure les follicules (Figure 16). L'identification des mécanismes, qui régulent l'attraction et la maturation des lymphocytes B dans les poumons, est une voie de recherche intéressante dans le cadre de l'asthme. Dans notre modèle nous montrons par exemple que CXCL13 est augmentée après exposition à l'allergène. Par ailleurs, les données bibliographiques démontrent que la neutralisation de cette chimiokine inhibe le recrutement des lymphocytes B et atténue l'inflammation bronchique (Baay-Guzman et al., 2012). Les mécanismes d'induction de CXCL13 en pathologie sont très peu décrits. Evidemment, la lymphotoxine joue un rôle primordial dans la production de celle-ci. Néanmoins, il est fort probable que d'autres molécules régulent la production de CXCL13. Dans notre modèle, des données préliminaires montrent par exemple que la neutralisation de l'IL-17 diminue l'infiltrat B dans les voies aériennes en réduisant l'expression et la production de CXCL13 (Figure 16).

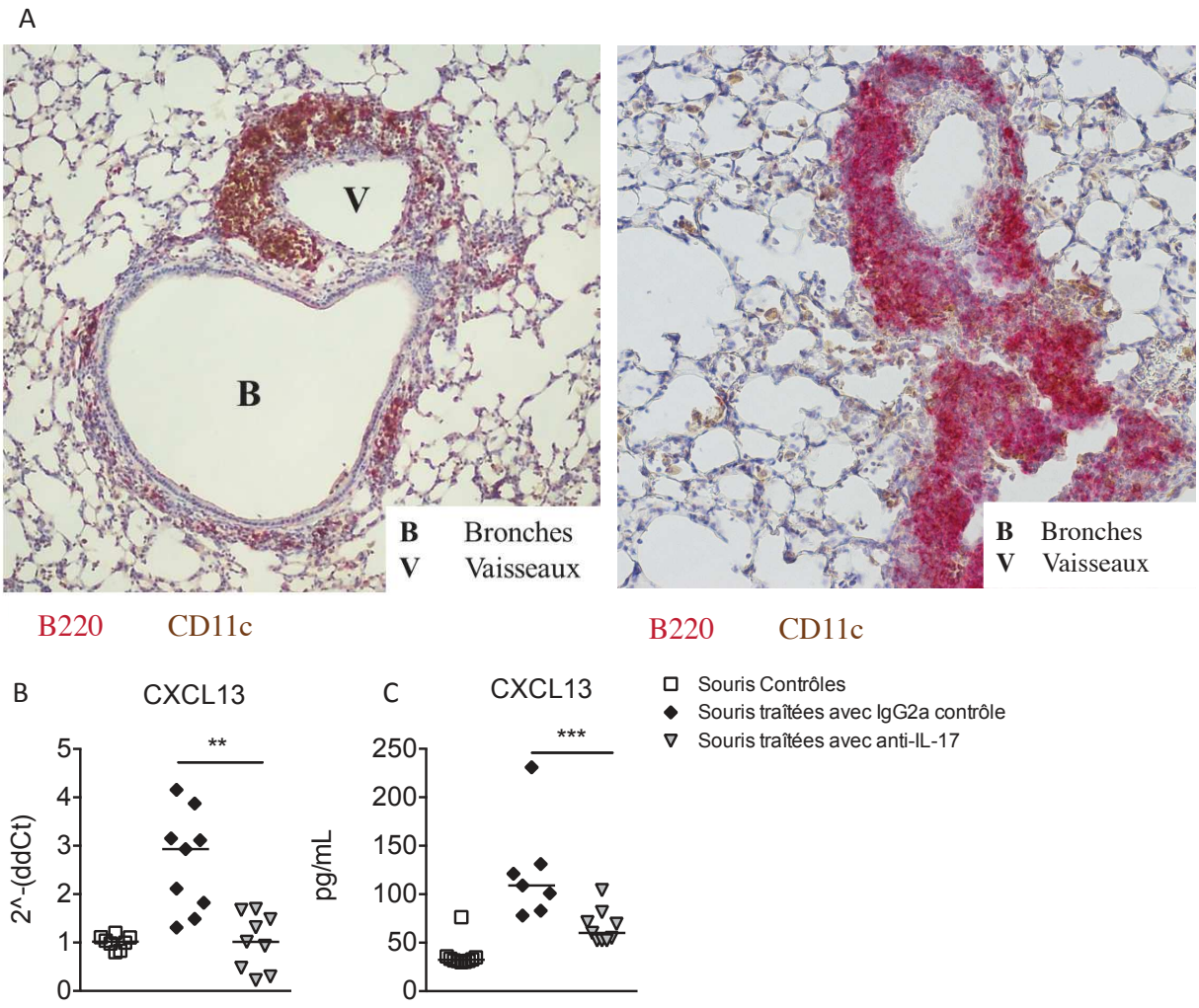

Figure 16 : Un rôle de l'IL-17 dans la maturation et l'attraction des cellules B ? A, Immunohistochimie sur coupes pulmonaires fixées. (Panel de gauche). Marquage des cellules B et cellules dendritiques sur des poumons de souris allergiques ayant reçu trois challenges. (Panel de droite). Marquage des cellules B et cellules dendritiques sur des poumons de souris allergiques ayant reçu deux challenges. Vous pouvez noter la plus grande intensité de marquage CD11c chez des souris ayant reçu trois challenges suggérant une plus grande structuration des follicules lymphoïdes. **B,** Expression dans le tissus pulmonaire et production dans le lavage bronchoalvéolaire de CXCL13 après blocage de l'IL-17 chez des souris ayant reçu deux challenges acariens.

L'IL-17 serait donc un facteur important dans l'attraction des cellules B. Des résultats similaires ont été récemment obtenus dans des modèle murins d'infections (Fleige et al., 2014; Rangel-Moreno et al., 2011). Dans le premier travail cité, l'exposition au LPS, chez des souriceaux, induit la formation d'organes lymphoïdes tertiaires dans les poumons. La génération de ces structures est fortement altérée chez des souris IL-17RA-/- (Rangel-Moreno et al., 2011). En neutralisant l'IL-17, les auteurs réduisent le

nombre de ces structures et diminuent fortement l'expression de CXCL13 (Rangel-Moreno et al., 2011). Dans une autre étude, l'infection à *P. aeruginosa* provoque la formation de structures lymphoïdes tertiaires dépendantes de l'IL-17. Cependant, dans ce modèle, l'IL-17 ne régule pas CXCL13 mais CXCL12, une chimiokine pro-B produite en grande quantité par les cellules stromales des poumons. Ceci démontre que l'IL-17 est bien centrale dans l'attraction des cellules B mais que les mécanismes sous-jacents dépendent du type de pathologie (Fleige et al., 2014). Par conséquent des études approfondies sur les mécanismes d'attraction et de maturation des cellules B dans les voies aériennes donneront de nouvelles pistes thérapeutiques dans l'asthme.

Le deuxième résultat important de ce travail est l'altération du profil cytokinique des cellules B. En effet, nous avons démontré que l'exposition à l'acarien induisait un profil pro-inflammatoire des cellules B du poumon et de la rate des souris. Ceci fait sens avec la description faite en introduction des lymphocytes Be et de leur rôle en immunopathologie. A notre connaissance c'est le premier travail qui rapporte ce type de profil dans un modèle d'asthme. L'augmentation de la production d'IL-4, IL-6 et de TNF-a suggère ainsi que les cellules B peuvent participer directement à la régulation des réponses T inflammatoires. L'utilisation de modèles KO conditionnels permettrait de répondre à cette hypothèse. L'application de notre modèle sur des souris $CD19^{Cre}IL\text{-}6^{flox}$ (Molnarfi et al., 2013) démontrerait le rôle des Be IL-6 dans la réponse T_H17, qui est par ailleurs prédominante dans notre modèle d'asthme aux acariens (Chesné et al., 2014). Ce profil B inflammatoire est associé à une altération dans le profil B régulateur. En effet nous observons que les cellules B de souris allergiques produisent moins d'IL-10 après activation *ex vivo*. La balance cytokinique des lymphocytes B est ainsi à la faveur des cytokines pro-inflammatoires. En accord avec cette hypothèse, nous démontrons que les cellules B des souris allergiques suppriment moins la prolifération des cellules T *in vitro*. Le mécanisme de suppression est dépendant de l'IL-10. Il est probable ici que la plus forte production de cytokines pro-inflammatoires vienne contrebalancer l'effet de

l'IL-10. Ainsi, on passerait d'un profil B tolérogène chez les souris contrôles à un profil B pathogénique chez les souris asthmatiques.

Dans une dernière partie nous nous sommes intéressés à la caractérisation des Bregs. Bien que certains marqueurs phénotypiques aient été décrits ces dernières années (Braza et al., 2014), nous sommes partis sans à priori et avons opté pour une approche pan-génomique. Le transcriptome des cellules productrices d'IL-10 a ainsi été analysé dans l'objectif d'identifier des marqueurs de surface spécifiques. De manière assez surprenante, les marqueurs classiquement décrits (CD5, CD1d, CD21, CD23, TIM-1, CD138) ne sont pas exprimés de façon différente entre les B IL-10^{+} et les B IL-10^{-}. Au contraire, nous avons identifié les molécules CD9, CD80, CD70, CD73 comme potentiels marqueurs phénotypiques des Bregs. Les expériences de confirmation, à l'échelle protéique, ont permis de proposer le CD9 comme marqueur d'enrichissement des Bregs à la fois chez la souris et chez l'homme. En effet, la molécule CD9 est surexprimée sur les cellules B immatures humaines, plusieurs fois décrites pour leurs capacités régulatrices (Blair et al., 2009 ; Flores-Broja et al., 2013). Chez la souris, l'injection de ces cellules permet de contrôler le développement de l'inflammation bronchique et l'hyperréactivité des voies aériennes. Ceci confirme ainsi l'utilité du CD9 pour identifier les Bregs. Malgré tout, nos résultats ne nous ont pas permis d'identifier « LE » marqueur ultime des Bregs. Et c'est bien la difficulté majeure : l'identification de ces cellules est un débat constant. Si l'on s'attarde sur la bibliographie, on se rend compte que les cellules B10 sont associées à un grand nombre de sous-populations chez la souris, amenant à quelques confusions. Et il est difficile de se faire une opinion sur leur profil phénotypique. Les travaux de Simon Fillatreau supportent l'idée que les Bregs sont des plasmocytes or, pour le moment ses travaux confirment ce phénotype seulement dans un modèle d'infection. Aucune donnée n'a encore permis de confirmer ces observations dans un autre modèle d'immunopathologie ou chez l'homme. Il est probable que ce phénotype soit du au modèle infectieux qui augmente la différenciation des B totaux en plasmocytes et donc des B10. Néanmoins en accord avec cette

hypothèse, de récents travaux démontrent que la production d'IL-10 par les cellules B précède et se maintient au cours de leur différenciation en plasmocytes (Maseda et al., 2012). Ce travail issu de l'équipe de Thomas Tedder reste assez contradictoire avec les autres données générées dans son modèle qui soutiennent un phénotype $CD5^+$ $CD1d^+$ pour les Bregs (Candando et al., 2014). Claudia Mauri décrit un phénotype T2-ZMP ($CD21^+$ $CD23^+$ IgM^+). De manière intéressante la molécule CD9 est exprimée dans les cellules ZM ($CD21^+$ $CD1d^+$), B1a ($CD5^+$) mais également sur les plasmocytes. Les cellules $CD9^+$ regrouperaient ainsi un ensemble de cellules B produisant préférentiellement de l'IL-10 après activation. Ce résultat expliquerait pourquoi avec seulement le CD9 nous identifions plus de B10 qu'avec les phénotypes déjà décrits. Chez l'homme, les données sont similaires avec l'identification de plusieurs phénotypes (Braza et al., 2014 ; Candando et al., 2014 ; Mauri et al., 2011). Ceci suggère fortement que les capacités régulatrices des cellules B peuvent être acquises ou perdues après stimulation, à tout stade de la différenciation lymphocytaire B, par une plasticité fonctionnelle comparable à celle décrite pour les lymphocytes T CD4. La diversité fonctionnelle des Bregs tend à soutenir cette hypothèse. En effet, aujourd'hui, on identifie d'autres sous-populations B régulatrices produisant de l'IL-35, du TGF-β ou du Granzyme (Braza et al., 2014; Chesneau et al., 2013). La stabilité des fonctions régulatrices des LB dans le temps est également une donnée inconnue. Dans les modèles murins, l'injection des B10 à un instant « t » permet de contrôler l'inflammation. Mais qu'en est-il dans le cadre d'une inflammation chronique ? Est ce qu'à l'instar des Tregs leur fonction suppressive s'amoindrie ou encore ces cellules se différencient-elles plutôt en lymphocytes Be ?

Il est clair que l'identification *in vivo* des mécanismes moléculaires, permettant ou inhibant la formation d'un sous type de cellules Breg, permettrait de répondre à certaines de ces questions.

Vaccination with Hypoallergenic Der p 2 peptides improves respiratory function and airway inflammation in a mouse model of house dust mite-induced asthma

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Letter to the editors

House dust mites (HDM) area major source of allergens affecting more than 50% of allergic patients [1, 2]. The *Dermatophagoides pteronyssinus* allergen, Der p 2, is one of the most representative HDM allergens, which is recognized by more than 90% of HDM-allergic patients [1, 2]. Continuous exposure to this allergen leads to the development of asthma, a pathology characterized by bronchoconstriction and airway inflammation. Genetic engineering allows the development of new therapeutic recombinant hypoallergenic derivatives with lower IgE and T cell reactivity [3]. Accordingly it was recently described that two recombinant hypoallergenic peptides derived from Der p 2 exhibited less *in vivo* allergenicity than the Der p 2 allergen but preserved immunogenicity [1, 2]. Thus, hypoallergenic peptides may represent candidates for specific immunotherapy especially in HDM allergic asthma.

To investigate whether Der p 2-derived peptides could modulate asthma in mice, we tested the therapeutic effect of three recombinant Der p 2 hypoallergenic derivatives in a mouse model of HDM-induced asthma: Der p 2.1 (aminoacids 1–53), Der p 2.2 (aminoacids 54–129) and a hybrid molecule: Der p 2.2+ 2.1. Mice have been vaccinated with one of these three peptides or an irrelevant peptide as control (major birch pollen allergen, Bet v 1) 10 days before a first sensitization to HDM extract and one day before the fourth sensitization (Figure 1A). All three recombinant peptides decreased airway hyperresponsiveness in response to metacholine suggesting that these peptides may influence the course of the disease. Interestingly the Der p 2.1 peptide showed stronger effect than other hypoallergenic derivatives (Figure 1B).

In a next step we analysed airway inflammation and found a dramatic decrease of cellular infiltrate in the bronchoalveolar lavage (BAL) of Der p 2.1 vaccinated mice with notably an important drop in eosinophil and neutrophil counts (Figure 1C-E). Altogether these results validate the therapeutic effect of the hypoallergenic Der p 2.1 peptide and suggest its influence in the development of HDM allergic asthma by controlling airway inflammation and restoring lung function.

To assess this hypothesis we focused on the Der p 2.1 peptide in our next experiments. Der p 2.1 peptide vaccination improved lung function and decreased inflammation (Figure 2). Indeed lung invasive experiments revealed a significant improvement in lung resistance and elastance of mice treated with Der p 2.1 derivate when compared to mice treated with control peptide (Figure 2A and B). Having found that Der p 2.1 vaccinations dampen immune cell recruitment in the airways, we analysed levels of inflammatory chemokines in BAL. We found a significant decrease in the production of MCP-1 (CCL2), Eotaxin (CCL11) and KC (CXCL1) explaining the dampened infiltrate observed in Der p 2.1 vaccinated mice (Figure 2C).

As this hypoallergenic peptide has been described as a poor inducer of T cell proliferation and activation [1, 2], we investigated by flow cytometry whether vaccination could modulate primary T-cell responses to inhaled allergen, primed in the lung tissue. Interestingly we found a decreased frequency of IL-4 and IL-17 producing $CD4^+$ T cells (Figure 2D), two T cell subsets described for their deleterious effects in allergic asthma [4]. No effect was observed in frequency of IL-13^+ or IFN-y^+ $CD4^+$ T cells (Figure 2D). In addition we found no IgE reactivity in the BAL of Der p 2.1-vaccinated mice confirming previous reports demonstrating the lack of IgE reactivity towards this recombinant hypoallergenic derivative (Figure 2E) [1, 2]. However, contrary to recent results we do not report on an increase of the inhibitory Der p 2 specific IgG_1 (Figure 2E) [5]. This result could be explained by B cells needs IL-4 to produce both IgG1 and IgE. Consequently by decreasing IL-4 producing T cells, vaccination with Derp 2.1 also reduce the production of T_H2 related immunoglobulin. Given their structural homology, Der p and Der f antigens demonstrate an important IgE cross-reactivity [6]. Based on this, we lastly investigated whether vaccination with Der p 2.1 peptide could control the development of Der f-induced airway hyperresponsiveness. Thus, vaccination with this peptide inhibited bronchial hyper-reactivity similarly to what obtained after vaccination in a Der p-induced asthma model (Figure 2F).

Collectively these data support a potent therapeutic effect of Der p 2.1 vaccination in the development of asthma by improving lung function, dampening airway inflammation,

influencing lung T cell responses and inhibiting IgE responses. By inhibiting production of IL-4 by T cells Der p 2.1 vaccination probably dampen IgE production leading to a global attenuation of airway inflammation. Lower levels of IgE leads to less allergen-IgE pathogenic complexes and so less activation of innate cells as eosinophils, mastocytes and basophils. The fact that IgG1 is also decreased is intriguing. It is probably due to the fact that IL-4 is also involved in the production of IgG1 in mice. Another interesting finding is the fact that vaccination also dampens the frequency of T_H17 cells in lungs. Given the potent role of these cells in severe asthma [7], this therapeutic strategy could represent an interesting alternative in some case of severe allergic asthma. Most interestingly we demonstrated that vaccination with Der p 2.1 could be effective in Der f asthmatic mice. Consequently this vaccine may reduce therefore IgE-mediated but also T cell-mediated allergic inflammation irrespectively of HDM species.

Bibliography

1. Chen KW, Blatt K, Thomas WR, Swoboda I, Valent P, Valenta R, et al. Hypoallergenic Der p 1/Der p 2 combination vaccines for immunotherapy of house dust mite allergy. J Allergy Clin Immunol 2012; 130:435-43 e4.
2. Chen KW, Focke-Tejkl M, Blatt K, Kneidinger M, Gieras A, Dall'Antonia F, et al. Carrier-bound nonallergenic Der p 2 peptides induce IgG antibodies blocking allergen-induced basophil activation in allergic patients. Allergy 2012; 67:609-21.
3. Valenta R, Ferreira F, Focke-Tejkl M, Linhart B, Niederberger V, Swoboda I, et al. From allergen genes to allergy vaccines. Annu Rev Immunol 2010; 28:211-41.
4. Botturi K, Langelot M, Lair D, Pipet A, Pain M, Chesne J, et al. Preventing asthma exacerbations: what are the targets? Pharmacol Ther 2011; 131:114-29.
5. Linhart B, Narayanan M, Focke-Tejkl M, Wrba F, Vrtala S, Valenta R. Prophylactic and therapeutic vaccination with carrier-bound Bet v 1 peptides lacking allergen-specific T cell epitopes reduces Bet v 1-specific T cell responses via blocking antibodies in a murine model for birch pollen allergy. Clin Exp Allergy 2014; 44:278-87.
6. Heymann PW, Chapman MD, Aalberse RC, Fox JW, Platts-Mills TA. Antigenic and structural analysis of group II allergens (Der f II and Der p II) from house dust mites (Dermatophagoides spp). J Allergy Clin Immunol 1989; 83:1055-67.
7. Chesne J, Braza F, Mahay G, Brouard S, Aronica M, Magnan A. IL-17 in Severe Asthma: Where Do We Stand? Am J Respir Crit Care Med 2014.

Materials and methods

Animal procedures - Balb/c female mice 6-8 weeks of age were purchased from Charles River breeding laboratories and used for all experiments. They were housed in UTE IRS-UN platform (Nantes, France). They were maintained under specific pathogen-free, temperature-controlled conditions with a strict 12 hours light-dark cycle and were given free access to food and water. All protocols were approved by the Regional Ethical Committee for Animal Experiments of Pays de la Loire (ceea.2012.77). Mice were sensitized on days 0, 7, 14 and 21 by percutaneous application of 500 µg of total extract of Dermatophagoïdes pternyssinus (Der p) or Dermatophagoïdes farinaes (Der f) (Stallergenes, Antony, France) in 20 µL of dimethylsulfoxyde (DMSO, Sigma-Aldrich, St. Louis, MO, United States) on the ears without any synthetic adjuvant. They were challenged intranasally with 250 µg of Der p in 40 µL of sterile PBS, once on D27 to induce asthma and once more on D34 to induce asthma exacerbation. Animals were anesthetized with 100µl of xylazine (ROMPUN® 2%, Bayer, 15mg/kg) intraperitoneally and 100µl of ketamine (IMALGENE®1000, Merial, 80 mg/kg) for both sensitizations and challenges. Mice were sacrificed by sublethal injection of dolethal (DOLETHAL®, vetoquinol, France) on D35 and D37 (Fig 1). For dynamic lung resistance measurements, mice were anesthetized with 200 µl of a ketamine-xylazine mix and paralyzed with 100 µl of rocuronium bromide (ESMERON®, Organon, 10 mg / ml) intraperitoneally.

Derivative peptide injection - Derivative peptides (Der p 2.1) and control (Bet v 1.2) peptides were purified as previously described by Chen et al (Mol Immunol, 2008). Peptides were injected 10 days before the first sensitization and 20 days later. Der p 2.1 was solubilized with 10mM NaH2PO4, pH 7 solution to a final concentration of 400 µg/ml. Bet v 1.2 was used as control and was solubilized with PBS to a final concentration of 150 µg/ml. 200ul of a solution of Alu-gel (Alu-Gel-S, SERVA Electrophoresis) containing 5 µg of peptides were injected subcutaneously in the neck of mice.

BAL preparation - 1 mL of sterile PBS was administered intra-tracheally in mice through flexible catheter. Cells and supernatants from removed fluid were separated by centrifugation. Total cell number was determined by optical microscopy and analyze of BAL cell composition was done by Flow cytometry (see below). Supernatants were stored at -80°C for cytokines assays.

Flow cytometry analysis - Lungs and spleen were recovered from asthmatic mice or controls 1 or 3 days after the last challenge and placed into RPMI. Cells were then isolated by passing tissues through 40 μm filter. Cells were first washed with RPMI and next with PBS-FBS 5%. Spleen cells were depleted of red blood cells by treatment with ammonium chloride prior to second wash. After counting, cells were stained for 20 minutes with titrated specific antibodies in PBS-FBS 5%. The anti-mouse antibodies used included: CD3-APC, CD19 PE-Cy7, CD11c PE-Cy7, F4/80 FITC, CD80 FITC, CD86 PE, Ly6G PerCP-Cy5.5 and CD45R PerCP-Cy5.5 (purchased from eBiosciences) ; CD8 APC-H7 and CD19 APC-H7 (purchased from BD Biosciences) ; CCR3 PE (purchased from R&D). Dead cells were excluded using DAPI. Stained cells resuspended in PBS were acquired on a BD LSR™ II (BD Biosciences) and analysed on BD FACSDiva™ software (BD Biosciences). The same protocol was used for BALF cell analysis.

Flow cytometry – For analysis of BAL cell, the following antibodies were used: CD3-APC, CD19 PE-Cy7, CD11c PE-Cy7, F4/80 FITC, CD80 FITC, CD86 PE, Ly6G PerCP-Cy5.5 and CD45R PerCP-Cy5.5 (purchased from eBiosciences) ; CD8 APC-H7 and CD19 APC-H7 (purchased from BD Biosciences) ; CCR3 PE (purchased from R&D). Dead cells were excluded using DAPI. Stained cells resuspended in PBS were acquired on a BD LSR™ II (BD Biosciences) and analysed on BD FACSDiva™ software (BD Biosciences). To study lung T cell responses, the lungs were removed, disrupted and digested at 37°C for 1H in 10 mL of PBS containing Dnase I (Roche) and collagenase II (Invitrogen). Lung cells were passed through a 40-µm cell strainer with a syringe plunger, and the strainer was washed with 10mL of RPMI 1640 media supplemented 10% FBS, 200 mg/mL penicillin, 200 U/mL streptomycin, 4 mM L-glutamine, and 0.05

mM β-mercaptoethanol. After red blood lysis, 5.10^5 lung cells were transferred to a 96-well round-bottom plate and were stimulated for 5H with a mix containing PMA (50 ng/mL) and ionomycin (500 ng/mL; Sigma-Aldrich) together with either monensin for T_H17 analysis (2 mg/mL; BD Bioscience) or brefeldin A for T_H2 assessment (1 mg/mL; BD Bioscience). For cytokine detection, FcRs were blocked with mouse CD16/CD32 mAbs (ebioscience, Paris, Fr). Prior to surface specific staining, the cells were stained with Fixable Viability Dye 450 (BD Biosciences) to exclude dead cells. The following antibodies were used for surface staining: CD3 PeCy7 (145-2C11;BD Biosciences), CD8 APC-H7 (53-6.7; BD Biosciences). The cells were then fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences) and stained intracellularly with anti-IL-4 (11B11; eBioscience), -IL-13 (eBio13A; eBiosciences), and -IL-17A (TC11-18H10; BD Biosciences).

Airway hyper-reactivity (AHR) measurement - Unrestrained mice were nebulized with increasing doses of methacholine (Mch) (Sigma-Aldrich) from 0 to 40 mg/mL. Enhanced pause (Penh) was measured by whole body plethysmography (Emka Technologies, Paris, France). Dynamic lung resistances were measured using a flexiVent® (SCIREQ). Mice were anesthetized and connected via the endotracheal cannula to a *flexiVent* system. After initiating mechanical ventilation, the mouse was paralyzed with ip injection of 0.1 ml of a 10 mg/ml solution of rocuronium bromide. The animal was ventilated at a respiratory rate of 150 breaths/min and tidal volume of 10 ml/kg against a positive end expiratory pressure (PEEP) of 3 cmH2O. Respiratory mechanics were assessed using a 1.2 second, 2.5 Hz single-frequency forced oscillation maneuver and a 3 second, broadband low frequency forced oscillation maneuver containing 13 mutually prime frequencies between 1 and 20.5 Hz. The settings of both perturbations were configured to ensure that onset transients were omitted and the oscillations had reached steady state in the analyzed portions of the maneuvers. Respiratory system resistance (R) were calculated in the *flexiVent* software by fitting the equation of motion of the linear single compartment model of lung mechanics using multiple linear regressions. Both maneuvers were executed every 15s in alternation after each MCh aerosol challenge to

capture the time course and the detailed response of the MCh-induced bronchoconstriction.

Cytokines quantification - Cytokines concentrations in BAL or spleen cell culture supernatants were quantified by Luminex® technology (BioPlex 200 system, Bio-Rad), using the Pro Mouse Cytokine 23-plex kit (Bio-Rad Laboratories, Munich, Germany). Assays were performed according to the manufacturer's specifications. Data analysis was performed using the Bio-Plex Manager Software version 4.0.

Statistical analysis - All statistical analysis were conducted using GraphPad Prism 4.0b (Graphpad Software Inc., La Jolla, CA USA). All data are expressed as mean ± standard error of the mean (SEM). Unless otherwise specified, differences in airway response between groups were tested for statistical significance using ANOVA. The Mann-Whitney test was used for other analysis. A *p* value less than 0.05 was considered statistically significant.

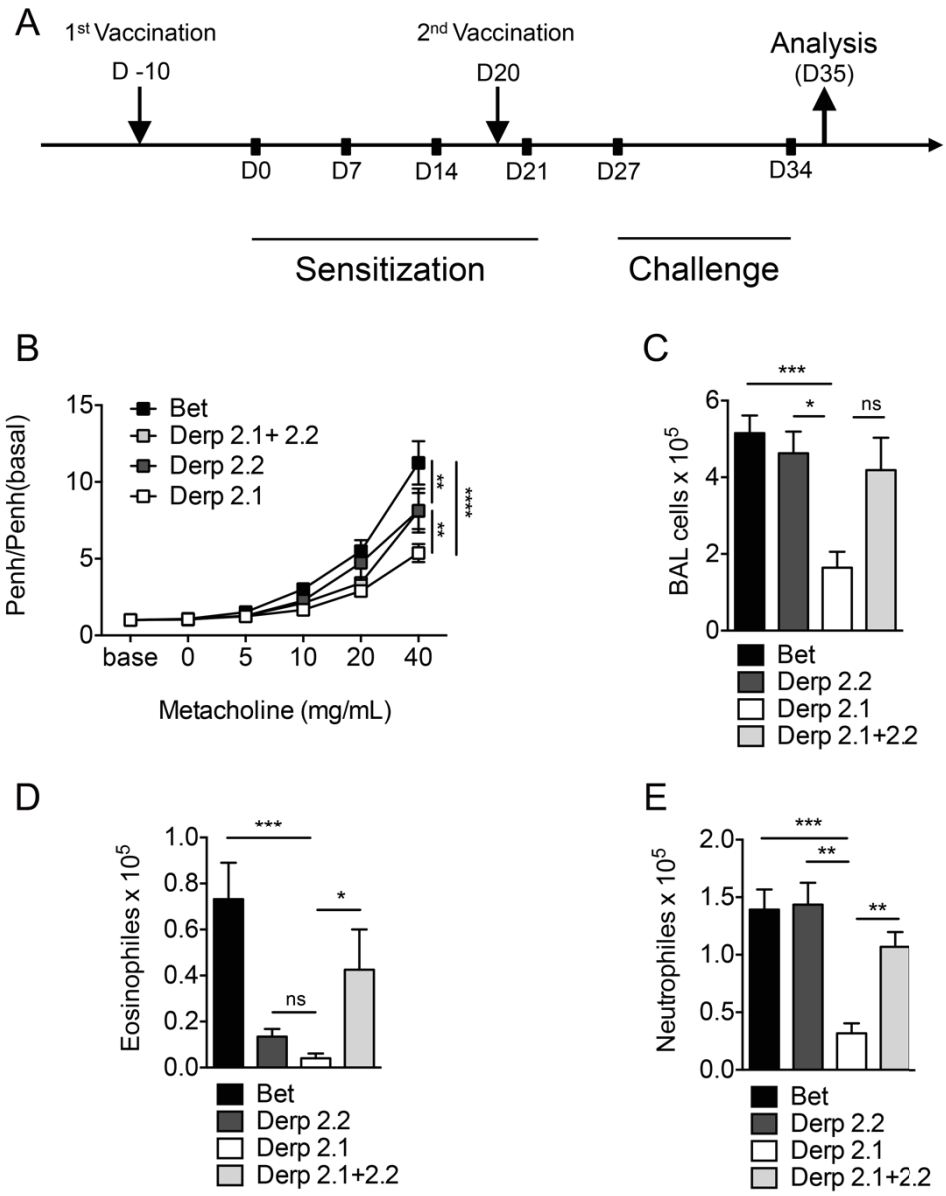

Figure 1: Derp 2.1 exhibits some therpeutic potential in a mouse model of Dermatophagoïdes pternyssinus induced asthma. A, Schematic representation of the HDM antigen–allergic model. B, Penh values (n=8-12 mice per group). C, Total BAL cell numbers (n=8-12 mice per group). D, BAL eosinophil values (n=8-12 mice per group). E, BAL neutrophils values (n=8-12 mice per group).

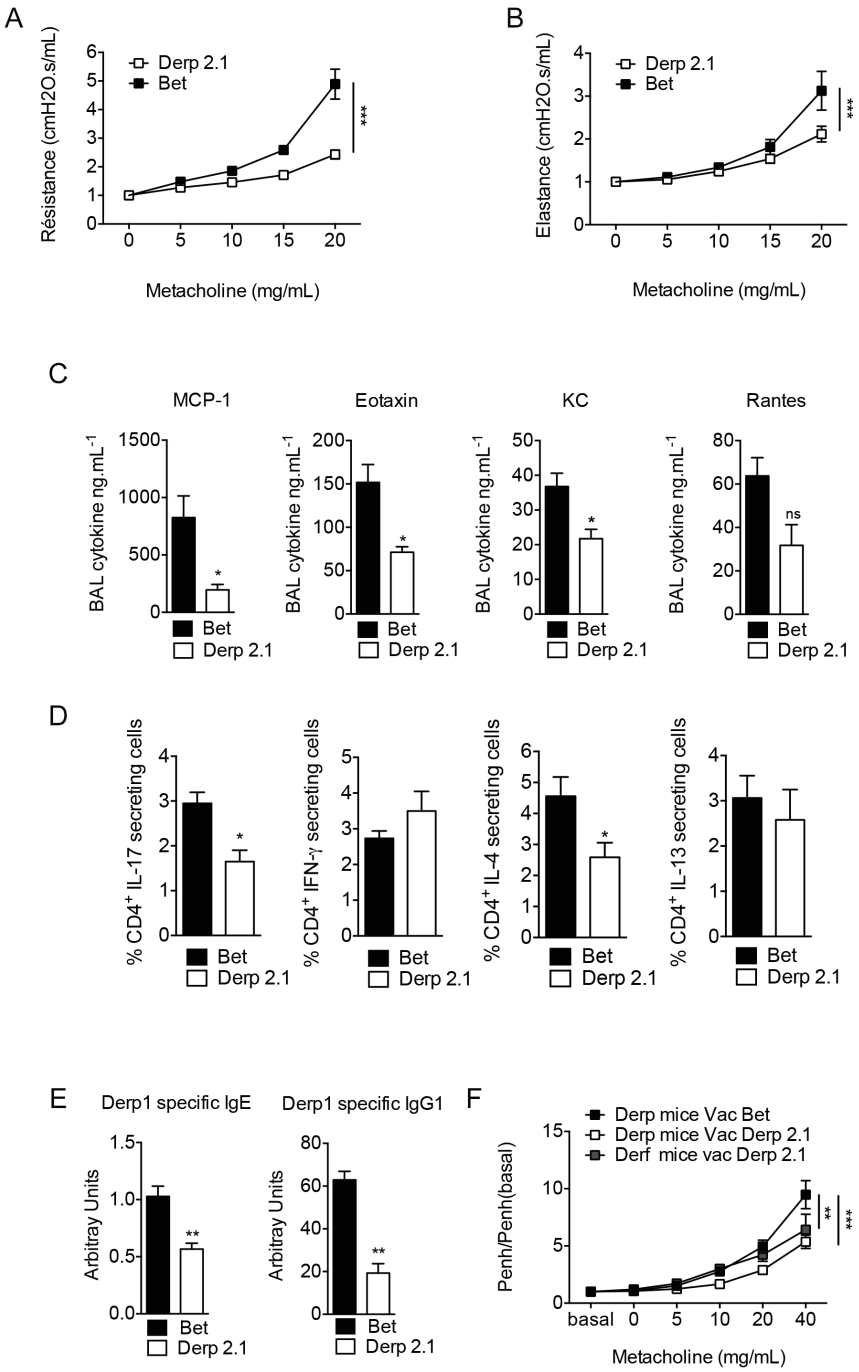

Figure 2: **Vaccination with Derp 2.1 normalizes lung fonction and influences airway inflammation**. A and B, Resistance and elastance values obtained by invasive methods. C, BAL levels of chemokines after vaccination. D, T cell responses in lung mucosa. E, Serum levels of Derp1 specific IgE after Derp2.1 vaccination. F, Penh values obtained after vaccination with Derp2.1 in mouse model of Dermatophagoïdes farinaes induced asthma.

IL-17 in severe asthma: where do we stand?

Chesné J*, **Braza F***, Mahay G, Brouard S, Aronica M, and Magnan A

In Press AJRCCM

Abstract

Asthma is a major chronic disease ranging from mild to severe refractory disease and is classified into various clinical phenotypes. Severe asthma is difficult to treat and frequently requires high doses of systemic steroids. In some cases, severe asthma even responds poorly to steroids. Several studies have suggested a central role of IL-17 (also called IL-17A) in severe asthma. Indeed, high levels of IL-17 are found in induced sputum and bronchial biopsies obtained from severe asthmatics. The recent identification of a steroid-insensitive pathogenic T_H17 pathway is therefore of major interest. In addition, IL-17A has been described in multiple aspects of asthma pathogenesis, including structural alterations of epithelial cells and smooth muscle contraction. In this perspective article, we frame the topic of IL-17A effects in severe asthma by reviewing updated information from human studies. We summarize and discuss the implications of IL-17 in the induction of neutrophilic airway inflammation, steroid insensitivity, the epithelial cell profile, and airway remodeling.

PULMONARY PERSPECTIVE

IL-17 in Severe Asthma
Where Do We Stand?

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Abstract

Asthma is a major chronic disease ranging from mild to severe refractory disease and is classified into various clinical phenotypes. Severe asthma is difficult to treat and frequently requires high doses of systemic steroids. In some cases, severe asthma even responds poorly to steroids. Several studies have suggested a central role of IL-17 (also called IL-17A) in severe asthma. Indeed, high levels of IL-17 are found in induced sputum and bronchial biopsies obtained from patients with severe asthma. The recent identification of a steroid-insensitive pathogenic Th17 pathway is therefore of major interest. In addition, IL-17A has been described in multiple aspects of asthma pathogenesis, including structural alterations of epithelial cells and smooth muscle contraction. In this perspective article, we frame the topic of IL-17A effects in severe asthma by reviewing updated information from human studies. We summarize and discuss the implications of IL-17 in the induction of neutrophilic airway inflammation, steroid insensitivity, the epithelial cell profile, and airway remodeling.

Keywords: severe asthma; IL-17; steroid insensitivity; neutrophil inflammation; airway remodeling; smooth muscle contraction

> "Truth has many faces" . . . so does asthma
>
> —Marion Zimmer Bradley, *The Mists of Avalon*

Asthma has long been considered a single homogenous disease characterized by chronic deregulated airway inflammation and bronchial hyperresponsiveness, leading to recurrent chest wheezing, cough, and shortness of breath. However, cluster analysis of large cohorts allowed the identification of several asthma phenotypes defined according to clinical and functional characteristics (1). These phenotypes roughly correspond to various pathophysiological pathways, or endotypes (1, 2). To what extent does this heterogeneity of asthma impact patient management and care? Oral (OCS) or inhaled (ICS) corticosteroids and long-acting bronchodilators are the only current therapies that are typically used for all types of asthma. However, high doses of ICS and sometimes OCS cannot control severe asthma in some patients. Moreover, steroid-insensitive or partially sensitive patients are found in some of these subgroups (3–5). For such patients, the precise description of their disease and their categorization into well-characterized subpopulations could facilitate the development of stratified and targeted therapies. Thus, the need to better characterize patients with asthma to improve their clinical management is greater than ever (6). The active development of "omics" as efficient tools with which to refine subgroups of patients together with the emergence in clinics of molecule-targeted biotherapies make this strategy of stratifying asthma care using a personalized medicine approach highly relevant.

The recent identification of patients with distinct $Th2^{high}$ and $Th2^{low}$ asthma is of particular interest based on this perspective of future stratified care (7). Th2-dependent allergic airway inflammation, first identified more than 25 years ago, appears to be the principal pathophysiological pathway. Patients with $Th2^{high}$ asthma appear to be easier to manage because they regularly respond to

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classical therapy; in addition, the patients in this subgroup with more severe asthma can be controlled with newly developed Th2-targeted therapies, such as monoclonal antibodies directed against IgE, IL-5, IL-13, IL-4, or their receptors (7–12). In contrast, $Th2^{low}$ asthma designates a patient subgroup with clinical needs that are poorly met by currently available and developing therapies (7, 13). The underlying mechanisms of the $Th2^{low}$ phenotype remain unknown, but these patients usually display predominant bronchial neutrophilic inflammation. IL-17 (formerly known as IL-17A), a key proinflammatory cytokine of the Th17 pathway, has been proposed to be involved in the neutrophilic inflammation and airway remodeling processes in severe asthma (14, 15). However, multiple new data have been accumulated, and in this paper we synthesize the knowledge on the role of IL-17 in asthma and, particularly, on the implications of IL-17 in neutrophilic inflammation, steroid insensitivity, the epithelial cell profile, and airway remodeling.

IL-17–Producing Cells in Asthma Are No Longer Limited to Th17 Cells

IL-17 is mainly secreted by a distinct $CD4^+$ T helper cell subset, characterized by the specific expression of the transcription factor retinoic acid–related orphan receptor-γt (RORγt). By secreting IL-17, Th17 cells orchestrate the recruitment of neutrophil granulocytes in the lungs and their activation directly through CXCL8 production (16) or indirectly by inducing the production of IL-6, colony stimulatory factors (CSFs) (e.g., G-CSF and GM-CSF), and the chemokines CXCL8 (IL-8), CXCL1, and CXCL5 by airway epithelial cells (17). Although Th17 cells represent a core source of IL-17, a newly identified population of innate lymphoid cells (ILCs) is also able to produce IL-17 (18). ILCs have a morphology that is typical of lymphocytes, but they lack rearranged antigen receptors (19). Currently, ILCs are broadly divided into three subsets depending on their ability to secrete Th1, Th2, and Th17 cell-associated cytokines, including group 1 ILCs (containing ILC1s and natural killer cells), group 2 ILCs (containing ILC2s), and group 3 ILCs (with ILC3s and lymphoid tissue inducer cells) (20). The important role of ILC2s in Th2-mediated allergic lung responses has been well addressed in animals (19, 21–23). However, a recent study provided new insight regarding the role of IL-17–producing ILC3s in asthma. These cells express CC chemokine receptor 6 (CCR6), produce IL-17 and sometimes IL-22, and are central to the development of asthma in obese mice. Increased numbers of this subset of cells have been found in the bronchoalveolar lavage (BAL) fluid of obese individuals with severe asthma (18, 24); however, further investigations in larger studies are needed to confirm this finding. Accumulating data demonstrated the ability of other immune cells to produce IL-17; these cells include B cells (25), neutrophils (26), γδ T cells (27), and natural killer T cells (28). However, their relevance in human asthma remains to be elucidated.

IL-17: A Clue for Severe Neutrophilic Asthma?

Neutrophilia in severe asthma was first described in bronchial biopsy studies that aimed to distinguish eosinophilic asthma from noneosinophilic asthma (29). More recent studies in large cohorts, such as the SARP (Severe Asthma Research Program) cohort, enabled the identification of neutrophilic inflammation through induced sputum as an important hallmark of a distinct cluster of patients with moderate to severe asthma (30). However, it is noteworthy that although neutrophilic inflammation predominates in this cluster, neutrophilia can also coexist with eosinophilia (3, 30, 31). This result emphasizes the complexity of the underlying mechanisms of severe asthma.

A large series of studies suggest that the presence of IL-17 in asthmatic airways is related to asthma severity (Table 1). Indeed, since the first identification of elevated levels of IL-17A in the sputum of patients with asthma (32), many reports have confirmed these findings and demonstrated a positive correlation between IL-17A production and asthma severity (15, 33, 34). IL-17A/F levels and the presence of Th17 cells, mixed Th17/Th2 cell infiltration, and IL-17–producing ILC3 cells in the sputum, BAL, lung biopsies, and peripheral blood have also been positively correlated with asthma severity in adults and children (14, 24, 35–42). Recently, a strong association between high diesel exhaust particle exposure, asthma severity, and the levels of serum IL-17A was reported in children with allergic asthma (43). Notably, although all children were atopic, no correlations between diesel exhaust particle exposure and serum IL-4, IL-5, and IL-13 were found, supporting a central role of IL-17A in these patients and the possible involvement of extrinsic factors in the modulation of asthma phenotypes.

A likely role of IL-17 in neutrophilic asthma is clearly supported by the important association between IL-17 and neutrophilic inflammation in other diseases, such as psoriasis, and by the efficiency of IL-17–targeted therapies against this pathology (44, 45). Regarding the relationships between IL-17 and neutrophilic asthma, a strong correlation between IL-17 and neutrophils was established in induced sputum and blood (15, 36), although the number of $IL\text{-}17^+$ cells did not correlate with the number of neutrophils or eosinophils in the bronchial mucosa assessed in biopsies (35). The link between neutrophilic inflammation and Th17 immunity is better established in mouse models of asthma. Indeed, allergic sensitization through the airways or the skin promotes bronchial Th17 responses and can induce airway hyperresponsiveness (AHR) in mice (46, 47). This response is associated with neutrophilic infiltration and up-regulation of proneutrophil chemokines. Deficiency in IL-17RA or IL-17 leads to an impaired neutrophilic response to allergens, lack of AHR, and reduced airway remodeling (46, 48), suggesting a central role of IL-17 in the physiopathology of certain types of asthma. The factors that drive the production of IL-17A in asthma are not well defined. Nevertheless, a recent study has shown a link between complement activation and the IL-23–Th17 axis in the development of severe asthma (49). This paper demonstrated that complement factor C5a reduces the frequency of IL-17–producing Th17 cells and AHR, whereas complement factor C3a enhances Th17 responses and AHR. A disruption of this delicate balance in favor of the C3a pathway promotes a strong AHR and Th17 response, as demonstrated in mice with severe asthma (49).

The high frequency of mixed neutrophilic-eosinophilic inflammation in

Table 1. Major Clinical Findings Serving as Evidence of a Role of IL-17 in Asthma Severity

Study	Experimental Approach	Results
Al-Ramli *et al.*, 2009 (14)	Immunostaining of IL-17A and IL-17F in lung biopsies from control patients and patients with severe asthma	Higher number of IL-17A–positive cells in patients with severe asthma compared with control groups
Bullens *et al.*, 2006 (15)	Measurement of mRNA levels of IL-17 in sputum of patients with moderate to severe asthma	Increased expression of IL-17 in severe asthma; correlation between IL-17 and neutrophilia
Kim *et al.*, 2014 (24)	Quantification of IL-17 secreting innate lymphoid cells in BAL of patients with severe asthma	Increased numbers of IL-17 secreting ILC3 in BAL of patients with severe asthma
Molet *et al.*, 2001 (32)	Quantification of IL-17 secreting cells in BAL and sputum of patients with asthma	Higher number of cells expressing IL-17 in sputum and BAL of subjects with asthma
Cosmi *et al.*, 2010 (39)	*Ex vivo* quantification of IL-4–, IL-17–producing T cells in patients with severe allergic asthma	Identification of a double IL-4/IL-17–producing T cells subset that is increased in patients with severe asthma
Irvin *et al.*, 2014 (42)	Analysis of Th2/Th17 cells in BAL compartment of patients with severe asthma	Increased frequency of dual-positive Th2/Th17 cells in BAL of patients with severe asthma
Brandt *et al.*, 2013 (43)	Quantification of IL-17 in sera from atopic children with asthma exposed or not to diesel exhaust particles	Significant higher levels of IL-17 in children with asthma exposed to diesel exhaust particles
Nanzer *et al.*, 2013 (63)	Quantification of IL-17–producing $CD4^+$ T cells after *in vitro* activation in presence of steroid	$CD4^+$ T cells from patients with steroid-resistant asthma produce higher levels of IL-17 than patients with steroid-sensitive asthma and healthy volunteers

Definition of abbreviation: BAL = bronchoalveolar lavage.

patients with severe asthma (3, 31) could be related to a mixed Th17/Th2 activation. Accordingly, high levels of periostin, a biomarker related to the $Th2^{high}$ phenotype of asthma, and high levels of IL-6, a pro-Th17 cytokine, were found in the blood of these patients (3). However, recent data obtained from phenotypic clustering of patients with asthma revealed that neutrophil infiltrates in patients with severe asthma are associated with systemic inflammation (50) and a higher expression of the NOD-like receptor family, pyrin domain containing 3 (NLRP3), IL-1β, and IL-6 (3, 4). Although IL-1β and IL-6 are crucial for the differentiation of Th17 cells (51) and the expansion of ILC3 (24), no increased expression of IL-17 was found in the sera or sputum of patients with asthma with neurophil infiltrate (3, 52, 53). Interestingly, a recent study revealed that dual Th2/Th17 cells were present at a higher frequency in BAL fluid from patients with asthma and were correlated with asthma severity and BAL eosinophilia but not neutrophilia (42).

The relationships between IL-17 and neutrophil activation are further illustrated in the two diseases that proved to be sensitive to IL-17 targeting, namely, ankylosing spondylitis and psoriasis. Although neutrophilia plays a role in these diseases that likely differs from its role in asthma, outcomes of IL-17 blockade in the former (54) or IL-17R neutralization in the latter produced a decrease in neutrophil influx and a down-regulation of the proneutrophilic pathways in the skin (44, 45). Some cases of neutropenia were also reported in these clinical trials, most likely due to a profound alteration of IL-17 receptor signaling pathways (54). Moreover, many studies demonstrated that IL-17 directly promotes the production of GM-CSF and IL-8 by lung epithelial cells (55, 56). The IL-17 neutrophil pathway is also well confirmed by the fact that patients with genetic IL-17–related disorders are characterized by neutropenia and susceptibility to fungal infections (57, 58).

Therefore, although the association between IL-17 and neutrophilic inflammation is well established, and although correlations between IL-17 production and disease severity and between bronchial neutrophilia and severity have been demonstrated, a cause and effect relationship between IL-17 production and neutrophilic asthma has not been determined.

A Role for IL-17 in the Development of Steroid Insensitivity

The daily administration of ICS remains the gold standard for controlling asthma symptoms and is efficient in the large majority of patients. However, in some patients with severe disease, OCS are necessary to achieve acceptable control of their symptoms. Still, other patients remain poorly controlled despite the use of high doses of OCS (59). These steroid-insensitive subjects may primarily be classified with a $Th2^{low}$ phenotype of asthma. As these individuals respond poorly to corticosteroid therapy, in the absence of validated Th2 biomarkers, the presence of Th17 cells may be responsible for the steroid insensitivity (7). This cortico-insensitivity has been well addressed in animal studies. The exposure of lymphocytes to dexamethasone *in vitro* importantly inhibits the release of Th2-related cytokines but has no effect on IL-17 production (60). In fact, dexamethasone may even promote and maintain Th17 differentiation *in vitro* (48). In addition, reconstitution of severe combined immunodeficient mice with ovalbumin allergen-specific Th2 cells induces the development of a steroid-sensitive allergic asthma. In contrast, the transfer of ovalbumin-specific Th17 cells induces a severe refractory asthma in mice, confirming the role of these cells in the mediation of cortico-resistance (60). Accordingly, mice overexpressing the transcription factor RORγT (and thus producing significant amounts of IL-17) exhibit a steroid-resistant neutrophilic asthma in response to antigen sensitization.

In this model, neutralization of IL-17 with a specific monoclonal antibody controlled the development of asthma (61). In humans, *in vitro* experiments support a role of IL-17 in steroid-insensitive asthma (62, 63); specifically, IL-17 up-regulates the expression of the glucocorticoid receptor β and induces steroid resistance in peripheral mononuclear cells (62), whereas vitamin D decreases IL-17 production in severe asthma in a steroid-independent manner (63). A mechanistic explanation of T cell–mediated steroid resistance has been recently proposed in severe asthma (42). Indeed, double-positive IL-4/IL-17 T cells can overexpress the MEK-extracellular signal-regulated kinase 1 pathway, a pathway shown to antagonize the inhibitory effect of glucosteroids (42, 64, 65). However, the higher levels of IL-17A produced by $CD4^+$ T cells from adults and children with severe asthma compared with those produced from corticosensitive patients with asthma and healthy volunteers could be due to pro-Th17 effects mediated by steroids. Consistent with this hypothesis, glucocorticoids have been shown to promote the production of IL-17A *in vitro* in patients with severe asthma (63, 66). These data imply that severe steroid-resistant asthma may arise from aberrant and uncontrolled IL-17 inflammation promoted by progressive doses of steroids (63, 66). The recent identification and characterization of human steroid-resistant and pathogenic Th17 cells support this hypothesis. These cells were restricted to a subset of $CCR6^+$ $CXCR3^{hi}$ $CCR4^{lo}$ $CCR10^-$ $CD161^+$ cells and express ABCB1, a transporter protein that is involved in drug resistance (67). ABCB1-expressing Th17 cells produce important levels of IL-17A, IL-17F, and IL-22 after TCR stimulation and are resistant to glucocorticoids. Interestingly, exposure to steroids even specifically favors the emergence of these cells within mixed T cell cultures (67).

The extent to which this Th17 subset is active and whether it is specifically selected under high-dose steroid therapy in patients with severe asthma remain to be elucidated. Collectively, these data suggest that inflammatory IL-17–producing cells are important in the pathogenesis of and drug resistance in severe asthma (Table 1) and that approaches to characterize, quantify, and manipulate these cells may be useful for identifying new severe asthmatic subgroups and developing new specific therapies.

IL-17 Modulates the Profile of Bronchial Epithelial Cells

Airway inflammation, including IL-17–dependent inflammation, is largely involved in the activation of epithelial cells 6 (Table 2). For example, IL-17 promotes the production of cytokines and chemokines by bronchial epithelial cells (68). Indeed, elevated levels of CXCL1, CXCL8, and IL-6 were found in cultured primary human bronchial epithelial cells after IL-17

Table 2. IL-17 Effects in Asthma Pathophysiology

Study	Cellular Type Used	Cellular Mechanisms Studied	IL-17 and Its Effects	Pathophysiologic Consequences
Cao *et al.*, 2011 (68) Zijlstra *et al.*, 2012 (70)	Primary human bronchial epithelial cells	Chemokine and cytokine production	Elevated levels of CXCL1, CXCL8, IL-6 proteins after IL-17 activation	Inflammation and steroid resistance
Huang *et al.*, 2007 (69)	Primary human bronchial epithelial cells	Gene induction	Induction by IL-17 of CXCL2, -3, -5, -6, and IL-19	Inflammation
Hallstrand *et al.*, 2013 (71)	Primary human bronchial epithelial cells	Phospholipase A2 group X activity	Increase sPLA2-X enzyme production by epithelial cells	Induction of AHR
Molet *et al.*, 2001 (32)	Primary bronchial fibroblasts	Profibrotic cytokine and chemokine production	IL-17 enhances production of IL-6, IL-11, and α-chemokines, IL-8	Subepithelial fibrosis
2 Bellini *et al.*, 2012 (73)	Human asthmatic circulating fibrocytes	The profibrotic and proinflammatory functions	IL-17A promotes the release of CXCL1, CXCL8, and TKF-α, IL-6, IL-11, LIF, α-SMA	Subepithelial fibrosis
Fujisawa *et al.*, 2009 (77)	Primary human bronchial epithelial cells	Secretion of mucins	Excessive synthesis of MUC5AC and MUC5B in response to IL-17	Mucus production
Ji *et al.*, 2013 (81)	Epithelial 16HBE cells	Expression of mesenchymal markers	IL-17 synergize with IL-4 and TGF-β to promote expression of α-SMA	(EMT
Dragon *et al.*, 2007 (85)	Human ASM cells	Chemokine and cytokine production	Production of IL-6, IL-Iβ, IL-11, Eotaxin, CXCL8 after Il-17 stimulation	ASM mass
Chang *et al.*, 2011 (87)	Human ASM cells	ASM cell proliferation and migration	Proliferative and migratory abilities are reduced after inactivation of IL-17-receptors	ASM mass

Definition of abbreviation: AHR = airway hyperresponsiveness; ASM = airway smooth muscle; EMT = epithelial–mesenchymal transition; TGF = transforming growth factor.

activation (68, 69). Additional genes, such as CXCL2, -3, -5, -6, and IL-19, are directly regulated by IL-17 in human airway epithelial cells. The induction of these genes is dependent on JAK-mediated PI3K signaling and ACT1/TRAF6/TAK1-dependent nuclear factor-κB activation (69). Interestingly, IL-17 can also counterbalance the effect of glucocorticoids on the human airway epithelium by inducing epigenetic changes and sustaining inflammatory cytokine production (70). Indeed, IL-17 prevents the decrease in tumor necrosis factor-α–induced IL-8 production in glucocorticoid-treated bronchial epithelial cells through the activation of the PI3K pathway and decreased histone deacetylase activity. This phenomenon may participate in the progressive establishment of steroid resistance (70).

In a subgroup of patients with exercise-induced asthma, recent data indicated that IL-17 is a key regulator of secreted phospholipase A2 group X (sPLA2-X) activity (71). sPLA2-X is the dominant sPLA2 and is expressed in the airway epithelium and BAL fluid in patients with asthma. This enzyme is strongly associated with bronchial hyperresponsiveness, and its expression can increase according to disease severity. Notably, sPLA2-X gene (PLA2G10) expression is directly regulated by IL-17 stimulation in primary airway epithelial cell cultures, indicating that IL-17 can indirectly potentiate AHR by inducing sPLA2-X enzymes expression from epithelial cells (71).

Effect of IL-17 on Airway Remodeling

Airway remodeling refers to bronchial structural changes that constitute an important feature of asthma pathophysiology. This pathological process involves the activation of different cell types, such as bronchial epithelial cells, fibroblasts, and smooth muscle cells. Recent reports support a functional role of IL-17 in this process (Table 2).

Indeed, recent studies support a contribution of IL-17A to the development of airway fibrosis during asthma by enhancing the production of profibrotic cytokines (32, 72–74), proangiogenic factors, and collagen. These fibrocyte-mediated responses in airway remodeling require cooperative interaction with Th17 cells in a CD40 signaling–dependent manner (75). The bronchial fibroblasts obtained from patients with asthma are able to specifically promote Th17 cells *in vitro* (76), demonstrating that the local environment in the airways could favor the emergence of Th17 cells in patients with asthma. IL-17 has also been described as a potent factor leading to hypersecretion of the mucins MUC5AC and MUC5B by goblet cells, a major physiopathological feature of airway remodeling (77, 78).

Moreover, IL-17 is also able to induce a phenotypic conversion from epithelial to mesenchymal morphology through the epithelial–mesenchymal transition (EMT) process. During this process, the bronchial epithelial cells lose their cellular polarization and their cell–cell contacts. This process is highly dependent on transforming growth factor-β (TGF-β) (79, 80). Interestingly, a recent study demonstrated that IL-17 synergizes with IL-4 and TGF-β to promote bronchial epithelial cell proliferation and morphological changes with the expression of mesenchymal markers (80, 81). However, the majority of studies investigating the role of EMT in asthma are based on stimulation of epithelial cells *in vitro* (82).

An elevated airway smooth muscle (ASM) mass is another feature of airway remodeling in severe asthma (71, 83, 84). The increase in ASM cell mass is provoked by an alteration of the secretory, proliferative, migratory, and contractile functions of ASM. Various inflammatory mediators can modulate these functions in asthma. Accordingly, recent reports suggest that IL-17A alters the function of ASM cells in asthma. Similar to the observation in epithelial cells, stimulation of ASM with IL-17 promotes the production of a large spectrum of inflammatory cytokines (IL-6, IL-1β, and IL-11) and chemokines (Eotaxin/CCL-11 and CXCL8), thus enhancing the inflammatory response (85). In addition, Th17-associated cytokines, including IL-17, promote ASM cell proliferation and migration. IL-17 also promotes ASM cell survival by inhibiting apoptosis (83, 86). Blockade of either IL-17RA or IL-17RC prevents the increase in proliferative and migratory capacities induced by exogenous administration of IL-17 (87). IL-17 increases the proliferation and migration of human ASM cells in an ERK1/2-MAP kinase–dependent manner (83, 86).

Finally, a very elegant study showed that IL-17A produced by Th17 cells enhances smooth muscle cell contraction in mouse tracheal rings and human bronchial tissue. This effect is mediated by the IL-17–induced activation of the RhoA-ROCK pathway in ASM cells, which are two prominent regulators of MLC phosphorylation involved in smooth muscle contractility (88, 89).

Clinical Studies on IL-17

Previous data support a functional role of IL-17 in the development of severe asthma. However, the role of this cytokine can only be confirmed in clinical trials. Recently, brodalumab, a humanized monoclonal antibody that binds to the IL-17 receptor, was evaluated in patients with inadequately controlled moderate to severe asthma (90). There was no effect of brodalumab on the primary outcome (the Asthma Control Questionnaire score), which suggests a marginal role, if any, of IL-17 in asthma. This questionnaire is the best instrument, according to the Global Initiative on Asthma guidelines, for evaluating the control of asthma. However, this questionnaire has limitations due to the variability of its score, especially in severe asthma. Another suitable outcome measurement would have been the exacerbation rate, but the study was too short to expect any improvement, especially in a population of patients with not only severe but also moderate asthma. Nevertheless, a subgroup analysis identified a group with high reversibility of FEV_1 in response to albuterol that demonstrated clinically meaningful treatment effects. Based on this result, this paper suggested a new phenotype (highly reversible patients) and a new endotype (IL-17R–dependent). The results of this clinical trial are in accordance with recent data showing a functional role of IL-17A in smooth muscle cell contraction in mouse tracheal rings and human bronchial tissue (88).

Given the putative role of IL-17 in neutrophilic inflammation and severity discussed above, one could argue that the negative result of the study could also be due to an inadequate selection of patients with asthma. Indeed, patients should have been

selected based on sputum neutrophilia, a biomarker for neutrophilic asthma, or the quantity of IL-17 in BAL, serum, or sputum to enrich the population of responding individuals (4, 50). The absence of selection based on an IL-17– or neutrophil-related criterion, and the inclusion of patients with both moderate and severe asthma resulted in the inclusion of many patients with $Th2^{high}$ asthma in the trial who are less likely to respond to an IL-17–targeted therapy.

Given the role of corticosteroids in the generation of Th17 cells (62, 63, 66, 67) and the survival of neutrophils (91), IL-17–related and neutrophilic asthma could be a consequence of high doses of ICS and OCS and do not play a role in disease severity. Consistent with this hypothesis, the neutrophilic phenotype is eliminated by controlled corticosteroid use (92). In addition, the presence of neutrophils in the airways of subjects with asthma can be caused by concurrent chronic sinopulmonary infections. Some studies reported an association between poor lung function, a history of lung infection, and the presence of neutrophils in patients with asthma (30, 93). Other factors, such as airborne pollution and obesity, are also associated with an IL-17/neutrophil-based phenotype (24, 43). Finally, there is a high frequency of atopy in severe neutrophilic and mixed granulocytic asthma, suggesting that the presence of atopy does not exclude a neutrophilic phenotype in subjects with severe asthma (3, 30). Similarly, patients with mixed Th2/Th17 inflammation are mainly atopic (39, 42). These considerations highlight the difficulty in properly defining any IL-17/neutrophil-related asthma phenotype in humans.

Conclusions and Future Directions

A growing body of evidence supports a pathological role of IL-17–producing cells in asthma, especially regarding severity, neutrophilic inflammation, steroid resistance, and immediate response to bronchodilators. However, it appears that the role of IL-17 in asthma differs among various patient subgroups. Further investigations at the cellular level in large cohorts of patients are still needed to develop a better understanding of the involvement of IL-17 in severe asthma. Notably, future research should also focus on the use of combined genomic, transcriptomic, proteomic, and cellular approaches. Indeed, *ex vivo* quantification of pathogenic IL-17–producing cells from the blood or bronchoalveolar fluid in combination with potent biomarkers could be a suitable method of selecting patients with $Th17^{predominant}$ asthma and elucidating the Th17-oriented endotypes of asthma. ■

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References

1. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. *Nat Med* 2012;18:716–725.
2. Moore WC, Meyers DA, Wenzel SE, Teague WG, Li H, Li X, D'Agostino R Jr, Castro M, Curran-Everett D, Fitzpatrick AM, *et al*.; National Heart, Lung, and Blood Institute's Severe Asthma Research Program. Identification of asthma phenotypes using cluster analysis in the Severe Asthma Research Program. *Am J Respir Crit Care Med* 2010;181:315–323.
3. Nagasaki T, Matsumoto H, Kanemitsu Y, Izuhara K, Tohda Y, Kita H, Horiguchi T, Kuwabara K, Tomii K, Otsuka K, *et al*. Integrating longitudinal information on pulmonary function and inflammation using asthma phenotypes. *J Allergy Clin Immunol* 2014;133:1474–1477.
4. Baines KJ, Simpson JL, Wood LG, Scott RJ, Fibbens NL, Powell H, Cowan DC, Taylor DR, Cowan JO, Gibson PG. Sputum gene expression signature of 6 biomarkers discriminates asthma inflammatory phenotypes. *J Allergy Clin Immunol* 2014;133:997–1007.
5. Wu W, Bleecker E, Moore W, Busse WW, Castro M, Chung KF, Calhoun WJ, Erzurum S, Gaston B, Israel E, *et al*. Unsupervised phenotyping of Severe Asthma Research Program participants using expanded lung data. *J Allergy Clin Immunol* 2014;133:1280–1288.
6. Arron JR, Scheerens H, Matthews JG. Redefining approaches to asthma. In: Advances in pharmacology. Elsevier; 2013. pp. 1–49.
7. Woodruff PG, Modrek B, Choy DF, Jia G, Abbas AR, Ellwanger A, Koth LL, Arron JR, Fahy JV. T-helper type 2-driven inflammation defines major subphenotypes of asthma. *Am J Respir Crit Care Med* 2009; 180:388–395.
8. Corren J, Lemanske RF Jr, Hanania NA, Korenblat PE, Parsey MV, Arron JR, Harris JM, Scheerens H, Wu LC, Su Z, *et al*. Lebrikizumab treatment in adults with asthma. *N Engl J Med* 2011;365:1088–1098.
9. Haldar P, Brightling CE, Hargadon B, Gupta S, Monteiro W, Sousa A, Marshall RP, Bradding P, Green RH, Wardlaw AJ, *et al*. Mepolizumab and exacerbations of refractory eosinophilic asthma. *N Engl J Med* 2009;360:973–984.
10. Haldar P, Brightling CE, Singapuri A, Hargadon B, Gupta S, Monteiro W, Bradding P, Green RH, Wardlaw AJ, Ortega H, *et al*. Outcomes after cessation of mepolizumab therapy in severe eosinophilic asthma: a 12-month follow-up analysis. *J Allergy Clin Immunol* 2014;133:921–923.
11. Ortega H, Chupp G, Bardin P, Bourdin A, Garcia G, Hartley B, Yancey S, Humbert M. The role of mepolizumab in atopic and nonatopic severe asthma with persistent eosinophilia. *Eur Respir J* 2014;44: 239–241.
12. Nair P, Pizzichini MMM, Kjarsgaard M, Inman MD, Efthimiadis A, Pizzichini E, Hargreave FE, O'Byrne PM. Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. *N Engl J Med* 2009;360:985–993.
13. De Boever EH, Ashman C, Cahn AP, Locantore NW, Overend P, Pouliquen IJ, Serone AP, Wright TJ, Jenkins MM, Panesar IS, *et al*. Efficacy and safety of an anti-IL-13 mAb in patients with severe asthma: a randomized trial. *J Allergy Clin Immunol* 2014;133: 989–996.
14. Al-Ramli W, Préfontaine D, Chouiali F, Martin JG, Olivenstein R, Lemière C, Hamid Q. T(H)17-associated cytokines (IL-17A and IL-17F) in severe asthma. *J Allergy Clin Immunol* 2009;123: 1185–1187.
15. Bullens DM, Truyen E, Coteur L, Dilissen E, Hellings PW, Dupont LJ, Ceuppens JL. IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? *Respir Res* 2006; 7:135.
16. Pelletier M, Maggi L, Micheletti A, Lazzeri E, Tamassia N, Costantini C, Cosmi L, Lunardi C, Annunziato F, Romagnani S, *et al*. Evidence for a cross-talk between human neutrophils and Th17 cells. *Blood* 2010; 115:335–343.
17. Nembrini C, Marsland BJ, Kopf M. IL-17-producing T cells in lung immunity and inflammation. *J Allergy Clin Immunol* 2009;123: 986–994. [Quiz pp. 995–996.].
18. Yu S, Kim HY, Chang Y-J, DeKruyff RH, Umetsu DT. Innate lymphoid cells and asthma. *J Allergy Clin Immunol* 2014;133:943–950. [Quiz p. 51].
19. Scanlon ST, McKenzie ANJ. Type 2 innate lymphoid cells: new players in asthma and allergy. *Curr Opin Immunol* 2012;24:707–712.
20. Walker JA, Barlow JL, McKenzie ANJ. Innate lymphoid cells—how did we miss them? *Nat Rev Immunol* 2013;13:75–87.
21. Bartemes KR, Iijima K, Kobayashi T, Kephart GM, McKenzie AN, Kita H. IL-33-responsive lineage- CD25+ CD44(hi) lymphoid cells mediate innate type 2 immunity and allergic inflammation in the lungs. *J Immunol* 2012;188:1503–1513.

22. Halim TYF, Steer CA, Mathä L, Gold MJ, Martinez-Gonzalez I, McNagny KM, McKenzie ANJ, Takei F. Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. *Immunity* 2014;40:425–435.
23. Kim HY, Chang YJ, Subramanian S, Lee HH, Albacker LA, Matangkasombut P, Savage PB, McKenzie AN, Smith DE, Rottman JB, *et al*. Innate lymphoid cells responding to IL-33 mediate airway hyperreactivity independently of adaptive immunity. *J Allergy Clin Immunol* 2012;129:216–227.e1–6.
24. Kim HY, Lee HJ, Chang Y-J, Pichavant M, Shore SA, Fitzgerald KA, Iwakura Y, Israel E, Bolger K, Faul J, *et al*. Interleukin-17-producing innate lymphoid cells and the NLRP3 inflammasome facilitate obesity-associated airway hyperreactivity. *Nat Med* 2014;20:54–61.
25. Vazquez-Tello A, Halwani R, Li R, Nadigel J, Bar-Or A, Mazer BD, Eidelman DH, Al-Muhsen S, Hamid Q. IL-17A and IL-17F expression in B lymphocytes. *Int Arch Allergy Immunol* 2012;157:406–416.
26. Taylor PR, Roy S, Leal SM Jr, Sun Y, Howell SJ, Cobb BA, Li X, Pearlman E. Activation of neutrophils by autocrine IL-17A-IL-17RC interactions during fungal infection is regulated by IL-6, IL-23, RORγt and dectin-2. *Nat Immunol* 2014;15:143–151.
27. Murdoch JR, Lloyd CM. Resolution of allergic airway inflammation and airway hyperreactivity is mediated by IL-17-producing γδT cells. *Am J Respir Crit Care Med* 2010;182:464–476.
28. Pichavant M, Goya S, Meyer EH, Johnston RA, Kim HY, Matangkasombut P, Zhu M, Iwakura Y, Savage PB, DeKruyff RH, *et al*. Ozone exposure in a mouse model induces airway hyperreactivity that requires the presence of natural killer T cells and IL-17. *J Exp Med* 2008;205:385–393.
29. Wenzel SE, Schwartz LB, Langmack EL, Halliday JL, Trudeau JB, Gibbs RL, Chu HW. Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. *Am J Respir Crit Care Med* 1999;160:1001–1008.
30. Moore WC, Hastie AT, Li X, Li H, Busse WW, Jarjour NN, Wenzel SE, Peters SP, Meyers DA, Bleecker ER, National Heart, Lung, and Blood Institute's Severe Asthma Research Program. Sputum neutrophil counts are associated with more severe asthma phenotypes using cluster analysis. *J Allergy Clin Immunol* 2014;133:1557–1563.e5.
31. Hastie AT, Moore WC, Meyers DA, Vestal PL, Li H, Peters SP, Bleecker ER; National Heart, Lung, and Blood Institute Severe Asthma Research Program. Analyses of asthma severity phenotypes and inflammatory proteins in subjects stratified by sputum granulocytes. *J Allergy Clin Immunol* 2010;125:1028–1036.e13.
32. Molet S, Hamid Q, Davoine F, Nutku E, Taha R, Pagé N, Olivenstein R, Elias J, Chakir J. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J Allergy Clin Immunol* 2001;108:430–438.
33. Barczyk A, Pierzchala W, Sozañska E. Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. *Respir Med* 2003;97:726–733.
34. Sun Y-C, Zhou Q-T, Yao W-Z. Sputum interleukin-17 is increased and associated with airway neutrophilia in patients with severe asthma. *Chin Med J (Engl)* 2005;118:953–956.
35. Doe C, Bafadhel M, Siddiqui S, Desai D, Mistry V, Rugman P, McCormick M, Woods J, May R, Sleeman MA, *et al*. Expression of the T helper 17-associated cytokines IL-17A and IL-17F in asthma and COPD. *Chest* 2010;138:1140–1147.
36. Agache I, Ciobanu C, Agache C, Anghel M. Increased serum IL-17 is an independent risk factor for severe asthma. *Respir Med* 2010;104: 1131–1137.
37. Zhao Y, Yang J, Gao Y-D, Guo W. Th17 immunity in patients with allergic asthma. *Int Arch Allergy Immunol* 2010;151:297–307.
38. Wang YH, Voo KS, Liu B, Chen CY, Uygungil B, Spoede W, Bernstein JA, Huston DP, Liu YJ. A novel subset of CD4(+) T(H)2 memory/effector cells that produce inflammatory IL-17 cytokine and promote the exacerbation of chronic allergic asthma. *J Exp Med* 2010;207:2479–2491.
39. Cosmi L, Maggi L, Santarlasci V, Capone M, Cardilicchia E, Frosali F, Querci V, Angeli R, Matucci A, Fambrini M, *et al*. Identification of a novel subset of human circulating memory CD4(+) T cells that produce both IL-17A and IL-4. *J Allergy Clin Immunol* 2010;125: 222–230.e1–e4.
40. Chien JW, Lin CY, Yang KD, Lin CH, Kao JK, Tsai YG. Increased IL-17A secreting CD4+ T cells, serum IL-17 levels and exhaled nitric oxide are correlated with childhood asthma severity. *Clin Exp Allergy* 2013; 43:1018–1026.
41. Pène J, Chevalier S, Preisser L, Vénéreau E, Guilleux M-H, Ghannam S, Molès J-P, Danger Y, Ravon E, Lesaux S, *et al*. Chronically inflamed human tissues are infiltrated by highly differentiated Th17 lymphocytes. *J Immunol* 2008;180:7423–7430.
42. Irvin C, Zafar I, Good J, Rollins D, Christianson C, Gorska MM, Martin RJ, Alam R. Increased frequency of dual-positive TH2/TH17 cells in bronchoalveolar lavage fluid characterizes a population of patients with severe asthma. *J Allergy Clin Immunol* [online ahead of print] 18 Jul 2014; DOI: 10.1016/j.jaci.2014.05.038.
43. Brandt EB, Kovacic MB, Lee GB, Gibson AM, Acciani TH, Le Cras TD, Ryan PH, Budelsky AL, Khurana Hershey GK. Diesel exhaust particle induction of IL-17A contributes to severe asthma. *J Allergy Clin Immunol* 2013;132:1194–1204.e2.
44. Papp KA, Leonardi C, Menter A, Ortonne J-P, Krueger JG, Kricorian G, Aras G, Li J, Russell CB, Thompson EH, *et al*. Brodalumab, an anti-interleukin-17-receptor antibody for psoriasis. *N Engl J Med* 2012; 366:1181–1189.
45. Krueger JG, Fretzin S, Suárez-Fariñas M, Haslett PA, Phipps KM, Cameron GS, McColm J, Katcherian A, Cueto I, White T, *et al*. IL-17A is essential for cell activation and inflammatory gene circuits in subjects with psoriasis. *J Allergy Clin Immunol* 2012;130:145–154.e9.
46. Wilson RH, Whitehead GS, Nakano H, Free ME, Kolls JK, Cook DN. Allergic sensitization through the airway primes Th17-dependent neutrophilia and airway hyperresponsiveness. *Am J Respir Crit Care Med* 2009;180:720–730.
47. He R, Oyoshi MK, Jin H, Geha RS. Epicutaneous antigen exposure induces a Th17 response that drives airway inflammation after inhalation challenge. *Proc Natl Acad Sci USA* 2007;104: 15817–15822.
48. Zhao J, Lloyd CM, Noble A. Th17 responses in chronic allergic airway inflammation abrogate regulatory T-cell-mediated tolerance and contribute to airway remodeling. *Mucosal Immunol* 2013;6: 335–346.
49. Lajoie S, Lewkowich IP, Suzuki Y, Clark JR, Sproles AA, Dienger K, Budelsky AL, Wills-Karp M. Complement-mediated regulation of the IL-17A axis is a central genetic determinant of the severity of experimental allergic asthma. *Nat Immunol* 2010;11: 928–935.
50. Wood LG, Baines KJ, Fu J, Scott HA, Gibson PG. The neutrophilic inflammatory phenotype is associated with systemic inflammation in asthma. *Chest* 2012;142:86–93.
51. Chung Y, Chang SH, Martinez GJ, Yang XO, Nurieva R, Kang HS, Ma L, Watowich SS, Jetten AM, Tian Q, *et al*. Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity* 2009;30: 576–587.
52. Baines KJ, Simpson JL, Bowden NA, Scott RJ, Gibson PG. Differential gene expression and cytokine production from neutrophils in asthma phenotypes. *Eur Respir J* 2010;35:522–531.
53. Manni ML, Trudeau JB, Scheller EV, Mandalapu S, Elloso MM, Kolls JK, Wenzel SE, Alcorn JF. The complex relationship between inflammation and lung function in severe asthma. *Mucosal Immunol* 2014;7:1186–1198.
54. Baeten D, Baraliakos X, Braun J, Sieper J, Emery P, van der Heijde D, McInnes I, van Laar JM, Landewé R, Wordsworth P, *et al*. Anti-interleukin-17A monoclonal antibody secukinumab in treatment of ankylosing spondylitis: a randomised, double-blind, placebo-controlled trial. *Lancet* 2013;382:1705–1713.
55. Jones CE, Chan K. Interleukin-17 stimulates the expression of interleukin-8, growth-related oncogene-alpha, and granulocyte-colony-stimulating factor by human airway epithelial cells. *Am J Respir Cell Mol Biol* 2002;26:748–753.
56. McAllister F, Henry A, Kreindler JL, Dubin PJ, Ulrich L, Steele C, Finder JD, Pilewski JM, Carreno BM, Goldman SJ, *et al*. Role of IL-17A, IL-17F, and the IL-17 receptor in regulating growth-related oncogene-alpha and granulocyte colony-stimulating factor in bronchial epithelium: implications for airway inflammation in cystic fibrosis. *J Immunol* 2005;175:404–412.

57. Boisson B, Wang C, Pedergnana V, Wu L, Cypowyj S, Rybojad M, Belkadi A, Picard C, Abel L, Fieschi C, *et al*. An ACT1 mutation selectively abolishes interleukin-17 responses in humans with chronic mucocutaneous candidiasis. *Immunity* 2013;39:676–686.
58. Drewniak A, Gazendam RP, Tool ATJ, van Houdt M, Jansen MH, van Hamme JL, van Leeuwen EMM, Roos D, Scalais E, de Beaufort C, *et al*. Invasive fungal infection and impaired neutrophil killing in human CARD9 deficiency. *Blood* 2013;121:2385–2392.
59. Bush A, Saglani S. Management of severe asthma in children. *Lancet* 2010;376:814–825.
60. McKinley L, Alcorn JF, Peterson A, Dupont RB, Kapadia S, Logar A, Henry A, Irvin CG, Piganelli JD, Ray A, *et al*. Th17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. *J Immunol* 2008;181:4089–4097.
61. Ano S, Morishima Y, Ishii Y, Yoh K, Yageta Y, Ohtsuka S, Matsuyama M, Kawaguchi M, Takahashi S, Hizawa N. Transcription factors GATA-3 and RORγt are important for determining the phenotype of allergic airway inflammation in a murine model of asthma. *J Immunol* 2013;190:1056–1065.
62. Vazquez-Tello A, Halwani R, Hamid Q, Al-Muhsen S. Glucocorticoid receptor-beta up-regulation and steroid resistance induction by IL-17 and IL-23 cytokine stimulation in peripheral mononuclear cells. *J Clin Immunol* 2013;33:466–478.
63. Nanzer AM, Chambers ES, Ryanna K, Richards DF, Black C, Timms PM, Martineau AR, Griffiths CJ, Corrigan CJ, Hawrylowicz CM. Enhanced production of IL-17A in patients with severe asthma is inhibited by 1α,25-dihydroxyvitamin D3 in a glucocorticoid-independent fashion. *J Allergy Clin Immunol* 2013;132:297–304.e3.
64. Liang Q, Guo L, Gogate S, Karim Z, Hanifi A, Leung DY, Gorska MM, Alam R. IL-2 and IL-4 stimulate MEK1 expression and contribute to T cell resistance against suppression by TGF-beta and IL-10 in asthma. *J Immunol* 2010;185:5704–5713.
65. Tsitoura DC, Rothman PB. Enhancement of MEK/ERK signaling promotes glucocorticoid resistance in CD4+ T cells. *J Clin Invest* 2004;113:619–627.
66. Gupta A, Dimeloe S, Richards DF, Chambers ES, Black C, Urry Z, Ryanna K, Xystrakis E, Bush A, Saglani S, *et al*. Defective IL-10 expression and in vitro steroid-induced IL-17A in paediatric severe therapy-resistant asthma. *Thorax* 2014;69:508–515.
67. Ramesh R, Kozhaya L, McKevitt K, Djuretic IM, Carlson TJ, Quintero MA, McCauley JL, Abreu MT, Unutmaz D, Sundrud MS. Pro-inflammatory human Th17 cells selectively express P-glycoprotein and are refractory to glucocorticoids. *J Exp Med* 2014;211:89–104.
68. Cao J, Ren G, Gong Y, Dong S, Yin Y, Zhang L. Bronchial epithelial cells release IL-6, CXCL1 and CXCL8 upon mast cell interaction. *Cytokine* 2011;56:823–831.
69. Huang F. Requirement for both JAK-mediated PI3K signaling and ACT1/TRAF6/TAK1-dependent NF-B activation by IL-17A in enhancing cytokine expression in human airway epithelial cells. *J Immunol* 2007;●●●:1–11.
70. Zijlstra GJ, Ten Hacken NH, Hoffmann RF, van Oosterhout AJ, Heijink IH. Interleukin-17A induces glucocorticoid insensitivity in human bronchial epithelial cells. *Eur Respir J* 2012;39:439–445.
71. Hallstrand TS, Lai Y, Altemeier WA, Appel CL, Johnson B, Frevert CW, Hudkins KL, Bollinger JG, Woodruff PG, Hyde DM, *et al*. Regulation and function of epithelial secreted phospholipase A2 group X in asthma. *Am J Respir Crit Care Med* 2013;188:42–50.
72. Bellini A, Marini MA, Bianchetti L, Barczyk M, Schmidt M, Mattoli S. Interleukin (IL)-4, IL-13, and IL-17A differentially affect the profibrotic and proinflammatory functions of fibrocytes from asthmatic patients. *Mucosal Immunol* 2012;5:140–149.
73. Al-Muhsen S, Letuve S, Vazquez-Tello A, Pureza MA, Al-Jahdali H, Bahammam AS, Hamid Q, Halwani R. Th17 cytokines induce pro-fibrotic cytokines release from human eosinophils. *Respir Res* 2013; 14:34.
74. Chakir J, Shannon J, Molet S, Fukakusa M, Elias J, Laviolette M, Boulet L-P, Hamid Q. Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF-β, IL-11, IL-17, and type I and type III collagen expression. *J Allergy Clin Immunol* 2003;111: 1293–1298.
75. Hayashi H, Kawakita A, Okazaki S, Yasutomi M, Murai H, Ohshima Y. IL-17A/F modulates fibrocyte functions in cooperation with CD40-mediated signaling. *Inflammation* 2013;36:830–838.
76. Loubaki L, Hadj-Salem I, Fakhfakh R, Jacques E, Plante S, Boisvert M, Aoudjit F, Chakir J. Co-culture of human bronchial fibroblasts and CD4+ T cells increases Th17 cytokine signature. *PLoS ONE* 2013;8: e81983.
77. Fujisawa T, Velichko S, Thai P, Hung LY, Huang F, Wu R. Regulation of airway MUC5AC expression by IL-1beta and IL-17A; the NF-kappaB paradigm. *J Immunol* 2009;183:6236–6243.
78. Chen Y, Thai P, Zhao Y-H, Ho Y-S, DeSouza MM, Wu R. Stimulation of airway mucin gene expression by interleukin (IL)-17 through IL-6 paracrine/autocrine loop. *J Biol Chem* 2003;278:17036–17043.
79. Pain M, Bermudez O, Lacoste P, Royer P-J, Botturi K, Tissot A, Brouard S, Eickelberg O, Magnan A. Tissue remodelling in chronic bronchial diseases: from the epithelial to mesenchymal phenotype. *Eur Respir Rev* 2014;23:118–130.
80. Vittal R, Fan L, Greenspan DS, Mickler EA, Gopalakrishnan B, Gu H, Benson HL, Zhang C, Burlingham W, Cummings OW, *et al*. IL-17 induces type V collagen overexpression and EMT via TGF-β-dependent pathways in obliterative bronchiolitis. *Am J Physiol Lung Cell Mol Physiol* 2013;304:L401–L414.
81. Ji X, Li J, Xu L, Wang W, Luo M, Luo S, Ma L, Li K, Gong S, He L, *et al*. IL4 and IL-17A provide a Th2/Th17-polarized inflammatory milieu in favor of TGF-β1 to induce bronchial epithelial-mesenchymal transition (EMT). *Int J Clin Exp Pathol* 2013;6:1481–1492.
82. Sohal SS, Ward C, Walters EH. Importance of epithelial mesenchymal transition (EMT) in COPD and asthma. *Thorax* 2014;69:768.
83. Chang Y, Al-Alwan L, Risse P-A, Halayko AJ, Martin JG, Baglole CJ, Eidelman DH, Hamid Q. Th17-associated cytokines promote human airway smooth muscle cell proliferation. *FASEB J* 2012;26: 5152–5160.
84. Girodet PO, Ozier A, Bara I, Tunon de Lara JM, Marthan R, Berger P. Airway remodeling in asthma: new mechanisms and potential for pharmacological intervention. *Pharmacol Ther* 2011;130:325–337.
85. Dragon S, Rahman MS, Yang J, Unruh H, Halayko AJ, Gounni AS. IL-17 enhances IL-1beta-mediated CXCL-8 release from human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2007;292: L1023–L1029.
86. Al-Alwan LA, Chang Y, Baglole CJ, Risse P-A, Halayko AJ, Martin JG, Eidelman DH, Hamid Q. Autocrine-regulated airway smooth muscle cell migration is dependent on IL-17-induced growth-related oncogenes. *J Allergy Clin Immunol* 2012;130:977–985.e6.
87. Chang Y, Al-Alwan L, Risse P-A, Roussel L, Rousseau S, Halayko AJ, Martin JG, Hamid Q, Eidelman DH. TH17 cytokines induce human airway smooth muscle cell migration. *J Allergy Clin Immunol* 2011; 127:1046–1053.e1–2.
88. Kudo M, Melton AC, Chen C, Engler MB, Huang KE, Ren X, Wang Y, Bernstein X, Li JT, Atabai K, *et al*. IL-17A produced by αβ T cells drives airway hyper-responsiveness in mice and enhances mouse and human airway smooth muscle contraction. *Nat Med* 2012;18: 547–554.
89. Loirand G, Sauzeau V, Pacaud P, Small G. Small G proteins in the cardiovascular system: physiological and pathological aspects. *Physiol Rev* 2013;93:1659–1720.
90. Busse WW, Holgate S, Kerwin E, Chon Y, Feng J, Lin J, Lin S-L. Randomized, double-blind, placebo-controlled study of brodalumab, a human anti-IL-17 receptor monoclonal antibody, in moderate to severe asthma. *Am J Respir Crit Care Med* 2013;188:1294–1302.
91. Kato T, Takeda Y, Nakada T, Sendo F. Inhibition by dexamethasone of human neutrophil apoptosis in vitro. *Nat Immun* 1995;14:198–208.
92. Cowan DC, Cowan JO, Palmay R, Williamson A, Taylor DR. Effects of steroid therapy on inflammatory cell subtypes in asthma. *Thorax* 2010;65:384–390.
93. Moore WC, Bleecker ER, Curran-Everett D, Erzurum SC, Ameredes BT, Bacharier L, Calhoun WJ, Castro M, Chung KF, Clark MP, *et al*.; National Heart, Lung, Blood Institute's Severe Asthma Research Program. Characterization of the severe asthma phenotype by the National Heart, Lung, and Blood Institute's Severe Asthma Research Program. *J Allergy Clin Immunol* 2007;119:405–413.

Prime role of IL-17A in neutrophilia and airway smooth muscle contraction in a house dust mite-induced allergic asthma model

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In revision in JACI

Background: Asthma is a heterogeneous inflammatory disorder with various endotypes leading to a series of distinct clinical phenotypes. Although there are emerging therapeutic candidates for the T_H2/eosinophilic phenotype, there are *subsets of severe asthma steroid insensitive patients* for which research needs to identify the *pathological processes involved.* A neutrophil inflammation, in which a T_H17 response predominates, is one of these. In this study, we address this in the context of a new asthma model induced by house dust mite (HDM). **Objective**: To determine *in vivo* the role of IL-17A and neutrophil in an HDM induced asthma model. **Methods**: Balb/c mice were sensitized to and challenged with HDM extract. Airway inflammation, airway hyperresponsiveness (AHR), *in vitro* airway contraction, bronchoalveolar lavage cytokines levels, and lung T cell activation were assessed. The respective roles of IL-17A, IL-13 and neutrophils were determined after intranasal administration of anti-IL-17A, anti-IL-13, and anti-Ly6G neutralizing antibodies. **Results**: Asthmatic mice exhibited AHR and a huge influx of neutrophils and eosinophils in their lungs. This pulmonary infiltrate was associated with a combined T_H2 and T_H17 activation, assessed by the secretion of IL-13, IL-4, and IL-17A. Unlike for IL-13, IL-17A neutralization abrogated AHR and decreased the neutrophil infiltrate. In addition to its role in the regulation of inflammation, IL-17A directly enhanced bronchial smooth muscle contraction through a RhoA-independent mechanism. **Conclusion**: This study describes an asthma model characterized by a combined T_H2/T_H17-driven inflammation equivalent to severe asthma. These results suggest that targeting IL-17A might be useful in reducing both inflammation and smooth muscle contraction.

Letter to the Editor

Prime role of IL-17A in neutrophilia and airway smooth muscle contraction in a house dust mite-induced allergic asthma model

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Key messages

- Skin sensitization followed by intranasal challenge drive T_H2-T_H17 airway inflammation
- IL-17A is central for the establishment of asthma in this model
- IL-17A directly modulates inflammation and enhances smooth muscle contraction

To the Editor:

Asthma is a bronchial inflammatory disease leading to airway hyperresponsiveness (AHR) and reversible airflow obstruction. Its incidence has considerably increased over the past 50 years, with about 300 million people affected worldwide [1]. The majority of animal studies, have demonstrated that CD4 T helper-2 (T_H2) cells are central in the orchestration and amplification of allergic asthma. However negative results obtained from T_H2-focused human clinical trials have highlighted the importance of considering the large variety of asthma phenotypes [2]. Indeed human asthma is highly heterogeneous and is not always only due to a T_H2 driven airway inflammation only [3,4]. Recently many studies suggest that IL-17A, a cytokine mainly produced by T_H17 cells, is involved in some severe cases of asthma by regulating neutrophilic inflammation and steroid resistance [5]. However support of this theory remains modest as some reports demonstrated that (i) IL-17A or T_H17 cells alone were not sufficient to trigger the disease and (ii) that IL-17A can intriguingly mediate anti-inflammatory mechanisms. Consequently we aimed to determine the contribution of IL-17A in a mouse model of acute asthma induced by house dust mites (HDM).

Asthma was induced in mice by percutaneous sensitization and intranasal challenge with a HDM extract (*Dermatophagoïdes farinae,* Der f, Figure E1A). Exposure to Der f results in the development of bronchial hyperreactivity and airway resistance, important production of Der f1-specific IgE and strong airway cellular infiltration (Figure E1, B, C, D). Cell subtype analysis by flow cytometry revealed a mixed influx of eosinophils, neutrophils and lymphocytes (Figure E2A). The recruitment of these inflammatory cells was associated with an upregulation of chemokines involved in eosinophil (Rantes, Eotaxin) and neutrophil (CXCL1, CXCL5) attraction (Figure E2B, C). Having found increased levels of eosinophils and neutrophils we investigated T_H2 and T_H17 responses. Elevated levels of IL-4, IL-5, IL13 and IL-17 cytokines in BAL associated with an expansion of T_H2 and T_H17 cells in lung

tissues were found in Der f asthmatic mice confirming the mixed T_H2-T_H17 driven inflammation in our model (Figure E2D, E). CCL20, IL-6 and IL-1β were increased in the airways of asthmatic mice 24h after the final challenge (Figure E2D). These molecules are known to attract and promote the differentiation of T_H17 cells. Furthermore, CCL20, IL-6 and IL-1β have been recently described as specific biomarkers for neutrophilic and mixed granulocytic inflammation in severe asthma [6,7].

To investigate the implication of T_H17 in the development of AHR and granulocytic infiltration, intranasal injections of a blocking anti-IL-17 antibody were performed during the challenges. Neutralization of IL-17 in Der f mice significantly reduces AHR (Figure 1A) and dampens neutrophils influx in BAL (Figure 1B). Interestingly, neutralization of IL-17 decreases the expression of pro-neutrophil chemokines CXCL1 and CXCL5 in the airways of anti-IL-17-treated mice (Figure 1C, D). In contrast, we showed similar levels of eosinophils and T_H2 cytokines in anti-IL-17 treated asthmatic mice (Figure 1D, E, F), suggesting no synergic effect between T_H2 and T_H17 responses. In addition, because IL-13 was crucial in the induction of asthma symptoms [8], we evaluated airway inflammation and AHR after IL-13 neutralization. Our results indicate a partial improvement of AHR (Figure E3A) but no modification on bronchial inflammation (Figure E3 B, C D). These results suggest a prime role of the IL-17-neutrophil axis in our model of allergic asthma.

Growing bodies of evidence support an intimate link between neutrophils and severe asthma. However the contribution of neutrophils in the development of AHR in T_H17-dependent allergic airway disease remains to be elucidated. Consequently in order to discover in what extent the complete disappearance of AHR induced after IL-17A neutralization was due to the decrease of neutrophil influx, neutrophils were depleted by administration of anti-Ly6G (Figure 2A). A lack of neutrophil partially decreases AHR in Der f mice (Figure 2B) with no impact on eosinophils levels (Figure 2C) demonstrating that the effect of IL-17A is definitely

not only neutrophil-mediated, and suggesting a direct role of IL-17 on bronchial contraction. To confirm this hypothesis, we incubated isolated bronchi from control and asthmatic mice with IL-17A for 12h and evaluated the contractile force generated in response to MCh or KCl *ex vivo*. Incubation with IL-17A significantly enhanced both MCh- and KCl-induced bronchial contraction in control mice with similar levels of contraction than Der f mice (Figure 2D). This direct role of IL-17 on AHR is of special interest since the recent clinical data showing an improvement of asthma control in response to anti-IL-17RA (brodalumab) in a group with high-reversibility of FEV1 in response to albuterol, a bronchial smooth muscle relaxant.

Small G-proteins from Rho family are central effectors in the smooth muscle contraction pathway [9]. Interestingly IL-17 has been described as an enhancer of smooth muscle contraction by boosting the RhoA pathway in mice [10]. Our molecular investigations reveal that *in vivo* neutralization of IL-17 does not alter the expression of RhoA in the bronchi of Der f mice (Figure 2E) but rather decreases the expression of Rac1 (Figure 2F), another small G-protein from Rho family involved in smooth muscle contraction [9]. Collectively these data demonstrate that IL-17 promotes AHR in asthmatic mice by regulating bronchial contraction through a Rac1-dependent signalling pathway.

Overall, our study demonstrates that Der f-induced allergic asthma after percutaneous sensitization and airway challenge induces AHR and a mixed T_H2/T_H17 driven inflammation close to the inflammatory phenotype observed in some severe cases of asthma. In this model, IL-17A neutralization is sufficient to prevent Der f-induced exacerbation. Consequently our data point to the necessity to develop therapies targeting IL-17A-mediated mechanisms to dampen airway inflammation and control refractory asthma.

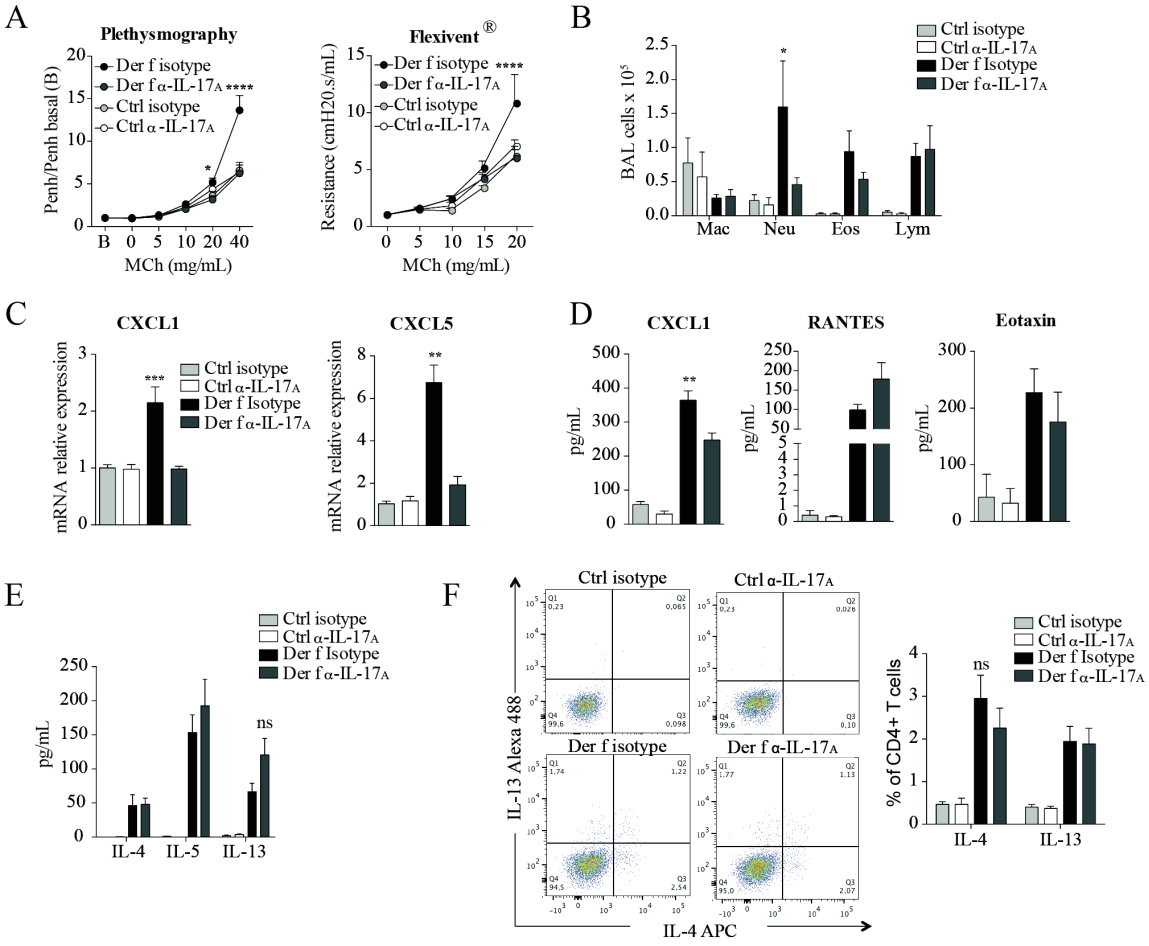

Figure 1: **IL-17A neutralization abrogates Der f-induced airway responses.** **A**, Measurement of AHR, airway resistance. **B**, BAL infiltrate cells, Mac: Macrophages; Polynuclear Neutrophils (Neu) and Eosinophils (Eos); Lym: Lymphocytes. **C**, CXCL1, CXCL5 expression in lung. D, CXCL1, Rantes, eotaxin production in BAL. **E**, Levels T_H2 cytokine in BAL. **F**, Frequency of lung IL-4 and IL-13 producing T cells from asthmatic mice (Der f) and control mice (Ctrl) were treated with isotype or anti-IL-17A. ****P < .0001, ***P < .001, **P < .01 *P = .05; n= 6-9 mice per group.

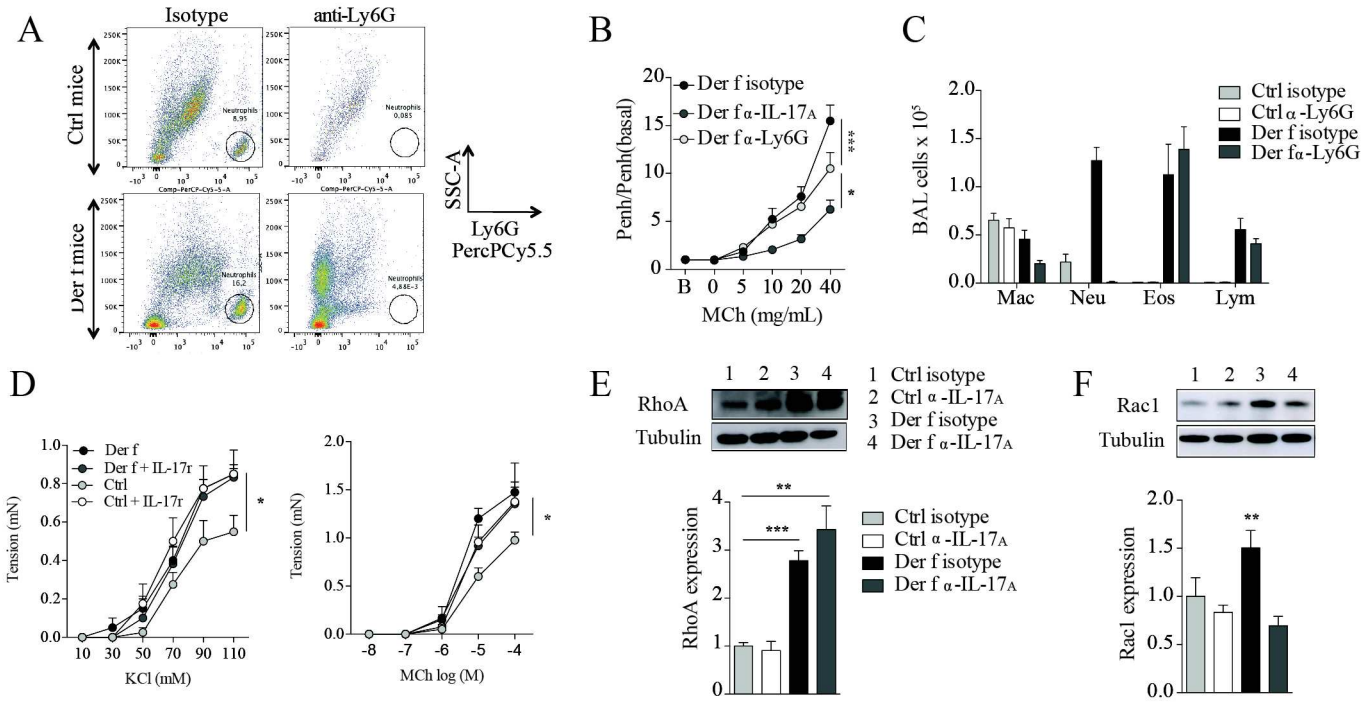

Figure 2: AHR depends partially on a direct contractile effect of IL-17A. A, Flow cytometry dot plots showing neutrophil depletion; **B and C**, AHR measurement (comparison with values after anti-IL-17A treatment) and BAL cellularity in control and Der f mice treated with isotype or anti-Ly6G; **D**, Bronchial contraction in response to KCl, MCh in asthmatic/control mice treated with or without IL-17A; **E and F**, Quantification of RhoA and Rac1 expression in Der f or Ctrl mice treated with isotype or anti-IL-17A.

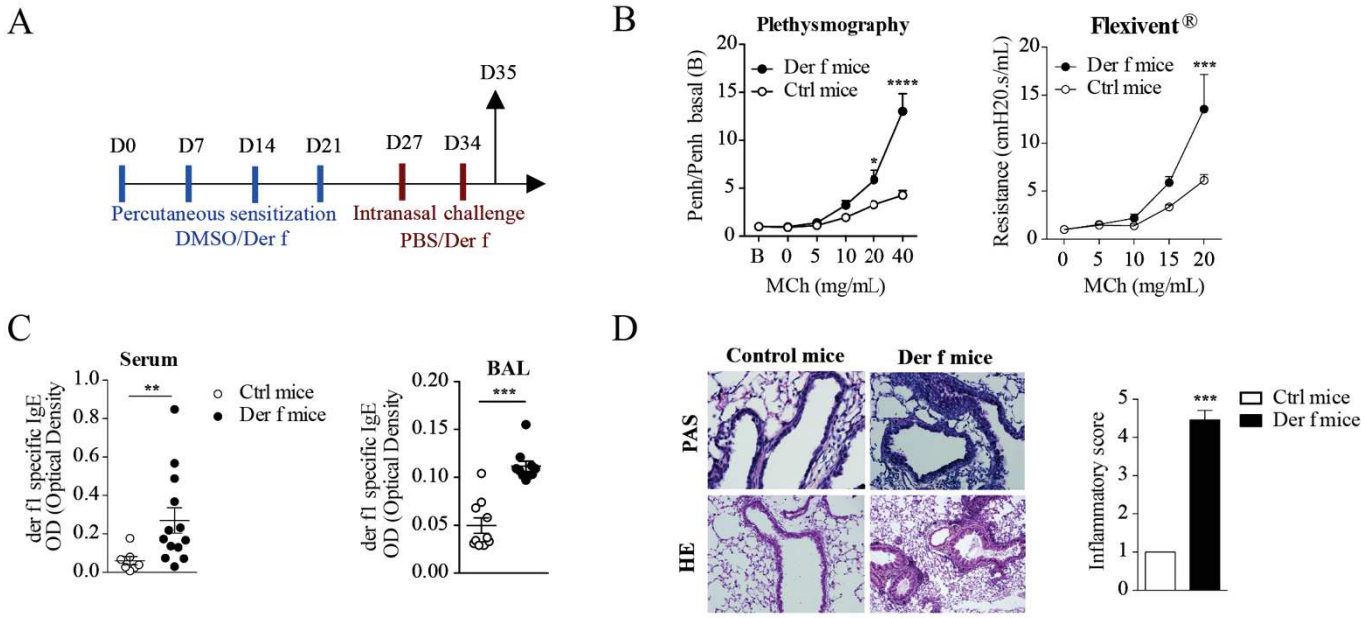

Figure E1: Percutaneous sensitization and intranasal challenge induce AHR and bronchial inflammation. A, Experimental model; **B**, Penh measurements values and airway resistance in response to methacholin. Data obtained from 2 separate experiments (n=10 mice per group; 2-way ANOVA; ****P <.0001, *P <.05); **C**, (derf 1)-specific IgE titers in serum and BAL; **D**, Representative PAS and HE staining in airways. Black columns: asthmatic mice; White columns: control mice.

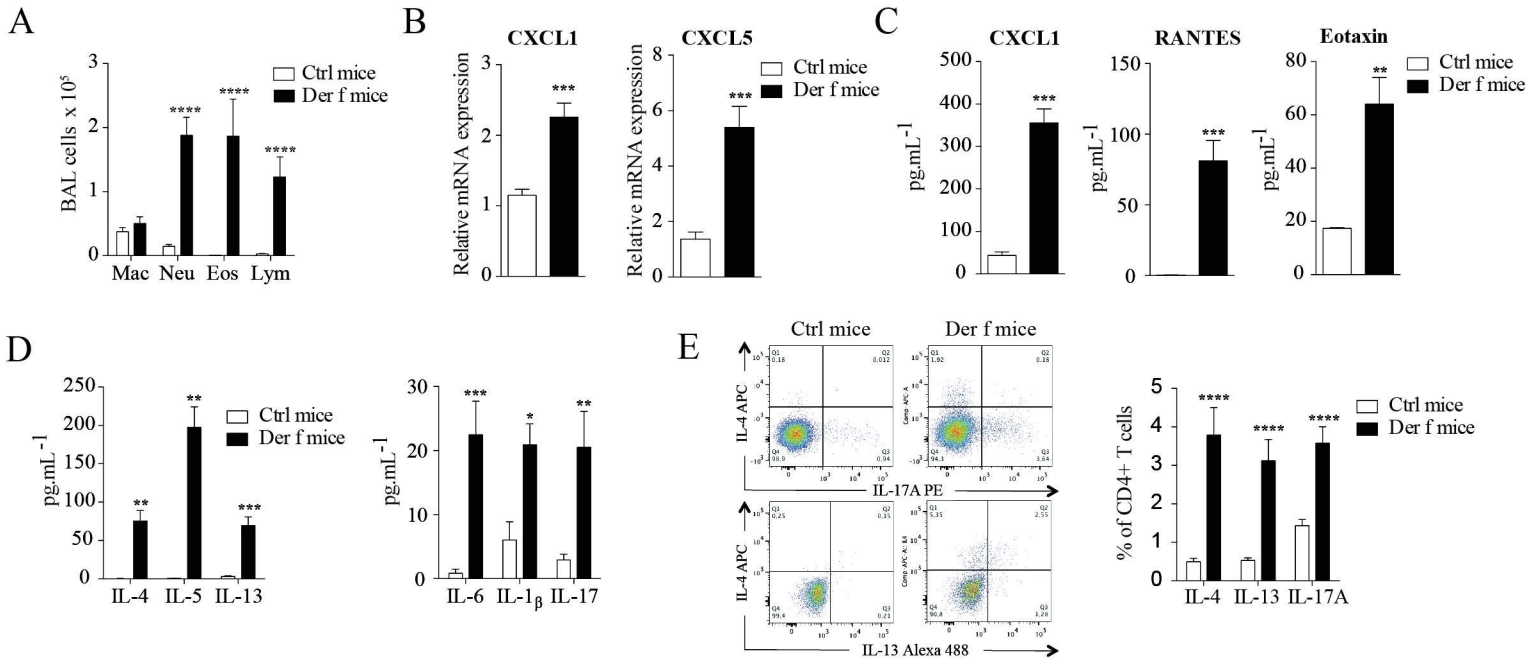

Figure E2: Allergen exposure generates a mixed bronchial inflammation. **A**, BAL cellularity (n=10-15 mice per group, Mac: Macrophages; Neu: Polynuclear Neutrophils; Eos: Polynuclear Eosinophils; Lym: Lymphocytes); **B**, Lung expression of CXCL1, CXCL5; **C**, BAL levels of CXCL1, eotaxin and Rantes; **D**, BAL secretions of T_H2 and T_H17 related cytokines (6-8 mice per group), **E**, Intracellular staining for $CD4^+$ T cells producing IL-13, IL-4 and IL-17A (n=15-20 mice per group).

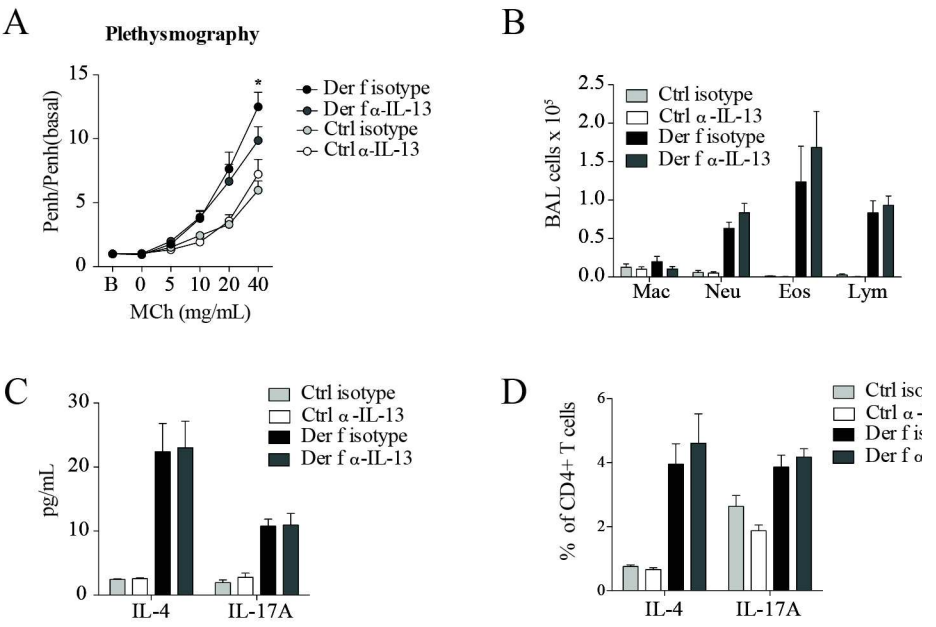

Figure E3: IL-13 blocking has a limited effect on AHR and airway inflammation. A the last methacholin dose. (n=5-9 mice per group, ***P < .001) B, Lung and BA after antibodies treatment (n=6-8 mice per group). C, Cytokines quantification us (n= 5-7 mice per group) D, Detection of intracellular cytokines in T CD4+ cells (per group).

The role of B lymphocytes in the progression from autoimmunity to autoimmune disease

Gabriela Franco Salinas, **Faouzi Braza**, Sophie Brouard, Paul-Peter Tak, Dominique Baeten

Abstract

Autoimmunity, defined as the presence of autoreactive T and/or B lymphocytes in the periphery, originates because central tolerance mechanisms are not perfect. Nonetheless, autoreactive cells do not lead automatically to autoimmune disease. The progression from autoimmunity to autoimmune disease is not only determined by the degree of central tolerance leakage, but also by peripheral mechanisms of activation and control of the autoreactive cells. In this review, we discuss the contribution of B lymphocytes to this process, ranging from activation of T cell and epitope spreading to regulation of the autoimmune process. We also discuss the similarities with the role of B cells in alloimmunity in the context of organ transplantation. Since peripheral mechanisms may be easier to modulate than central tolerance, a thorough understanding of the role of peripheral B cells in the progression from autoimmunity to autoimmune disease may open new avenues for treatment and prevention of autoimmune disorders.

REVIEW

The role of B lymphocytes in the progression from autoimmunity to autoimmune disease

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Abstract Autoimmunity, defined as the presence of autoreactive T and/or B lymphocytes in the periphery, is a frequent and probably even physiological condition. It is mainly caused by the fact that the central tolerance mechanisms, which are responsible for counter-selection of autoreactive lymphocytes, are not perfect and thus a limited number of these autoreactive cells can mature and enter the periphery. Nonetheless, autoreactive cells do not lead automatically to autoimmune disease as evidenced by a multitude of experimental and human data sets. Interestingly, the progression from autoimmunity to autoimmune disease is not only determined by the degree of central tolerance leakage and thus the amount of autoreactive lymphocytes in the periphery, but also by peripheral mechanism of activation and control of the autoreactive cells. In this review, we discuss the contribution of peripheral B lymphocytes in this process, ranging from activation of T cells and epitope spreading to control of the autoimmune process by regulatory mechanisms. We also discuss the parallels with the role of B cells in the induction and control of alloimmunity in the context of organ transplantation, as more precise knowledge of the pathogenic antigens and time of initiation of the immune response in allo- versus auto-immunity allows better dissection of the exact role of B cells. Since peripheral mechanisms may be easier to modulate than central tolerance, a more thorough understanding of the role of peripheral B cells in the progression from autoimmunity

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to autoimmune disease may open new avenues for treatment and prevention of autoimmune disorders.

Contents

1. Introduction

Autoimmune diseases are defined by B and/or T cells specific for self reactive antigens, that upon activation lead to chronic tissue inflammation and often irreversible structural and functional damage. Conceptually, different stages in the development of autoimmune disease can be defined. In a first stage, the regulatory mechanisms of central tolerance fail and allow an increased number of autoreactive cells to enter the periphery [1]. A key determinant of B cell central tolerance is the strength of B cell receptor (BCR) signaling: a strong BCR signal by binding with high affinity to an autoantigen will lead to deletion or receptor editing while an intermediate binding affinity will permit B cells to survive and progress to the periphery [1]. This concept has been elegantly demonstrated in several animal models. As an example: bringing in not only the heavy chain against the myelin oligodendrocyte glycoprotein (MOG) autoantigen but also the light chain leads to a block in central development and does not increase the number of MOG-specific B cells relative to transgenic animals in which only the heavy chain is self reactive [2,3]. However, if the signaling potential of the BCR is affected for example by PTPN22 mutations or CD19 overexpression, the autoreactive B cells will not be deleted and leak to the periphery [4,5]. Impairment of this B cell central tolerance checkpoint will thus increase the frequency of autoreactive cells in the periphery and, as a consequence, the chance of developing autoimmune disease [6].

2. From autoimmunity to autoimmune disease

Remarkably, however, autoimmunity (defined as the presence of autoreactive T or B cells in the periphery) does not lead automatically to autoimmune disease (Fig. 1A). Using reactivity to the central nervous system autoantigen MOG as an example, more than 90% of the peripheral T cells are autoreactive in MOG-specific T cell receptor (TCR) transgenic mice but only a small minority of animals (<4%) will develop spontaneous experimental autoimmune encephalomyelitis (EAE) [7]. In heterozygous and homozygous knock-in IgH transgenic mice, 30% and 50% of the IgM positive B cells bind recombinant MOG, respectively. These autoreactive B cells colonize the immune organs in the animals and undergo normal isotype switching and affinity maturation after immunization, producing MOG-specific antibodies. Nevertheless, they are not pathogenic by themselves as mice do not develop any neurological signs of disease [3]. Serological analysis of congenic strains of NOD mice, the most studied animal model of spontaneous type 1 diabetes (T1D), reveals that high level of insulin autoantibodies (IAAs) may occur in the absence of diabetes progression. Moreover, there is no statistically significant difference in the positive IAA indexes between the NOD mice and the diabetes-resistant strains [8].

Also in humans, clear signs of autoreactivity can be observed in healthy individuals in the absence of autoimmune disease. Anti-nuclear antibodies (ANAs) and rheumatoid factor (RF) can be observed in healthy individuals and their prevalence increases with age. More strikingly, vaccination against influenza, hepatitis A and hepatitis B can trigger the production of ANAs, anti-cardiolipin antibodies (aCL) and anti-extractable nuclear antigen (ENA) antibodies in healthy individuals but this autoimmunity does not lead to autoimmune disease and tends to resolve by itself in most cases [9–11]. Another example is the induction of ANAs, anti-double stranded DNA and aCL antibodies by TNF blockade in rheumatoid arthritis (RA) and spondyloarthritis (SpA). However, this largely IgM restricted autoimmune response is not related to the development of systemic lupus erythematosus (SLE) or any other autoimmune disorder and tends to resolve spontaneously upon withdrawal of anti-TNF treatment [12]. Similarly, ANA titers in patients with IgA deficiency do not predict whether patients will develop autoimmune disease [13].

A series of other studies, however, has clearly demonstrated that the onset of clinical autoimmune disease is often preceded by the presence of autoantibodies. Among children

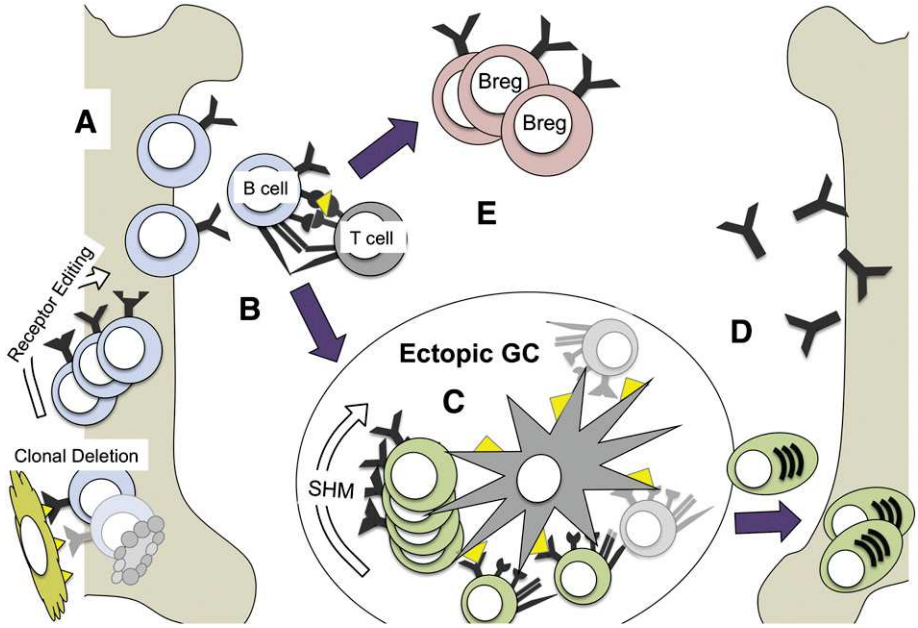

Figure 1 Autoreactive B lymphocytes that escape the central tolerance mechanisms are not sufficient to cause overt autoimmune disease (A), but other factors are necessary. B lymphocytes may contribute to drive the progression from autoimmunity to autoimmune diseases in different ways. B lymphocytes are very efficient antigen presenting cells that activate T lymphocytes (B). T lymphocytes in turn activate B cells, enabling them to start ectopic germinal center reactions (C) in the target tissue of autoimmunity. In the germinal center, B lymphocytes undergo somatic hypermutation and class switch recombination, amplifying the autoimmune response and shaping the pathogenic autoimmune memory (D). Remarkably, not only B lymphocytes drive autoimmune disease, but may also control it via regulatory B lymphocytes (E) which suppress autoimmunity by different mechanisms such as the production of IL-10 and TGFβ, and the induction of apoptosis in effector cells.

without type 1 diabetes (T1D) family history, autoantibodies to pancreatic islets (ICAS), human glutamic acid decarboxylase (GAD), receptor-type protein tyrosine phosphatase IA-2 and insulin were established at early age and prospectively identified all individuals who developed T1D [14]. In a retrospective study of 130 SLE patients, at least one disease specific autoantibody was present in all individuals months to years before clinical diagnosis [15]. In two seminal studies in RA, approximately half of the patients were positive for rheumatoid factor or anti-citrullinated protein antibodies (ACPA) up to 10 years before the onset of symptoms [16,17]. In absolute terms, however, only a minority of healthy individuals with RF or ACPA will develop RA (around 16%) [18], even when preselected on their attendance to a rheumatology outpatient clinic.

Taken together, these experimental and human data clearly indicate that leaky central tolerance increases the risk for subsequent development of autoimmune disease (Fig. 1A) but that a number of additional factors control this progression from autoimmunity to autoimmune disease. This clinical concept is in agreement with the existence of immunological peripheral tolerance checkpoints as described by Wardemann, Meffre and colleagues [1]. In this review, we will focus on the role of antigen specific B cells in the progression from autoimmunity to autoimmune disease. B lymphocytes interact with cognate autoreactive T cells. In this context, several aspects of the B cell biology uniquely endow them to amplify the autoimmune response and drive the progression towards autoimmune disease. In addition to this enhancing behavior, nonetheless, recent studies in experimental autoimmune disease have also revealed novel immunoregulatory roles for B cells. We will discuss here a number of key mechanisms by which autoreactive B cells can promote and control the progression from autoimmunity to clinical disease in experimental and human autoimmune disorders.

3. Autoantigen presentation and activation of pathogenic T cells

B lymphocytes are uniquely endowed to drive autoimmunity as antigen presenting cells because they can bind native self proteins through their BCR, process them and present them to T lymphocytes (Fig. 1B). In murine EAE, B lymphocytes are dispensable when disease is induced by MOG peptides but absolutely required for disease to develop if mice are immunized with MOG protein [19]. In MOG-specific TCR and BCR double transgenic mice, autoreactive B cells cause severe EAE by presentation of endogenous MOG protein to autoreactive T cells rather than by production of autoantibodies [20,21]. In the transgenic mIgM.MRL-FAS^{lpr} mouse, whose B lymphocytes cannot secrete antibodies but can present antigen, lupus develops spontaneously and T cell activation is comparable to MRL/lpr controls [22]. In the same way, NOD mice with a mutant IgM heavy chain that cannot be secreted, demonstrate that increased insulitis and spontaneous diabetes may occur in the absence of the antibody production but require antigen presentation by B cells [23].

The unique ability of B cells to bind autoantigens through their BCR allows them to act as potent antigen presenting

cells at very low protein concentrations. In vitro experiments show that B cells specific for tetanus toxoid native protein and rabbit anti-mouse immunoglobulin are able to present their cognate antigen to antigen specific T cells at 2000–10,000 fold lower concentrations that non-specific B cells [24,25]. B cells recognizing TNP conjugated to a terpolymer of glutamic acid, lysine and phenylalanine (GLØ) are able to stimulate GLØ-reactive T cells at a concentration of 0.1 μg/ml. Moreover, at this low concentration, as few as 7000 B cells can induce 70% of the T cell maximal response. In contrast, the same B cells can only present unconjugated, non-BCR specific GLØ at 1000 times higher concentrations (100 μg/ml) and higher cell numbers (20,000 B cells) [26]. The efficiency of antigen presentation by B cells at low concentrations of antigen has also been demonstrated in the context of autoimmune disease. In the MOG-specific TCR and BCR double transgenic mice, antigen specific B cells selectively process and present MOG protein to T cells at concentrations 100 fold lower than B cells with other BCR specificities [20,21]. Moreover, non-antigen specific B cells can prime T cells to some degree in a proteoglycan (PG)-induced arthritis model, but that level of activation is not sufficient to transfer disease to SCID mice. In contrast, when antigen specific B cells prime T cells, these become efficiently activated and competent to transfer arthritis [27].

4. Amplification of the autoimmune response by epitope spreading

B cells bind to a specific epitope in antigens via their BCR. Subsequently, protein and even protein complexes can be internalized and processed for antigen presentation. The protein may, however, contain several other epitopes besides the epitope originally recognized by the BCR, which eventually can fit in the binding grooves of the MHCII molecules of the B cell. As a consequence, the B cells can present not only the original epitope but also other epitopes of the same protein or protein complex to T lymphocytes and thereby trigger different T cell specificities [28]. This phenomenon, known as epitope spreading, allows self antigens that were not the initial targets of autoreactive lymphocytes at the onset of autoimmunity to become antigens at later stages [29].

Epitope spreading is documented in almost all immune diseases and is frequently associated with disease progression. Studies of the SLE related antigens, Ro and La ribonucleoproteins, have demonstrated that both intra-molecular and inter-molecular epitope spreading leads to autoimmunity in mice, the latter occurring when the two antigens are physically linked in vivo [30,31]. However, not only autoimmunity but also overt autoimmune disease can be triggered by epitope spreading. SJL/J mice immunized with protelipid (PLP) protein develop T cell responses specific for different epitopes in the molecule. These distinct T cell responses contribute to the relapse phases of the EAE and can initiate disease upon secondary adoptive transfer to naïve animals [32]. In the NOD mouse model of spontaneous diabetes, T cell responses and antibodies to the T1D autoantigens GAD65 and GAD67 isoforms of GAD (glutamic acid decarboxylase) are observed in mice at 4 weeks of age. At 6 weeks of age, T and B lymphocyte responses for other β cell antigens: peripherin, carboxypeptidase H and Hsp60, are also detected. By 8 weeks of age, responses to all former antigens are enhanced. Remarkably, the initial GAD specific reactivity in this model coincides with the onset of insulitis whereas the progression of insulitis to β cell destruction with age correlates to the epitope spreading of B and T cells [33]. Temporal progression of autoreactivity to autoimmune disease by epitope spreading also occurs in humans. In childhood T1D diabetes, insulin autoantibodies (IAA) are the first autoantibodies detected. IAA-positive children that sequentially develop antibodies to other β cell antigens such as GAD and protein tyrosine phosphatase-like proteins IA-2 and IA-3β, usually progress to T1D. In contrast, children that remain positive for only IAAs rarely develop the disease [34]. Also in RA, several reports have shown that the number of antibody specificities increases with time. Similarly to T1D patients, individuals with a broad ACPA repertoire, e.g. antibodies recognizing more citrullinated peptides, have a higher risk of developing arthritis [35,36]. In SLE, ANAs, anti-Ro, anti-La and antiphospholipid antibodies appear first, followed by anti-dsDNA antibodies, and later by anti-Sm and anti-nuclear ribonucleoproteins. The number of autoantibodies and their absolute concentrations increase until the onset of clinical symptoms and remain stable after clinical diagnosis [15].

5. Induction of novel autoreactivities by somatic hypermutation

During the germinal center (GC) reaction, B cells undergo somatic hypermutation (SHM) to increase the affinity of their BCR for their cognate antigen (Fig. 1C). This process is not only relevant for immune defense against pathogens and malignancies but can also contribute to enhance autoimmune responses. In the lupus-prone MRL/lpr mouse, B cell clones evolve towards higher affinity to dsDNA by somatic hypermutations [37]. Besides, analysis of the ratio of replacement to silent mutations in the V regions of the Ig genes of rheumatoid factors secreted by hybridomas from this strain, also demonstrates that SHM is an antigen-driven selection process [38]. Accordingly, high replacement to silent mutation ratio in the CDR regions, and hence, affinity maturation, is documented in patients with multiple sclerosis (MS) [39–41], RA [42], Type 1 diabetes and Sjögren's syndrome [43,44].

Interestingly, SHM does not only drive autoimmune disease by mediating antigen-driven affinity maturation (Fig. 1C) but also generates new autoreactive BCRs from non self-reactive B cells. Indeed, random mutations of non self-reactive BCRs during this affinity maturation process generate BCRs of new specificities, some of which may be self-reactive and hence have the potential to drive autoimmune disease. Much of the knowledge about the capacity of SHM to generate autoreactivities de novo is derived from the study of anti-dsDNA antibodies, the hallmark of SLE. Hybridoma studies have shown that somatic mutation of one amino acid in an IgA murine anti-phosphocholine antibody, which mediates protection against *Streptococcus pneumoniae*, results in acquired reactivity to dsDNA [45,46]. More recently, mutation reversion analysis in the lupus prone C57BL/6.NZB-Nba2 mouse has revealed that ANA originate from non autoreactive B cells that have diversified their Ig genes via SHM [47]. In humans, analyses using site directed mutagenesis to revert the somatic mutations in human anti-dsDNA antibodies have also shown that high affinity binding to dsDNA is acquired in a stepwise

manner through SHM from non self-reactive B cells. Furthermore, self reactivity to other nuclear epitopes from apoptotic cells is acquired in the same somatic hypermutation process that generates high affinity dsDNA binding from non self reactive germ-line V regions [48]. Reversion analysis of two autoantibodies with high reactivity for the nuclear autoantigens Ro52 and La in SLE also demonstrated that they originated from naïve cells that were not self-reactive, probably as a consequence of SHM [49].

6. Diversification of the effector functions by isotype switching

During a germinal center reaction, B cells undergo not only somatic hypermutation but also isotype switching (Fig. 1C) [50]. The isotype determines part of the effector function of autoantibodies [51–54], shaping the immunological pathogenic memory (Fig. 1D). In general terms, a broader isotype usage of autoantibodies in autoimmunity not only indicates a more extensive B cell activation but, more importantly, also implies that different effector functions can cooperate to drive or aggravate autoimmune disease. Much of our current understanding about how class switching enhances autoimmune disease is derived from SLE as prototypical autoantibody driven disorder. Low affinity IgM antibodies to dsDNA, RNA, La and Ro are present in sera from healthy relatives of SLE patients [55] and in the sera of RA and SpA patients treated with TNF blockers [12]. Nonetheless, for SLE to occur, self reactive antibodies must undergo affinity maturation and class switching to IgG [56]. In patients with both IgM and IgG anti-dsDNA antibodies in serum, predominant IgM titers relate to less active disease, milder or no renal involvement and longer survival [57]. Conversely, IgG anti-dsDNA antibodies are associated with more severe clinical disease, immunoglobulin and complement deposits in renal glomeruli and impaired renal function [57,58]. Regarding immunoglobulin subclasses in SLE, most studies indicate that IgG1 and IgG3 are the most common IgG subclasses in anti-dsDNA autoantibodies. IgG1 is also the predominant subclass for anti-RNP, anti-Ro, anti-La and anti-SM antibodies [59–62]. The facts that IgG1 and IgG3 fix complement better than other subclasses and are associated with clinical nephritis in SLE patients, confirm the hypothesis that different isotypes with different effector functions can enhance autoimmune disease [62]. Also in RA a varied isotype usage by autoantibodies is associated with a more severe disease course. Undifferentiated arthritis patients who progress to RA have a more diverse autoantibody response consisting of IgM, IgG1, IgG3 and IgA anti-CCP antibodies than those who do not progress to RA [63]. The presence of IgM, IgG and IgA ACPAs in RA patients indicates higher risk of future radiographic damage [64,65] and resistance to biologic therapy [66]. More recently, IgE antibodies against citrullinated fibrinogen have been demonstrated in ACPA+ RA patients. These IgE antibodies activate rat basophils expressing human FcεRI in vitro and bind to mast cells in synovial tissue in vivo. As mast cells are degranulated and high histamine levels are found in SF from ACPA+ patients, IgE could be responsible for a different effector mechanism of inflammation in RA [67]. Anti-CCP autoantibodies of the IgA isotype are significantly correlated with smoking and may predict a more severe disease course in RA [68]. The same association of autoantibodies of the IgA isotype and a more severe disease is also observed for RF as patients with positive IgA RF and IgM RF had more radiological damage than IgA RF negative patients [69,70]. Moreover, high levels of IgA RF are associated with poor clinical response to TNF blockers [71].

7. Immunomodulation by regulatory B cells

An important aspect in the progression from autoimmunity to autoimmune inflammation is that antigen specific B cells may have a regulatory function and thereby keep autoreactive lymphocytes in check (Fig. 1E). Regulatory B cells (Bregs) that suppress inflammation by producing IL-10 (so-called B10 cells) were first identified in murine models. Phenotypically, they are CD1d high, CD5+, CD19 high, CD21 high/int, CD23 low, CD24 high, CD43+/− and CD93− [72], but their characterization is often debated as they share surface markers with the CD5+ B-1a, CD21+ CD23− and the CD1d+ CD21+ CD23+ T2 marginal zone precursor B cells [73,74]. Studies in mice have demonstrated that regulatory B cells expand with age independently of T cell or environmental gut flora derived signals. BCR diversity, CD19 and MyD88 signals are critical for the development of B10 cells and CD22 deficiency and CD40L overexpression enhance their numbers. Importantly, regulatory B cells are in principle, antigen specific, as they require antigen stimulation via the BCR plus CD40 ligation to produce IL-10 [72,75,76]. They can proliferate in vivo and their mechanisms of suppressing inflammation include the suppression of activated CD4 T cells by means of IL-10 production [73,77], the conversion of effector CD4 T cells to IL-10 producing Tr1 cells [78,79], and the induction of regulatory T cells by means of TGFβ [80] and B7-mediated costimulation [81]. Because regulatory B regs express Fas-L, they can also directly eliminate effector CD4 T cells via Fas mediated apoptosis [82]. Finally, they can also play a role in the homeostasis of iNKT cells [83].

Numerous studies have analyzed the role of regulatory B cells in murine models of autoimmune disease. Downregulation of inflammation by IL-10 producing regulatory B cells is observed in collagen induced arthritis [73,76] and in inflammatory bowel disease like-chronic intestinal inflammation [84]. Accordingly, IL-10 producing CD1d high CD5+ regulatory B cells downregulate contact hypersensitivity responses [77] and dextran-sulfate sodium induced intestinal injury, which is a model for ulcerative colitis [85]. Regulatory CD1d+ CD5+ B cells located in the spleen inhibit EAE initiation in mice early in the pathogenesis by producing IL-10 [86], and are also important in the recovery phase [75]. Finally, intravenous transfusion of BCR-activated B cells cultured with anti-IgM antibodies protects NOD mice from T1D in an IL-10 dependent manner by reducing the incidence and delaying the onset of disease and polarizing T cells to the Th2 phenotype [87].

A number of studies have also described a regulatory role of B cells in murine models of asthma and allergy. In a cockroach-allergy model, the subpopulation CD5 B-1a, positive for Fas-L, attenuates inflammatory cytokine production and pulmonary inflammation by binding to the Fas receptor on activated $CD4^+$ T cells, hence inducing apoptosis [88]. In an OVA-induced mouse model of allergic airway disease, continuous exposure to allergens leads to the development of local inhalational tolerance (LIT) mediated by B cells. B cells transferred from LIT mice lead to a diminution in pulmonary eosinophilic

infiltration, bronchial hyperreactivity, lung inflammation and promoted the generation of Foxp3+ regulatory T cells. Mechanisms of tolerance involve in the generation of Foxp3+ Tregs were mediated by the production of TGFβ by B cells [89]. Also, helminth infections in an OVA-asthma model induce a strong accumulation of B cells in the spleen and stop the allergic airway inflammation [90]. Contrary to the previous studies, B cells mediated their regulatory functions by the secretion of IL-10. Surface phenotype of IL-10 producing B cells demonstrated that they were CD5 CD21 high CD23 CD1d high and IgM high resembling T2-MZ precursor B cells. Furthermore the transfer of IL-10 Bregs in sensitized mice leads to a diminution of Th2 cytokines in broncho-alveolar liquid (BAL) and the generation of $Foxp3^+$ regulatory T cells [90].

Contrary to the wealth of information on the phenotype and function of regulatory B cells in mice, these cells remain relatively poorly characterized in humans. IL-10 producing B cells have been detected in CD24 high CD27+ [91] and CD24 high CD38 high B cell compartments [92], but their exact origin and induction requirements are uncertain at this point. Also in humans this specific B cell subset was suggested to have immunomodulatory capacities as IL-10 produced upon combined CpG and anti-IgG stimulation can effectively downregulate CD4 activation in vitro [93]. In vivo, helminth infections induce a B cell population producing high levels of IL-10 which could counterbalance neurological damage in MS patients [94]. Moreover, different autoimmune diseases have been associated with decreased numbers or impaired function of IL-10 producing B cells: relapsing remitting MS patients have a decreased frequency of IL-10+ B cells in comparison with healthy controls [95] and the regulatory capacities of CD19+ CD24 high CD38 high B cells are impaired in SLE [92].

8. Lessons from alloimmunity and graft rejection

A major issue in studying the progression from autoimmunity to autoimmune disease in general and the role of B cells in this process in particular is that we do not know exactly in humans when autoimmunity starts and when it progresses to autoimmune disease. In alloimmunity, we can of course not study 'central tolerance' but we know exactly when the alloantigen is appearing and thus the alloresponse is initiated. Also here, some individuals do develop a clear alloresponse but do not reject their graft, called operational tolerance [96]. B cells have now also emerged to play a key role in these processes.

The central role of B cells in graft rejection was originally suggested by the link between circulating donor-specific antibodies (DSA) and chronic rejection [97]. This link has not only been confirmed in multiple experimental models [98,99] but also in humans: a recent large prospective study demonstrated that transplant patients with DSA had more risk to develop graft failure compared to those who did not have DSA [100]. Development of specific immunoassay demonstrated that highly polymorphic mismatched HLA molecules were the most representative target of DSA [101] with a dominant contribution of alloantibody against HLA-II rather than anti-HLA I [102,103]. Besides anti-HLA antibodies, immunoglobulins against non-HLA antigens have also been identified to contribute to the pathogenesis of antibody-mediated rejection [104–106]. The targets of humoral responses against non-HLA antigens are very diverse, including endothelial cells (AEC) [107–110] and MICA molecules [111–116].

Interestingly, it is currently well recognized that alloimmune responses act together with tissue-specific autoimmune responses to promote graft rejection. Indeed, graft lesions induced by alloreactive cytotoxic T lymphocytes and alloantibodies induce the release of not only alloantigens but also sequestrated self-tissue antigens that can be presented via the indirect pathway to generate pathogenic autoreactive cellular and humoral immune responses [117]. For example, autoantibodies against vimentin, a non-polymorphic intermediate filament expressed in cytosol of a wide variety of cells including endothelial cells and vascular smooth muscle cells, contribute to both acute and chronic rejection of cardiac allografts [118–120]. Other autoantigens targeted by humoral responses in chronic rejection include collagen V [121,122], the heparan sulfate proteoglycan agrin [123], the Angiotensin II receptor type I [124], and intra-nuclear antigens [125]. The exact mechanisms underlying the breach in self tolerance during chronic graft rejection remain poorly understood but probably include epitope spreading and generation of pathogenic autoreactive T cells [117,126], the formation of tertiary lymphoid tissues in the graft [127–129], and enhanced plasma cell generation and survival mediated by factors such as BAFF [129]. Taken together, these data clearly indicate the role of not only alloreactive but also autoreactive B cells in the initiation and amplification of the graft rejection and demonstrate striking mechanistic parallels with the B cell biology in autoimmune diseases.

9. Tolerogenic B lymphocytes in organ transplantation

Although B cells are commonly described for their pathogenic effector functions, recent evidence emphasizes the potential tolerogenic role of B cells in the long term graft survival [130,131]. In kidney transplantation, a small proportion of recipients can tolerate their graft after stopping their immunosuppressive therapy [96]. These patients called operationally tolerant display a particular B cell signature in their blood [130,132–134]. Three aspects of this B cell signature are of particular interest.

Firstly, transplant tolerance was associated with a strong expression of inhibitory molecules such as FcγRIIb receptor and BANK-1 by B cells [132]. These observations were confirmed in a long-term cardiac allograft tolerance model, where it was shown that B cells of tolerant rats displayed an inhibitory phenotype and could transfer tolerance in newly transplanted rats [135]. Secondly, a higher proportion and number of naive and transitional B cells were found in operationally tolerant patients compared to healthy donors and stable patients under immunosuppression [130,132,133,136]. This naïve/transitional signature was also found in patients undergoing depletion strategies or minimization of immunosuppression [137,138]. From a functional perspective, it was shown that naïve and transitional B cells are poor stimulators for T cells [139–141] and preferentially generate regulatory T cells [142–144]. This may explain the induction of acceptance of skin allografts by transfer of naïve B cells [140]. In addition, polyclonal activation of transitional B cells induces more

secretion of IL-10 in tolerant patients compared to stable patients [130]. These data suggest that the re-population by immature and naïve B cells could be a feature facilitating tolerance in kidney transplantation.

Evidence from animal models of neonatal tolerance [145] and from ABO-incompatible heart transplantation in children [146] additionally indicates that clonal depletion could also contribute to the appearance of B cell tolerance. In these settings, tolerance is promoted by elimination of donor-reactive B lymphocytes [147,148] which may be dependent of the persistence of alloantigen during B cell development. We can hypothesize that B cell depleting therapies could have a similar effect as repopulation with naïve and transitional B cells in the presence of allograft antigens may promote clonal deletion of alloreactive clones.

Taken together, these data indicate that B cell tolerance in transplantation can be achieved by different mechanisms including inhibition of effector mechanisms, receptor editing, generation of regulatory T cells, production of IL-10, and clonal deletion [149,150]. As such, the induction of B cell tolerance in transplantation mirrors the progression from subclinical B cell autoreactivity to overt inflammatory disease in autoimmunity.

10. Clinical implications and future perspectives

The global picture emerging from these studies in experimental models and human disorders indicates that cognate interaction between B and T cells plays a crucial role in the progression from autoimmunity to autoimmune disease. In particular, autoreactive B cells contribute to the activation of T cells reactive against the original autoantigen as well as against linked antigens and, with the help of T cells, increase their affinity for the original autoantigen, mutate to novel autoreactivities and diversify their effector functions by isotype switching. Taken together, all these mechanisms lead to amplification and progression of the autoimmune response. Better mechanistic understanding of the different facets of this process is of great clinical relevance as it may help us to design novel and better tailored treatments for a wide variety of autoimmune diseases.

Firstly, detection of autoantibodies and/or autoreactive lymphocytes in healthy individuals may offer a window of opportunity to halt the progression from autoimmunity to autoimmune disease. However, many individuals with autoantibodies will never develop overt autoimmune disease. The major challenge here will be to determine either additional risk factors or specific features of this early autoimmune response allowing one to predict who will and who will not develop overt autoimmune disease. Better definition of both the fine specificity and the breath of the autoimmune response may be of importance in this context. Secondly, in such individuals we could try to interrupt cognate T–B cell interactions with established T or B cell directed drugs such as abatacept and rituximab. Even more attractive targets here are key molecules involved in the cognate T–B cell interactions such as CD40-CD40L. The original anti-CD40L antibodies were highly effective but had thromboembolic side effects by affecting platelets [151]. New anti-CD154 antibodies as well as other co-stimulation blockers are currently in clinical development and could be particularly useful in these pre-clinical conditions. Thirdly, better understanding of the features and specificities of autoreactive T and B cells at this stage may open novel perspective for antigen-specific therapies. Tolerance induction protocols have yielded disappointing results in full-blown autoimmune disease [152,153] but may turn out to be much more promising in pre-clinical disease. Alternatively, the development of novel technologies to detect and identify expanded lymphocyte clones [154,155] could lead to more specific targeting of these specific cell populations rather than global B or T cell targeting [156]. Finally, further description and functional analysis of IL-10 producing B cells subsets may yield novel insights in how to expand these cells and thereby counter-regulate developing or established autoimmune disease. IFNβ, a validated treatment for MS, has recently been shown to enhance IL-10 production by B cells [157]. Moreover, we recently demonstrated that, in sharp contrast with BAFF which promotes autoimmune disease, overexpression of the B cell growth factor APRIL leads to an expansion of the B10 pool and suppression of a variety of immune-mediated inflammatory disease models. Translation of these findings to the human setting will open new perspectives for the immunotherapy of autoimmune diseases.

Conflict of interest statement

The author(s) declare that there are no conflicts of interest.

References

[1] S. Yurasov, H. Wardemann, J. Hammersen, M. Tsuiji, E. Meffre, V. Pascual, M.C. Nussenzweig, Defective B cell tolerance checkpoints in systemic lupus erythematosus, J. Exp. Med. 201 (2005) 703–711.

[2] T. Litzenburger, H. Bluthmann, P. Morales, D. Pham-Dinh, A. Dautigny, H. Wekerle, A. Iglesias, Development of myelin oligodendrocyte glycoprotein autoreactive transgenic B lymphocytes: receptor editing in vivo after encounter of a self-antigen distinct from myelin oligodendrocyte glycoprotein, J. Immunol. 165 (2000) 5360–5366.

[3] T. Litzenburger, R. Fassler, J. Bauer, H. Lassmann, C. Linington, H. Wekerle, A. Iglesias, B lymphocytes producing demyelinating autoantibodies: development and function in gene-targeted transgenic mice, J. Exp. Med. 188 (1998) 169–180.

[4] L. Menard, D. Saadoun, I. Isnardi, Y.S. Ng, G. Meyers, C. Massad, C. Price, C. Abraham, R. Motaghedi, J.H. Buckner, P.K. Gregersen, E. Meffre, The PTPN22 allele encoding an R620W variant interferes with the removal of developing autoreactive B cells in humans, J. Clin. Invest. 121 (2011) 3635–3644.

[5] M. Inaoki, S. Sato, B.C. Weintraub, C.C. Goodnow, T.F. Tedder, CD19-regulated signaling thresholds control peripheral tolerance and autoantibody production in B lymphocytes, J. Exp. Med. 186 (1997) 1923–1931.

[6] T. Habib, A. Funk, M. Rieck, A. Brahmandam, X. Dai, A.K. Panigrahi, E.T. Luning Prak, A. Meyer-Bahlburg, S. Sanda, C. Greenbaum, D.J. Rawlings, J.H. Buckner, Altered B cell homeostasis is associated with type I diabetes and carriers of the PTPN22 allelic variant, J. Immunol. 188 (2012) 487–496.

[7] E. Bettelli, M. Pagany, H.L. Weiner, C. Linington, R.A. Sobel, V.K. Kuchroo, Myelin oligodendrocyte glycoprotein-specific T cell receptor transgenic mice develop spontaneous autoimmune optic neuritis, J. Exp. Med. 197 (2003) 1073–1081.

[8] D.T. Robles, G.S. Eisenbarth, N.J. Dailey, L.B. Peterson, L.S. Wicker, Insulin autoantibodies are associated with islet

inflammation but not always related to diabetes progression in NOD congenic mice, Diabetes 52 (2003) 882–886.
[9] N. Toplak, T. Kveder, A. Trampus-Bakija, V. Subelj, S. Cucnik, T. Avcin, Autoimmune response following annual influenza vaccination in 92 apparently healthy adults, Autoimmun. Rev. 8 (2008) 134–138.
[10] Z. Karali, S.T. Basaranoglu, Y. Karali, B. Oral, S.S. Kilic, Autoimmunity and hepatitis A vaccine in children, J. Investig. Allergol. Clin. Immunol. 21 (2011) 389–393.
[11] P.J. Martinuc, T. Avcin, B. Bozic, M. Kuhar, S. Cucnik, M. Zupancic, K. Prosenc, T. Kveder, B. Rozman, Anti-phospholipid antibodies following vaccination with recombinant hepatitis B vaccine, Clin. Exp. Immunol. 142 (2005) 377–380.
[12] L. DeRycke, D. Baeten, E. Kruithof, F. Van den Bosch, E.M. Veys, K.F. De, Infliximab, but not etanercept, induces IgM anti-double-stranded DNA autoantibodies as main antinuclear reactivity: biologic and clinical implications in autoimmune arthritis, Arthritis Rheum. 52 (2005) 2192–2201.
[13] N. Gulez, N.E. Karaca, G. Aksu, N. Kutukculer, Increased percentages of autoantibodies in immunoglobulin A-deficient children do not correlate with clinical manifestations, Autoimmunity 42 (2009) 74–79.
[14] J.M. LaGasse, M.S. Brantley, N.J. Leech, R.E. Rowe, S. Monks, J.P. Palmer, G.T. Nepom, D.K. McCulloch, W.A. Hagopian, Successful prospective prediction of type 1 diabetes in schoolchildren through multiple defined autoantibodies: an 8-year follow-up of the Washington State Diabetes Prediction Study, Diabetes Care 25 (2002) 505–511.
[15] M.R. Arbuckle, M.T. McClain, M.V. Rubertone, R.H. Scofield, G.J. Dennis, J.A. James, J.B. Harley, Development of autoantibodies before the clinical onset of systemic lupus erythematosus, N. Engl. J. Med. 349 (2003) 1526–1533.
[16] M.M. Nielen, S.D. van, H.W. Reesink, R.J. van de Stadt, I.E. van der Horst-Bruinsma, M.H. de Koning, M.R. Habibuw, J.P. Vandenbroucke, B.A. Dijkmans, Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors, Arthritis Rheum. 50 (2004) 380–386.
[17] S. Rantapaa-Dahlqvist, B.A. de Jong, E. Berglin, G. Hallmans, G. Wadell, H. Stenlund, U. Sundin, W.J. van Venrooij, Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis, Arthritis Rheum. 48 (2003) 2741–2749.
[18] J. Ursum, W.H. Bos, R.J. van de Stadt, B.A. Dijkmans, S.D. van, Different properties of ACPA and IgM-RF derived from a large dataset: further evidence of two distinct autoantibody systems, Arthritis Res. Ther. 11 (2009) R75.
[19] J.A. Lyons, M. San, M.P. Happ, A.H. Cross, B cells are critical to induction of experimental allergic encephalomyelitis by protein but not by a short encephalitogenic peptide, Eur. J. Immunol. 29 (1999) 3432–3439.
[20] E. Bettelli, D. Baeten, A. Jager, R.A. Sobel, V.K. Kuchroo, Myelin oligodendrocyte glycoprotein-specific T and B cells cooperate to induce a Devic-like disease in mice, J. Clin. Invest. 116 (2006) 2393–2402.
[21] G. Krishnamoorthy, H. Lassmann, H. Wekerle, A. Holz, Spontaneous opticospinal encephalomyelitis in a double-transgenic mouse model of autoimmune T cell/B cell cooperation, J. Clin. Invest. 116 (2006) 2385–2392.
[22] O.T. Chan, L.G. Hannum, A.M. Haberman, M.P. Madaio, M.J. Shlomchik, A novel mouse with B cells but lacking serum antibody reveals an antibody-independent role for B cells in murine lupus, J. Exp. Med. 189 (1999) 1639–1648.
[23] F.S. Wong, L. Wen, M. Tang, M. Ramanathan, I. Visintin, J. Daugherty, L.G. Hannum, C.A. Janeway Jr., M.J. Shlomchik, Investigation of the role of B-cells in type 1 diabetes in the NOD mouse, Diabetes 53 (2004) 2581–2587.
[24] A. Lanzavecchia, Antigen-specific interaction between T and B cells, Nature 314 (1985) 537–539.
[25] T. Kakiuchi, R.W. Chesnut, H.M. Grey, B cells as antigen-presenting cells: the requirement for B cell activation, J. Immunol. 131 (1983) 109–114.
[26] K.L. Rock, B. Benacerraf, A.K. Abbas, Antigen presentation by hapten-specific B lymphocytes. I. Role of surface immunoglobulin receptors, J. Exp. Med. 160 (1984) 1102–1113.
[27] S.K. O'Neill, M.J. Shlomchik, T.T. Glant, Y. Cao, P.D. Doodes, A. Finnegan, Antigen-specific B cells are required as APCs and autoantibody-producing cells for induction of severe autoimmune arthritis, J. Immunol. 174 (2005) 3781–3788.
[28] G. Tchernev, C.E. Orfanos, Antigen mimicry, epitope spreading and the pathogenesis of pemphigus, Tissue Antigens 68 (2006) 280–286.
[29] C.A. Janeway Jr., T. Paul, M. Walport, M. Shlomchik, Immunobiology: The Immune System in Health and Disease, Garland Science Publishing, 2005..
[30] F. Topfer, T. Gordon, J. McCluskey, Intra- and intermolecular spreading of autoimmunity involving the nuclear self-antigens La (SS-B) and Ro (SS-A), Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 875–879.
[31] C.L. Keech, T.P. Gordon, J. McCluskey, The immune response to 52-kDa Ro and 60-kDa Ro is linked in experimental autoimmunity, J. Immunol. 157 (1996) 3694–3699.
[32] B.L. McRae, C.L. Vanderlugt, M.C. Dal Canto, S.D. Miller, Functional evidence for epitope spreading in the relapsing pathology of experimental autoimmune encephalomyelitis, J. Exp. Med. 182 (1995) 75–85.
[33] R. Tisch, X.D. Yang, S.M. Singer, R.S. Liblau, L. Fugger, H.O. McDevitt, Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice, Nature 366 (1993) 72–75.
[34] A.G. Ziegler, M. Hummel, M. Schenker, E. Bonifacio, Autoantibody appearance and risk for development of childhood diabetes in offspring of parents with type 1 diabetes: the 2-year analysis of the German BABYDIAB Study, Diabetes 48 (1999) 460–468.
[35] D. van der Woude, S. Rantapaa-Dahlqvist, A. Ioan-Facsinay, C. Onnekink, C.M. Schwarte, K.N. Verpoort, J.W. Drijfhout, T.W. Huizinga, R.E. Toes, G.J. Pruijn, Epitope spreading of the anti-citrullinated protein antibody response occurs before disease onset and is associated with the disease course of early arthritis, Ann. Rheum. Dis. 69 (2010) 1554–1561.
[36] L.A. van de Stadt, A.R. van der Horst, M.H. de Koning, W.H. Bos, G.J. Wolbink, R.J. van de Stadt, G.J. Pruijn, B.A. Dijkmans, S.D. van, D. Hamann, The extent of the anti-citrullinated protein antibody repertoire is associated with arthritis development in patients with seropositive arthralgia, Ann. Rheum. Dis. 70 (2011) 128–133.
[37] M. Shlomchik, M. Mascelli, H. Shan, M.Z. Radic, D. Pisetsky, A. Marshak-Rothstein, M. Weigert, Anti-DNA antibodies from autoimmune mice arise by clonal expansion and somatic mutation, J. Exp. Med. 171 (1990) 265–292.
[38] M.J. Shlomchik, A. Marshak-Rothstein, C.B. Wolfowicz, T.L. Rothstein, M.G. Weigert, The role of clonal selection and somatic mutation in autoimmunity, Nature 328 (1987) 805–811.
[39] G.P. Owens, A.M. Ritchie, M.P. Burgoon, R.A. Williamson, J.R. Corboy, D.H. Gilden, Single-cell repertoire analysis demonstrates that clonal expansion is a prominent feature of the B cell response in multiple sclerosis cerebrospinal fluid, J. Immunol. 171 (2003) 2725–2733.
[40] M. Colombo, M. Dono, P. Gazzola, S. Roncella, A. Valetto, N. Chiorazzi, G.L. Mancardi, M. Ferrarini, Accumulation of clonally related B lymphocytes in the cerebrospinal fluid of multiple sclerosis patients, J. Immunol. 164 (2000) 2782–2789.

[41] Y. Qin, P. Duquette, Y. Zhang, P. Talbot, R. Poole, J. Antel, Clonal expansion and somatic hypermutation of V(H) genes of B cells from cerebrospinal fluid in multiple sclerosis, J. Clin. Invest. 102 (1998) 1045–1050.
[42] K. Itoh, V. Patki, R.A. Furie, E.K. Chartash, R.I. Jain, L. Lane, S.E. Asnis, N. Chiorazzi, Clonal expansion is a characteristic feature of the B-cell repertoire of patients with rheumatoid arthritis, Arthritis Res. 2 (2000) 50–58.
[43] D.I. Stott, F. Hiepe, M. Hummel, G. Steinhauser, C. Berek, Antigen-driven clonal proliferation of B cells within the target tissue of an autoimmune disease. The salivary glands of patients with Sjogren's syndrome, J. Clin. Invest. 102 (1998) 938–946.
[44] S. Gellrich, S. Rutz, A. Borkowski, S. Golembowski, E. Gromnica-Ihle, W. Sterry, S. Jahn, Analysis of V(H)-D-J(H) gene transcripts in B cells infiltrating the salivary glands and lymph node tissues of patients with Sjogren's syndrome, Arthritis Rheum. 42 (1999) 240–247.
[45] D. Eilat, M. Hochberg, J. Pumphrey, S. Rudikoff, Monoclonal antibodies to DNA and RNA from NZB/NZW F1 mice: antigenic specificities and NH2 terminal amino acid sequences, J. Immunol. 133 (1984) 489–494.
[46] B. Diamond, M.D. Scharff, Somatic mutation of the T15 heavy chain gives rise to an antibody with autoantibody specificity, Proc. Natl. Acad. Sci. U. S. A. 81 (1984) 5841–5844.
[47] W. Guo, D. Smith, K. Aviszus, T. Detanico, R.A. Heiser, L.J. Wysocki, Somatic hypermutation as a generator of antinuclear antibodies in a murine model of systemic autoimmunity, J. Exp. Med. 207 (2010) 2225–2237.
[48] U. Wellmann, M. Letz, M. Herrmann, S. Angermuller, J.R. Kalden, T.H. Winkler, The evolution of human anti-double-stranded DNA autoantibodies, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 9258–9263.
[49] B. Mietzner, M. Tsuiji, J. Scheid, K. Velinzon, T. Tiller, K. Abraham, J.B. Gonzalez, V. Pascual, D. Stichweh, H. Wardemann, M.C. Nussenzweig, Autoreactive IgG memory antibodies in patients with systemic lupus erythematosus arise from nonreactive and polyreactive precursors, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 9727–9732.
[50] Y.J. Liu, F. Malisan, B.O. de, C. Guret, S. Lebecque, J. Banchereau, F.C. Mills, E.E. Max, H. Martinez-Valdez, Within germinal centers, isotype switching of immunoglobulin genes occurs after the onset of somatic mutation, Immunity 4 (1996) 241–250.
[51] T. Kurosaki, Y. Aiba, K. Kometani, S. Moriyama, Y. Takahashi, Unique properties of memory B cells of different isotypes, Immunol. Rev. 237 (2010) 104–116.
[52] A.J. Macpherson, M.B. Geuking, K.D. McCoy, Immunoglobulin A: a bridge between innate and adaptive immunity, Curr. Opin. Gastroenterol. 27 (2011) 529–533.
[53] F. Nimmerjahn, J.V. Ravetch, Divergent immunoglobulin g subclass activity through selective Fc receptor binding, Science 310 (2005) 1510–1512.
[54] J. Kalesnikoff, S.J. Galli, New developments in mast cell biology, Nat. Immunol. 9 (2008) 1215–1223.
[55] N. Talal, R.J. Pillarisetty, R.J. DeHoratius, R.P. Messner, Immunologic regulation of spontaneous antibodies to DNA and RNA I. Significance of IgM and IgG antibodies in SLE patients and asymptomatic relatives, Clin. Exp. Immunol. 25 (1976) 377–382.
[56] N.F. Rothfield, B.D. Stollar, The relation of immunoglobulin class, pattern of anti-nuclear antibody, and complement-fixing antibodies to DNA in sera from patients with systemic lupus erythematosus, J. Clin. Invest. 46 (1967) 1785–1794.
[57] J.B. Pennebaker, J.N. Gilliam, M. Ziff, Immunoglobulin classes of DNA binding activity in serum and skin in systemic lupus erythematosus, J. Clin. Invest. 60 (1977) 1331–1338.
[58] H. Hashimoto, Y. Utagawa, J. Yamagata, T. Okada, Y. Shiokawa, S. Suzuki, T. Tomiyama, The relationship of renal histopathological lesions to immunoglobulin classes and complement fixation of anti-native DNA antibodies in systemic lupus erythematosus, Scand. J. Rheumatol. 12 (1983) 209–214.
[59] W.J. Yount, P. Cohen, R.A. Eisenberg, Distribution of IgG subclasses among human autoantibodies to Sm, RNP, dsDNA, SS-B and IgG rheumatoid factor, Monogr. Allergy 23 (1988) 41–56.
[60] R. Elenitsas, E. Abell, Y.Y. Lee, J. Huang, J.S. Deng, Comparison of IgG subclass autoantibodies in patients with systemic lupus erythematosus and subacute cutaneous lupus erythematosus, J. Dermatol. Sci. 1 (1990) 207–215.
[61] M. Wahren, N.R. Ringertz, I. Pettersson, IgM and IgG subclass distribution of human anti-Ro/SSA 60 kDa autoantibodies, Scand. J. Immunol. 39 (1994) 179–183.
[62] P.H. Schur, M. Monroe, N. Rothfield, The gammaG subclass of antinuclear and antinucleic acid antibodies, Arthritis Rheum. 15 (1972) 174–182.
[63] K.N. Verpoort, C.M. Jol-van der Zijde, E.A. Papendrecht-van der Voort, A. Ioan-Facsinay, J.W. Drijfhout, M.J. van Tol, F.C. Breedveld, T.W. Huizinga, R.E. Toes, Isotype distribution of anti-cyclic citrullinated peptide antibodies in undifferentiated arthritis and rheumatoid arthritis reflects an ongoing immune response, Arthritis Rheum. 54 (2006) 3799–3808.
[64] D. van der Woude, S.W. Syversen, E.I. van der Voort, K.N. Verpoort, G.L. Goll, M.P. van der Linden, A.H. van der Helm-van Mil, D.M. van der Heijde, T.W. Huizinga, T.K. Kvien, R.E. Toes, The ACPA isotype profile reflects long-term radiographic progression in rheumatoid arthritis, Ann. Rheum. Dis. 69 (2010) 1110–1116.
[65] H. Kokkonen, M. Mullazehi, E. Berglin, G. Hallmans, G. Wadell, J. Ronnelid, S. Rantapaa-Dahlqvist, Antibodies of IgG, IgA and IgM isotypes against cyclic citrullinated peptide precede the development of rheumatoid arthritis, Arthritis Res. Ther. 13 (2011) R13.
[66] R.H. Syed, B.E. Gilliam, T.L. Moore, Prevalence and significance of isotypes of anti-cyclic citrullinated peptide antibodies in juvenile idiopathic arthritis, Ann. Rheum. Dis. 67 (2008) 1049–1051.
[67] A.J. Schuerwegh, A. Ioan-Facsinay, A.L. Dorjee, J. Roos, I.M. Bajema, E.I. van der Voort, T.W. Huizinga, R.E. Toes, Evidence for a functional role of IgE anticitrullinated protein antibodies in rheumatoid arthritis, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 2586–2591.
[68] A. Svard, A. Kastbom, A. Reckner-Olsson, T. Skogh, Presence and utility of IgA-class antibodies to cyclic citrullinated peptides in early rheumatoid arthritis: the Swedish TIRA project, Arthritis Res. Ther. 10 (2008) R75.
[69] D.A. Houssien, T. Jonsson, E. Davies, D.L. Scott, Rheumatoid factor isotypes, disease activity and the outcome of rheumatoid arthritis: comparative effects of different antigens, Scand. J. Rheumatol. 27 (1998) 46–53.
[70] E. Lindqvist, K. Eberhardt, K. Bendtzen, D. Heinegard, T. Saxne, Prognostic laboratory markers of joint damage in rheumatoid arthritis, Ann. Rheum. Dis. 64 (2005) 196–201.
[71] F. Bobbio-Pallavicini, R. Caporali, C. Alpini, S. Avalle, O.M. Epis, C. Klersy, C. Montecucco, High IgA rheumatoid factor levels are associated with poor clinical response to tumour necrosis factor alpha inhibitors in rheumatoid arthritis, Ann. Rheum. Dis. 66 (2007) 302–307.
[72] K. Yanaba, J.D. Bouaziz, T. Matsushita, T. Tsubata, T.F. Tedder, The development and function of regulatory B cells expressing IL-10 (B10 cells) requires antigen receptor diversity and TLR signals, J. Immunol. 182 (2009) 7459–7472.
[73] J.G. Evans, K.A. Chavez-Rueda, A. Eddaoudi, A. Meyer-Bahlburg, D.J. Rawlings, M.R. Ehrenstein, C. Mauri, Novel suppressive

function of transitional 2 B cells in experimental arthritis, J. Immunol. 178 (2007) 7868–7878.
[74] P. Lenert, R. Brummel, E.H. Field, R.F. Ashman, TLR-9 activation of marginal zone B cells in lupus mice regulates immunity through increased IL-10 production, J. Clin. Immunol. 25 (2005) 29–40.
[75] S. Fillatreau, C.H. Sweenie, M.J. McGeachy, D. Gray, S.M. Anderton, B cells regulate autoimmunity by provision of IL-10, Nat. Immunol. 3 (2002) 944–950.
[76] C. Mauri, D. Gray, N. Mushtaq, M. Londei, Prevention of arthritis by interleukin 10-producing B cells, J. Exp. Med. 197 (2003) 489–501.
[77] K. Yanaba, J.D. Bouaziz, K.M. Haas, J.C. Poe, M. Fujimoto, T.F. Tedder, A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses, Immunity 28 (2008) 639–650.
[78] P.A. Blair, K.A. Chavez-Rueda, J.G. Evans, M.J. Shlomchik, A. Eddaoudi, D.A. Isenberg, M.R. Ehrenstein, C. Mauri, Selective targeting of B cells with agonistic anti-CD40 is an efficacious strategy for the generation of induced regulatory T2-like B cells and for the suppression of lupus in MRL/lpr mice, J. Immunol. 182 (2009) 3492–3502.
[79] M. Gray, K. Miles, D. Salter, D. Gray, J. Savill, Apoptotic cells protect mice from autoimmune inflammation by the induction of regulatory B cells, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 14080–14085.
[80] J. Tian, D. Zekzer, L. Hanssen, Y. Lu, A. Olcott, D.L. Kaufman, Lipopolysaccharide-activated B cells down-regulate Th1 immunity and prevent autoimmune diabetes in nonobese diabetic mice, J. Immunol. 167 (2001) 1081–1089.
[81] M.K. Mann, K. Maresz, L.P. Shriver, Y. Tan, B.N. Dittel, B cell regulation of CD4+CD25+ T regulatory cells and IL-10 via B7 is essential for recovery from experimental autoimmune encephalomyelitis, J. Immunol. 178 (2007) 3447–3456.
[82] S.K. Lundy, D.A. Fox, Reduced Fas ligand-expressing splenic CD5+ B lymphocytes in severe collagen-induced arthritis, Arthritis Res. Ther. 11 (2009) R128.
[83] A. Bosma, A. Abdel-Gadir, D.A. Isenberg, E.C. Jury, C. Mauri, Lipid-antigen presentation by CD1d(+) B cells is essential for the maintenance of invariant natural killer T cells, Immunity 36 (2012) 477–490.
[84] A. Mizoguchi, E. Mizoguchi, H. Takedatsu, R.S. Blumberg, A.K. Bhan, Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation, Immunity 16 (2002) 219–230.
[85] K. Yanaba, A. Yoshizaki, Y. Asano, T. Kadono, T.F. Tedder, S. Sato, IL-10-producing regulatory B10 cells inhibit intestinal injury in a mouse model, Am. J. Pathol. 178 (2011) 735–743.
[86] T. Matsushita, K. Yanaba, J.D. Bouaziz, M. Fujimoto, T.F. Tedder, Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression, J. Clin. Invest. 118 (2008) 3420–3430.
[87] S. Hussain, T.L. Delovitch, Intravenous transfusion of BCR-activated B cells protects NOD mice from type 1 diabetes in an IL-10-dependent manner, J. Immunol. 179 (2007) 7225–7232.
[88] S.K. Lundy, A.A. Berlin, T.F. Martens, N.W. Lukacs, Deficiency of regulatory B cells increases allergic airway inflammation, Inflamm. Res. 54 (2005) 514–521.
[89] A. Singh, W.F. Carson, E.R. Secor Jr., L.A. Guernsey, R.A. Flavell, R.B. Clark, R.S. Thrall, C.M. Schramm, Regulatory role of B cells in a murine model of allergic airway disease, J. Immunol. 180 (2008) 7318–7326.
[90] S. Amu, S.P. Saunders, M. Kronenberg, N.E. Mangan, A. Atzberger, P.G. Fallon, Regulatory B cells prevent and reverse allergic airway inflammation via FoxP3-positive T regulatory cells in a murine model, J. Allergy Clin. Immunol. 125 (2010) 1114–1124.
[91] Y. Iwata, T. Matsushita, M. Horikawa, D.J. Dilillo, K. Yanaba, G.M. Venturi, P.M. Szabolcs, S.H. Bernstein, C.M. Magro, A.D. Williams, R.P. Hall, E.W. St Clair, T.F. Tedder, Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells, Blood 117 (2011) 530–541.
[92] P.A. Blair, L.Y. Norena, F. Flores-Borja, D.J. Rawlings, D.A. Isenberg, M.R. Ehrenstein, C. Mauri, CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients, Immunity 32 (2010) 129–140.
[93] J.D. Bouaziz, S. Calbo, M. Maho-Vaillant, A. Saussine, M. Bagot, A. Bensussan, P. Musette, IL-10 produced by activated human B cells regulates CD4(+) T-cell activation in vitro, Eur. J. Immunol. 40 (2010) 2686–2691.
[94] J. Correale, M. Farez, G. Razzitte, Helminth infections associated with multiple sclerosis induce regulatory B cells, Ann. Neurol. 64 (2008) 187–199.
[95] S. Knippenberg, E. Peelen, J. Smolders, M. Thewissen, P. Menheere, J.W. Cohen Tervaert, R. Hupperts, J. Damoiseaux, Reduction in IL-10 producing B cells (Breg) in multiple sclerosis is accompanied by a reduced naive/memory Breg ratio during a relapse but not in remission, J. Neuroimmunol. 239 (2011) 80–86.
[96] G. Roussey-Kesler, M. Giral, A. Moreau, J.F. Subra, C. Legendre, C. Noel, E. Pillebout, S. Brouard, J.P. Soulillou, Clinical operational tolerance after kidney transplantation, Am. J. Transplant. 6 (2006) 736–746.
[97] M. Jeannet, V.W. Pinn, M.H. Flax, H.J. Winn, P.S. Russell, Humoral antibodies in renal allotransplantation in man, N. Engl. J. Med. 282 (1970) 111–117.
[98] O. Thaunat, L. Louedec, J. Dai, F. Bellier, E. Groyer, S. Delignat, A.T. Gaston, G. Caligiuri, E. Joly, D. Plissonnier, J.B. Michel, A. Nicoletti, Direct and indirect effects of alloantibodies link neointimal and medial remodeling in graft arteriosclerosis, Arterioscler. Thromb. Vasc. Biol. 26 (2006) 2359–2365.
[99] P.S. Russell, C.M. Chase, R.B. Colvin, Alloantibody- and T cell-mediated immunity in the pathogenesis of transplant arteriosclerosis: lack of progression to sclerotic lesions in B cell-deficient mice, Transplantation 64 (1997) 1531–1536.
[100] P.I. Terasaki, M. Ozawa, R. Castro, Four-year follow-up of a prospective trial of HLA and MICA antibodies on kidney graft survival, Am. J. Transplant. 7 (2007) 408–415.
[101] P.I. Terasaki, J. Cai, Humoral theory of transplantation: further evidence, Curr. Opin. Immunol. 17 (2005) 541–545.
[102] N. Issa, F.G. Cosio, J.M. Gloor, S. Sethi, P.G. Dean, S.B. Moore, S. DeGoey, M.D. Stegall, Transplant glomerulopathy: risk and prognosis related to anti-human leukocyte antigen class II antibody levels, Transplantation 86 (2008) 681–685.
[103] G. Einecke, B. Sis, J. Reeve, M. Mengel, P.M. Campbell, L.G. Hidalgo, B. Kaplan, P.F. Halloran, Antibody-mediated microcirculation injury is the major cause of late kidney transplant failure, Am. J. Transplant. 9 (2009) 2520–2531.
[104] S. Sumitran-Karuppan, G. Tyden, F. Reinholt, U. Berg, E. Moller, Hyperacute rejections of two consecutive renal allografts and early loss of the third transplant caused by non-HLA antibodies specific for endothelial cells, Transpl. Immunol. 5 (1997) 321–327.
[105] G. Opelz, Non-HLA transplantation immunity revealed by lymphocytotoxic antibodies, Lancet 365 (2005) 1570–1576.
[106] P. Amico, G. Honger, D. Bielmann, D. Lutz, D. Garzoni, J. Steiger, M.J. Mihatsch, D. Dragun, S. Schaub, Incidence and prediction of early antibody-mediated rejection due to non-human leukocyte antigen-antibodies, Transplantation 85 (2008) 1557–1563.
[107] A.M. Ismail, R.M. Badawi, A.E. El-Agroudy, M.A. Mansour, Pretransplant detection of anti-endothelial cell antibodies could predict renal allograft outcome, Exp. Clin. Transplant. 7 (2009) 104–109.

[108] N. Lucchiari, N. Panajotopoulos, C. Xu, H. Rodrigues, L.E. Ianhez, J. Kalil, D. Glotz, Antibodies eluted from acutely rejected renal allografts bind to and activate human endothelial cells, Hum. Immunol. 61 (2000) 518–527.

[109] S. Le Bas-Bernardet, M. Hourmant, S. Coupel, J.D. Bignon, J.P. Soulillou, B. Charreau, Non-HLA-type endothelial cell reactive alloantibodies in pre-transplant sera of kidney recipients trigger apoptosis, Am. J. Transplant. 3 (2003) 167–177.

[110] A. Bordron, R. Revelen, F. D'Arbonneau, M. Dueymes, Y. Renaudineau, C. Jamin, P. Youinou, Functional heterogeneity of anti-endothelial cell antibodies, Clin. Exp. Immunol. 124 (2001) 492–501.

[111] K.G. Hankey, C.B. Drachenberg, J.C. Papadimitriou, D.K. Klassen, B. Philosophe, S.T. Bartlett, V. Groh, T. Spies, D.L. Mann, MIC expression in renal and pancreatic allografts, Transplantation 73 (2002) 304–306.

[112] I. Quiroga, M. Salio, D.D. Koo, L. Cerundolo, D. Shepherd, V. Cerundolo, S.V. Fuggle, Expression of MHC class I-related Chain B (MICB) molecules on renal transplant biopsies, Transplantation 81 (2006) 1196–1203.

[113] K. Mizutani, P. Terasaki, A. Rosen, V. Esquenazi, J. Miller, R.N. Shih, R. Pei, M. Ozawa, J. Lee, Serial ten-year follow-up of HLA and MICA antibody production prior to kidney graft failure, Am. J. Transplant. 5 (2005) 2265–2272.

[114] B. Suarez-Alvarez, A. Lopez-Vazquez, M.Z. Gonzalez, J.L. Fdez-Morera, B. Diaz-Molina, M.A. Blanco-Gelaz, D. Pascual, J. Martinez-Borra, M. Muro, M.R. Alvarez-Lopez, C. Lopez-Larrea, The relationship of anti-MICA antibodies and MICA expression with heart allograft rejection, Am. J. Transplant. 7 (2007) 1842–1848.

[115] Y. Zou, F. Mirbaha, A. Lazaro, Y. Zhang, B. Lavingia, P. Stastny, MICA is a target for complement-dependent cytotoxicity with mouse monoclonal antibodies and human alloantibodies, Hum. Immunol. 63 (2002) 30–39.

[116] A. Alvarez-Marquez, I. Aguilera, M.A. Gentil, J.L. Caro, G. Bernal, A.J. Fernandez, M.J. Acevedo, V. Cabello, I. Wichmann, M.F. Gonzalez-Escribano, A. Nunez-Roldan, Donor-specific antibodies against HLA, MICA, and GSTT1 in patients with allograft rejection and C4d deposition in renal biopsies, Transplantation 87 (2009) 94–99.

[117] N. Fukami, S. Ramachandran, D. Saini, M. Walter, W. Chapman, G.A. Patterson, T. Mohanakumar, Antibodies to MHC class I induce autoimmunity: role in the pathogenesis of chronic rejection, J. Immunol. 182 (2009) 309–318.

[118] B. Mahesh, H.S. Leong, A. McCormack, P. Sarathchandra, A. Holder, M.L. Rose, Autoantibodies to vimentin cause accelerated rejection of cardiac allografts, Am. J. Pathol. 170 (2007) 1415–1427.

[119] S. Jurcevic, M.E. Ainsworth, A. Pomerance, J.D. Smith, D.R. Robinson, M.J. Dunn, M.H. Yacoub, M.L. Rose, Antivimentin antibodies are an independent predictor of transplant-associated coronary artery disease after cardiac transplantation, Transplantation 71 (2001) 886–892.

[120] L.D. Barber, A. Whitelegg, J.A. Madrigal, N.R. Banner, M.L. Rose, Detection of vimentin-specific autoreactive CD8+ T cells in cardiac transplant patients, Transplantation 77 (2004) 1604–1609.

[121] T. Iwata, A. Philipovskiy, A.J. Fisher, R.G. Presson Jr., M. Chiyo, J. Lee, E. Mickler, G.N. Smith, I. Petrache, D.B. Brand, W.J. Burlingham, B. Gopalakrishnan, D.S. Greenspan, J.D. Christie, D.S. Wilkes, Anti-type V collagen humoral immunity in lung transplant primary graft dysfunction, J. Immunol. 181 (2008) 5738–5747.

[122] W.J. Burlingham, R.B. Love, E. Jankowska-Gan, L.D. Haynes, Q. Xu, J.L. Bobadilla, K.C. Meyer, M.S. Hayney, R.K. Braun, D.S. Greenspan, B. Gopalakrishnan, J. Cai, D.D. Brand, S. Yoshida, O.W. Cummings, D.S. Wilkes, IL-17-dependent cellular immunity to collagen type V predisposes to obliterative bronchiolitis in human lung transplants, J. Clin. Invest. 117 (2007) 3498–3506.

[123] S.A. Joosten, Y.W. Sijpkens, V. van H., L.A. Trouw, V. d. van, B. van den Heuvel, K.C. van, L.C. Paul, Antibody response against the glomerular basement membrane protein agrin in patients with transplant glomerulopathy, Am. J. Transplant. 5 (2005) 383–393.

[124] D. Dragun, D.N. Muller, J.H. Brasen, L. Fritsche, M. Nieminen-Kelha, R. Dechend, U. Kintscher, B. Rudolph, J. Hoebeke, D. Eckert, I. Mazak, R. Plehm, C. Schonemann, T. Unger, K. Budde, H.H. Neumayer, F.C. Luft, G. Wallukat, Angiotensin II type 1-receptor activating antibodies in renal-allograft rejection, N. Engl. J. Med. 352 (2005) 558–569.

[125] O. Thaunat, S. Graff-Dubois, N. Fabien, A. Duthey, V. Attuil-Audenis, A. Nicoletti, N. Patey, E. Morelon, A stepwise breakdown of B-cell tolerance occurs within renal allografts during chronic rejection, Kidney Int. 81 (2012) 207–219.

[126] A. Valujskikh, O. Lantz, S. Celli, P. Matzinger, P.S. Heeger, Cross-primed CD8(+) T cells mediate graft rejection via a distinct effector pathway, Nat. Immunol. 3 (2002) 844–851.

[127] F.K. Baddoura, I.W. Nasr, B. Wrobel, Q. Li, N.H. Ruddle, F.G. Lakkis, Lymphoid neogenesis in murine cardiac allografts undergoing chronic rejection, Am. J. Transplant. 5 (2005) 510–516.

[128] S. Segerer, G.A. Bohmig, M. Exner, D. Kerjaschki, H. Regele, D. Schlondorff, Role of CXCR3 in cellular but not humoral renal allograft rejection, Transpl. Int. 18 (2005) 676–680.

[129] O. Thaunat, Humoral immunity in chronic allograft rejection: puzzle pieces come together, Transpl. Immunol. 26 (2012) 101–106.

[130] K.A. Newell, A. Asare, A.D. Kirk, T.D. Gisler, K. Bourcier, M. Suthanthiran, W.J. Burlingham, W.H. Marks, I. Sanz, R.I. Lechler, M.P. Hernandez-Fuentes, L.A. Turka, V.L. Seyfert-Margolis, Identification of a B cell signature associated with renal transplant tolerance in humans, J. Clin. Invest. 120 (2010) 1836–1847.

[131] F. Braza, J.P. Soulillou, S. Brouard, Gene expression signature in transplantation tolerance, Clin. Chim. Acta 413 (2012) 1414–1418.

[132] A. Pallier, S. Hillion, R. Danger, M. Giral, M. Racape, N. Degauque, E. Dugast, J. Ashton-Chess, S. Pettre, J.J. Lozano, R. Bataille, A. Devys, A. Cesbron-Gautier, C. Braudeau, C. Larrose, J.P. Soulillou, S. Brouard, Patients with drug-free long-term graft function display increased numbers of peripheral B cells with a memory and inhibitory phenotype, Kidney Int. 78 (2010) 503–513.

[133] P. Sagoo, E. Perucha, B. Sawitzki, S. Tomiuk, D.A. Stephens, P. Miqueu, S. Chapman, L. Craciun, R. Sergeant, S. Brouard, F. Rovis, E. Jimenez, A. Ballow, M. Giral, I. Rebollo-Mesa, M.A. Le, C. Braudeau, R. Hilton, B. Gerstmayer, K. Bourcier, A. Sharif, M. Krajewska, G.M. Lord, I. Roberts, M. Goldman, K.J. Wood, K. Newell, V. Seyfert-Margolis, A.N. Warrens, U. Janssen, H.D. Volk, J.P. Soulillou, M.P. Hernandez-Fuentes, R.I. Lechler, Development of a cross-platform biomarker signature to detect renal transplant tolerance in humans, J. Clin. Invest. 120 (2010) 1848–1861.

[134] S. Brouard, E. Mansfield, C. Braud, L. Li, M. Giral, S.C. Hsieh, D. Baeten, M. Zhang, J. Ashton-Chess, C. Braudeau, F. Hsieh, A. Dupont, A. Pallier, A. Moreau, S. Louis, C. Ruiz, O. Salvatierra, J.P. Soulillou, M. Sarwal, Identification of a peripheral blood transcriptional biomarker panel associated with operational renal allograft tolerance, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 15448–15453.

[135] T.L. Le, P. Thebault, A. Lavault, C. Usal, E. Merieau, T. Quillard, B. Charreau, J.P. Soulillou, M.C. Cuturi, S. Brouard, E. Chiffoleau, Long-term allograft tolerance is characterized by the accumulation of B cells exhibiting an inhibited profile, Am. J. Transplant. 11 (2011) 429–438.

[136] S. Louis, C. Braudeau, M. Giral, A. Dupont, F. Moizant, N. Robillard, A. Moreau, J.P. Soulillou, S. Brouard, Contrasting CD25hiCD4+T cells/FOXP3 patterns in chronic rejection and operational drug-free tolerance, Transplantation 81 (2006) 398–407.

[137] S. Heidt, J. Hester, S. Shankar, P.J. Friend, K.J. Wood, B cell repopulation after alemtuzumab induction-transient increase in transitional B cells and long-term dominance of naive B cells, Am. J. Transplant. 12 (2012) 1784–1792.

[138] S.J. Knechtle, J. Pascual, D.D. Bloom, J.R. Torrealba, E. Jankowska-Gan, W.J. Burlingham, J. Kwun, R.B. Colvin, V. Seyfert-Margolis, K. Bourcier, H.W. Sollinger, Early and limited use of tacrolimus to avoid rejection in an alemtuzumab and sirolimus regimen for kidney transplantation: clinical results and immune monitoring, Am. J. Transplant. 9 (2009) 1087–1098.

[139] O. Lassila, O. Vainio, P. Matzinger, Can B cells turn on virgin T cells? Nature 334 (1988) 253–255.

[140] E.J. Fuchs, P. Matzinger, B cells turn off virgin but not memory T cells, Science 258 (1992) 1156–1159.

[141] E.E. Eynon, D.C. Parker, Small B cells as antigen-presenting cells in the induction of tolerance to soluble protein antigens, J. Exp. Med. 175 (1992) 131–138.

[142] P. Reichardt, B. Dornbach, S. Rong, S. Beissert, F. Gueler, K. Loser, M. Gunzer, Naive B cells generate regulatory T cells in the presence of a mature immunologic synapse, Blood 110 (2007) 1519–1529.

[143] S. Morlacchi, C. Soldani, A. Viola, A. Sarukhan, Self-antigen presentation by mouse B cells results in regulatory T-cell induction rather than anergy or clonal deletion, Blood 118 (2011) 984–991.

[144] X. Chen, P.E. Jensen, Cutting edge: primary B lymphocytes preferentially expand allogeneic FoxP3+ CD4 T cells, J. Immunol. 179 (2007) 2046–2050.

[145] R.E. Billingham, L. Brent, P.B. Medawar, Actively acquired tolerance of foreign cells, Nature 172 (1953) 603–606.

[146] X. Fan, A. Ang, S.M. Pollock-Barziv, A.I. Dipchand, P. Ruiz, G. Wilson, J.L. Platt, L.J. West, Donor-specific B-cell tolerance after ABO-incompatible infant heart transplantation, Nat. Med. 10 (2004) 1227–1233.

[147] R.F. Parsons, K. Vivek, R.R. Redfield, T.S. Migone, M.P. Cancro, A. Naji, H. Noorchashm, B-cell tolerance in transplantation: is repertoire remodeling the answer? Expert Rev. Clin. Immunol. 5 (2009) 703.

[148] R.F. Parsons, K. Vivek, S.Y. Rostami, G. Zekavat, S.M. Ziaie, Y. Luo, B. Koeberlein, R.R. Redfield, M.P. Cancro, A. Naji, H. Noorchashm, Acquisition of humoral transplantation tolerance upon de novo emergence of B lymphocytes, J. Immunol. 186 (2011) 614–620.

[149] M.P. Cancro, Signalling crosstalk in B cells: managing worth and need, Nat. Rev. Immunol. 9 (2009) 657–661.

[150] C. Mauri, P.A. Blair, Regulatory B cells in autoimmunity: developments and controversies, Nat. Rev. Rheumatol. 6 (2010) 636–643.

[151] D.T. Boumpas, R. Furie, S. Manzi, G.G. Illei, D.J. Wallace, J.E. Balow, A. Vaishnaw, A short course of BG9588 (anti-CD40 ligand antibody) improves serologic activity and decreases hematuria in patients with proliferative lupus glomerulonephritis, Arthritis Rheum. 48 (2003) 719–727.

[152] R.B. Landewe, J.G. Houbiers, F. Van den Bosch, H.J. in't, P.C. Verschueren, J.H. Meijerink, F.H. van den Hoogen, B.A. Masek, G.A. Bruyn, J.M. Wouters, A.E. Voskuyl, J.M. van Laar, J.J. Bijlsma, D.M. van der Heijde, F.C. Breedveld, L.B. van de Putte, A.M. Miltenburg, K.F. De, Intranasal administration of recombinant human cartilage glycoprotein-39 as a treatment for rheumatoid arthritis: a phase II, multicentre, double-blind, randomised, placebo-controlled, parallel-group, dose-finding trial, Ann. Rheum. Dis. 69 (2010) 1655–1659.

[153] H.L. Weiner, G.A. Mackin, M. Matsui, E.J. Orav, S.J. Khoury, D.M. Dawson, D.A. Hafler, Double-blind pilot trial of oral tolerization with myelin antigens in multiple sclerosis, Science 259 (1993) 1321–1324.

[154] T. Cantaert, S. Brouard, R.M. Thurlings, A. Pallier, G.F. Salinas, C. Braud, P.L. Klarenbeek, V.N. de, Y. Zhang, J.P. Soulillou, P.P. Tak, D. Baeten, Alterations of the synovial T cell repertoire in anti-citrullinated protein antibody-positive rheumatoid arthritis, Arthritis Rheum. 60 (2009) 1944–1956.

[155] P.L. Klarenbeek, M.J. de Hair, M.E. Doorenspleet, B.D. van Schaik, R.E. Esveldt, M.G. van de Sande, T. Cantaert, D.M. Gerlag, D. Baeten, A.H. van Kampen, F. Baas, P.P. Tak, V.N. de, Inflamed target tissue provides a specific niche for highly expanded T-cell clones in early human autoimmune disease, Ann. Rheum. Dis. 71 (2012) 1088–1093.

[156] J.B. Sun, C. Czerkinsky, J. Holmgren, B lymphocytes treated in vitro with antigen coupled to cholera toxin B subunit induce antigen-specific Foxp3(+) regulatory T cells and protect against experimental autoimmune encephalomyelitis, J. Immunol. 188 (2012) 1686–1697.

[157] V.S. Ramgolam, Y. Sha, K.L. Marcus, N. Choudhary, L. Troiani, M. Chopra, S. Markovic-Plese, B cells as a therapeutic target for IFN-beta in relapsing–remitting multiple sclerosis, J. Immunol. 186 (2011) 4518–4526.

MicroRNAs, majors players in B cells homeostasis and functions

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Abstract

As a main actor in humoral immunity, B cells participate in various antibody-related disor- ders. However, a deeper understanding of B-cell differentiation and function is needed in order to decipher their immune-modulatory roles, notably with the recent highlighting of regulatory B cells. microRNAs (miRNAs), key factors in various biological and pathological processes, have been shown to be essential for B-cell homeostasis, and therefore under- standing their participation in B-cell biology could help identify biomarkers and contribute toward curing B-cell-related immune disorders. This review aims to report studies casting light on the roles played by miRNAs in B-cell lineage and function and B-cell-related immune pathologies.

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MicroRNAs, major players in B cells homeostasis and function

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As a main actor in humoral immunity, B cells participate in various antibody-related disorders. However, a deeper understanding of B-cell differentiation and function is needed in order to decipher their immune-modulatory roles, notably with the recent highlighting of regulatory B cells. microRNAs (miRNAs), key factors in various biological and pathological processes, have been shown to be essential for B-cell homeostasis, and therefore understanding their participation in B-cell biology could help identify biomarkers and contribute toward curing B-cell-related immune disorders. This review aims to report studies casting light on the roles played by miRNAs in B-cell lineage and function and B-cell-related immune pathologies.

Keywords: B cell, immunology, immune disorder, microRNA, gene-expression regulation

INTRODUCTION

MicroRNAs (miRNAs) are the most studied class of non-coding RNAs and their gene-expression regulating role is key in various biological and pathological processes. MiRNAs play a role in immune processes such as the development of immune cells, inflammation, and tolerance (1, 2). Evidence that miRNAs are needed for B-cell development is given by mice where B-cell-specific deletion of the endoribonuclease Dicer results in a lack of B cells (3). Furthermore, miRNAs finely tune the differentiation and activation programs of B cells, thus influencing their function. B cells are also central mediators in humoral immunity and play an important role in transplantation, autoimmunity, and reaction to infectious diseases. Consequently, it is important to understand in what circumstances miRNAs can influence B-cell function, and therefore immuno-pathology. In the present review, we describe recent studies shedding light on the roles played by miRNAs in B-cell biology and B-cell-related immune pathologies (major miRNA roles in B cells are reported in **Table 1** and **Figure 1**).

MicroRNA CHARACTERISTICS

BIOGENESIS

Discovered in *Caenorhabditis elegans* in 1993 by Ambros and Ruvkun's teams, miRNAs are endogenous small (19–23 nucleotides in length) non-coding RNAs that perform post transcriptional regulation by targeting messenger RNAs (mRNAs) for degradation or translational inhibition (30, 31). Since their first description, miRNAs have been extensively studied. The 20th release (June 2013) of the official miRNA registry, miRbase, contains 2, 578 and 1, 908 mature miRNAs for human and mouse, respectively (32). MiRNA biogenesis has been reported in detail (33). The canonical miRNA biogenesis involves the transcription of long primary transcripts (pri-miRNA) by the RNA polymerase II which allows transcription factor regulation (34). This pri-miRNA is processed by the microprocessor complex, including the endoribonucleases Drosha/Di George syndrome critical region protein 8 (DGCR8). The resulting precursor miRNA (pre-miRNA) is transported into the cytoplasm where it is processed and cleaved by the Dicer RNase III. This process leads to the formation of a short double-stranded RNA containing the miRNA and its complementary sequence. Finally, the mature miRNA is unwrapped and packed in the RNA-induced silencing complex (RISC). This complex is composed of several proteins including the Argonaute proteins (AGO) and allows a stable conservation of the miRNA. This RISC complex guides the miRNA to the target mRNA containing miRNA Recognition Elements (MRE) (35).

MECHANISMS OF ACTION

The most widely accepted model for miRNA targeting is based on the seed region, a 6 nucleotide region in the 5′ end of the miRNA, where miRNA/mRNA matching is perfect, whereas an uncomplimentary region, or "bulge" sequence, is present between the miRNA/mRNA hybrid (36). Due to this short recognition sequence, miRNAs are predicted to target hundreds to thousands of genes. This is confirmed in different reports where deletion or over-expression of miRNAs regulate the expression of numerous genes and proteins (37, 38). Consequently, a lot of predictive bioinformatic tools have been developed to identify potential direct miRNA targets [reviewed in Ref. (39)]. However, even the most accurate software gives a high rate of false positives and false negatives and predictions have to be experimentally validated.

The exact mechanisms by which miRNAs repress gene-expression still remain unknown. Recent experiments suggest that miRNAs act as protein transcriptional repressors, preventing ribosome association with mRNAs, leading to mRNA destabilization

Table 1 | Major miRNAs playing a role in B cells.

miRNA	Targets	Biological effects	Associated disorders
miR-17-92 Cluster	*Bim* (3, 4) *Pten* (4)	Participate in B-cell proliferation and cell-death control (3, 4)	
miR-24	*Bim* and Caspase 9 (5)	Inhibit B-cell development, under the control of PU.1 (5, 6)	
miR-29a	*TCL1*, *MCL10*, and *CDK6* (7)		Up-regulated in indolent B-cell chronic lymphocytic leukemia (CLL) compared to normal B cells (8).
miR-34a	*Foxp1* (9)	Induces block of B-cell development whereas its deletion induces high number of mature B cells (9)	
miR-146a	*Irak1* and *Traf6* (10) *Fas* (11).	Participate in B-cell development, over-expression causes spontaneous autoimmune disorders in mice (10, 11)	Over-expressed in patients suffering from rheumatoid arthritis and psoriasis (12–14) Over-expressed in kidney biopsy and urine from patients with IgA nephropathy (15) Induced by EBV and inhibits the expression of interferon related genes (16)
miR-150	*c-Myb* (17).	Its over-expression in B-cell progenitors results in a partial block of B-cell development and a reduction in B1-cell numbers (17).	Under-expressed in peripheral B cells from SLE patients (18)
miR-155	Pu.1 (*Sfpi1* gene) (19) *Shp1* (21) *Aid* (20, 22)	Reduced generation of high-affinity antibodies against a T-cell-dependent antigen (19, 20)	Over-expressed in peripheral B cells from SLE patients (18) Is induced by EBV, through LMP1 and: -targets BMP signaling cascade suggesting an inhibition of the antitumor effects of BMP signaling (23) -contributes to the resistance toward Rituximab in inducing cell survival signal (24)
miR-181a	*Bim* (25)	Its over-expression inhibits the pro-apoptotic protein BIM (25) and increases number of B-lineage cells (26)	
miR-181b	*Aid* (27)	Impairs the class-switch recombination (27)	
miR-210		Control of immunoglobin class-switch and under the control of Oct-2 (28)	
miR-221		Implicated in the retention of early B-lineage cells in bone marrow and under the control of PAX5 (29).	

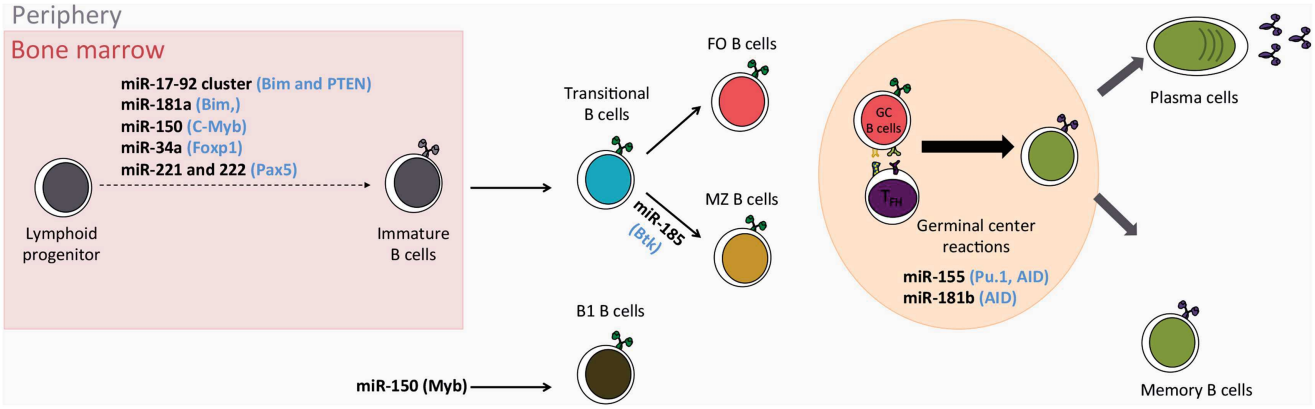

FIGURE 1 | Role of miRNAs in the development of B cells. (in Blue: miRNA targets; FO Follicular B cells; MZ Marginal zone B cells).

and degradation (40–42). This would explain the absence of the rapid diminution of mRNA levels after miRNA induction. This would further mean that miRNAs, not requiring translation, could be active in inhibiting mRNA translation more quickly than transcription factors.

Another important property of miRNAs is that they have distinct functions in different cell types, the transcript levels differing depending on the cell, and number of mRNA containing MRE also differing. This is the case for miR-155, which represses the expression of the factor transcription c-MAF and the IFNγ receptor 1 (IFNGR1) in activated naïve CD4 positive cells, whereas it represses the expression of the PU.1 transcription factor and the phosphatidyl inositol 5'-phosphatase SHIP1 in B-lymphocytes [(21, 43, 44); reviewed in Ref. (45)]. Few miRNAs are cell-specific. Some lymphoid miRNAs have been identified, such as miR-150, that have been shown to be expressed in B cells and also in T and NK cells (46). Furthermore, miRNAs have been found expressed in various body fluids including plasma, sera, urine, saliva (47, 48), and their resistance to degradation, either by enzymatic (RNases) or physic (freezing/defreezing) processes make them good biomarkers.

Finally, while the large number of miRNA targets, their possible rapid intervention, and their multifactorial function explain why miRNAs are important in cell biology, the exact mechanisms of miRNA are complex and as yet undiscovered. MiRNAs can directly induce gene-expression (49, 50) despite being mainly described as gene-expression repressors. They can also act in the 5′ untranslated region (UTR), and not only in the 3′ UTR (51). Finally miRNAs can themselves be regulated by long non-coding RNA (52).

miRNAs AND B-CELL LINEAGE

SPECIFIC miRNAs HIGHLIGHTED IN B-CELL LINEAGE

Knock-out (KO) experiments have shown that miRNAs are involved in, and even indispensable to, normal hematopoiesis (3, 53) (**Figure 1**). Indeed, reconstitution of irradiated mice with *Ago2*-deleted bone marrow cells, which induces a reduction in miRNA levels, impairs generation of pre-B cells and subsequently peripheral B cells (53). Specifically in B cells, the *Dicer* ablation at an early stage blocks cells at the pro-B to pre-B-cell transition (3) whereas the *Dicer* ablation at a later stage, in antigen-activated B cells, results in a severe impairment of antibody response with prevention of germinal center (GC) B-cell, long-lived plasma cell, and memory B-cell formation (54). Analysis of up-regulated genes in blocked pro-B cells highlights gene-bearing seed motifs for miRNAs such as miR-142-3p and the miR-17–92 cluster (including miR-17, -19a, -19b, -20a, and -92), suggesting that at least these miRNAs are important for B-cell development (3). Among genes targeted by miRNAs from the miR-17–92 cluster, the authors showed that *Bim* (BCL2-like 11), a pro-apoptotic gene, is increased in *Dicer* KO mice and could be responsible, at least in part, for the massive apoptosis observed at the pre-B stage. In contrast, the over-expression of this miR-17–92 cluster in transgenic mice results in lymphoproliferative and autoimmune phenotypes due to a reduced PTEN (phosphatase and tensin homolog) and BIM protein expression (4). Altogether, these data suggest that the miR-17–92 cluster has a role in the proliferation control of B cells.

Similarly, the over-expression of miR-181a in hematopoietic stem cells induces an increase in the number of B-lineage cells in both tissue-culture and adult mice after re-implantation in bone marrow (26). A deep-sequencing experiment confirms the preferential expression of the miR-181 family in early and transitional B-cell stages (55). Furthermore, the inhibition of the pro-apoptotic gene *BIM* by miR-181a, reducing B-cell apoptosis, could explain the observed increased number of B cells when miR-181a is over-expressed (25). This sequencing study also confirms the expression of miRNAs related to B-cell differentiation, such as miR-146a, miR-150, miR-155, and miR-34a. MiR-150 is indispensable for B-cell development as it controls, in a dose-dependent manner, the expression of *c-Myb* (v-myb avian myeloblastosis viral oncogene homolog, officially called *Myb*), coding for a transcription factor important in multiple steps of lymphocyte development and notably the generation of B1 cells, considered as innate immune cells producing immunoglobulin M and A (IgM and IgA) (17). The ectopic expression of miR-150 in B-cell progenitors results in a partial blockade of B-cell development and a reduction in B1-cell numbers whereas splenic B1 cells are four-times as numerous in miR-150 KO mice (17). Interestingly, in mice with miR-150 ectopic expression, no obvious adverse physiological effect is observed in non-hematopoietic lineage cells, demonstrating that miRNAs display their functions with cell-specificity. Similarly to miR-150, the constitutive expression of miR-34a results in a partial blockade of B-cell development, whereas its deletion induces high number of mature B cells (9). Indeed, miR-34a constitutive expression inhibits the transition of pro-B cells into pre-B cells by targeting the gene coding the transcription factor *Foxp1* (Forkhead box P1). Spierings et al. described high levels of expression of miR-146a in B1 lineage and in to a lesser extent in marginal zone (MZ) B cells (55). Considering that this miRNA has been described as playing a role in a negative feedback loop on NF-kB activity by down-regulating IL-1 receptor associated kinase 1 (*Irak1*) and TNF receptor–associated factor 6 (*Traf6*) upon lipopolysaccharide stimulation in monocyte, miR-146a could be implicated in B1-cell development. The possibility of this role is reinforced by the fact that miR-146a controls *Irak1* and *Traf6* in splenic B cells (10) and furthermore by the association of increased miR-146a expression in *c-Myc* related lymphoma models (56) and in splenic MZ lymphoma (57). Thus, it is clear that miRNAs play key roles in B-cell development.

As for other RNAs, the expression of miRNAs is under the control of transcription factors, which are definitely involved in B-cell development. Starting from the fact that PAX5 (paired box 5) participate in B-cell fate, Knoll et al. have shown the down-regulation of miR-221 and miR-222 during B-lymphocyte development, and the involvement of miR-221 in the retention of early B-lineage cells in bone marrow (29). A similar procedure has been shown with the transcription factor PU.1 {encoded by the *Sfpi1* gene [SPI1 spleen focus forming virus (SFFV) proviral integration oncogene]}, because high ectopic expression of *Pu.1* in multipotent progenitors promotes myeloid cell development at the expense of B-cell development (6). Using a $Pu.1^{-/-}$ myeloblast cell line and model of bone marrow transplantation, the authors identified miR-24 as a transcriptional target of PU.1 able to inhibit B-cell development *in vivo* as well as *in vitro* (6). In contrast, the

same group report that miR-24 enhances cell survival in both the myeloid and pre-B-cell lines, inhibiting pro-apoptotic molecules such as BIM and Caspase 9 (5). It remains unclear why this miRNA inhibits B lymphopoiesis or enhances lymphocyte survival but is likely due to a change in the cellular environment.

Regarding the other roles of miRNAs in B cells, it is clear that they partly control proliferation and apoptosis in B cells and numerous miRNAs have been highlighted in lymphoma. B lymphoma-related miRNAs were reviewed recently (58). Their study highlighted the role of miRNAs in normal B cells. For example, miR-155, which was initially described within the non-coding B-cell integration cluster (*BIC*) gene, is over-expressed in various lymphoma, and has also been shown to be a major miRNA involved in B-cell maturation. This is also the case for miR-29a, highly expressed in B cells and up-regulated in indolent B-cell chronic lymphocytic leukemia (CLL) compared to normal B cells (8). Its B-cell-specific over-expression induces a CLL-like disease in mice with an expanded $CD5^+CD19^+$ B-cell population suggesting that miR-29a acts as an oncomiR (8). However, this miRNA is expressed less in aggressive CLL compared to indolent CLL, and it is speculated that a reduction of control of miR-29a targets, including several oncogenes such as T-cell leukemia/lymphoma 1A (*TCL1*), myeloid cell leukemia sequence 1 (*MCL10*) and cyclin-dependent kinase 6 (*CDK6*) participate to the aggressive CLL phenotype (7). Thus, miR-29a could act either as an oncogene or a tumor suppressor, demonstrating that miRNAs can play different roles depending on the cellular context. Finally, the role of miR-29a in normal B cells has not yet been described, but considering the enrichment of B-cell signaling pathways among its targets, it is also likely to have an important function in normal B cells [reviewed in Ref. (59)].

ENRICHED miRNAs IN B-CELL LINEAGE

Overall, most miRNAs are ubiquitously expressed, only some of them being preferentially expressed, in restricted cell types (60, 61); this is the case for miR-122 in liver and miR-1 in muscle. Global profiling studies focusing on hematopoietic cell lineage and particularly in B cells also foreground enriched miRNAs such as miR-16, miR-30c, miR-34a, miR-142-3 and-5p, miR-150, miR-155, miR-181, and miR-223 (46, 55, 62–65). These profiling studies are very useful tools in a first attempt to appreciate the role of a particular miRNA in conjunction with cells or tissues expressing this miRNA. This is the case for the deep-sequencing study by Spierings et al. which shows the expression of 232 known miRNAs in 10 developmental stages (55). Altogether, these studies demonstrate that miRNAs with distinct expression in B cells have to be investigated for the complete understanding of B-cell biology.

IMPLICATION OF miRNAs IN PERIPHERAL B-CELL DEVELOPMENT

ROLES OF miRNAs IN FO MZ FATE DECISION

Primary antibody diversification takes places during B-cell differentiation in the bone marrow through somatic DNA rearrangement of Ig by V(D)J recombination. This process leads to a high diversity of B-cell antigen receptors that can recognize self-antigens and potentially induce autoimmunity. Control of B-cell auto-reactivity is guaranteed by intrinsic tolerance mechanisms (66, 67). Two checkpoints ensure B-cell tolerance in bone marrow and the periphery, where strongly self-reactive B cells might undergo receptor editing or clonal deletion (66, 67). In mice, peripheral immature IgM^+ B cells start to express IgD and terminate their differentiation into follicular (FO) or MZ B cells (11, 68). FO B cells are currently associated with a T dependent-response whereas MZ B cells, located in the spleen marginal sinus, can mount independent humoral responses. The differentiation of immature B cells into FO or MZ B cells depends on BCR intensity signals and cleavage of Notch2. For their differentiation MZ B cells need weak BCR signals, allowing the activation of Notch2 pathways, whereas FO B cells are generated after strong, tonic BCR interactions associated with BAFF survival signals (11). MiRNA are involved in the FO vs. MZ fate decision (69). Mice with conditional KO of *Dicer* in B cells exhibit a total switch of their B-cell subsets, with a higher proportion of immature and MZ B cells and a strong alteration of the FO B cells (69). This alteration is specific to *Dicer* deletion and not due to any compensatory homeostatic mechanisms. Mixed chimeric mice, with 50% wild-type bone marrow and 50% conditional *Dicer* KO bone marrow, exhibit an impairment of FO B-cell generation and an increase in the MZ compartment only in the KO part. This confirms that the augmentation of MZ B cells in *Dicer*-deficient mice is not a homeostatic response but instead reflects an altered process in B-cell fate induced by the absence of miRNAs (69). Remarkably, FO B cells have a higher expression of *Dicer* than MZ B cells, suggesting a central role for miRNAs in this population. Indeed, among the 177 measured, 31 differentially expressed miRNAs have been highlighted in FO and MZ B cells. Among them, miR-185 was identified as central for the differentiation into the FO compartment (69). MiR-185 targets burton tyrosine kinase (*BTK*), which transduces signals downstream of BCR by phosphorylating Erk pathway. Consequently, by targeting *BTK*, miR-185 modulates BCR signals and its activation threshold. Thus, in physiological conditions, miR-185 dampens BCR signals, confirming that immature B cells needs strong BCR activation to differentiate into FO B cells (70). In *Dicer*-deficient mice, BCR signals are not diminished and so immature B cells preferentially differentiate into the MZ compartment. In addition, *Dicer* KO mice exhibit autoimmune features with a skewed antibody repertoire enriched in self-reactive specificities that lead to the development of autoimmune diseases (69). Concentration of IgG against dsDNA, ssDNA, and cardiolipin autoantigens are increased in *Dicer* KO mice, suggesting a passive biased selection of the BCR repertoire, probably due to alterations in BCR signals. More interestingly, these autoimmune features are only found in older female mice, mimicking thus the etiology of autoimmune diseases in human where older women are more susceptible to developing such pathologies (71).

ROLES OF miRNAs IN THE CONTROL OF MEMORY AND HUMORAL RESPONSES

Memory and plasma cells are generated during the primary immune response against foreign antigens. This process is initiated when naïve FO and MZ B cells expressing surface Ig bind the antigen in secondary lymphoid organs, receive or do not receive signals from helper T cells, and proliferate. This proliferation produces short-lived plasmablasts and GC cells. A secondary

diversification process occurs in the GC where B cells switch their Ig constant region from IgM to IgG, IgA, or IgE and generate somatic mutations in their variable regions. B cells expressing high affinity Ig survive and emerge from the GC reaction and differentiate into plasma cells. Recently, a number of studies have identified the involvement of miRNAs in the GC reaction and B-cell memory responses. Mice lacking miR-155 exhibit substantial immune defects with reductions in GC B cells and dampened B-cell memory responses accompanied by an alteration in the function of T lymphocytes and dendritic cells (43, 72). miR-155 deficiency in B cells leads to a reduced generation of high-affinity antibodies against a T-cell-dependent antigen (19). The authors have identified the transcription factor PU.1 among targets of this miRNA. Furthermore, they have shown that *Pu.1* over-expression in B cells results in reduced numbers of IgG1-switched cells, reinforcing the evidence of this factor in B-cell maturation. In addition, the gene coding for the activation-induced cytidine deaminase (*Aid* gene) regulating the class-switch recombination and somatic hypermutation, has also been shown to be a miR-155 target (20, 22). *Aid* is also a target gene for miR-181b, impairing the class-switch recombination, and its expression is down-regulated upon B-cell activation, allowing efficient antibody maturation (27). Although the majority of miRNAs are down-regulated upon B-cell activation, miR-210 has been shown to be up-regulated in these circumstances (28). In models of KO and transgenic mice, miR-210, itself under the control of the transcription factor OCT-2, is involved in the control of immunoglobin class-switch preventing autoimmunity (28) and mice deficient in miR-210 spontaneously produce high levels of autoantibodies.

Collectively these data demonstrate that miRNAs have a key role in the differentiation of peripheral mature B cells and in humoral responses. In addition, the data suggest that deregulation of miRNA expression can alter B-cell homeostasis and break tolerance by favoring the generation of autoantibodies. Regarding the significance of miRNAs in B-cell biology, we can also assume that miRNAs are involved in other essential functions of B-cells. However, to our knowledge, no miRNA has yet been described as playing a direct role in B cells functions such as antigen presentation to T cells, cytokine secretion or regulatory functions (73).

miRNA DYSREGULATION IN B-CELL-RELATED DISORDERS

B-CELL-RELATED miRNAs IN AUTOIMMUNE DISORDERS

B-cells are involved in autoimmune diseases due to their primary function of antibody production. That miRNA plays a role in the establishment of autoimmunity has been strongly suggested in rodents (69), but few studies have been performed specifically on human B cells. Stagakis et al. found seven miRNAs with differential expression in peripheral B cells in a small group of 5 patients with SLE, compared to three healthy controls (18). Three were under-expressed in SLE (miR-150, miR-16, miR-15a) and the four others were up-regulated (miR-155, miR-25, miR-21, miR-106b). Interestingly, miR-21, a pleiotropic miRNA described as controlling major cell functions, had also been shown to be over-expressed in splenic B cells from two mice models of SLE, the MRL/lpr and the B6.Sle123 mouse strains, suggesting it has a role in this pathology (74, 75). Among others, miR-15a modulation has also been described in another SLE model, B/W mice enhanced by IFNλ, with a significant correlation between miR-15a expression and autoantibody production in SLE prone-B/W mice (76). Surprisingly, miR-15a expression is essentially found in the regulatory B-cell subset under steady state and its expression progressively increases in other B-cell subsets along the course of the disease. However, in this study, miR-15a is over-expressed in splenic B cells, although it has previously been described as down-expressed in human blood B cells (18). Further investigation is required to decipher whether this discrepancy is due to differences in the origins of the analyzed B cells (peripheral blood vs. splenic B cells) or to differences between the mice model and human SLE. Even if miRNA sequences are well conserved between species, their expression profiles could be different from one species to another. For example, among 12 analyzed miRNAs which were expressed both in human and mouse, only 6 had similar expression profiles in their lymphocyte subsets (mainly $CD4^+$ cells) (77). Using a hypothesis-driven approach, miR-30a was shown to directly target Lyn, a member of the Src family preferentially expressed in B cells (78). Gene and protein levels of Lyn are lower in SLE patients and negatively correlate with the expression of miR-30a in blood $CD19^+$ purified cells (78). Unlike other members of the miR-30 family, only miR-30a exhibits regulatory capacity upon B-cell proliferation and antibody production in the two B-cell lines studied (Daudi and Raji). Interestingly, miR-142-3p and -5p, which are down-regulated in $CD4^+$ T cells from SLE patients, have an indirect effect on IgG production; the over-expression of these miRNAs in SLE $CD4^+$ T-cells induces a decrease in IgG production (79). In multiple sclerosis (MS), two studies from the same group show two sets of modulated miRNAs in isolated B cells from relapsing-remitting MS patients compared to healthy controls (80, 81). Despite no overlapping miRNAs, five differential miRNAs from the second study have been validated in a second set of samples and could thus be investigated to decipher B-cell deregulation in MS (miR-106b, miR-19b, miR-181a, miR-25, and miR-93) (81).

The over-expression of miR-146a has been reported in synovial tissue from patients with rheumatoid arthritis. *In situ* hybridization analysis reveals that $CD79A^+$ B cells express high amounts of miR-146a in these synovial tissues (13). Similarly, miR-146a is over-expressed in kidney biopsy and urine from patients with IgA nephropathy, although its specific expression has not been proven (15). The involvement of miR-146a in immune disorders is further suggested by the generation of transgenic mice over-expressing miR-146a with immune disorders including enlarged spleens and lymph nodes, increased frequency and numbers of T and B cells, accumulation of GC B cells, and an increase in Ig serum levels (11). MiR-146a mediates its effect by repressing the expression of Fas in B cells, a molecule essentially expressed on GCB cells and which promotes their apoptosis during GC reaction. Thus over-expression of this miRNA enhances homeostatic B-lymphocyte proliferation leading to the development of autoimmune lymphoproliferative syndrome. In concordance with these observations, higher levels of miR-146a have been found in patients suffering from rheumatoid arthritis and psoriasis (12–14).

B-CELL-RELATED miRNAs IN SOLID ORGAN TRANSPLANTATION

An increasing number of articles dealing with miRNAs and solid organ transplantation, including kidney, liver, and lung, suggest their significant role in organ acceptance or rejection and their usefulness as biomarkers (82, 83). Regarding B cells, several miRNAs have been identified in biopsy or peripheral blood mononuclear cells from patients with antibody-mediated rejection (AMR) in renal transplantation (84–86). The link between these miRNAs and B cells is probable, but because B cells can exercise their function at a distance from the graft and because other factors participate in the AMR process, further investigation is required to demonstrate that these miRNAs are really involved. To our knowledge, we were the first to report miRNA dysregulation specifically in B cells after transplantation. We reported the over-expression of miR-142-3p in blood B cells from patients with operational tolerance, a specific situation where transplant patients maintain a well-functioning graft having stopped their immunosuppressive treatment (87). The over-expression of miR-142-3p in the Raji B-cell line induces the modulation of genes previously described as associated with renal tolerance, suggesting that it may contribute to the maintenance of tolerance in B cells. These observations were reinforced by the observation that miR-142-3p may play a regulatory role in T lymphocytes by controlling leukocyte activation (79). In addition, miR-155 has been shown to contribute to resistance to Rituximab in inducing cell survival signals through AKT and myeloid cell leukemia sequence 1 (MCL1) since its inhibition resulted in a significant decrease in the survival of EBV-positive cells treated with Rituximab (24). These results indicate that the inhibition of miR-155 could be a valuable approach for treating EBV-induced PTLD.

Of course, other phenomena not directly related to B cells could occur during transplantation, including ischemia and reperfusion, cellular rejection, and recurrence of the initial disease, and miRNAs could be associated with these. In renal transplantation, miR-142-5p, shown to be present during chronic AMR, has been previously indicated as over-expressed in biopsies from patients with cellular acute rejection (85, 88). Similarly, miR-142-3p, related to B cells in renal operational tolerance, has been found to be associated with interstitial fibrosis and tubular atrophy in urine (87, 89).

B-CELL-RELATED miRNA USEFULNESS IN THERAPY

Since miRNAs can be detected and measured in various body fluids, they may represent ideal non-invasive biomarkers. Among recent, numerous examples, miR-210 has been proposed as an urinary biomarker of acute rejection in renal transplantation (48), miR-142-5p to diagnose chronic AMR in renal allograft (85), and miR-155 to predict patients with CLL who fail to achieve a complete response in plasma samples collected before treatment initiation (90).

As a result, the use of miRNA inhibitors, otherwise known as antagomirs, may be promising as therapeutic tools. One particular example is the use of miRNA inhibitor against miR-122, a liver specific microRNA required by the hepatitis C virus (HCV) for replication. The use of this miR-122 inhibitor, "miravirsen," induced a decrease in HCV RNA levels in a dose-dependent manner in a clinical phase II study (91). Other inhibitors of miRNAs have been proposed for various diseases and it can be assumed that B-cell targeting ones will be designed in the future, for example to impair their production of antibodies. However, the targeted miRNAs generally have several functions in several different cell types and while their inhibition could provide powerful remedies, they could also have wide-reaching side-effects and caution is mandatory.

Finally, it has recently been proposed to use B cells as producers and delivers of therapeutic miRNAs in $CD8^+$ T cells (92). After *in vitro* transfection of B cells with a plasmid coding for miR-155, these B cells delivered miR-155 in $CD8^+$ T cells during antigen cross-priming only (92). The authors suggest this cell-based strategy could be used in inflammation and autoimmune diseases.

CONCLUSION

Recent studies have clearly demonstrated that miRNAs are involved in B-cell development and function. With the current interest in miRNAs, as well as the renewed emphasis on B-cell function, notably triggered by the recent discovery of regulatory B cells, it seems clear that further discoveries will be made in the near future. Ideally, theses insights would allow the use of miRNAs as disease biomarkers, but may also allow modulation of miRNA expression as master gene modulators to cure B-cell-related immune disorders.

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REFERENCES

1. Stefani G, Slack FJ. Small non-coding RNAs in animal development. *Nat Rev Mol Cell Biol* (2008) **9**:219–30. doi:10.1038/nrm2347
2. Williams AE. Functional aspects of animal microRNAs. *Cell Mol Life Sci* (2008) **65**:545–62. doi:10.1007/s00018-007-7355-9
3. Koralov SB, Muljo SA, Galler GR, Krek A, Chakraborty T, Kanellopoulou C, et al. Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage. *Cell* (2008) **132**:860–74. doi:10.1016/j.cell.2008.02.020
4. Xiao C, Srinivasan L, Calado DP, Patterson HC, Zhang B, Wang J, et al. Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes. *Nat Immunol* (2008) **9**:405–14. doi:10.1038/ni1575
5. Nguyen T, Rich A, Dahl R. MiR-24 promotes the survival of hematopoietic cells. *PLoS One* (2013) **8**:e55406. doi:10.1371/journal.pone.0055406

6. Kong KY, Owens KS, Rogers JH, Mullenix J, Velu CS, Grimes HL, et al. MIR-23A microRNA cluster inhibits B-cell development. *Exp Hematol* (2010) **38**:629.e–40.e. doi:10.1016/j.exphem.2010.04.004
7. Pekarsky Y, Santanam U, Cimmino A, Palamarchuk A, Efanov A, Maximov V, et al. Tcl1 expression in chronic lymphocytic leukemia is regulated by miR-29 and miR-181. *Cancer Res* (2006) **66**:11590–3. doi:10.1158/0008-5472.CAN-06-3613
8. Santanam U, Zanesi N, Efanov A, Costinean S, Palamarchuk A, Hagan JP, et al. Chronic lymphocytic leukemia modeled in mouse by targeted miR-29 expression. *Proc Natl Acad Sci U S A* (2010) **107**:12210–5. doi:10.1073/pnas.1007186107
9. Rao DS, O'connell RM, Chaudhuri AA, Garcia-Flores Y, Geiger TL, Baltimore D. MicroRNA-34a perturbs B lymphocyte development by repressing the forkhead box transcription factor Foxp1. *Immunity* (2010) **33**:48–59. doi:10.1016/j.immuni.2010.06.013
10. Boldin MP, Taganov KD, Rao DS, Yang L, Zhao JL, Kalwani M, et al. miR-146a is a significant brake on autoimmunity, myeloproliferation, and cancer in mice. *J Exp Med* (2011) **208**:1189–201. doi:10.1084/jem.20101823
11. Guo Q, Zhang J, Li J, Zou L, Zhang J, Xie Z, et al. Forced miR-146a expression causes autoimmune lymphoproliferative syndrome in mice via down-regulation of Fas in germinal center B cells. *Blood* (2013) **121**:4875–83. doi:10.1182/blood-2012-08-452425
12. Sonkoly E, Wei T, Janson PC, Saaf A, Lundeberg L, Tengvall-Linder M, et al. MicroRNAs: novel regulators involved in the pathogenesis of psoriasis? *PLoS One* (2007) **2**:e610. doi:10.1371/journal.pone.0000610
13. Nakasa T, Miyaki S, Okubo A, Hashimoto M, Nishida K, Ochi M, et al. Expression of microRNA-146 in rheumatoid arthritis synovial tissue. *Arthritis Rheum* (2008) **58**:1284–92. doi:10.1002/art.23429
14. Stanczyk J, Pedrioli DM, Brentano F, Sanchez-Pernaute O, Kolling C, Gay RE, et al. Altered expression of MicroRNA in synovial fibroblasts and synovial tissue in rheumatoid arthritis. *Arthritis Rheum* (2008) **58**:1001–9. doi:10.1002/art.23386
15. Wang G, Kwan BC, Lai FM, Chow KM, Li PK, Szeto CC. Elevated levels of miR-146a and miR-155 in kidney biopsy and urine from patients with IgA nephropathy. *Dis Markers* (2011) **30**:171–9. doi:10.3233/DMA-2011-0766
16. Cameron JE, Yin Q, Fewell C, Lacey M, McBride J, Wang X, et al. Epstein-Barr virus latent membrane protein 1 induces cellular MicroRNA miR-146a, a modulator of lymphocyte signaling pathways. *J Virol* (2008) **82**(4):1946–58. doi:10.1128/JVI.02136-07
17. Xiao C, Calado DP, Galler G, Thai TH, Patterson HC, Wang J, et al. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. *Cell* (2007) **131**:146–59. doi:10.1016/j.cell.2007.07.021
18. Stagakis E, Bertsias G, Verginis P, Nakou M, Hatziapostolou M, Kritikos H, et al. Identification of novel microRNA signatures linked to human lupus disease activity and pathogenesis: miR-21 regulates aberrant T cell responses through regulation of PDCD4 expression. *Ann Rheum Dis* (2011) **70**:1496–506. doi:10.1136/ard.2010.139857
19. Vigorito E, Perks KL, Abreu-Goodger C, Bunting S, Xiang Z, Kohlhaas S, et al. microRNA-155 regulates the generation of immunoglobulin class-switched plasma cells. *Immunity* (2007) **27**:847–59. doi:10.1016/j.immuni.2007.10.009
20. Teng G, Hakimpour P, Landgraf P, Rice A, Tuschl T, Casellas R, et al. MicroRNA-155 is a negative regulator of activation-induced cytidine deaminase. *Immunity* (2008) **28**:621–9. doi:10.1016/j.immuni.2008.03.015
21. Pedersen IM, Otero D, Kao E, Miletic AV, Hother C, Ralfkiaer E, et al. Onco-miR-155 targets SHIP1 to promote TNFalpha-dependent growth of B cell lymphomas. *EMBO Mol Med* (2009) **1**:288–95. doi:10.1002/emmm.200900028
22. Dorsett Y, Mcbride KM, Jankovic M, Gazumyan A, Thai TH, Robbiani DF, et al. MicroRNA-155 suppresses activation-induced cytidine deaminase-mediated Myc-Igh translocation. *Immunity* (2008) **28**:630–8. doi:10.1016/j.immuni.2008.04.002
23. Yin Q, Wang X, Fewell C, Cameron J, Zhu H, Baddoo M, et al. MicroRNA miR-155 inhibits bone morphogenetic protein (BMP) signaling and BMP-mediated Epstein-Barr virus reactivation. *J Viro* (2010) **84**(13):6318–27. doi:10.1128/JVI.00635-10
24. Kim JH, Kim WS, Park C. Epstein-Barr virus latent membrane protein-1 protects B-cell lymphoma from rituximab-induced apoptosis through miR-155-mediated Akt activation and up-regulation of Mcl-1. *Leuk Lymphoma* (2012) **53**:1586–91. doi:10.3109/10428194.2012.659736
25. Lwin T, Lin J, Choi YS, Zhang X, Moscinski LC, Wright KL, et al. Follicular dendritic cell-dependent drug resistance of non-Hodgkin lymphoma involves cell adhesion-mediated Bim down-regulation through induction of microRNA-181a. *Blood* (2010) **116**:5228–36. doi:10.1182/blood-2010-03-275925
26. Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate hematopoietic lineage differentiation. *Science* (2004) **303**:83–6. doi:10.1126/science.1091903
27. de Yebenes VG, Belver L, Pisano DG, Gonzalez S, Villasante A, Croce C, et al. miR-181b negatively regulates activation-induced cytidine deaminase in B cells. *J Exp Med* (2008) **205**:2199–206. doi:10.1084/jem.20080579
28. Mok Y, Schwierzeck V, Thomas DC, Vigorito E, Rayner TF, Jarvis LB, et al. MiR-210 is induced by Oct-2, regulates B cells, and inhibits autoantibody production. *J Immunol* (2013) **191**(6):3037–48. doi:10.4049/jimmunol.1301289
29. Knoll M, Simmons S, Bouquet C, Grun JR, Melchers F. miR-221 redirects precursor B cells to the BM and regulates their residence. *Eur J Immunol* (2013) **43**(9):2497–506. doi:10.1002/eji.201343367
30. Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* (1993) **75**:843–54. doi:10.1016/0092-8674(93)90529-Y
31. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. *Cell* (1993) **75**:855–62. doi:10.1016/0092-8674(93)90530-4
32. Kozomara A, Griffiths-Jones S. miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res* (2011) **39**:D152–7. doi:10.1093/nar/gkq1027
33. Kim VN, Han J, Siomi MC. Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol* (2009) **10**:126–39. doi:10.1038/nrm2632
34. Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH, et al. MicroRNA genes are transcribed by RNA polymerase II. *EMBO J* (2004) **23**:4051–60. doi:10.1038/sj.emboj.7600385
35. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* (2004) **116**:281–97. doi:10.1016/S0092-8674(04)00045-5
36. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. *Cell* (2003) **115**:787–98. doi:10.1016/S0092-8674(03)01018-3
37. Baek D, Villén J, Shin C, Camargo FD, Gygi SP, Bartel DP. The impact of microRNAs on protein output. *Nature* (2008) **455**:64–71. doi:10.1038/nature07242
38. Guo H, Ingolia NT, Weissman JS, Bartel DP. Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature* (2010) **466**:835–40. doi:10.1038/nature09267
39. Maziere P, Enright AJ. Prediction of microRNA targets. *Drug Discov Today* (2007) **12**:452–8. doi:10.1016/j.drudis.2007.04.002
40. Mathonnet G, Fabian MR, Svitkin YV, Parsyan A, Huck L, Murata T, et al. MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F. *Science* (2007) **317**:1764–7. doi:10.1126/science.1146067
41. Bazzini AA, Lee MT, Giraldez AJ. Ribosome profiling shows that miR-430 reduces translation before causing mRNA decay in zebrafish. *Science* (2012) **336**:233–7. doi:10.1126/science.1215704
42. Djuranovic S, Nahvi A, Green R. miRNA-mediated gene silencing by translational repression followed by mRNA deadenylation and decay. *Science* (2012) **336**:237–40. doi:10.1126/science.1215691
43. Rodriguez A, Vigorito E, Clare S, Warren MV, Couttet P, Soond DR, et al. Requirement of bic/microRNA-155 for normal immune function. *Science* (2007) **316**:608–11. doi:10.1126/science.1139253
44. Banerjee A, Schambach F, Dejong CS, Hammond SM, Reiner SL. Micro-RNA-155 inhibits IFN-gamma signaling in CD4+ T cells. *Eur J Immunol* (2010) **40**:225–31. doi:10.1002/eji.200939381
45. Schott J, Stoecklin G. Networks controlling mRNA decay in the immune system. *Wiley Interdiscip Rev RNA* (2010) **1**:432–56. doi:10.1002/wrna.13
46. Allantaz F, Cheng DT, Bergauer T, Ravindran P, Rossier MF, Ebeling M, et al. Expression profiling of human immune cell subsets identifies miRNA-mRNA regulatory relationships correlated with cell type specific expression. *PLoS One* (2012) **7**:e29979. doi:10.1371/journal.pone.0029979
47. Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, et al. The microRNA spectrum in 12 body fluids. *Clin Chem* (2010) **56**:1733–41. doi:10.1373/clinchem.2010.147405
48. Lorenzen JM, Volkmann I, Fiedler J, Schmidt M, Scheffner I, Haller H, et al. Urinary miR-210 as a mediator of acute T-cell mediated rejection in renal allograft

recipients. *Am J Transplant* (2011) **11**:2221–7. doi:10.1111/j.1600-6143.2011.03679.x

49. Vasudevan S, Tong Y, Steitz JA. Switching from repression to activation: microRNAs can up-regulate translation. *Science* (2007) **318**:1931–4. doi:10.1126/science.1149460
50. Place RF, Li LC, Pookot D, Noonan EJ, Dahiya R. MicroRNA-373 induces expression of genes with complementary promoter sequences. *Proc Natl Acad Sci U S A* (2008) **105**:1608–13. doi:10.1073/pnas.0707594105
51. Lytle JR, Yario TA, Steitz JA. Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. *Proc Natl Acad Sci U S A* (2007) **104**:9667–72. doi:10.1073/pnas.0703820104
52. Cesana M, Cacchiarelli D, Legnini I, Santini T, Sthandier O, Chinappi M, et al. A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. *Cell* (2011) **147**:358–69. doi:10.1016/j.cell.2011.09.028
53. O'Carroll D, Mecklenbrauker I, Das PP, Santana A, Koenig U, Enright AJ, et al. A slicer-independent role for argonaute 2 in hematopoiesis and the microRNA pathway. *Genes Dev* (2007) **21**:1999–2004. doi:10.1101/gad.1565607
54. Xu S, Guo K, Zeng Q, Huo J, Lam KP. The RNase III enzyme Dicer is essential for germinal center B-cell formation. *Blood* (2012) **119**:767–76. doi:10.1182/blood-2011-05-355412
55. Spierings DC, Mcgoldrick D, Hamilton-Easton AM, Neale G, Murchison EP, Hannon GJ, et al. Ordered progression of stage-specific miRNA profiles in the mouse B2 B-cell lineage. *Blood* (2011) **117**:5340–9. doi:10.1182/blood-2010-10-316034
56. Chang TC, Yu D, Lee YS, Wentzel EA, Arking DE, West KM, et al. Widespread microRNA repression by Myc contributes to tumorigenesis. *Nat Genet* (2008) **40**:43–50. doi:10.1038/ng.2007.30
57. Peveling-Oberhag J, Crisman G, Schmidt A, Doring C, Lucioni M, Arcaini L, et al. Dysregulation of global microRNA expression in splenic marginal zone lymphoma and influence of chronic hepatitis C virus infection. *Leukemia* (2012) **26**:1654–62. doi:10.1038/leu.2012.29
58. Di Lisio L, Martinez N, Montes-Moreno S, Piris-Villaespesa M, Sanchez-Beato M, Piris MA. The role of miRNAs in the pathogenesis and diagnosis of B-cell lymphomas. *Blood* (2012) **120**:1782–90. doi:10.1182/blood-2012-05-402784
59. Liston A, Papadopoulou AS, Danso-Abeam D, Dooley J. MicroRNA-29 in the adaptive immune system: setting the threshold. *Cell Mol Life Sci* (2012) **69**:3533–41. doi:10.1007/s00018-012-1124-0
60. Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, et al. A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell* (2007) **129**:1401–14. doi:10.1016/j.cell.2007.04.040
61. Liang Y, Ridzon D, Wong L, Chen C. Characterization of microRNA expression profiles in normal human tissues. *BMC Genomics* (2007) **8**:166. doi:10.1186/1471-2164-8-166
62. Ramkissoon SH, Mainwaring LA, Ogasawara Y, Keyvanfar K, Mccoy JP Jr, Sloand EM, et al. Hematopoietic-specific microRNA expression in human cells. *Leuk Res* (2006) **30**:643–7. doi:10.1016/j.leukres.2005.09.001
63. Merkerova M, Belickova M, Bruchova H. Differential expression of microRNAs in hematopoietic cell lineages. *Eur J Haematol* (2008) **81**:304–10. doi:10.1111/j.1600-0609.2008.01111.x
64. Basso K, Sumazin P, Morozov P, Schneider C, Maute RL, Kitagawa Y, et al. Identification of the human mature B cell miRNome. *Immunity* (2009) **30**:744–52. doi:10.1016/j.immuni.2009.03.017
65. Malumbres R, Sarosiek KA, Cubedo E, Ruiz JW, Jiang X, Gascoyne RD, et al. Differentiation stage-specific expression of microRNAs in B lymphocytes and diffuse large B-cell lymphomas. *Blood* (2009) **113**:3754–64. doi:10.1182/blood-2008-10-184077
66. Meffre E, Casellas R, Nussenzweig MC. Antibody regulation of B cell development. *Nat Immunol* (2000) **1**:379–85. doi:10.1038/80816
67. Meffre E, Wardemann H. B-cell tolerance checkpoints in health and autoimmunity. *Curr Opin Immunol* (2008) **20**:632–8. doi:10.1016/j.coi.2008.09.001
68. Carsetti R, Rosado MM, Wardmann H. Peripheral development of B cells in mouse and man. *Immunol Rev* (2004) **197**:179–91. doi:10.1111/j.0105-2896.2004.0109.x
69. Belver L, De Yebenes VG, Ramiro AR. MicroRNAs prevent the generation of autoreactive antibodies. *Immunity* (2010) **33**:713–22. doi:10.1016/j.immuni.2010.11.010
70. Pillai S, Cariappa A. The follicular versus marginal zone B lymphocyte cell fate decision. *Nat Rev Immunol* (2009) **9**:767–77. doi:10.1038/nri2656
71. Whitacre CC. Sex differences in autoimmune disease. *Nat Immunol* (2001) **2**:777–80. doi:10.1038/ni0901-777
72. Thai TH, Calado DP, Casola S, Ansel KM, Xiao C, Xue Y, et al. Regulation of the germinal center response by microRNA-155. *Science* (2007) **316**:604–8. doi:10.1126/science.1141229
73. Lund FE, Randall TD. Effector and regulatory B cells: modulators of CD4+ T cell immunity. *Nat Rev Immunol* (2010) **10**:236–47. doi:10.1038/nri2729
74. Pan W, Zhu S, Yuan M, Cui H, Wang L, Luo X, et al. MicroRNA-21 and microRNA-148a contribute to DNA hypomethylation in lupus CD4+ T cells by directly and indirectly targeting DNA methyltransferase 1. *J Immunol* (2010) **184**:6773–81. doi:10.4049/jimmunol.0904060
75. Garchow BG, Bartulos Encinas O, Leung YT, Tsao PY, Eisenberg RA, Caricchio R, et al. Silencing of microRNA-21 in vivo ameliorates autoimmune splenomegaly in lupus mice. *EMBO Mol Med* (2011) **3**:605–15. doi:10.1002/emmm.201100171
76. Yuan Y, Kasar S, Underbayev C, Vollenweider D, Salerno E, Kotenko SV, et al. Role of microRNA-15a in autoantibody production in interferon-augmented murine model of lupus. *Mol Immunol* (2012) **52**:61–70. doi:10.1016/j.molimm.2012.04.007
77. Rossi RL, Rossetti G, Wenandy L, Curti S, Ripamonti A, Bonnal RJ, et al. Distinct microRNA signatures in human lymphocyte subsets and enforcement of the naive state in CD4+ T cells by the microRNA miR-125b. *Nat Immunol* (2011) **12**:796–803. doi:10.1038/ni.2057
78. Liu Y, Dong J, Mu R, Gao Y, Tan X, Li Y, et al. MicroRNA-30a promotes B cell hyperactivity in patients with systemic lupus erythematosus by direct interaction with Lyn. *Arthritis Rheum* (2013) **65**:1603–11. doi:10.1002/art.37912
79. Ding S, Liang Y, Zhao M, Liang G, Long H, Zhao S, et al. Decreased microRNA-142-3p/5p expression causes CD4+ T cell activation and B cell hyperstimulation in systemic lupus erythematosus. *Arthritis Rheum* (2012) **64**:2953–63. doi:10.1002/art.34505
80. Lindberg RL, Hoffmann F, Mehling M, Kuhle J, Kappos L. Altered expression of miR-17-5p in CD4+ lymphocytes of relapsing-remitting multiple sclerosis patients. *Eur J Immunol* (2010) **40**:888–98. doi:10.1002/eji.200940032
81. Sievers C, Meira M, Hoffmann F, Fontoura P, Kappos L, Lindberg RL. Altered microRNA expression in B lymphocytes in multiple sclerosis: towards a better understanding of treatment effects. *Clin Immunol* (2012) **144**:70–9. doi:10.1016/j.clim.2012.04.002
82. Anglicheau D, Muthukumar T, Suthanthiran M. MicroRNAs: small RNAs with big effects. *Transplantation* (2010) **90**:105–12. doi:10.1097/TP.0b013e3181e913c2
83. Sarma NJ, Tiriveedhi V, Ramachandran S, Crippin J, Chapman W, Mohanakumar T. Modulation of immune responses following solid organ transplantation by microRNA. *Exp Mol Pathol* (2012) **93**:378–85. doi:10.1016/j.yexmp.2012.09.020
84. Ben-Dov IZ, Muthukumar T, Morozov P, Mueller FB, Tuschl T, Suthanthiran M. MicroRNA sequence profiles of human kidney allografts with or without tubulointerstitial fibrosis. *Transplantation* (2012) **94**:1086–94. doi:10.1097/TP.0b013e3182751efd
85. Danger R, Paul C, Giral M, Lavault A, Foucher Y, Degauque N, et al. Expression of miR-142-5p in peripheral blood mononuclear cells from renal transplant patients with chronic antibody-mediated rejection. *PLoS One* (2013) **8**(4):e60702. doi:10.1371/journal.pone.0060702
86. Wilflingseder J, Regele H, Perco P, Kainz A, Soleiman A, Muhlbacher F, et al. miRNA profiling discriminates types of rejection and injury in human renal allografts. *Transplantation* (2013) **95**:835–41. doi:10.1097/TP.0b013e318280b385
87. Danger R, Pallier A, Giral M, Martinez-Llordella M, Lozano JJ, Degauque N, et al. Upregulation of miR-142-3p in peripheral blood mononuclear cells of operationally tolerant patients with a renal transplant. *J Am Soc Nephrol* (2012) **23**:597–606. doi:10.1681/ASN.2011060543
88. Anglicheau D, Sharma VK, Ding R, Hummel A, Snopkowski C, Dadhania D, et al. MicroRNA expression profiles predictive of human renal allograft status. *Proc Natl Acad Sci U S A* (2009) **106**:5330–5. doi:10.1073/pnas.0813121106
89. Scian MJ, Maluf DG, David KG, Archer KJ, Suh JL, Wolen AR, et al. MicroRNA profiles in allograft tissues and paired urines associate with chronic allograft dysfunction with IF/TA. *Am J Transplant* (2011) **11**:2110–22. doi:10.1111/j.1600-6143.2011.03666.x
90. Ferrajoli A, Shanafelt TD, Ivan C, Shimizu M, Rabe KG, Nouraee N, et al. Prognostic value of miR-155 in individuals with monoclonal B-cell lymphocytosis

and patients with B chronic lymphocytic leukemia. *Blood* (2013) **122**:1891–9. doi:10.1182/blood-2013-01-478222
91. Janssen HL, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres M, Patel K, et al. Treatment of HCV infection by targeting microRNA. *N Engl J Med* (2013) **368**:1685–94. doi:10.1056/NEJMoa1209026
92. Almanza G, Anufreichik V, Rodvold JJ, Chiu KT, Delaney A, Akers JC, et al. Synthesis and delivery of short, noncoding RNA by B lymphocytes. *Proc Natl Acad Sci U S A* (2013) **110**:20182–7. doi:10.1073/pnas.1311145110

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Central role of CD45RA$^-$ Foxp3^{hi} memory Tregs in clinical kidney transplantation tolerance

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Abstract

The role of Foxp3$^+$ regulatory T cells in operational tolerance remains elusive as initial results have shown increased frequency of the subset in tolerant patients but no functional differences compared to immunosuppressed recipients. In addition, recent studies in regulatory B cells strongly suggest that Tregs may not play a central role in kidney transplantation tolerance. However recent work on (i) the crucial role of Foxp3 demethylation in Treg function, and (ii) the possibility of identifying distinct Foxp3 T-cell subsets prompted us to attempt to characterize more deeply regulatory T cells in operational tolerant patients. We thus studied the level of demethylation of the Foxp3 Treg-specific demethylated region (TSDR) in circulating CD4$^+$ T cells and analyzed Treg-subset frequency in tolerant patients, healthy volunteers, patients with stable graft function under immunosuppression, and chronically rejecting recipients. We found a higher proportion of CD4$^+$ T cells with demethylated Foxp3 and a specific expansion of CD4$^+$ CD45RA$^-$ Foxp3^{hi} memory Tregs exclusively in tolerant patients. The memory Treg of tolerant recipients display increased Foxp3 TSDR demethylation and greater suppressive properties than memory Treg from patients with stable graft function. Taken together, our data show that operationally tolerant patients mobilize a whole array of potentially suppressive cells including not only Breg but also Treg cells. Our results also indicate that tolerant patients display potent additional CD4$^+$CD45RA$^-$ Foxp3^{hi} memory Tregs with a specific Foxp3 TSDR demethylation pattern which may contribute to the maintenance of graft tolerance in these patients.

Central role of CD45RA^{-} Foxp3^{hi} memory Tregs in clinical kidney transplantation tolerance

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Abbreviations

CD: Cluster of Differentiation

HV: Healthy Volunteers

RC: Patients with Chronic rejection

STA: Patients stable under immunosuppressive therapy

TOL: Operational Tolerant Patients

Tregs: Regulatory T cells

Memory Tregs: mTregs

Naïve Tregs: nTregs

Bregs: Regulatory B cells

PBMC: Peripheral Blood Mononuclear Cells

PTLD: Post-Transplant Lymphoproliferative Disorder

TSDR: Treg-specific demethylated region

Foxp3: Forkhead Box P3

TGF-β: Transforming Growth Factor beta

DTH: Delayed type Hypersensitivity

GITR: glucocorticoid-induced TNFR-related protein

Lag3: Lymphocyte-activation gene 3

CTLA4: cytotoxic T-lymphocyte-associated protein 4

Abstract

The role of Foxp3^{+} regulatory T cells in operational tolerance remains elusive as initial results have shown increased frequency of the subset in tolerant patients but no functional differences compared to immunosuppressed recipients. In addition, recent studies in regulatory B cells strongly suggest that Tregs may not play a central role in kidney transplantation tolerance. However recent work on (i) the crucial role of Foxp3 demethylation in Treg function, and (ii) the possibility of identifying distinct Foxp3 T-cell subsets prompted us to attempt to characterize more deeply regulatory T cells in operational tolerant patients. We thus studied the level of demethylation of the Foxp3 Treg-specific demethylated region (TSDR) in circulating CD4^{+} T cells and analyzed Treg-subset frequency in tolerant patients, healthy volunteers, patients with stable graft function under immunosuppression, and chronically rejecting recipients. We found a higher proportion of CD4^{+} T cells with demethylated Foxp3 and a specific expansion of CD4^{+} CD45RA^{-} Foxp3^{hi} memory Tregs exclusively in tolerant patients. The memory Treg of tolerant recipients display increased Foxp3 TSDR demethylation, express higher lever of CD39 and GITR and harbor greater suppressive properties than memory Treg from patients with stable graft function. Taken together, our data show that operationally tolerant patients mobilize a whole array of potentially suppressive cells including not only Breg but also Treg cells. Our results also indicate that tolerant patients display potent additional CD4^{+}CD45RA^{-} Foxp3^{hi} memory Tregs with a specific Foxp3 TSDR demethylation pattern which may contribute to the maintenance of graft tolerance in these patients.

Introduction

Transplantation tolerance is defined as the maintenance of a stable allograft function in the absence of immunosuppressive therapy. An increasing number of "operationally tolerant patients" have been described in the literature showing that this state exists in humans [1-3]. Analyses performed on these patients suggest the potential involvement of multiple cell subsets contributing to the development and/or maintenance of tolerance, including B- and T-cell subsets. We, and others, have reported an increased number of B cells [4] and the over-expression of numerous genes related to their proliferation, maturation and differentiation [5-8]. In parallel, some studies, including our own, also suggest a potential role for regulatory T cells (Tregs) in these patients, with over-expression of several genes associated with regulatory functions [6, 8-10]. In particular, stable patients and recipients with ongoing chronic rejection display lower levels and proportions of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells compared to operationally tolerant patients and healthy volunteers [9, 11]. However Tregs from tolerant patients displayed no increased suppressive properties compared to those of other patients when polyclonally stimulated [9]. Thus, although the potency of Tregs in preventing graft rejection and inducing transplant tolerance in experimental models has been clearly demonstrated [12-14], their role in clinical settings still remains to be precisely defined.

Despite the central role of Foxp3 in Treg homeostasis, recent data has demonstrated that its expression is not sufficient to generate stable and functional Tregs [15-17]. In fact, the epigenetic status of the Foxp3 gene locus is closely linked to the differentiation and stability of Tregs [18]. Epigenetic modifications including histone modifications, chromatin interactions and DNA methylations, play important roles in Tregs cell differentiation [18, 19]. Mounting evidences demonstrate that specific DNA demethylation of the Treg-specific demethylated region (TSDR) and chromatin modifications of the Foxp3 gene occur in Tregs [20, 21]. Demethylation of the Foxp3 region is not only important for the induction and stabilization of Foxp3 expression, but also ensures the differentiation and the suppressive function of Tregs [22, 23]. In the present paper, we characterize the profile of Tregs in circulating blood from operationally tolerant patients and we investigate their Foxp3 demethylation level in conjunction with their suppressive properties.

For the first time, we report on an enhanced frequency of CD45RA^{-} Foxp3^{hi} memory Tregs (mTregs) expressing CD39 and GITR in operational tolerance and displaying increased Foxp3 TSDR demethylation and higher suppressive properties.

116 **Results**

117 ***Clinical characteristics of transplant patients*** **-** Three groups of transplant recipients were
118 included in the study: 13 operationally tolerant (TOL), 33 with stable graft function under
119 immunosuppression (STA), and 19 with symptoms of chronic rejection and functional
120 degradation (RC). There were also 15 healthy volunteers (HV) as a control (See "patients" in
121 material and methods section). TOL are defined as patients with a stable kidney graft function
122 (creatinemia <150 mmol/l and proteinuria <1g/24 h) in the absence of immunosuppression for
123 at least 1 year. Age and genders were not significantly different between TOL, STA, RC and HV
124 (Table 1). A well functioning graft is defined by stable levels of creatinine <150µM/L and
125 proteinuria <1g/24h for at least 3 years. Immunosuppressive treatment was stopped in TOL due
126 to non-compliance (n=8), post-transplant lymphoproliferative disorder (PTLD) (n=3), calcineurin
127 inhibitor toxicity (n=1) and meningio-encephalitis (n=1). RC recipients had a creatinemia
128 >150µM/L and/or proteinuria >1g/24h and were histopathologically and serologically classified
129 according to the updated Banff classification criteria [24, 25]: 17/19 patients display graft
130 glomerulopathy with 11 RC patients having positive C4d staining and 4 with anti-donor specific
131 antibodies (Table 1). Apart from 2 TOL, all renal grafts had been obtained from cadaveric
132 donors. No significant differences in cancer, infection, HLA mismatches and numbers of acute
133 rejection episodes were found between the three groups of patients. All clinical parameters are
134 provided in the Table 1.
135
136 ***CD4$^+$ T cells from tolerant patients have higher level of DNA demethylation of the Treg-***
137 ***specific Foxp3 region –*** As Foxp3 demethylation is correlated with the function of regulatory T
138 cells, we analyzed Foxp3 TSDR demethylation in the purified CD4$^+$ T cells from the transplanted
139 patients and healthy volunteers. The percentage of CD4$^+$ T cells with Foxp3 demethylation was
140 significantly higher in TOL (n=11; 13.33% +/-1.276) compared to STA (n=17; 5.21% +/-0.677;
141 p<0.001), RC (n=8; 5.788% +/-0.806; p<0.01) and HV (n=9; 8.996% +/-0.978; p<0.05) (Figure 1A).
142 To investigate if Foxp3 demethylation levels were age-dependent, we analyzed the % of CD4$^+$
143 cells with demethylated TSDR according to the age of the 4 groups of patients. No correlation
144 was found between age, time post-transplantation and demethylation of the Foxp3 region in

our groups (figure 1B). Interestingly, no correlation was found between %$CD4^+$ T cells with a demethylated TSDR region and length of time since cessation of immunosuppressors, showing that %$CD4^+$ with demethylated TSDR does not result from the reduced immunosuppression of patients further post-transplant (Figure 1C). The level of Foxp3 demethylation inversely correlated with the level of creatinemia (p=0.0312 r=-0.3596) suggesting a close link between graft integrity and Treg function (Figure 1D). Because Tregs have been mainly defined by the expression of CD25, CD127 and Foxp3, we then quantified the frequency of circulating Tregs based on the expression of these markers [26]. A higher proportion of $CD4^+$ $CD25^+$ $CD127^{lo}$ $Foxp3^+$ Tregs was found in blood of TOL (3.11% +/-0.47) compared to STA (1.88% +/-0.14 p<0.05) (Figure 2A). A similar trend was observed with comparison to RC samples, but was not statistically significant (p=0.054). Finally, total $CD4^+$ $CD25^+$ $CD127^{lo}$ Treg frequency in all transplant recipients correlated with Foxp3 demethylation observed in circulating $CD4^+$ cells (Figure 2B). Because of this correlation, we investigated the TSDR demethylation of Foxp3 in FACS-sorted $CD4^+$ $CD25^+$ $CD127^{lo}$ T cells. No difference in Foxp3 demethylation was found between the 3 groups of patients and HV (Figure 2C).

Operationally tolerant patients have a significantly higher level of circulating $CD4^+$ $CD45RA^-$ $Foxp3^{hi}$ mTregs **-** Many studies have attempted to identify distinct Tregs subsets based on their phenotypic and functional properties [16, 27, 28]. Based on the expression of CD45RA, CD25, CD127 and Foxp3, one can distinguish $CD45RA^+$ $Foxp3^{lo}$ $CD25^+$ $CD127^{lo}$ naive Tregs (nTregs), $CD45RA^-$ $Foxp3^{hi}$ $CD25^{hi}$ $CD127^{lo}$ mTregs and $CD45RA^-$ $Foxp3^{lo}$ $CD25^+$ $CD127^+$ non-Tregs [16] (Supplemental Figure 1A and 1B). Each population has been reported to exhibit specific Foxp3 epigenetic modifications and functional properties [16]. To investigate if there was a difference in Treg subsets in our cohort of patients, we quantified them using by flow cytometry. This revealed a significant and specific higher level in the frequency of $CD45RA^-$ $Foxp3^{hi}$ mTregs in the TOL group (3A and 3B). Interestingly, the percentage of mTregs strongly correlates with the percentage of $CD4^+$ T cells with demethylated Foxp3 observed in our patients (p<0.0001 r=0.7010) (Figure 3C), suggesting a potential role of this subset in operational tolerance.

174 ***Circulating mTregs from tolerant recipients, express higher levels of CD39 GITR and exhibit***
175 ***higher Foxp3 demethylation levels and greater suppressive function –*** Given the greater
176 proportion of mTregs in TOL, its correlation with Foxp3 demethylation in total $CD4^+$ T cells, we
177 characterized this Treg subset in transplant recipients in more detail. As a first step, we studied
178 the expression of four Treg-molecules previously described as overexpressed on activated
179 mTregs, GITR, Lag3, CD39 and CTLA4 [29]. Meta-analysis of our previous transcriptomic studies [6,
180 30] reveals higher expression of CTLA4, CD39 and GITR in the blood of TOL, compared to other
181 groups (Figure 4A). Consistent with this, using flow cytometry we confirmed the overexpression
182 of CD39 and GITR at the surface of mTregs of TOL compared to STA and HV (Figure 4B and 4C).
183 In a second step, we assessed the *in vitro* suppressive potency of these cells. MTregs were
184 purified and sorted based on their $CD4^+$ $CD45RA^-$ $CD25^{hi}$ phenotype [16] (Supplemental Figure 1B).
185 Then we measured the extent of cell trace dilution of FACS-sorted labeled $CD4^+$ $CD25^-$ effector T
186 cells cocultured with $CD4^+$ $CD45RA^-$ $CD25^{hi}$ mTregs (ratio 1:1 and 2:1) and stimulated for 4 days
187 with coated anti-CD3 in presence of feeder cells. We found that, at both ratios, $CD4^+$ $CD45RA^-$
188 $CD25^{hi}$ mTregs from TOL and HV were highly suppressive whereas they were not suppressive
189 and unable to control $CD4^+$ $CD25^-$ effector T cell proliferation in STA (Figure 4A, B). Linked to
190 this, increased demethylation of Foxp3 locus was observed in mTregs from TOL when compared
191 to STA (TOL; 72.4% +/- 12.1 vs STA 36.3% +/- 7.1, $p<0.05$). Collectively these data demonstrate
192 that mTregs from TOL are fully functional and overexpress CD39 and GITR, this being linked to a
193 higher level of demethylation of their Foxp3 locus, whereas they are non-functional in STA.
194

195 **Discussion**

196 Induction of tolerance in graft recipients is a keenly explored field. However, identification of
197 mechanisms of tolerance in stable grafts recipients would significantly improve long-term
198 clinical outcomes by decreasing immunosuppressive drugs co-morbidity [31, 32]. Although the
199 phenomenon of operational tolerance offers a unique model for the regulation of immune
200 response in humans, the mechanisms responsible for the maintenance of tolerance in these
201 patients remains unclear. We and others have demonstrated a possible involvement of
202 regulatory cells, including Bregs [33] and Tregs [4, 8, 9, 34]. We initially reported on an greater number
203 of circulating $CD4^+$ $CD25^{hi}$ $Foxp3^+$ cells in blood from patients with operational tolerance [9] and
204 previous transcriptomic studies have highlighted higher mRNA levels of Foxp3 in the blood,
205 graft and urine of tolerant patients compared with transplant recipients with different clinical
206 situations, and with healthy volunteers [6, 7, 35]. In accord with this, lower frequencies of
207 regulatory T cells have been correlated with poor graft outcomes [4, 11, 34, 36]. Despite the
208 accumulating data from various experimental models suggesting a vital role for Tregs, the
209 present clinical results are insufficient to prove that Tregs play a role in the maintenance of
210 tolerance in operationally tolerant recipients. In this paper, we confirm and further extend our
211 previous observation of a greater level of Tregs in the blood of tolerant patients. We also report
212 on an increased frequency of total circulating mTregs in the blood of these recipients compared
213 to non-tolerant equivalents and healthy controls. Furthermore, our data give more support to a
214 possible involvement of Tregs in the maintenance of operational tolerance by providing the first
215 evidence that operationally tolerant patients harbor Treg cells exhibiting a specific pattern of
216 Foxp3 demethylation, a key regulator of the Treg function.
217 Foxp3 has been clearly recognized for years as the main regulator of regulatory T-cell function
218 [37-39]. However, recent evidences suggest that the expression of Foxp3 alone may not be
219 sufficient to maintain the suppressive function of Tregs over time [40]. As a further evidence,
220 $CD4^+$ $CD45RA^-$ cells, which express low levels of Foxp3, do not exhibit Treg activity and may
221 display a pro-inflammatory profile [16]. In line with this, the ectopic expression of Foxp3 by T cells
222 using retrovirus is not sufficient to induce functional regulatory T cells [15]. Other factors, such as
223 DNA demethylation of the Foxp3 gene locus, have been reported to be crucial for the stability

and function of Treg cells [18]. Interestingly we report here that operationally tolerant patients have significantly higher levels of $CD4^+$ T cells with demethylated Foxp3 when compared with stable patients, patients with chronic rejection and also with healthy volunteers, confirming that this feature is not treatment dependent but specifically associated with operational tolerance. This is confirmed by the correlation found between graft function (assessed by levels of creatinemia) and percentage of demethylated TSDR in $CD4^+$ T cells from operationally tolerant recipients. Accordingly high levels of TSDR in $CD4^+$ T cells in the kidney graft were associated with a favourable long-term graft outcome [41]. We also found a higher frequency of total circulating $CD4^+$ $CD25^+$ $Foxp3^+$ $CD127^{lo}$ in blood of tolerant recipients. However, Foxp3 demethylation was similar in Tregs counted overall in all groups of patients and the healthy volunteers. This may explain why previous studies on Treg from operationally tolerant recipients do not indicate a higher suppressive function for these cells *in vitro* [9]. These studies, including our own, were essentially based on an overall characterization of Treg cells using a consensual phenotype based mostly on higher expression of CD25 and lower expression of CD127 [8, 9, 11]. This is most likely over-simplistic considering the known heterogeneity and plasticity of Tregs [16, 28, 42].

In humans, a recent study identified three distinct Foxp3 expressing T-cells subsets [16]. We can distinguish two distinct memory $CD4^+$ $CD45RA^-$ $FOXP3^{hi}$ and naïve $CD4^+$ $CD45RA^+$ $FOXP3^{lo}$ Treg-cell subsets with strong suppressive functions as along with $CD4^+$ $CD45RA^-$ $FOXP3^{lo}$ non-suppressive T cells able to secrete inflammatory cytokines after polyclonal activation. The suppressive activities of mTregs are positively correlated with the DNA methylation status of their Foxp3 locus [16]. Similar observations have been made in human skin-resident mTregs characterized by (i) strong Foxp3 demethylation and (ii) inability to produce inflammatory cytokines [43]. Consistent with this, our data demonstrate that the percentage of $CD4^+$ with demethylated Foxp3 is strongly correlated to the level of mTregs in transplant patients. Further investigations into mTregs demonstrate that this subset is increased in the blood of tolerant recipients and not other groups. These differences between tolerant, other transplant patients and healthy volunteers support that this phenomenon is independent of immunosuppressive

253 therapy. In addition, higher levels of Foxp3 demethylation have been observed in purified
254 mTregs from tolerant patients and healthy volunteers and not in patients with stable graft
255 function. Accordingly, mTregs from tolerant recipients exhibit greater suppressive function than
256 cells from stable patients. This could be a result of the effect of immunosuppressive therapy on
257 Tregs. However studies describing the effects of immunosuppression on these cells are
258 contradictory [41, 44]. In paediatric liver recipients, high doses of CNI seem to alter TSDR
259 demethylation and Treg function [44]. On the other hand, the presence of demethylated Foxp3$^+$
260 Treg cells in kidney recipients with subclinical rejection **(**even with IF/TA) is associated with a
261 favorable long-term allograft outcome independently of immunosuppressive therapy [41]. This
262 second result is in accordance with our findings. Moreover the fact that mTregs are specifically
263 increased in TOL and not in HV suggests the generation and expansion of alloantigen-specific
264 mTregs in TOL. This may explain why their mTreg frequencies are higher than in HV individuals,
265 but that on a per cell level, their suppressive functions are similar. This is of special interest for
266 graft tolerance given the therapeutic potential of Tregs in transplantation [45, 46]. Our work also
267 demonstrates that phenotypic, functional and also epigenetic investigations of Tregs are
268 complementary to the evaluation of their role in immunopathology. A recent comprehensive
269 genome wide study in human, revealed that mTregs are also characterized by a specific
270 signature consisting of upregulated genes involved in immune regulation such as GITR, CTLA-4,
271 CD39, TGF-β1, LAG3 and IL-10 [29]. Some of these genes have already been described as
272 upregulated in blood from operational tolerant patients reinforcing our observations [6]. We
273 found a higher expression of GITR and CD39 at the surface of mTregs from TOL compared to
274 STA and HV. The fact that CTLA4 and Lag3 were downregulated would suggest that transplant
275 operational tolerance could favor the emergence of a specific functional GITR$^+$ CD39$^+$ mTreg
276 subset. This result is consistent with the growing body of evidence demonstrating the role of
277 CD39 immunological tolerance [47]. Indeed, it has been shown that CD39$^+$ Tregs catalyse cleavage
278 of adenosine triphosphate (ATP) to adenosine monophosphate (AMP), which is then further
279 cleaved to adenosine, leading to control of T cell activation [48]. Interestingly some reports show
280 a role for this molecule in the control of inflammation and graft rejection [49, 50]. These data taken
281 together with our own suggest a potential role for CD39$^+$ Tregs in transplantation tolerance.

Although an overall understanding of operational tolerance remains to be elaborated. We know that operationally tolerant patients display a specific transcriptomic and cellular-signature biais towards regulatory T cells and B cells with inhibitory transcriptional and phenotypic profiles [6-8, 10]. This suggests that tolerance may arise from the action of multiple mechanisms working in concert leading to the emergence of well-functioning suppressive cells. In this paper, we have demonstrated that mTregs with regulatory properties and high level of TSDR demethylation may play a role in this complex system of regulation. Foxp3 expression, demethylation and Treg function are critically affected by many mechanisms [18]. For example, the duration of TCR stimulation is instrumental for Treg DNA demethylation [23]. In addition, other signals favor the stabilization and the suppressive phenotype of Tregs [18]. This is the case for TGF-β, known to induce suppressive T cells *in vitro* [51]. Interestingly, TGF-β-induced Tregs are less stable as regards the expression of Treg-cell-function-associated molecules [15, 23] and fail to induce a complete demethylation of the Foxp3 locus [23]. However continuous exposure to TGF-β, in conjunction with TCR stimulation, can progressively induce complete Foxp3 demethylation in Tregs *in vivo* [23]. One could hypothesize that continuous exposure to alloantigens in a pro-TGF-β environment could contribute to the generation of *in vivo* antigen-specific Tregs in operationally tolerant patients. Indeed, we previously reported that more than 40% of differential expression of genes in blood from tolerant patients is dependent on TGF-β pathway [6] and that regulatory mechanisms such as mir143-3p, which is expressed at higher levels in blood from tolerant recipients, are also dependent on TGF-β [52, 53]. Consistent with this, recent data demonstrate that immune cells from tolerant patients display the greatest suppressive responses in a DTH model and that the regulatory mechanism is TGF-β dependent [54]. Finally, B cells are also potential significant candidates in transplantation tolerance [33]. The recent description of regulatory B cells able to induce, maintain and expand regulatory T cells strengthens this idea [55] A link between regulatory B and T cells in transplantation tolerance has been suggested in recent work [54]. All these mechanisms could be responsible for an amplification loop leading to the stabilization of Tregs through continuous maintenance of Foxp3 demethylation.

In summary, these data reveals new findings concerning Foxp3 epigenetic modifications in Tregs with a specific phenotype and immune-regulatory properties in patients with operational tolerance. Future investigation should focus on the identification of mechanisms which ensure the stabilization of the Treg phenotype and the role of the extrinsic signals necessary for the maintenance of Treg in operational tolerance. How these factors and extracellular stimuli interact with each other to maintain functional Tregs in transplantation tolerance remains to be established. Undoubtedly, understanding the mechanisms regulating the stability of Tregs and their epigenetic modifications will allow the identification of new ways to promote Treg function and tolerance in organ transplant recipients.

Concise Methods

Patients, study cohorts – The University Hospital Ethical Committee and the Committee for the Protection of Patients from Biological Risks approved the study. All patients who participated in this study gave informed consent. A total of 65 age-matched kidney transplant patients were included in the study (summary of clinical data in Table I). Criteria for operational tolerance have been described in detailed elsewhere [1]. TOL are defined as patients with a stable kidney graft function (creatinemia <150 mmol/l and proteinuria <1g/24 h) in the absence of immunosuppression for at least 1 year.

Antibodies, flow cytometry and cell sorting - The following antibodies and reagents were used for flow cytometry and cell sorting: CD45-PO (Caltag, Invitrogen, Darmstadt, Germany), CD3-AlexaFluor-700, anti-CD45-PE and CD4-PB (both from BD Biosciences, Heidelberg, Germany), CD3 Brillant-Violet-605, CD4 APC-Cy7, CD45RA-PeCy7, CCR7-PE, CD25 Brillant-Violet-421, CD127-AlexaFluor-647 (Biolegend, San Diego, USA) Foxp3-PercpCy5.5, GITR-APC, CD39-APC, Lag3-Pe and CTLA4-Pe (eBioscience, San Diego, USA) anti-CD4-FITC (Miltenyi Biotec, Bergisch Gladbach, Germany) and anti-CD25-Alexa Fluor 647 (anti-CD25 from Immunotech, Marseille, France).

Foxp3 TSDR quantification - Genomic DNA was isolated from PBMC samples, purified CD4+ T cells and FACS-sorted regulatory T cells using the QIAamp^{R} DNA Blood Mini kit (Qiagen, Hilden, Germany). Real time-PCR was performed to quantify % Foxp3 TSDR as described eslewhere [56].

Suppression assay - 96-well round-bottom plates were coated with 0.5µg/mL of anti-CD3 (OKT3 mAb) and then 1 x 10^5 $CD4^-$ irradiated feeders cells were added to the plates. $CD4^+CD25^-$ responder T cells were labeled with Cell Trace Violet (Invitrogen, Cergy Pontoise, France) following the manufacturer's instructions and 1 x 10^4 cells were seeded. Suppressive activity was assessed by the addition of 1 x 10^4 or 2 x 10^4 $CD4^+CD25^{hi}CD45RA^-$ unlabeled mTreg cells per well. After 96 hours of culture the cells were stained with the anti-CD4-FITC antibody and the

proliferation of $CD4^+$ Cell Trace Violet labeled cells was assessed by flow cytometry using an LSRII cytometer and FACSDiva™ software (BD Parmingen, Mountain View, CA).

Statistical analysis – We compared subject group characteristics using chi-square testing as appropriate. The non-parametric Kruskal Wallis test was used for comparison of more than 2 groups. Differences were defined as statistically significant when $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***). Correlations were analyzed by linear regression. All statistical analysis was performed in GraphPad Prism v6.

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Bibliography

1. Brouard, S, Pallier, A, Renaudin, K, Foucher, Y, Danger, R, Devys, A, Cesbron, A, Guillot-Guegen, C, Ashton-Chess, J, Le Roux, S, Harb, J, Roussey, G, Subra, JF, Villemain, F, Legendre, C, Bemelman, FJ, Orlando, G, Garnier, A, Jambon, H, Le Monies De Sagazan, H, Braun, L, Noel, C, Pillebout, E, Moal, MC, Cantarell, C, Hoitsma, A, Ranbant, M, Testa, A, Soulillou, JP, Giral, M: The natural history of clinical operational tolerance after kidney transplantation through twenty-seven cases. *Am J Transplant,* 12**:** 3296-3307, 2012.
2. Orlando, G, Hematti, P, Stratta, RJ, Burke, GW, 3rd, Di Cocco, P, Pisani, F, Soker, S, Wood, K: Clinical operational tolerance after renal transplantation: current status and future challenges. *Ann Surg,* 252**:** 915-928, 2010.
3. Roussey-Kesler, G, Giral, M, Moreau, A, Subra, JF, Legendre, C, Noel, C, Pillebout, E, Brouard, S, Soulillou, JP: Clinical operational tolerance after kidney transplantation. *Am J Transplant,* 6**:** 736-746, 2006.
4. Louis, S, Braudeau, C, Giral, M, Dupont, A, Moizant, F, Robillard, N, Moreau, A, Soulillou, JP, Brouard, S: Contrasting CD25hiCD4+T cells/FOXP3 patterns in chronic rejection and operational drug-free tolerance. *Transplantation,* 81**:** 398-407, 2006.
5. Pallier, A, Hillion, S, Danger, R, Giral, M, Racape, M, Degauque, N, Dugast, E, Ashton-Chess, J, Pettre, S, Lozano, JJ, Bataille, R, Devys, A, Cesbron-Gautier, A, Braudeau, C, Larrose, C, Soulillou, JP, Brouard, S: Patients with drug-free long-term graft function display increased numbers of peripheral B cells with a memory and inhibitory phenotype. *Kidney Int,* 78**:** 503-513, 2010.
6. Brouard, S, Mansfield, E, Braud, C, Li, L, Giral, M, Hsieh, SC, Baeten, D, Zhang, M, Ashton-Chess, J, Braudeau, C, Hsieh, F, Dupont, A, Pallier, A, Moreau, A, Louis, S, Ruiz, C, Salvatierra, O, Soulillou, JP, Sarwal, M: Identification of a peripheral blood transcriptional biomarker panel associated with operational renal allograft tolerance. *Proc Natl Acad Sci U S A,* 104**:** 15448-15453, 2007.
7. Newell, KA, Asare, A, Kirk, AD, Gisler, TD, Bourcier, K, Suthanthiran, M, Burlingham, WJ, Marks, WH, Sanz, I, Lechler, RI, Hernandez-Fuentes, MP, Turka, LA, Seyfert-Margolis, VL, Immune Tolerance Network, STSG: Identification of a B cell signature associated with renal transplant tolerance in humans. *J Clin Invest,* 120**:** 1836-1847, 2010.
8. Sagoo, P, Perucha, E, Sawitzki, B, Tomiuk, S, Stephens, DA, Miqueu, P, Chapman, S, Craciun, L, Sergeant, R, Brouard, S, Rovis, F, Jimenez, E, Ballow, A, Giral, M, Rebollo-Mesa, I, Le Moine, A, Braudeau, C, Hilton, R, Gerstmayer, B, Bourcier, K, Sharif, A, Krajewska, M, Lord, GM, Roberts, I, Goldman, M, Wood, KJ, Newell, K, Seyfert-Margolis, V, Warrens, AN, Janssen, U, Volk, HD, Soulillou, JP, Hernandez-Fuentes, MP, Lechler, RI: Development of a cross-platform biomarker signature to detect renal transplant tolerance in humans. *J Clin Invest,* 120**:** 1848-1861, 2010.
9. Braudeau, C, Racape, M, Giral, M, Louis, S, Moreau, A, Berthelot, L, Heslan, M, Ashton-Chess, J, Soulillou, JP, Brouard, S: Variation in numbers of CD4+CD25highFOXP3+ T cells with normal immuno-regulatory properties in long-term graft outcome. *Transpl Int,* 20**:** 845-855, 2007.
10. Moraes-Vieira, PM, Takenaka, MC, Silva, HM, Monteiro, SM, Agena, F, Lemos, F, Saitovitch, D, Kalil, J, Coelho, V: GATA3 and a dominant regulatory gene expression profile discriminate operational tolerance in human transplantation. *Clin Immunol,* 142**:** 117-126, 2012.
11. Moraes-Vieira, PM, Silva, HM, Takenaka, MC, Monteiro, SM, Lemos, F, Saitovitch, D, Kalil, J, Coelho, V: Differential monocyte STAT6 activation and CD4(+)CD25(+)Foxp3(+) T cells in kidney operational tolerance transplanted individuals. *Hum Immunol,* 71**:** 442-450, 2010.

12. Lin, CY, Graca, L, Cobbold, SP, Waldmann, H: Dominant transplantation tolerance impairs CD8+ T cell function but not expansion. *Nat Immunol,* 3**:** 1208-1213, 2002.
13. Waldmann, H, Graca, L, Cobbold, S, Adams, E, Tone, M, Tone, Y: Regulatory T cells and organ transplantation. *Semin Immunol,* 16**:** 119-126, 2004.
14. Graca, L, Cobbold, SP, Waldmann, H: Identification of regulatory T cells in tolerated allografts. *J Exp Med,* 195**:** 1641-1646, 2002.
15. Hill, JA, Feuerer, M, Tash, K, Haxhinasto, S, Perez, J, Melamed, R, Mathis, D, Benoist, C: Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. *Immunity,* 27**:** 786-800, 2007.
16. Miyara, M, Yoshioka, Y, Kitoh, A, Shima, T, Wing, K, Niwa, A, Parizot, C, Taflin, C, Heike, T, Valeyre, D, Mathian, A, Nakahata, T, Yamaguchi, T, Nomura, T, Ono, M, Amoura, Z, Gorochov, G, Sakaguchi, S: Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity,* 30**:** 899-911, 2009.
17. Gavin, MA, Rasmussen, JP, Fontenot, JD, Vasta, V, Manganiello, VC, Beavo, JA, Rudensky, AY: Foxp3-dependent programme of regulatory T-cell differentiation. *Nature,* 445**:** 771-775, 2007.
18. Ohkura, N, Kitagawa, Y, Sakaguchi, S: Development and maintenance of regulatory T cells. *Immunity,* 38**:** 414-423, 2013.
19. Morikawa, H, Ohkura, N, Vandenbon, A, Itoh, M, Nagao-Sato, S, Kawaji, H, Lassmann, T, Carninci, P, Hayashizaki, Y, Forrest, AR, Standley, DM, Date, H, Sakaguchi, S, Consortium, F: Differential roles of epigenetic changes and Foxp3 expression in regulatory T cell-specific transcriptional regulation. *Proc Natl Acad Sci U S A,* 111**:** 5289-5294, 2014.
20. Polansky, JK, Kretschmer, K, Freyer, J, Floess, S, Garbe, A, Baron, U, Olek, S, Hamann, A, von Boehmer, H, Huehn, J: DNA methylation controls Foxp3 gene expression. *Eur J Immunol,* 38**:** 1654-1663, 2008.
21. Zheng, Y, Josefowicz, S, Chaudhry, A, Peng, XP, Forbush, K, Rudensky, AY: Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature,* 463**:** 808-812, 2010.
22. Toker, A, Engelbert, D, Garg, G, Polansky, JK, Floess, S, Miyao, T, Baron, U, Duber, S, Geffers, R, Giehr, P, Schallenberg, S, Kretschmer, K, Olek, S, Walter, J, Weiss, S, Hori, S, Hamann, A, Huehn, J: Active demethylation of the Foxp3 locus leads to the generation of stable regulatory T cells within the thymus. *J Immunol,* 190**:** 3180-3188, 2013.
23. Ohkura, N, Hamaguchi, M, Morikawa, H, Sugimura, K, Tanaka, A, Ito, Y, Osaki, M, Tanaka, Y, Yamashita, R, Nakano, N, Huehn, J, Fehling, HJ, Sparwasser, T, Nakai, K, Sakaguchi, S: T cell receptor stimulation-induced epigenetic changes and Foxp3 expression are independent and complementary events required for Treg cell development. *Immunity,* 37**:** 785-799, 2012.
24. Racusen, L, C. , Solez, K, Colvin, R, B. , Bonsib, S, M. , Castro, M, C. , Cavallo, T, Croker, B, P. , Demetris, A, Jake , Drachenberg, C, B. , Fogo, A, B. , Furness, P, Gaber, L, W. , Gibson, I, W., Glotz, D, Goldberg, J, C. , Grande, J, Halloran, P, F. , Hansen, H, E. , Hartley, B, Hayry, P, J. , Hill, C, M. , Hoffman, E, O. , Hunsicker, L, G. , Lindblad, A, S. , Marcussen, N, Mihatsch, M, J. , Nadasdy, T, Nickerson, P, Olsen, T, Steen , Papadimitriou, J, C. , Randhawa, P, S. , Rayner, D, C. , Roberts, I, Rose, S, Rush, D, Salinas-Madrigal, L, Salomon, D, R. , Sund, S, Taskinen, E, Trpkov, K, Yamaguchi, Y: The Banff 97 working classification of renal allograft pathology. *Kidney International*, 1999.
25. Haas, M, Sis, B, Racusen, LC, Solez, K, Glotz, D, Colvin, RB, Castro, MC, David, DS, David-Neto, E, Bagnasco, SM, Cendales, LC, Cornell, LD, Demetris, AJ, Drachenberg,

CB, Farver, CF, Farris, AB, 3rd, Gibson, IW, Kraus, E, Liapis, H, Loupy, A, Nickeleit, V, Randhawa, P, Rodriguez, ER, Rush, D, Smith, RN, Tan, CD, Wallace, WD, Mengel, M, Banff meeting report writing, c: Banff 2013 meeting report: inclusion of c4d-negative antibody-mediated rejection and antibody-associated arterial lesions. *Am J Transplant,* 14**:** 272-283, 2014.
26. Liu, W, Putnam, AL, Xu-Yu, Z, Szot, GL, Lee, MR, Zhu, S, Gottlieb, PA, Kapranov, P, Gingeras, TR, Fazekas de St Groth, B, Clayberger, C, Soper, DM, Ziegler, SF, Bluestone, JA: CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med,* 203**:** 1701-1711, 2006.
27. Vukmanovic-Stejic, M, Zhang, Y, Cook, JE, Fletcher, JM, McQuaid, A, Masters, JE, Rustin, MH, Taams, LS, Beverley, PC, Macallan, DC, Akbar, AN: Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo. *J Clin Invest,* 116**:** 2423-2433, 2006.
28. Valmori, D, Merlo, A, Souleimanian, NE, Hesdorffer, CS, Ayyoub, M: A peripheral circulating compartment of natural naive CD4 Tregs. *J Clin Invest,* 115**:** 1953-1962, 2005.
29. Schmidl, C, Hansmann, L, Lassmann, T, Balwierz, PJ, Kawaji, H, Itoh, M, Kawai, J, Nagao-Sato, S, Suzuki, H, Andreesen, R, Hayashizaki, Y, Forrest, AR, Carninci, P, Hoffmann, P, Edinger, M, Rehli, M, consortium, F: The enhancer and promoter landscape of human regulatory and conventional T-cell subpopulations. *Blood,* 123**:** e68-78, 2014.
30. Braud, C, Baeten, D, Giral, M, Pallier, A, Ashton-Chess, J, Braudeau, C, Chevalier, C, Lebars, A, Leger, J, Moreau, A, Pechkova, E, Nicolini, C, Soulillou, JP, Brouard, S: Immunosuppressive drug-free operational immune tolerance in human kidney transplant recipients: Part I. Blood gene expression statistical analysis. *J Cell Biochem,* 103**:** 1681-1692, 2008.
31. Braza, F, Soulillou, JP, Brouard, S: Gene expression signature in transplantation tolerance. *Clin Chim Acta,* 413**:** 1414-1418, 2012.
32. Dantal, J, Soulillou, JP: Immunosuppressive drugs and the risk of cancer after organ transplantation. *N Engl J Med,* 352**:** 1371-1373, 2005.
33. Chesneau, M, Michel, L, Degauque, N, Brouard, S: Regulatory B cells and tolerance in transplantation: from animal models to human. *Front Immunol,* 4**:** 497, 2013.
34. Schaier, M, Seissler, N, Schmitt, E, Meuer, S, Hug, F, Zeier, M, Steinborn, A: DR(high+)CD45RA(-)-Tregs potentially affect the suppressive activity of the total Treg pool in renal transplant patients. *PLoS One,* 7**:** e34208, 2012.
35. Becker, LE, de Oliveira Biazotto, F, Conrad, H, Schaier, M, Kihm, LP, Gross-Weissmann, ML, Waldherr, R, Bierhaus, A, Nawroth, PP, Zeier, M, Morath, C: Cellular infiltrates and NFkappaB subunit c-Rel signaling in kidney allografts of patients with clinical operational tolerance. *Transplantation,* 94**:** 729-737, 2012.
36. Zuber, J, Brodin-Sartorius, A, Lapidus, N, Patey, N, Tosolini, M, Candon, S, Rabant, M, Snanoudj, R, Panterne, C, Thervet, E, Legendre, C, Chatenoud, L: FOXP3-enriched infiltrates associated with better outcome in renal allografts with inflamed fibrosis. *Nephrol Dial Transplant,* 24**:** 3847-3854, 2009.
37. Bennett, CL, Christie, J, Ramsdell, F, Brunkow, ME, Ferguson, PJ, Whitesell, L, Kelly, TE, Saulsbury, FT, Chance, PF, Ochs, HD: The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet,* 27**:** 20-21, 2001.
38. Hori, S, Nomura, T, Sakaguchi, S: Control of regulatory T cell development by the transcription factor Foxp3. *Science,* 299**:** 1057-1061, 2003.
39. Fontenot, JD, Gavin, MA, Rudensky, AY: Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol,* 4**:** 330-336, 2003.

40. Allan, SE, Crome, SQ, Crellin, NK, Passerini, L, Steiner, TS, Bacchetta, R, Roncarolo, MG, Levings, MK: Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. *Int Immunol,* 19**:** 345-354, 2007.
41. Bestard, O, Cunetti, L, Cruzado, JM, Lucia, M, Valdez, R, Olek, S, Melilli, E, Torras, J, Mast, R, Goma, M, Franquesa, M, Grinyo, JM: Intragraft regulatory T cells in protocol biopsies retain foxp3 demethylation and are protective biomarkers for kidney graft outcome. *Am J Transplant,* 11**:** 2162-2172, 2011.
42. Seddiki, N, Santner-Nanan, B, Tangye, SG, Alexander, SI, Solomon, M, Lee, S, Nanan, R, Fazekas de Saint Groth, B: Persistence of naive CD45RA+ regulatory T cells in adult life. *Blood,* 107**:** 2830-2838, 2006.
43. Sanchez Rodriguez, R, Pauli, ML, Neuhaus, IM, Yu, SS, Arron, ST, Harris, HW, Yang, SH, Anthony, BA, Sverdrup, FM, Krow-Lucal, E, MacKenzie, TC, Johnson, DS, Meyer, EH, Lohr, A, Hsu, A, Koo, J, Liao, W, Gupta, R, Debbaneh, MG, Butler, D, Huynh, M, Levin, EC, Leon, A, Hoffman, WY, McGrath, MH, Alvarado, MD, Ludwig, CH, Truong, HA, Maurano, MM, Gratz, IK, Abbas, AK, Rosenblum, MD: Memory regulatory T cells reside in human skin. *J Clin Invest,* 124**:** 1027-1036, 2014.
44. Akimova, T, Kamath, BM, Goebel, JW, Meyers, KE, Rand, EB, Hawkins, A, Levine, MH, Bucuvalas, JC, Hancock, WW: Differing effects of rapamycin or calcineurin inhibitor on T-regulatory cells in pediatric liver and kidney transplant recipients. *Am J Transplant,* 12**:** 3449-3461, 2012.
45. Wood, KJ, Bushell, A, Hester, J: Regulatory immune cells in transplantation. *Nat Rev Immunol,* 12**:** 417-430, 2012.
46. Di Ianni, M, Falzetti, F, Carotti, A, Terenzi, A, Castellino, F, Bonifacio, E, Del Papa, B, Zei, T, Ostini, RI, Cecchini, D, Aloisi, T, Perruccio, K, Ruggeri, L, Balucani, C, Pierini, A, Sportoletti, P, Aristei, C, Falini, B, Reisner, Y, Velardi, A, Aversa, F, Martelli, MF: Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood,* 117**:** 3921-3928, 2011.
47. Antonioli, L, Pacher, P, Vizi, ES, Hasko, G: CD39 and CD73 in immunity and inflammation. *Trends Mol Med,* 19**:** 355-367, 2013.
48. Borsellino, G, Kleinewietfeld, M, Di Mitri, D, Sternjak, A, Diamantini, A, Giometto, R, Hopner, S, Centonze, D, Bernardi, G, Dell'Acqua, ML, Rossini, PM, Battistini, L, Rotzschke, O, Falk, K: Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood,* 110**:** 1225-1232, 2007.
49. Crikis, S, Lu, B, Murray-Segal, LM, Selan, C, Robson, SC, D'Apice, AJ, Nandurkar, HH, Cowan, PJ, Dwyer, KM: Transgenic overexpression of CD39 protects against renal ischemia-reperfusion and transplant vascular injury. *Am J Transplant,* 10**:** 2586-2595, 2010.
50. Deaglio, S, Dwyer, KM, Gao, W, Friedman, D, Usheva, A, Erat, A, Chen, JF, Enjyoji, K, Linden, J, Oukka, M, Kuchroo, VK, Strom, TB, Robson, SC: Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med,* 204**:** 1257-1265, 2007.
51. Chen, W, Jin, W, Hardegen, N, Lei, KJ, Li, L, Marinos, N, McGrady, G, Wahl, SM: Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med,* 198**:** 1875-1886, 2003.
52. Danger, R, Pallier, A, Giral, M, Martinez-Llordella, M, Lozano, JJ, Degauque, N, Sanchez-Fueyo, A, Soulillou, JP, Brouard, S: Upregulation of miR-142-3p in peripheral blood mononuclear cells of operationally tolerant patients with a renal transplant. *J Am Soc Nephrol,* 23**:** 597-606, 2012.
53. Lei, Z, Xu, G, Wang, L, Yang, H, Liu, X, Zhao, J, Zhang, HT: MiR-142-3p represses TGF-beta-induced growth inhibition through repression of TGFbetaR1 in non-small cell lung cancer. *FASEB J,* 28**:** 2696-2704, 2014.

54. Haynes, LD, Jankowska-Gan, E, Sheka, A, Keller, MR, Hernandez-Fuentes, MP, Lechler, RI, Seyfert-Margolis, V, Turka, LA, Newell, KA, Burlingham, WJ: Donor-specific indirect pathway analysis reveals a B-cell-independent signature which reflects outcomes in kidney transplant recipients. *Am J Transplant,* 12**:** 640-648, 2012.
55. Rosser, EC, Blair, PA, Mauri, C: Cellular targets of regulatory B cell-mediated suppression. *Mol Immunol*, 2014.
56. Wieczorek, G, Asemissen, A, Model, F, Turbachova, I, Floess, S, Liebenberg, V, Baron, U, Stauch, D, Kotsch, K, Pratschke, J, Hamann, A, Loddenkemper, C, Stein, H, Volk, HD, Hoffmuller, U, Grutzkau, A, Mustea, A, Huehn, J, Scheibenbogen, C, Olek, S: Quantitative DNA methylation analysis of FOXP3 as a new method for counting regulatory T cells in peripheral blood and solid tissue. *Cancer Res,* 69**:** 599-608, 2009.

Figures and table legends

Table 1: Demographic and clinical characteristics of individuals and patients enrolled in this study. (HV= Healthy volunteers; TOL= Tolerant patients; STA= Stable patients under immunosuppressive treatment; RC= Patients with chronic rejection)

Figure 1: Operational tolerance is associated with higher percentage of $CD4^+$ T cells with demethylated Foxp3. Purified $CD4^+$ circulating T cells from HV (n=9), TOL (n=11) STA (n=17) and RC (n=8) have been subjected to quantitative PCR to determine percentage of TSDR demethylation (A). Correlation between percentage of TSDR demethylation and age (B). Correlation between percentage of TSDR demethylation and time after cessation of immunosuppressors (C). Correlation between percentage of TSDR demethylation and time post-transplantation (D). Correlation between percentage of TSDR demethylation and levels of creatinine (E). $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***).

Figure 2: Study of $CD25^{hi}$ $CD127^{low}$ Tregs. Gating strategy for the identification of total Tregs (A). Percentage of Tregs in the blood of HV (n=5), TOL (n=10) STA (n=26) and RC (n=15) (B). Correlation between percentage of TSDR demethylation in $CD4^+$ T cells and percentage of total Tregs, TOL (n=8) STA (n=11) and RC (n=5) (C). Percentage of purified circulating $CD25^{hi}$ $CD127^{low}$

Tregs with TSDR demethylation was measured by quantitative PCR, TOL (n=8) STA (n=11) and RC (n=5) (D). p<0.05 (*), p<0.01 (**), and p<0.001 (***).

Figure 3: CD45RA$^-$ Foxp3^{hi} mTregs are significantly expanded in the blood of TOL. Quantification of Tregs subsets in the blood of HV (n=5), TOL (n=10), STA (n=26) and RC (n=15) (A, B). Correlation between the percentage of TSDR demethylation in CD4$^+$ T cells and the percentage of CD45RA$^+$ Foxp3$^+$ mTregs TOL (n=8) STA (n=11) and RC (n=5) (C). p<0.05 (*), p<0.01 (**), and p<0.001 (***).

Figure 4: Expression of Treg-related molecules in TOL recipients. Expression of CD39, GITR, CTLA4 and Lag3 in PBMC of TOL, HV, STA and RC. Data have been obtained from a meta-analysis of previous microarray studies (A). Expression of CD39, GITR, CTLA4 and Lag3 at the surface of circulating mTregs from TOL, HV, STA and RC (B, C).

Figure 5: Memory Tregs from TOL are fully functional. Coculture of mTregs with activated trace-labeled CD4$^+$ CD25$^-$ T responder cells in the presence of feeders at the indicated suppressor/responder cell ratio (A). Percentage of inhibition of T cells proliferation in TOL (n=6), HV (n=6) and STA (n=6) (B). Percentage of TSDR demethylation in CD25^{hi} CD45RA$^-$ mTregs from of HV (n=3), TOL (n=4) and STA (n=14) (D). p<0.05 (*), p<0.01 (**).

Supplemental Figure 1: Phenotyping of Foxp3 expressing T cells subsets. Isotype control for Foxp3 staining (A). Three distinct subsets of Foxp3 expressing-cells are defined by the expression of CD45RA, Foxp3, CD25 and CD127: Orange line (II), naïve Tregs Foxp3^{lo} CD45RA$^+$ CD25$^+$ CD127^{lo} cells; Green line (III), non-Tregs Foxp3^{lo} CD45RA$^-$ CD25$^+$ CD127^{lo} cells; Red line (IV), memory Tregs Foxp3^{hi} CD45RA$^-$ CD25^{hi} CD127^{lo} (B, C).

Supplemental Table 1: Clinical characteristic of operational tolerant patients

Demographics and transplant characteristics of HV, TOL, STA and RC

	HV	TOL	STA	RC	*p* value
Donor type, n					
Cadaveric	-	13	33	19	
Living	-	2	-	-	
Recipients age, yr	41 (37-45)	55 (40-84)	51 (18-73)	51 (25-80)	**0,8781**
Gender					**0,3317**
Female	4	6	16	11	
Male	11	7	17	8	
Time post-transplantation	-	226 (114-347)	129 (67-285)	89 (26-241)	**< 0,0001**
Creatinine level µmol/L	**-**	105 (63-216)	107 (80-214)	187 (109-957)	**< 0,0001**
Proteinuria level g/24h	**-**	0.2 (0-1.69)	0.13 (0.03-0.39)	1.6 (0.2-4.61)	**< 0,0001**
HLA mismatch	**-**	3 (0-5)	3 (0-6)	4 (0-6)	**0,2253**
CMV infection, % (n)	**-**	46.1% (6/13)	27.3% (9/33)	21.05% (4/19)	**0,3785**
Cancer, % (n)	**-**	36.4% (5/13)	31.0% (9/29)	15.8% (3/19)	**0,3248**
Time of immunosuppression cessation, yr	**-**	8 (2-15)	-	-	
Episode of acute rejection, % (n)	**-**	15.4% (2/13)	15.2% (5/33)	10.5% (2/19)	**0,8832**
Reason of cessation, % (n)					
Medical condition	-	38.5% (5/13)	-	-	
Non-compliance	-	61.5% (8/13)	-	-	
Chronic rejection					
C4d staining	-	-	-	57.9% (11/19)	
Graft glomerulopathy	-	-	-	89.5% (17/19)	
Donor specific antibodies, n	-	15.4% (2/13)	-	21.1% (4/19)	
Immunossuppressive therapy					
Calcineurin inhibitor		-	33	16	
Mycophenolate mofetil/Mycophenolate sodium		-	9	9	
Corticotherapy		-	11	10	
Azathioprine		-	8	-	

Figure 1

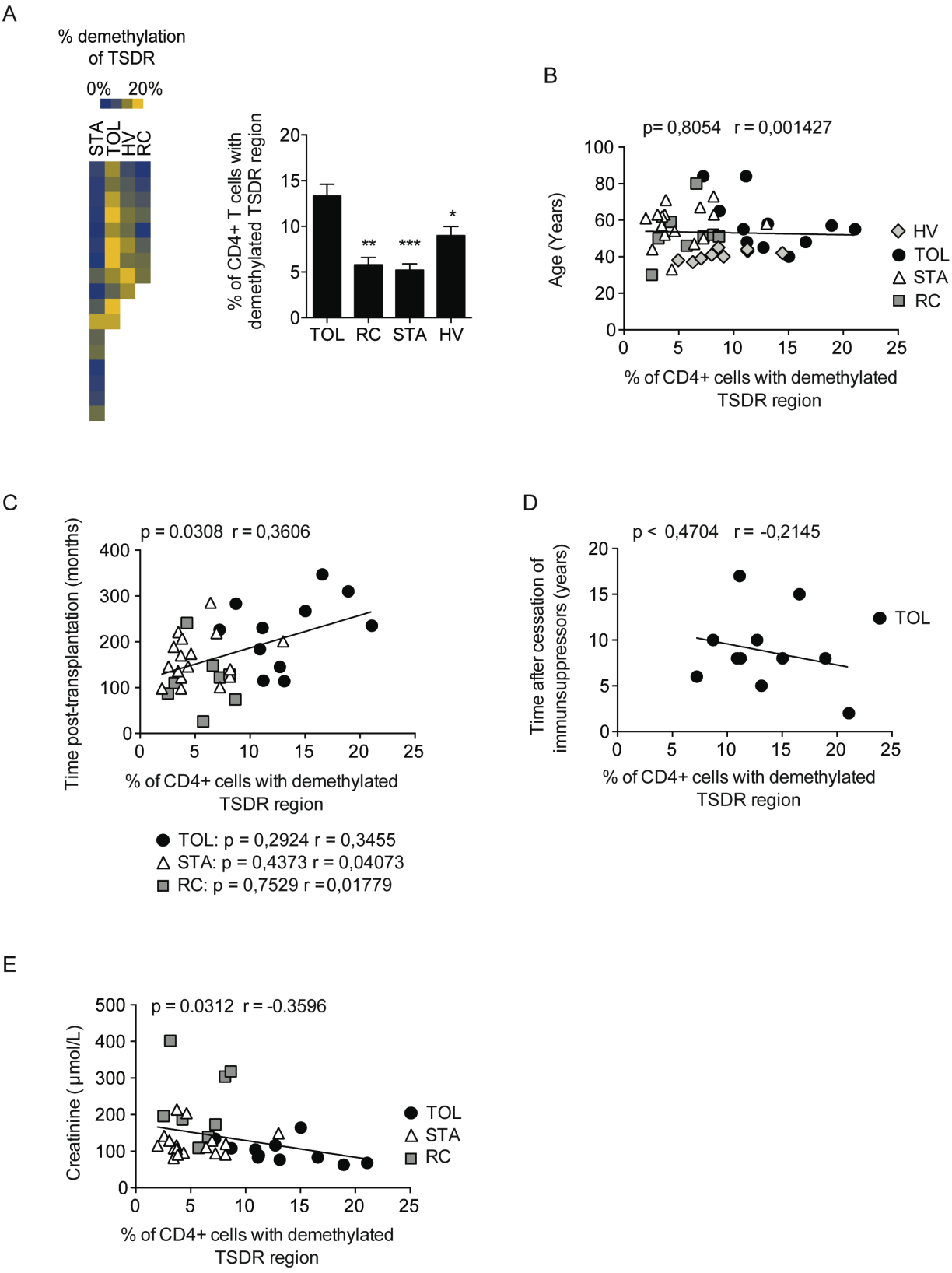

A
% demethylation of TSDR
0% 20%
STA
TOL
HV
RC
% of CD4+ T cells with demethylated TSDR region
TOL RC STA HV
B
p= 0,8054 r = 0,001427
Age (Years)
% of CD4+ cells with demethylated TSDR region
HV
TOL
STA
RC
C
p = 0.0308 r = 0,3606
Time post-transplantation (months)
% of CD4+ cells with demethylated TSDR region
TOL: p = 0,2924 r = 0,3455
STA: p = 0,4373 r = 0,04073
RC: p = 0,7529 r =0,01779
D
p < 0,4704 r = -0,2145
Time after cessation of immunsuppressors (years)
% of CD4+ cells with demethylated TSDR region
TOL
E
p = 0.0312 r = -0.3596
Creatinine (µmol/L)
% of CD4+ cells with demethylated TSDR region
TOL
STA
RC

Figure 2

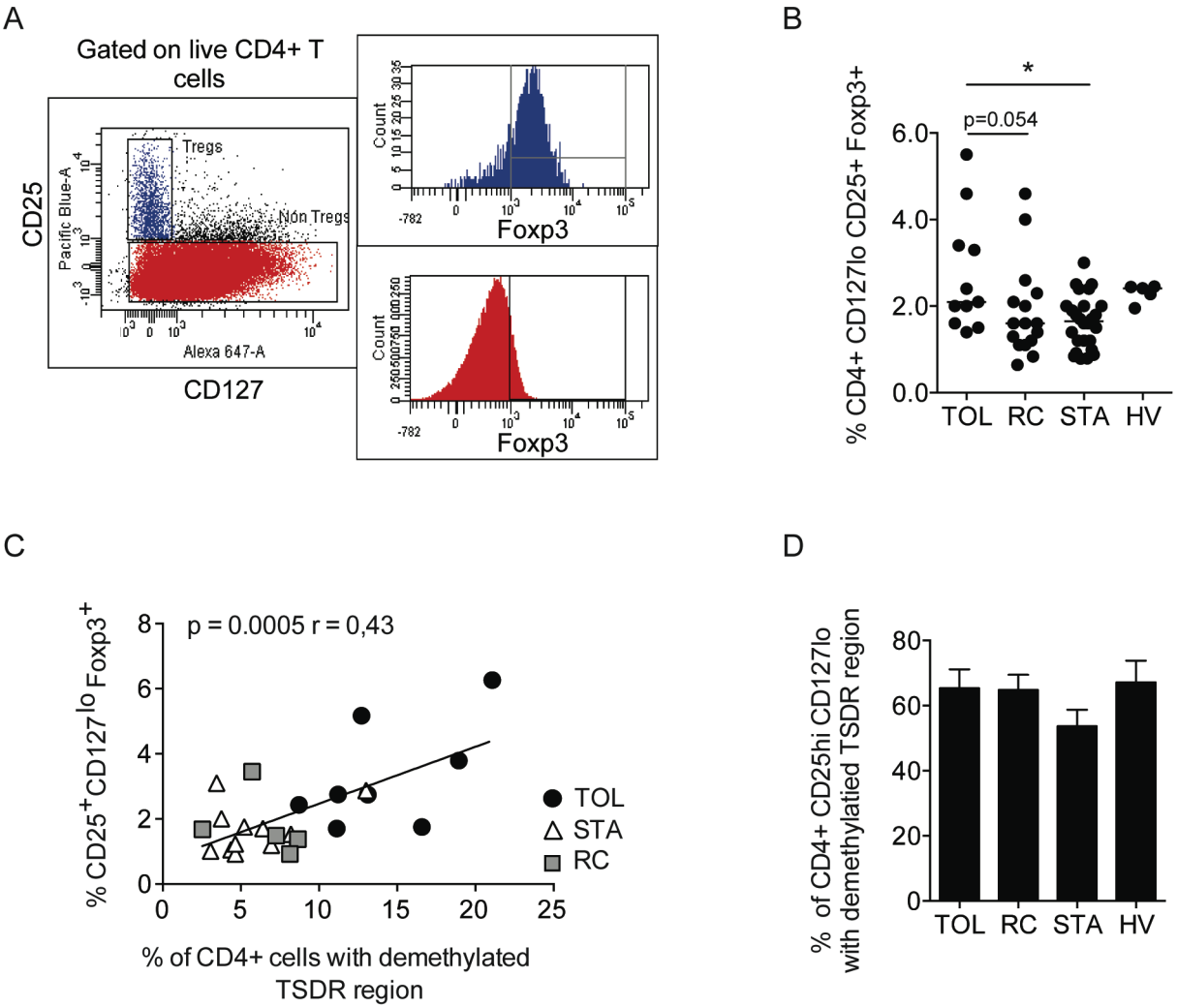

A
Gated on live CD4+ T cells
Tregs
Non Tregs
CD25
Pacific Blue-A
Alexa 647-A
CD127
Count
Foxp3
B
*
p=0.054
% CD4+ CD127lo CD25+ Foxp3+
TOL RC STA HV
C
p = 0.0005 r = 0,43
% CD25+CD127lo Foxp3+
TOL
STA
RC
% of CD4+ cells with demethylated TSDR region
D
% of CD4+ CD25hi CD127lo with demethylatied TSDR region
TOL RC STA HV

Figure 3

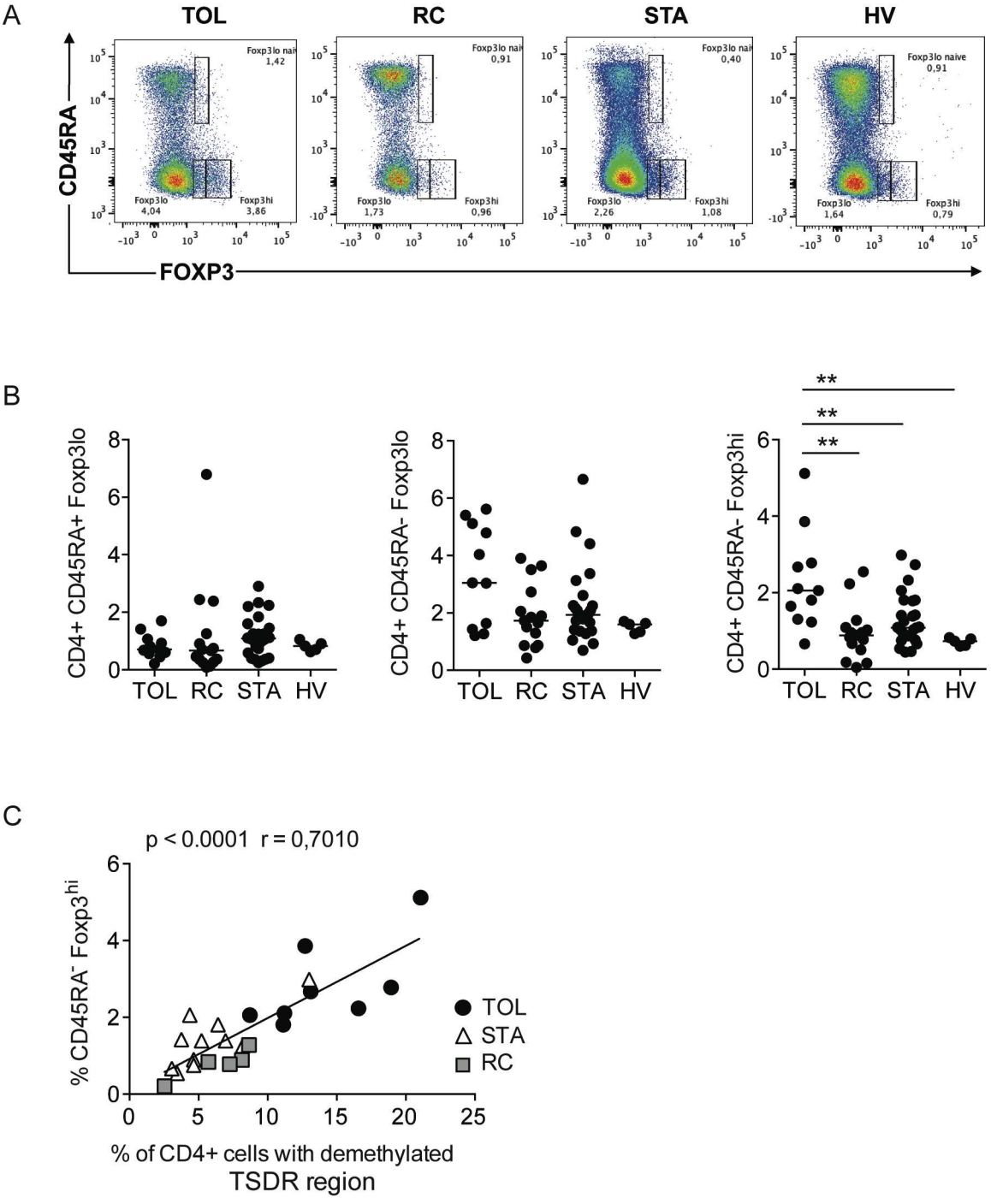

A
TOL
RC
STA
HV
CD45RA
FOXP3
Foxp3lo naïve 1,42
Foxp3lo 4,04
Foxp3hi 3,86
Foxp3lo naïve 0,91
Foxp3lo 1,73
Foxp3hi 0,96
Foxp3lo naïve 0,40
Foxp3lo 2,26
Foxp3hi 1,08
Foxp3lo naive 0,91
Foxp3lo 1,64
Foxp3hi 0,79
B
CD4+ CD45RA+ Foxp3lo
CD4+ CD45RA- Foxp3lo
CD4+ CD45RA- Foxp3hi
TOL RC STA HV
**
**
**
C
p < 0.0001 r = 0,7010
% CD45RA- Foxp3hi
% of CD4+ cells with demethylated TSDR region
TOL
STA
RC

Figure 4

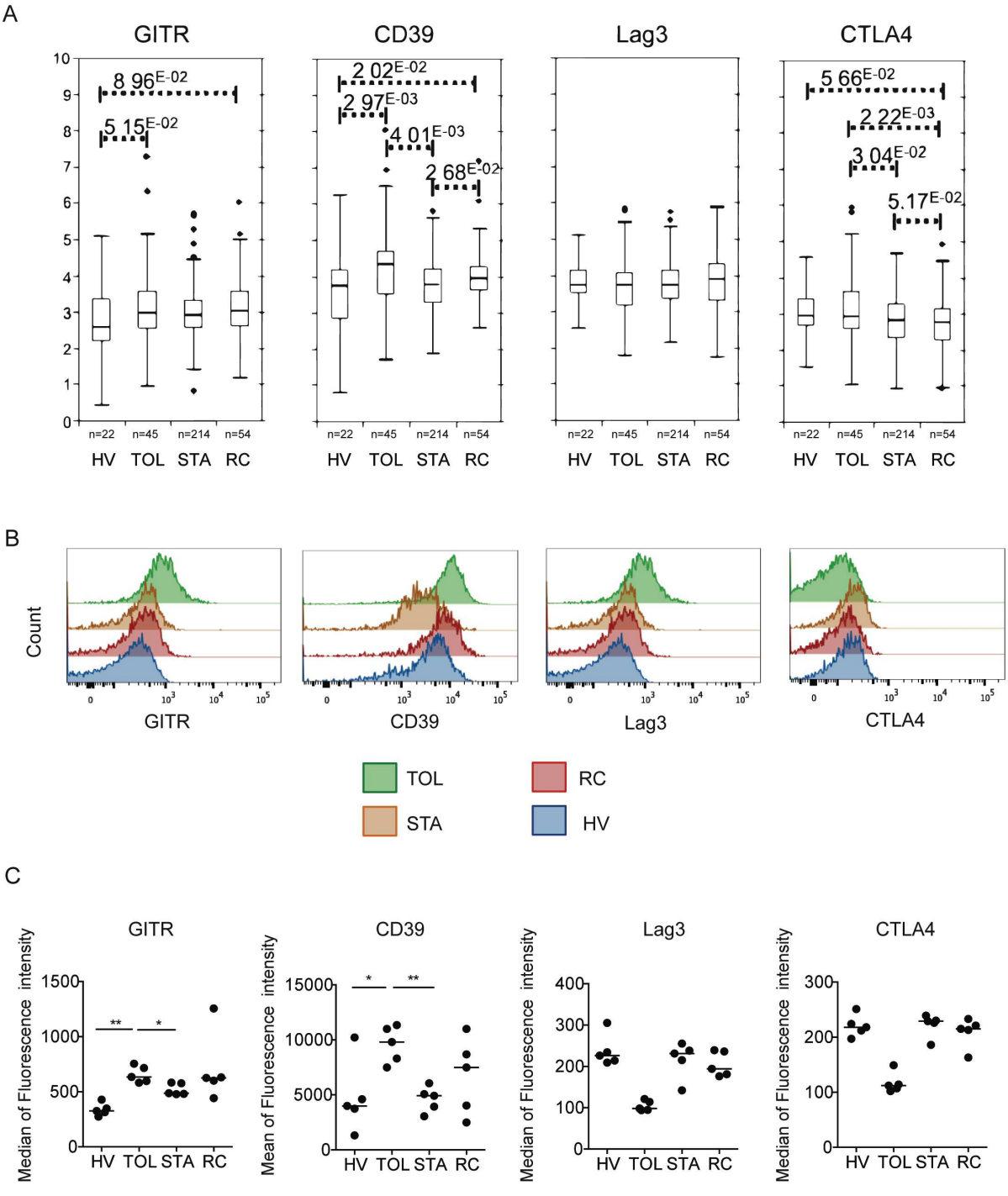
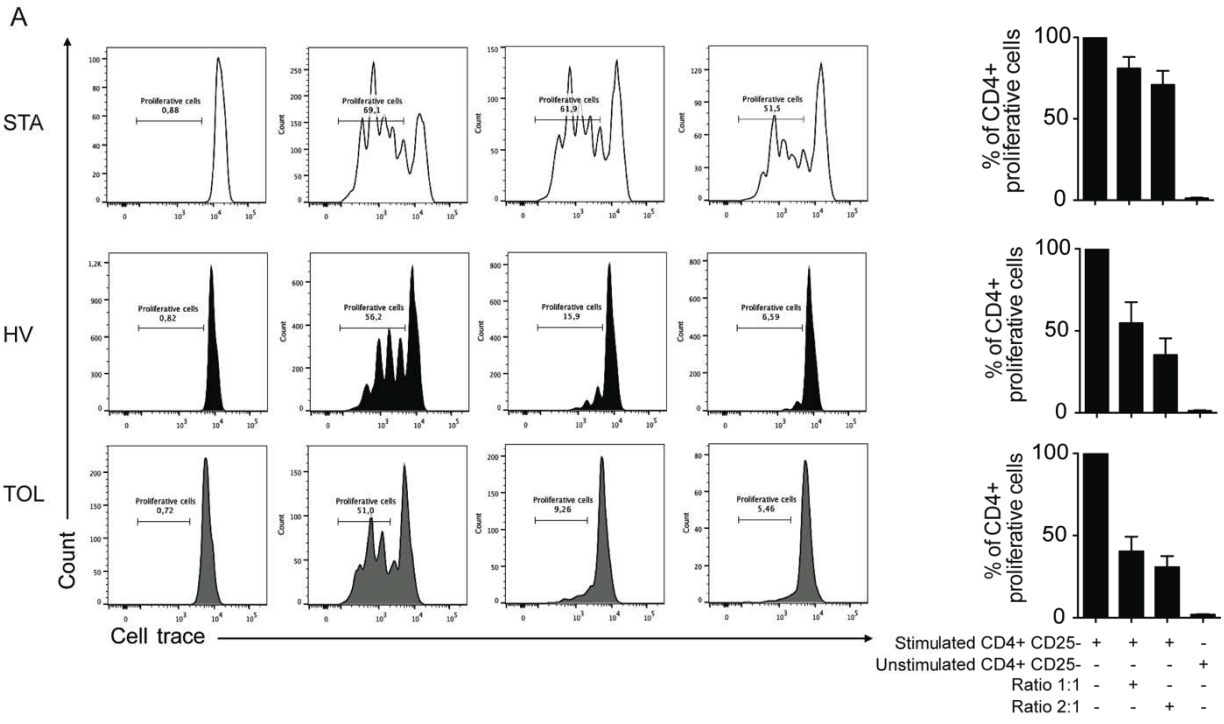
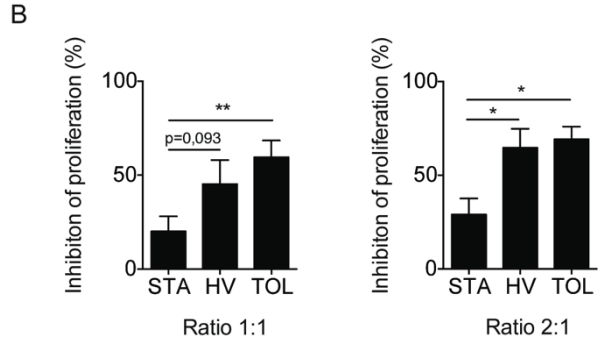
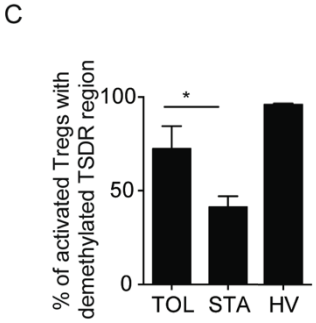

A
GITR
CD39
Lag3
CTLA4
8.96E-02
5.15E-02
2.02E-02
2.97E-03
4.01E-03
2.68E-02
5.66E-02
2.22E-03
3.04E-02
5.17E-02
n=22 n=45 n=214 n=54
HV TOL STA RC
B
Count
GITR
CD39
Lag3
CTLA4
TOL
RC
STA
HV
C
GITR
CD39
Lag3
CTLA4
Median of Fluorescence intensity
Mean of Fluorescence intensity
HV TOL STA RC

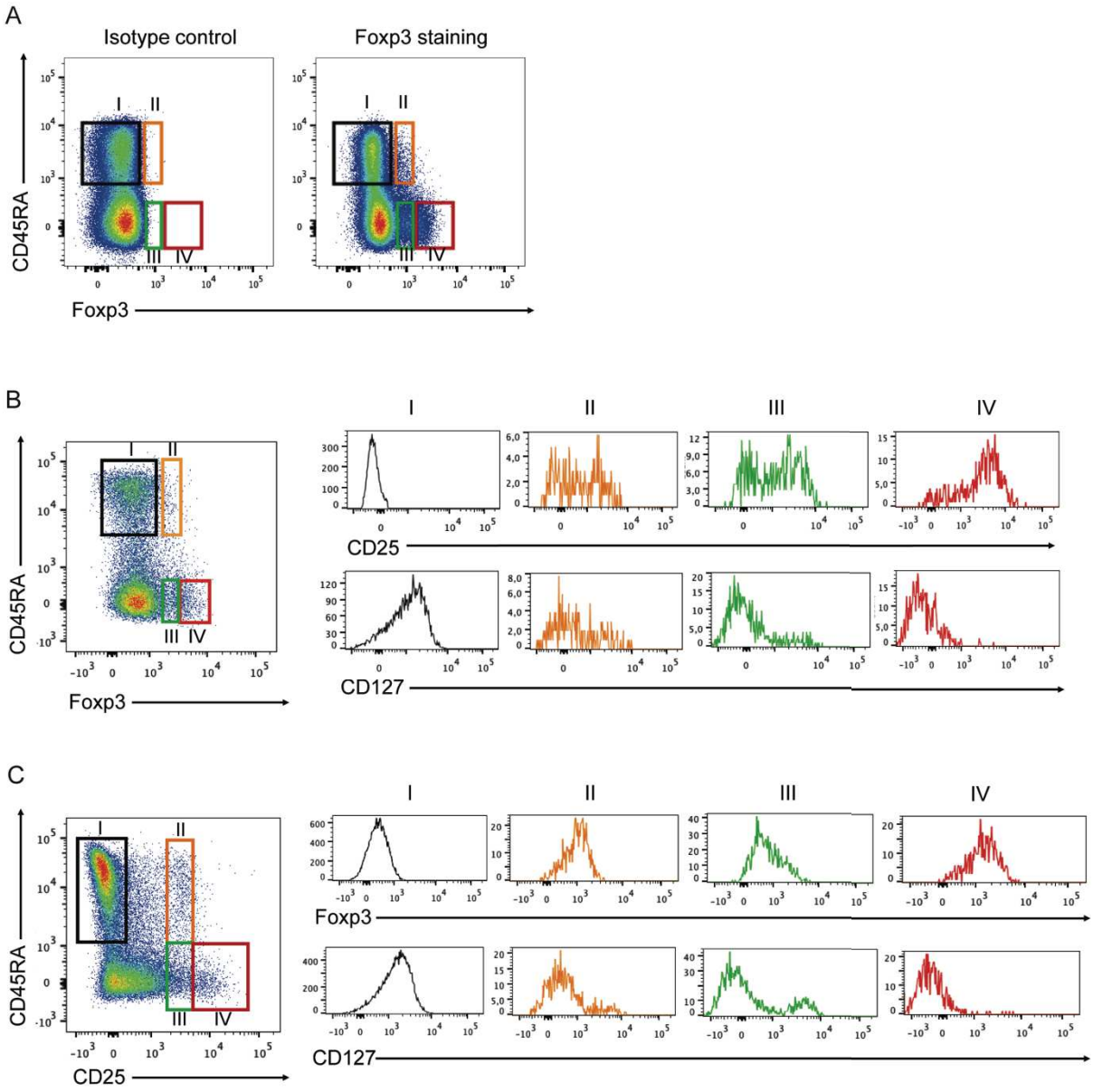
Figure 5


A
STA
HV
TOL
Count
Cell trace
Proliferative cells 0,88
Proliferative cells 69,1
Proliferative cells 61,9
Proliferative cells 51,5
Proliferative cells 0,82
Proliferative cells 56,2
Proliferative cells 15,9
Proliferative cells 6,59
Proliferative cells 0,72
Proliferative cells 51,0
Proliferative cells 9,26
Proliferative cells 5,46
% of CD4+ proliferative cells
100
50
0
Stimulated CD4+ CD25- + + + -
Unstimulated CD4+ CD25- - - - +
Ratio 1:1 - + - -
Ratio 2:1 - - + -


B
Inhibiton of proliferation (%)
100
50
0
**
p=0,093
STA HV TOL
Ratio 1:1
Inhibiton of proliferation (%)
100
50
0
*
*
STA HV TOL
Ratio 2:1


C
% of activated Tregs with demethylated TSDR region
100
50
0
*
TOL STA HV

Supplemental Figure 1



Gene expression signature in transplantation tolerance

Faouzi Braza, Jean-Paul Soulillou and Sophie Brouard

Abstract

Human kidney transplantation tolerance exists, but the definition of true tolerance as defined by Billingham and colleagues suffers several key elements that cannot be demonstrated in humans. Indeed, human tolerance in transplantation is defined by different functional and clinical parameters; this is why in clinical transplantation we preferentially talk about operational tolerance. These patients are very rare and defined in the literature as immunocompetent patients with stable graft function without any immunosuppressive treatments. These patients are characterized by a stable graft function in the absence of histologic information since the biopsy is often lacking. In kidney transplantation, this state of operational tolerance is observed in two situations. It is sometimes detected by chance in patients who stop their immunosuppressive treatments due to incompliance or because of secondary effects (cancer, opportunistic infections). The principal goal in kidney transplantation and one of the reasons why so many people are interested in cases of operational tolerance in humans, is to be able to identify patients who are developing spontaneous tolerance to their transplants while under classical immunosuppression. Consequently, there is an increasing need to develop an assay to identify and differentiate such patients with specific and noninvasive methods. In this review we will discuss the various studies that attempt to identify these new biomarkers of tolerance thanks to gene expression profiling using microarrays or quantitative PCR that have become a benchmark for research in novel and informative transplant monitoring assays.

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Invited critical review

Gene expression signature in transplantation tolerance

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ABSTRACT

Human kidney transplantation tolerance exists, but the definition of true tolerance as defined by Billingham and colleagues suffers several key elements that cannot be demonstrated in humans. Indeed, human tolerance in transplantation is defined by different functional and clinical parameters, this is why in clinical transplantation we preferentially talk about operational tolerance. These patients are very rare and defined in the literature as immunocompetent patients with stable graft function without any immunosuppressive treatments. These patients are characterized by a stable graft function in the absence of histologic information since the biopsy is often lacking. In kidney transplantation, this state of operational tolerance is observed in two situations. It is sometimes detected by chance in patients who stop their immunosuppressive treatments due to incompliance or because of secondary effects (cancer, opportunistic infections). The principal goal in kidney transplantation and one of the reasons why so many people are interested in cases of operational tolerance in humans, is to be able to identify patients who are developing spontaneous tolerance to their transplants while under classical immunosuppression. Consequently, there is an increasing need to develop an assay to identify and differentiate such patients with specific and noninvasive methods. In this review we will discuss the various studies that attempt to identify these new biomarkers of tolerance thanks to gene expression profiling using microarrays or quantitative PCR that have become a benchmark for research in novel and informative transplant monitoring assays.

Contents

1. Introduction

Advances in immunosuppressive treatments led us to better control acute rejection and improve graft survival in organ transplantation. However, immunosuppressive drugs do not master long term chronic rejection [1]. Moreover, due to their toxicity, they are responsible of many side effects as opportunistic infections, renal failure, cardiovascular disease and malignancy [2–5]. Thus, inducing tolerance is becoming a main goal in transplantation. Several studies in rodents and humans described the possibility to induce tolerance by

Abbreviations: ADORA, Adenosine A3 Receptor; BANK, B Cell Scaffold Protein with Ankyrin Repeats; BCR, B Cell Receptor; Bregs, B Regulatory Cells; FOXP3, Forkhead Box P3; IFN, Inteferon Gamma; KTFS, Kidney Transplant Failure Score; NK-Cells, Natural Killer Cells; PAM, Prediction Analysis of Microarray; PBMC, Peripheral Blood Mononuclear Cells; SAM, Significance Analysis of Microarray; SOCS, Suppressor of Cytokine Signaling; TGF, Transforming Growth Factor; TNF, Tumor Necrosis Factor Alpha.

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manipulating host immunological mechanisms as central tolerance [6–8] and peripheral process blocking costimulation and cytokines [9–12]. However, most tries at bringing these strategies into the clinic have proven unsuccessful. Maintenance of a normal allograft function despite complete discontinuation of all immunosuppressive drugs was reported notably in human liver [13] and more rarely in kidney [14] transplantation providing a proof of concept that a process of tolerance can be achieved in humans. Many studies have tried to dissect the phenotype of tolerance in liver and kidney transplantation in order to understand its mechanisms but also to be able to identify patients under conventional immunosuppression that could have develop tolerance to their transplants. In that case, tolerant-identified-patients would benefit of partial (at least) stopping of immunosuppression. Consequently, there was an increasing need to develop assay and identify a gene signature of tolerance.

2. DNA microarray in transplantation tolerance, a useful tool…with limits

Currently, patients' follow-up includes blood and urine tests on creatinine and protein levels for renal transplantation and liver chemistries (enzymes ± serum bilirubin) for liver transplantation to identify graft dysfunction and biopsy to establish the diagnosis. This monitoring allows modulation of the treatment but can hardly prevent chronic dysfunction of the transplant. Various studies have shown that it is possible to intentionally withdraw immunosuppressive treatment from stable liver transplant patients [15] but it seems to be more difficult and risky in kidney transplantation. The reasons are various and unclear yet. While peripheral blood might not be the optimal tissue source to dissect the molecular mechanisms responsible for allograft tolerance, the use of non-invasive blood monitoring has obviously clinical advantages, and it has been widely validated for diagnostic purposes in transplantation studies employing gene expression profiling [16–21].

The aim of these studies was to determine the differentially expressed genes between different groups of transplanted patients and predict clinical outcome of one patient on the basis of its transcriptomic profile. Basically, it works in two great steps, identify a signature and test it in training and validation groups of patients using for example significance analysis of microarray (SAM) and prediction analysis of microarray (PAM) respectively. Very stringent statistical tools are used to perform the analysis of data. Once a gene signature is identified, it's interesting to explore functions of the genes involved in order to interpret mechanisms responsible for tolerance. Multiple bioinformatic tools are available to assist scientists in this functional analysis. But a lot of genes remain without attributed functions due notably to the lack of annotations in bioinformatic databases. Another key limit of DNA microarray is the sensitivity to experimental biases notably during hybridation (inter and intra-platform variability) [22]. Consequently, gene signature must be validated by another transcriptomic approach such as quantitative real time PCR [23].

Another issues, and particularly when applied in tolerance in transplantation, is linked to the small sample sizes that is statistically challenging, particularly in class prediction studies. There is a large disproportion between the high number of genes analyzed and the limited number of samples available, so there are always one or more sets of genes capable of accurately classifying the samples from which the gene models were derived even when data are completely random. The experience accumulated in cancer research, where much larger microarray studies have been performed, indicate that at least 200 samples are required to generate accurate and stable predictive signatures. In human transplantation tolerance studies of such sample sizes are very difficult to achieve. Also, the problem of adequate comparators has remained unsolved. This paradox is due to the clinical situation of tolerant patients that display stable graft function but no longer receive immunosuppression, a state that, until now, was only achievable in rodents. Healthy donors share with tolerant patients the absence of immunosuppression but have not received a transplant. Patients with chronic rejection have a transplant but are under immunosuppression and likely display a contrasted inflammatory response. Stable patients who share graft function stability with DF patients are probably the best controls but are under immunosuppression [20].

Despite these methodological limitations of microarray technology, the use of these tools to clinical transplantation in the identification and characterization of tolerant patients has been very useful for the identification of potential biomarkers. Indeed the use of a non-invasive monitoring of tolerance has many clinical advantages notably to identify biomarkers.

3. Biomarkers in kidney transplantation tolerance

Using microarray analysis our team identified 49 genes differentially expressed in tolerant recipients. This signature, designated as the "tolerance footprint", could accurately discriminate tolerant recipients from patients under chronic rejection. Interestingly, we were able to identify immunosuppressed stable recipients among the "test-group" as potential tolerant recipients suggesting that these patients could benefit of a weaning of the immunosuppressive therapy [16]. On a larger cohort of immunosuppressed patients, we reported that 3.5% of patients under classical immunosuppression exhibited a gene expression profile compatible with a profile of tolerance suggesting that the frequency of tolerance is lower in kidney than in liver transplantation [24].

Bioinformatical analysis of the differentially expressed genes shows that tolerant state was associated with a reduced immune activation. Indeed, classical markers for T cell activation and genes characterizing pro-inflammatory Th1/Th2 responses were reduced in tolerant patients. Indeed, approximately 90% of known pro-inflammatory cytokines were down-regulated in tolerant patients compared to chronic rejection suggesting a global quiescent and non-deleterious immune response against the graft. No difference was observed for the expression of TGF-β, whereas it regulates the function of 27% of the peripheral blood genes that differentiate tolerance from chronic rejection [16].

A recent study used a panel of regulatory/inflammatory molecules (FOXP3, GATA3, IL-10, TGF-β1, TGF-βR1/TBX21, TNF and IFN-γ) to investigate by real-time PCR the gene expression profile of five tolerant patients [25]. They showed a predominant regulatory profile in tolerant recipients compared to patients under immunosuppression and chronic rejection. This regulatory profile was stable over time and associated with a strong GATA3 transcript and protein expression suggesting a potential Th2 deviation that may be a relevant pathway to tolerance in kidney transplantation [25]. Nevertheless to be confirmed, this study should be performed on a larger cohort of tolerant recipients.

Three additional studies reported the presence of a strong B cell signature in tolerant renal recipients. As previously described [26], cellular analysis by flow cytometry reported an increase in absolute number of B cells in tolerant patients compared to immunosuppressed recipients [18–20]. This was associated with a enrichment in naive and transitional B cells subsets in the peripheral blood mononuclear cells of tolerant patients [18,19]. Phenotypic analysis identifies a global inhibitory profile with a diminution of FcγRIIA/FcγRIIB ratio, increased expression of BANK1 and augmentation of CD1d CD5 expressing B cells [20]. Polyclonal activation of total B cells revealed no difference in cytokines secretion in all groups of transplanted patients [19,20], but stimulation of transitional B cells showed an enrichment in IL-10 secreting B cells in tolerant and healthy donors compared to stable patients [18]. These results could be explained by the fact that transitional B cells constituted only 0-5% of total B cells [27]. Consequently this response could be undetectable in total B cells.

In addition, our group and other have analyzed the transcriptome of peripheral blood mononuclear cells. We revealed an increased expression of B cells related-genes in tolerant patients compared to stable

patients [18–20]. These genes were involved in the activation and maturation of B cells [20]. Also many genes expressed by naive and immature B cells were over-represented in tolerant patients confirming the enrichment of these subsets in this group [18]. Finally transcriptomic examination of urinary sediment cells showed that only CD20, a B cell marker, was over-expressed in tolerant compared to both stable recipients and healthy donors.

4. Do B cells play a role in tolerance to renal allograft ?

Based on recent reports there is a growing interest in the role of B cell in the development of tolerance following renal transplantation. One of specific feature characterizing tolerant patients is the increased number of B cells in their blood. How could we explain this phenomenon? Firstly we must keep in mind that patients with end-stage renal disease have a significant reduction in the peripheral total B-cell count [28–30]. Secondly, after transplantation, patients are treated with a strong immunosuppressive therapy which guarantees a stable function of the graft but strongly alters the immune system. But some patients who stopped their treatments managed to tolerate their kidney allograft and are subject to a strong immune reconstitution with an increase of naïve or immature B cells in their blood compared to stable patients. This phenomenon is observed in kidney transplantation tolerance mediated by mixed chimerism with a re-population of transitional B cells in tolerant recipients [31]. These data suggest that the re-population by immature and naïve B cells could be a feature facilitating tolerance in kidney transplantation. But in order to strengthen these observations, large studies will be necessary to follow and study changes in B cell compartment of patients who have successfully stopped their immunosuppressive therapy.

B cells can mediate tolerance through different mechanisms involving clonal deletion, receptor editing, competition for survival factors [32–34]. It is currently known that immature and transitional B cells underwent clonal deletion, editing and anergy after B cell receptor engagement with self antigens, and it is interesting to notice that tolerant patients over-express genes involved in class switch and receptor editing suggesting a remodeling of BCR repertoire in tolerant recipients [18]. Moreover, recent data in rodents demonstrated that depletion of the B cell compartment, at the time of transplantation, can induce tolerance by the deletion of alloreactive clones and so remodeling of the BCR repertoire [34,35]. In clinical setting B cell tolerance has also been observed in the developing immune system of heart transplanted-children under ABO-incompatible conditions [36]. Together, these data suggest that the presence of alloantigens could remodel the humoral repertoire of tolerant recipients.

A recent report has investigated the B cell compartment during the development of tolerance in a rat model of long-term cardiac allograft tolerance. Tolerant rodents displayed an increased B cells number in their blood and graft, a blockade in the IgM to IgG switch recombination process and over-expressed BANK-1 and FcγRIIB. Most important, B cells from tolerant rats were able to transfer tolerance. Thus, as observed in humans [20], tolerant rats have a accumulation of B cells exhibiting an inhibited and regulatory profile [37] strengthening their role in the maintenance of transplantation tolerance. More investigations will be necessary to dissect mechanisms responsible for tolerance in this model.

Regulatory B cell secreting IL-10 (Breg) has recently been described in both rodents and humans. Bregs are able to regulate immune response by suppressing proliferation and Th1 differentiation of T cells [38–40] and impairing monocytes and dendritic cells maturation [41]. Phenotype of IL-10 secreting B cells is under many speculations. Indeed regulatory B cell compartment was associated to transitional [38–40] or memory B cell compartment [39,41]. Importantly, each B regulatory subsets identified in these studies expressed the molecule CD5, and it was reported that CD5 was associated to IL-10 secretion in human [42]. Consequently, regulatory functions by the B cell compartment may, thus, result from appropriate stimulation of any B cell population expressing CD5 [40]. In transplantation tolerance, our team showed that tolerant patients were characterized by higher number of $CD5^+$ B cells in their blood than stable patients under immunosuppression, suggesting a potential role of this population in the long-term graft survival. Thus, moving forward, it will be important to assess specifically the $CD5^+$ B cell population as a potential Breg population and as an inductor of Treg cells.

Observations of altered B cells in tolerant recipients can be interpreted as a consequence of the immune reconstitution after a long immunosuppressive therapy. But now a lot of studies demonstrate that B cells could be the primary drivers of the tolerant state. This hypothesis challenges the prevailing paradigm that regulatory T cells (Tregs) are the principal mediator of transplantation tolerance and is consistent with emerging data on the role of B cells in other immune processes. But we need to confirm and define those situations in which B cells play a role in transplantation tolerance, the mechanisms by which they can act, how they can interact with other components of the immune system and how to best promote their development [43].

5. Biomarkers in liver transplantation tolerance

In contrast to the B cell signature reported by many groups in kidney transplantation tolerance, tolerant liver recipients have been identified by transcriptional fingerprint enriched in NK cells and γδ T cells. Indeed a first study employing microarray gene expression in liver transplantation, have compared 16 tolerant patients with 16 non tolerant patients who underwent a failed immunosuppressors weaning. The comparison between the two groups resulted in 628 differentially expressed genes putting on light an over-expression of γδ T cells and NK cells genes pathway [15,44]. A second report, by the same group, on a larger group of recipients containing 17 tolerants, 21 non-tolerant exhibit three specific predictive gene signature of tolerance that could accurately classify most samples included in the training group [15,44]. Also, these transcriptomic fingerprints were able to identify the clinical state of patients in an independent cohort of 11 tolerant and 12 non-tolerant patients. The expression of the "tolerance genes" was measured on stable patients showing that 26% of them were potentially tolerant recipients. As previously described, they confirmed the enrichment in NK cells and γδ T cells genes with an increased numbers of these cellular subsets in peripheral blood of tolerant patients [15,44].

In order to find a common immunologic ground shared by recipients operationally tolerant to different organs, we directly compared on the same microarray platform blood samples collected from operationally tolerant and non tolerant liver and kidney recipients [21]. As described by previous studies, liver and kidney operationally tolerant recipients exhibited distinct blood transcriptional and cell phenotypic patterns, which were related to NK and B cells, respectively. No or minimal overlaps in blood cell phenotype and whole-genome expression patterns were observed between the two groups of tolerant recipients. These data suggest that different PBMC subsets contribute to tolerance-related expression patterns in liver and in kidney transplantation and does not support the existence of a common immunological ground between recipients spontaneously developing tolerance to liver and kidney allografts. This is in accordance with the view of the liver as a "particular organ" for "tolerance facilitation" and this is further illustrated by the higher difficulty to induce tolerance in kidney allograft models compared to liver allograft models.

A recent study has defined the blood and intra-graft molecular patterns discriminating between tolerant and non-tolerant patients [45]. In this study, investigators analyzed sequential blood and liver tissue samples collected from liver transplant recipients enrolled in a prospective multicenter immunosuppressive drug withdrawal clinical trial. First they confirm their NK cells signature in the peripheral blood of tolerant patients. Also their immunophenotype study demonstrated that before

the start of drug minimization, tolerant recipients exhibited an increased proportion of NK lymphocytes and a decreased proportion of Vδ2-TCR γδT cells as compared with non-tolerant patients [45]. Moreover, they showed that PBMC of tolerant recipients contained a higher proportion of circulating $CD4^+$ $CD25^+$ $CD127^-$ $Foxp3^+$ T cells than non-tolerant recipients after 12 months of drug withdrawal. Microarray study before initiation of drug minimization on liver tissues showed that tolerant and non-tolerant recipients differed in the intragraft expression of genes involved in the regulation of iron homeostasis. Moreover this "iron signature" accurately predicts the outcome of drug withdrawal independent of any clinical parameter. Furthermore, as compared with non tolerant recipients, operationally tolerant patients exhibited higher serum levels of hepcidin and ferritin and increased hepatocyte iron deposition. These results point to a critical role for iron metabolism in the regulation of intra-graft alloimmune responses in humans and provide a set of biomarkers to conduct drug-weaning trials in liver transplantation [45].

6. Tolerance mechanisms in liver transplantation: just iron homoeostasis?

The liver is continuously exposed to food and microbial antigens through the portal venous blood.

This result in a microenvironment biased toward tolerance rather than immunity [46,47] and likely contributes to the systemic liver tolerance effect observed in most animal models of liver transplantation and in selected human recipients who require no immunosuppressive drugs. A lot of mechanisms responsible for this tolerance effect were described. Indeed, resident liver dendritic cells display an immature phenotype and produce immunoregulatory cytokines as IL-10 [46]. Also, Kupffer cells depletion induces impaired tolerance toward alloantigens suggesting their role in transplantation tolerance [48]. Regulatory T cells surely play a role in the maintenance of liver tolerance as described in rodents [49]. Studies on peripheral blood of tolerant recipients have displayed a strong NK cells and γδ T cells signature suggesting a role of these populations in the long term graft survival [15,44] but no data have demonstrated their immunosuppressive functions in the context of liver tolerance. All these data left us suppose during long time that liver tolerance were essentially based on immunological parameters. But the last study by Bohne and colleagues demonstrated that transplantation tolerance doesn't rely exclusively on the immune system and that other parameters should be studied as accommodation mechanisms. In their study they showed that the modulation of hepcidine expression and redistribution of intra graft iron stores could be involved in the capacity of the liver graft to restrain immune response. The finding of Bohne and his colleagues suggest that other mechanisms and molecules may be involved in this process to prevent inflammatory tissue destruction such as SOCS1 pathway described for his cytoprotective role after LPS stimulation [50], or CD26 and ADORA3 involved in the metabolism of adenosine, an immunosuppressive mediator responsible in part for the suppressive function of regulatory T cells [51]. Their findings suggest that the capacity of the liver allograft to resist immune-mediated injuries is likely to be a central mechanism in the maintenance of operational tolerance rather than regulation of immune adaptive mechanisms [45].

7. Conclusion

Several reports have associated a specific transcriptomic and cellular profile with tolerance in liver and kidney transplantation. Although there are no reliable markers for identifying tolerant patients, we and others have found that tolerant recipients exhibit unique peripheral blood transcriptional patterns. The difference in expression patterns is related to the immune response and could constitute the basis for a future diagnostic test of tolerance in order to adapt the immunosuppressive therapy. But we must keep in mind that other variables may play a role in the development of tolerance. Indeed today we can't just rely on the genetic profile of recipients to determine if they can be weaned of suppressive therapy. Thus clinical parameters must be considered before reducing or weaning immunosuppression. A previous non-invasive composite clinical tool, the KTFS [52] was described as a surrogate for long-term graft outcome. But to increase the sensitivity and the specificity of the prediction, the composite score needs to be infusing with immunological parameters. Consequently we must associate the "transcriptome/immunome" with the "clinicome" in order to accurately detect potential tolerant recipients and begin gradual diminution of the suppressive therapy. Also the time post transplantation before weaning must be considered in both liver and kidney recipients. In liver transplantation, tolerance has been reported in approximately 20% of liver transplant recipients after weaning, although it could be much more prevalent at very late time points after transplantation [15]. In kidney transplantation, few studies exist concerning the factors associated with the increased risk of rejection relating to weaning immunosuppression in stable renal transplant patients [53–55]. in all these studies the diminution of immunosuppressive therapy was associated with a significant increase of rejection. However, most of these studies were conducted soon after transplantation, after one year or less, without any clinical selection for minimizing the risk of rejection. All these clinical experience suggest indeed that the time post-transplant may "plays an important role as a predictive variable of the tolerance development in transplant recipient". Based on these experiments, our hypothesis is that the strict screening of patients presenting with significant clinical and biological graft stability, of more than 4 years, should make it possible to investigate gradual weaning of immunosuppression. But now there is a need to validate these signature using larger multicentric cohorts in order to move from potential biomarkers into clinically useful biomarkers. Large cohort of patients have to be enrolled in order to take into account potential confounding factors when evaluating diagnostic or prognostic biomarkers. Finally, it will be necessary to move from snapshot studies into longitudinal studies in order to define the fluctuation of these biomarkers and better evaluate, understand and, why not, induce tolerance in transplantation.

References

[1] Nankivell BJ, Borrows RJ, Fung CL-S, O'Connell PJ, Allen RDM, Chapman JR. The natural history of chronic allograft nephropathy. N Engl J Med déc 11 2003;349(24):2326–33.
[2] Stegall MD, Everson GT, Schroter G, et al. Prednisone withdrawal late after adult liver transplantation reduces diabetes, hypertension, and hypercholesterolemia without causing graft loss. Hepatology janv 1997;25(1):173–7.
[3] Soulillou JP, Giral M. Controlling the incidence of infection and malignancy by modifying immunosuppression. Transplantation déc 27 2001;72(12 Suppl.): S89–93.
[4] Ojo AO, Held PJ, Port FK, et al. Chronic renal failure after transplantation of a non-renal organ. N Engl J Med sept 4 2003;349(10):931–40.
[5] Fishman JA. Infection in solid-organ transplant recipients. N Engl J Med déc 20 2007;357(25):2601–14.
[6] Sharabi Y, Sachs DH. Mixed chimerism and permanent specific transplantation tolerance induced by a nonlethal preparative regimen. J Exp Med févr 1 1989;169(2):493–502.
[7] Kawai T, Cosimi AB, Spitzer TR, et al. HLA-mismatched renal transplantation without maintenance immunosuppression. N Engl J Med janv 24 2008;358(4): 353–61.
[8] Scandling JD, Busque S, Dejbakhsh-Jones S, et al. Tolerance and chimerism after renal and hematopoietic-cell transplantation. N Engl J Med janv 24 2008;358(4):362–8.
[9] Cosimi AB, Burton RC, Colvin RB, et al. Treatment of acute renal allograft rejection with OKT3 monoclonal antibody. Transplantation déc 1981;32(6):535–9.
[10] Vincenti F, Nashan B, Light S. Daclizumab: outcome of phase III trials and mechanism of action. Double Therapy and the Triple Therapy Study Groups. Transplant Proc août 1998;30(5):2155–8.
[11] Vincenti F, Larsen C, Durrbach A, et al. Costimulation blockade with belatacept in renal transplantation. N Engl J Med août 25 2005;353(8):770–81.
[12] Poirier N, Azimzadeh AM, Zhang T, et al. Inducing CTLA-4-dependent immune regulation by selective CD28 blockade promotes regulatory T cells in organ transplantation. Sci Transl Med févr 3 2010;2(17):17ra10.

[13] Lerut J, Sanchez-Fueyo A. An appraisal of tolerance in liver transplantation. Am J Transplant août 2006;6(8):1774–80.
[14] Roussey-Kesler G, Giral M, Moreau A, et al. Clinical operational tolerance after kidney transplantation. Am J Transplant avr 2006;6(4):736–46.
[15] Sánchez-Fueyo A. Hot-topic debate on tolerance: immunosuppression withdrawal. Liver Transpl nov 2011;17(Suppl. 3):S69–73.
[16] Brouard S, Mansfield E, Braud C, et al. Identification of a peripheral blood transcriptional biomarker panel associated with operational renal allograft tolerance. Proc Natl Acad Sci U S A sept 25 2007;104(39):15448–53.
[17] Martínez-Llordella M, Lozano JJ, Puig-Pey I, et al. Using transcriptional profiling to develop a diagnostic test of operational tolerance in liver transplant recipients. J Clin Invest août 2008;118(8):2845–57.
[18] Newell KA, Asare A, Kirk AD, et al. Identification of a B cell signature associated with renal transplant tolerance in humans. J Clin Invest juin 2010;120(6):1836–47.
[19] Sagoo P, Perucha E, Sawitzki B, et al. Development of a cross-platform biomarker signature to detect renal transplant tolerance in humans. J Clin Invest juin 2010;120(6):1848–61.
[20] Pallier A, Hillion S, Danger R, et al. Patients with drug-free long-term graft function display increased numbers of peripheral B cells with a memory and inhibitory phenotype. Kidney Int sept 2010;78(5):503–13.
[21] Lozano JJ, Pallier A, Martinez-Llordella M, et al. Comparison of transcriptional and blood cell-phenotypic markers between operationally tolerant liver and kidney recipients. Am J Transplant sept 2011;11(9):1916–26.
[22] Canales RD, Luo Y, Willey JC, et al. Evaluation of DNA microarray results with quantitative gene expression platforms. Nat Biotechnol sept 2006;24(9):1115–22.
[23] Patel AC. Basic science for the practicing physician: gene expression microarrays. Ann Allergy Asthma Immunol sept 2008;101(3):325–32.
[24] Brouard S, Le Bars A, Dufay A, et al. Identification of a gene expression profile associated with operational tolerance among a selected group of stable kidney transplant patients. Transpl Int juin 2011;24(6):536–47.
[25] Moraes-Vieira PMM, Takenaka MCS, Silva HM, et al. GATA3 and a dominant regulatory gene expression profile discriminate operational tolerance in human transplantation. Clin Immunol févr 2012;142(2):117–26.
[26] Louis S, Braudeau C, Giral M, et al. Contrasting CD25hiCD4+T cells/FOXP3 patterns in chronic rejection and operational drug-free tolerance. Transplantation févr 15 2006;81(3):398–407.
[27] Perez-Andres M, Paiva B, Nieto WG, et al. Human peripheral blood B-cell compartments: a crossroad in B-cell traffic. Cytometry B Clin Cytom 2010;78(Suppl. 1):S47–60.
[28] Girndt M, Sester M, Sester U, Kaul H, Köhler H. Molecular aspects of T- and B-cell function in uremia. Kidney Int Suppl févr 2001;78:S206–11.
[29] Smogorzewski M, Massry SG. Defects in B-cell function and metabolism in uremia: role of parathyroid hormone. Kidney Int Suppl févr 2001;78:S186–9.
[30] Pahl MV, Gollapudi S, Sepassi L, Gollapudi P, Elahimehr R, Vaziri ND. Effect of end-stage renal disease on B-lymphocyte subpopulations, IL-7, BAFF and BAFF receptor expression. Nephrol Dial Transplant janv 2010;25(1):205–12.
[31] Porcheray F, Wong W, Saidman SL, et al. B-cell immunity in the context of T-cell tolerance after combined kidney and bone marrow transplantation in humans. Am J Transplant sept 2009;9(9):2126–35.
[32] Cancro MP. Signalling crosstalk in B cells: managing worth and need. Nat Rev Immunol sept 2009;9(9):657–61.
[33] Scholz JL, Cancro MP. Resolve, revise, and relax: The 3 Rs of B cell repertoire adjustment. Immunol Lett mars 30 2012;143(1):2–8.
[34] Parsons RF, Vivek K, Rostami SY, et al. Acquisition of humoral transplantation tolerance upon de novo emergence of B lymphocytes. J Immunol janv 1 2011;186(1):614–20.
[35] Parsons RF, Redfield III RR, Rodriguez E, et al. Primary B cell repertoire remodeling to achieve humoral transplantation tolerance. Semin Immunol avr 2012;24(2):109–14.
[36] Fan X, Ang A, Pollock-Barziv SM, et al. Donor-specific B-cell tolerance after ABO-incompatible infant heart transplantation. Nat Med nov 2004;10(11):1227–33.
[37] Le Texier L, Thebault P, Lavault A, et al. Long-term allograft tolerance is characterized by the accumulation of B cells exhibiting an inhibited profile. Am J Transplant mars 2011;11(3):429–38.
[38] Blair PA, Noreña LY, Flores-Borja F, et al. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. Immunity janv 29 2010;32(1):129–40.
[39] Bouaziz J-D, Calbo S, Maho-Vaillant M, et al. IL-10 produced by activated human B cells regulates CD4(+) T-cell activation in vitro. Eur J Immunol oct 2010;40(10):2686–91.
[40] Lemoine S, Morva A, Youinou P, Jamin C. Human T cells induce their own regulation through activation of B cells. J Autoimmun mai 2011;36(3–4):228–38.
[41] Iwata Y, Matsushita T, Horikawa M, et al. Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. Blood janv 13 2011;117(2):530–41.
[42] Garaud S, Morva A, Lemoine S, et al. CD5 promotes IL-10 production in chronic lymphocytic leukemia B cells through STAT3 and NFAT2 activation. J Immunol avr 15 2011;186(8):4835–44.
[43] Newell KA, Chong AS. Making a B-line for transplantation tolerance. Am J Transplant mars 2011;11(3):420–1.
[44] Martínez-Llordella M, Puig-Pey I, Orlando G, et al. Multiparameter immune profiling of operational tolerance in liver transplantation. Am J Transplant févr 2007;7(2):309–19.
[45] Bohne F, Martínez-Llordella M, Lozano J-J, et al. Intra-graft expression of genes involved in iron homeostasis predicts the development of operational tolerance in human liver transplantation. J Clin Invest janv 3 2012;122(1):368–82.
[46] Tiegs G, Lohse AW. Immune tolerance: what is unique about the liver. J Autoimmun févr 2010;34(1):1–6.
[47] Thomson AW, Knolle PA. Antigen-presenting cell function in the tolerogenic liver environment. Nat Rev Immunol nov 2010;10(11):753–66.
[48] Roland CR, Mangino MJ, Duffy BF, Flye MW. Lymphocyte suppression by Kupffer cells prevents portal venous tolerance induction: a study of macrophage function after intravenous gadolinium. Transplantation mai 1993;55(5):1151–8.
[49] He F, Chen Z, Xu S, et al. Increased CD4+CD25+Foxp3+ regulatory T cells in tolerance induced by portal venous injection. Surgery juin 2009;145(6):663–74.
[50] Fujimoto M, Naka T. SOCS1, a negative regulator of cytokine signals and TLR responses, in human liver diseases. Gastroenterol Res Pract 2010;2010(pii):470468.
[51] Gri G, Piconese S, Frossi B, et al. CD4+CD25+ regulatory T cells suppress mast cell degranulation and allergic responses through OX40-OX40L interaction. Immunity nov 14 2008;29(5):771–81.
[52] Foucher Y, Daguin P, Akl A, et al. A clinical scoring system highly predictive of long-term kidney graft survival. Kidney Int déc 2010;78(12):1288–94.
[53] Abramowicz D, Del Carmen Rial M, Vitko S, et al. Cyclosporine withdrawal from a mycophenolate mofetil-containing immunosuppressive regimen: results of a five-year, prospective, randomized study. J Am Soc Nephrol juill 2005;16(7):2234–40.
[54] Ekberg H, Tedesco-Silva H, Demirbas A, et al. Reduced exposure to calcineurin inhibitors in renal transplantation. N Engl J Med déc 20 2007;357(25):2562–75.
[55] Pallardó LM, Oppenheimer F, Guirado L, et al. Calcineurin inhibitor reduction based on maintenance immunosuppression with mycophenolate mofetil in renal transplant patients: POP study. Transplant Proc sept 2007;39(7):2187–9.

Reconsidering the bio-detection of tolerance in renal transplantation

Faouzi Braza, Jean-Paul Soulillou and Sophie Brouard

Abstract

Operational tolerance in kidney transplantation tolerance is rare phenomenon. It concerns recipients who keep a good function of their graft without immunosuppressors for more than one year. A critical need in the field of transplantation tolerance is the identification of biomarkers able to detect precociously tolerance phenotype in stable recipient in order to adapt treatment and progressively stop immunosuppressive therapy. But many limitations in these studies slow the application in clinics of such tolerance signature. In this addendum article we talk about these limitations and potential new directions to improve our approach in the quest of tolerance biomarkers.

ARTICLE ADDENDUM

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Reconsidering the bio-detection of tolerance in renal transplantation

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Operational tolerance in kidney transplantation tolerance is rare phenomenon. It concerns recipients who keep a good function of their graft without immunosuppressors for more than one year. A critical need in the field of transplantation tolerance is the identification of biomarkers able to detect precociously tolerance phenotype in stable recipient in order to adapt treatment and progressively stop immunosuppressive therapy. But many limitations in these studies slow the application in clinics of such tolerance signature. In this addendum article we talk about these limitations and potential new directions to improve our approach in the quest of tolerance biomarkers.

Keywords: transplantation, tolerance, signature, kidney, DNA microarray, operational tolerance

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Transplantation tolerance is defined as the absence of deleterious responses with a stable maintenance of allograft function without any immunosuppressive therapy. In clinical settings, patients are referred to as "operationally tolerant" when the function of their graft is stable despite having stopped all immunosuppression for at least one year.[1] Operational tolerance has been reported in renal transplantation,[2] but is more common in liver transplantation.[3] Immunosuppressive therapies have strongly improved long-term graft survival but are associated with severe side effects.[4] Thus, cardiovascular disease, nephrotoxicity, development of cancer and opportunistic infections have all detrimentally affected graft survival and patient health.[5,6] Our team and colleagues have developed tools to identify specific signatures of tolerance in renal transplant recipients.[7] These tools should help to identify patients who have developed tolerance to their transplants and who could benefit from partial or complete withdrawal of immunosuppression. The use of DNA microarrays and multi-color flow cytometry has led to the identification of a B cell transcriptomic and phenotypic signature of tolerance in renal transplantation.[8,9,10,11,12] This tolerance signature has been tested for its capacity to classify transplant recipients according to their clinical state.[9,10,11] We tried to detect a potential tolerance fingerprint in a cohort of 111 immunosuppressed patients with stable graft function after 5 years of transplantation, with the 20 most informative genes selected among a signature of 49 blood genes specific for tolerance.[9,13] Nearly five percent of patients were found to exhibit a molecular phenotype of tolerance.[13] This result confirms the rarity of tolerance in kidney transplantation, but also suggests that the current blood transcriptomic signature may not be sufficient to detect the tolerance phenotype. One other possibility is that immunosuppressors, by altering the expression of gene, can mask or hide the tolerance signature and so biased the possibility to identify potential tolerant patients. Nevertheless, in our previous report,[9,13] we showed that the signature of tolerance was identified in blood from some patients under immunosuppression suggesting that the signature is not masked by immunosuppression. In the same study, we also show that 50% of patients under minimal dose of corticosteroids (< 10 mg/day) exhibit a transcriptomic signature of tolerance whereas only 8% (1/12) of stable recipients under classic immunosuppression matched with tolerance fingerprint, suggesting that corticosteroids may not influence "too much" this

signature of tolerance. However, a recent work demonstrated in an elegant manner that steroid monotherapy could interfere with the B cell signature and B cell function. Indeed by analyzing the T cell indirect pathway in transplanted patients, authors suggest a regulatory role of B cells in the graft. They suggest that immunosuppression impact on B cells function and homeostasis and that progressive weaning could allow patient to reach a tolerance threshold. Repopulation by naïve, transitional and regulatory B cells would be a central mechanism for the establishment of transplant tolerance.[14]

So how this approach could be improved upon, to identify robust signatures and detect potentially tolerant recipients? First, the immunophenotyping and microarray technologies should be standardized in these studies in order to limit variability. Second, in addition to analyses on peripheral blood samples, other compartments should also be considered, such as serum or urine, for the identification of new relevant biomarkers of kidney transplantation tolerance.[10] Third the development and use of bioinformatic tools must be pursued in order to strengthen and improve data analysis. For example, a meta-analysis would enable the investigation of microarray data from various studies using different platforms, thereby identifying the most relevant differentially expressed genes. We have already undertaken such an analysis integrating the recent studies in kidney tolerance and identified very few overlapping genes.[15] Nevertheless we confirmed the B cell signature of tolerance and its specificity for kidney transplantation.[15] Finally, there is a clear necessity to progress toward longitudinal studies with larger cohorts of patients, to test the stability of these transcriptomic signatures over time. Newell and colleagues identified a very stringent transcriptomic signature composed of three genes coding for immunoglobulin variable regions. The expression of these genes could accurately distinguish tolerant patients from stable patients and healthy donors. This signature was stable in time and correlated with the recipient's clinical state (Newell, personal communication, ATC 2012). Nevertheless, the ability of this signature to identify potentially tolerant patients in a new and independent cohort of immunosuppressed recipients has not yet been demonstrated.

Obviously, omics-based classification of recipient clinical states could lead to more efficient targeting or even personalization of immunosuppressive therapies by identifying surrogate markers of tolerance. But although these approaches have accelerated the development of mechanistic hypotheses and have provided clinical insight, a global systems-level understanding of kidney transplantation tolerance has not yet emerged. This limitation, which is neither technological nor ethical in nature, could be due to the reductionist approach currently taken. This approach is not adapted to the added levels of complexity in human transplantation tolerance. Transcriptomic signatures alone are powerful, but reflect pathway biology only in a very limited way due to the remarkable heterogeneity of tolerant recipients, the lack of distinct clinical features (age, anti-DSA antibodies, infections) and the lack of comprehensive molecular mechanisms.[16] Consequently integrative approaches must be used, by combining transcriptomic patterns with clinical, cellular, proteomic and phenotypic data in order to create a model of tolerance. This type of approach is well described in the field of systems biology or systems pathology where mathematical tools are used to predict the evolution of pathology.[17-19] Moreover, bio-mathematical and statistical models could offer the potential to link clinical and molecular-level knowledge to the outcome on an individual scale.[20,21] Thus, multi-states and multi-dimensional approaches, such as Cox and semi-Markov models, could become central to analyzing graft outcome.[20,21] Obviously these approaches would not necessarily provide a definitive answer, but together they could contribute to making informed decisions during the medical management of transplant recipients. Thus, the use of dynamic models as predictive tools could potentially help to reduce weaning failures in kidney transplantation. However, to apply this kind of method, the identified biomarkers or clinical parameters related to transplantation tolerance need to be validated. The validity of these biomarkers and their predictive capacity will be tested in drug minimization trials conducted in subgroups of kidney recipients with low "immunological risk." One such study is currently in progress in which CNI weaning is being performed (http://clinicaltrials.gov/ct2/show/NCT01292525). This study is being conducted in the context of a European consortium (FP7 BioDrim) and with centers from the CENTAURE network (www.fondation-centaure.org/) (Londono et al., 2012).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Roussey-Kesler G, Giral M, Moreau A, Subra J-F, Legendre C, Noël C, et al. Clinical operational tolerance after kidney transplantation. Am J Transplant 2006; 6:736-46; PMID:16539630; http://dx.doi.org/10.1111/j.1600-6143.2006.01280.x.
2. Orlando G, Hematti P, Stratta RJ, Burke GW 3rd, Di Cocco P, Pisani F, et al. Clinical operational tolerance after renal transplantation: current status and future challenges. Ann Surg 2010; 252:915-28; PMID:21107102; http://dx.doi.org/10.1097/SLA.0b013e3181f3efb0.
3. Martínez-Llordella M, Puig-Pey I, Orlando G, Ramoni M, Tisone G, Rimola A, et al. Multiparameter immune profiling of operational tolerance in liver transplantation. Am J Transplant 2007; 7:309-19; PMID:17241111; http://dx.doi.org/10.1111/j.1600-6143.2006.01621.x.
4. Sola E, Gonzalez-Molina M, Cabello M, Burgos D, Ramos J, Gutierrez C, et al. Long-term improvement of deceased donor renal allograft survival since 1996: a single transplant center study. Transplantation 2010; 89:714-20; PMID:20035250; http://dx.doi.org/10.1097/TP.0b013e3181c892dd.
5. Dantal J, Hourmant M, Cantarovich D, Giral M, Blancho G, Dreno B, et al. Effect of long-term immunosuppression in kidney-graft recipients on cancer incidence: randomised comparison of two cyclosporin regimens. Lancet 1998; 351:623-8; PMID:9500317; http://dx.doi.org/10.1016/S0140-6736(97)08496-1.
6. Soulillou J-P, Giral M. Controlling the incidence of infection and malignancy by modifying immunosuppression. Transplantation 2001; 72(Suppl):S89-93; PMID:11833147.
7. Braza F, Soulillou J-P, Brouard S. Gene expression signature in transplantation tolerance. Clin Chim Acta 2012; 413:1414-8; PMID:22580152; http://dx.doi.org/10.1016/j.cca.2012.04.024.
8. Louis S, Braudeau C, Giral M, Dupont A, Moizant F, Robillard N, et al. Contrasting CD25hiCD4+T cells/FOXP3 patterns in chronic rejection and operational drug-free tolerance. Transplantation 2006; 81:398-407; PMID:16477227; http://dx.doi.org/10.1097/01.tp.0000203166.44968.86.
9. Brouard S, Mansfield E, Braud C, Li L, Giral M, Hsieh S-C, et al. Identification of a peripheral blood transcriptional biomarker panel associated with operational renal allograft tolerance. Proc Natl Acad Sci U S A 2007; 104:15448-53; PMID:17873064; http://dx.doi.org/10.1073/pnas.0705834104.

10. Newell KA, Asare A, Kirk AD, Gisler TD, Bourcier K, Suthanthiran M, et al.; Immune Tolerance Network ST507 Study Group. Identification of a B cell signature associated with renal transplant tolerance in humans. J Clin Invest 2010; 120:1836-47; PMID:20501946; http://dx.doi.org/10.1172/JCI39933.
11. Sagoo P, Perucha E, Sawitzki B, Tomiuk S, Stephens DA, Miqueu P, et al. Development of a cross-platform biomarker signature to detect renal transplant tolerance in humans. J Clin Invest 2010; 120:1848-61; PMID:20501943; http://dx.doi.org/10.1172/JCI39922.
12. Pallier A, Hillion S, Danger R, Giral M, Racapé M, Degauque N, et al. Patients with drug-free long-term graft function display increased numbers of peripheral B cells with a memory and inhibitory phenotype. Kidney Int 2010; 78:503-13; PMID:20531452; http://dx.doi.org/10.1038/ki.2010.162.
13. Brouard S, Le Bars A, Dufay A, Gosselin M, Foucher Y, Guillet M, et al. Identification of a gene expression profile associated with operational tolerance among a selected group of stable kidney transplant patients. Transpl Int 2011; 24:536-47; PMID:21457359; http://dx.doi.org/10.1111/j.1432-2277.2011.01251.x.
14. Haynes LD, Jankowska-Gan E, Sheka A, Keller MR, Hernandez-Fuentes MP, Lechler RI, et al. Donor-specific indirect pathway analysis reveals a B-cell-independent signature which reflects outcomes in kidney transplant recipients. Am J Transplant 2012; 12:640-8; PMID:22151236; http://dx.doi.org/10.1111/j.1600-6143.2011.03869.x.
15. Lozano JJ, Pallier A, Martinez-Llordella M, Danger R, López M, Giral M, et al. Comparison of transcriptional and blood cell-phenotypic markers between operationally tolerant liver and kidney recipients. Am J Transplant 2011; 11:1916-26; PMID:21827613; http://dx.doi.org/10.1111/j.1600-6143.2011.03638.x.
16. Brouard S, Pallier A, Renaudin K, Foucher Y, Danger R, Devys A, et al. The Natural History of Clinical Operational Tolerance After Kidney Transplantation Through Twenty-Seven Cases. Am J Transplant 2012; 12:3296-307; PMID:22974211; http://dx.doi.org/10.1111/j.1600-6143.2012.04249.x.
17. Faratian D, Clyde RG, Crawford JW, Harrison DJ. Systems pathology--taking molecular pathology into a new dimension. Nat Rev Clin Oncol 2009; 6:455-64; PMID:19581910; http://dx.doi.org/10.1038/nrclinonc.2009.102.
18. Nicholson JK, Holmes E, Lindon JC, Wilson ID. The challenges of modeling mammalian biocomplexity. Nat Biotechnol 2004; 22:1268-74; PMID:15470467; http://dx.doi.org/10.1038/nbt1015.
19. Tomlin CJ, Axelrod JD. Biology by numbers: mathematical modelling in developmental biology. Nat Rev Genet 2007; 8:331-40; PMID:17440530; http://dx.doi.org/10.1038/nrg2098.
20. Foucher Y, Giral M, Soulillou J-P, Daures JP. A flexible semi-Markov model for interval-censored data and goodness-of-fit testing. Stat Methods Med Res 2010; 19:127-45; PMID:18765502; http://dx.doi.org/10.1177/0962280208093889.
21. Foucher Y, Daguin P, Akl A, Kessler M, Ladrière M, Legendre C, et al. A clinical scoring system highly predictive of long-term kidney graft survival. Kidney Int 2010; 78:1288-94; PMID:20861817; http://dx.doi.org/10.1038/ki.2010.232.

Unique B cell differentiation profile in tolerant kidney transplant patients.

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Abstract

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.

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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 FlowCytomix 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.

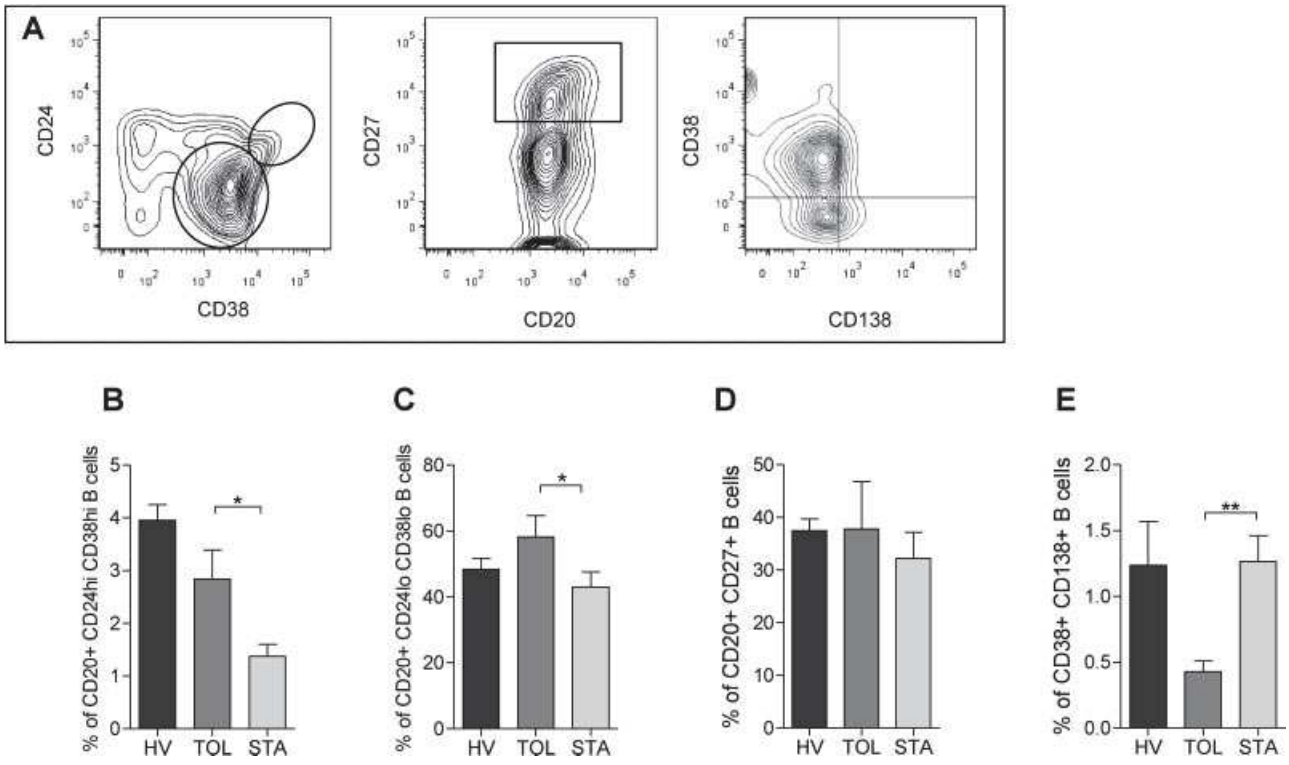

Figure 1: B cell phenotype of tolerant patients. B cell subsets were analyzed by flow cytometry as follows (A). Frequency of transitional B cells $CD20^{+}CD24^{hi}CD38^{hi}$ (B). Frequency of naïve B cells $CD20^{+}CD24^{lo}CD38^{lo}$ (C). Frequency of memory B cells $CD20^{+}CD27^{+}$ (D). Frequency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

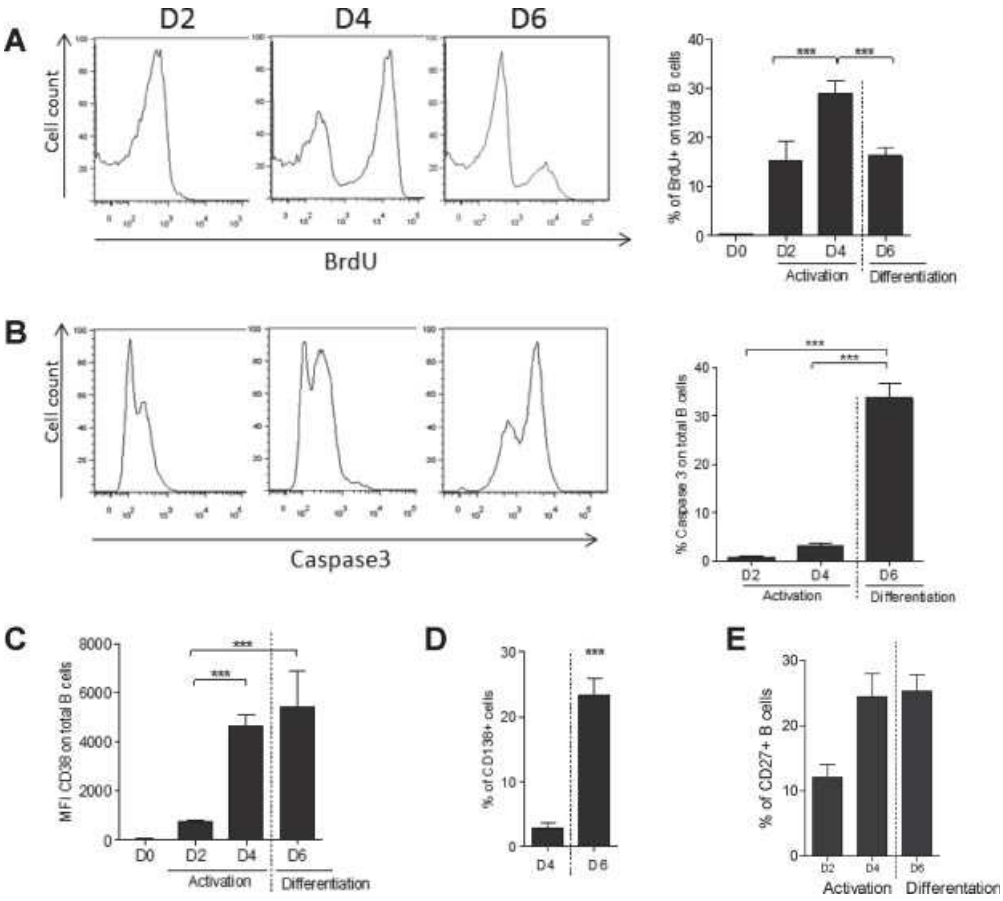

Figure 2: Validation of B cell activation and differentiation model in 18 healthy volunteers. For the activation phase, purified human B cells were cultured at 3.75×10^5 cells/mL and activated with 2 μg/mL $F(ab')^2$ Fragment Goat anti-Human IgA+ IgG+ IgM+ (H + L) (Jackson ImmunoResearch Laboratories, 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

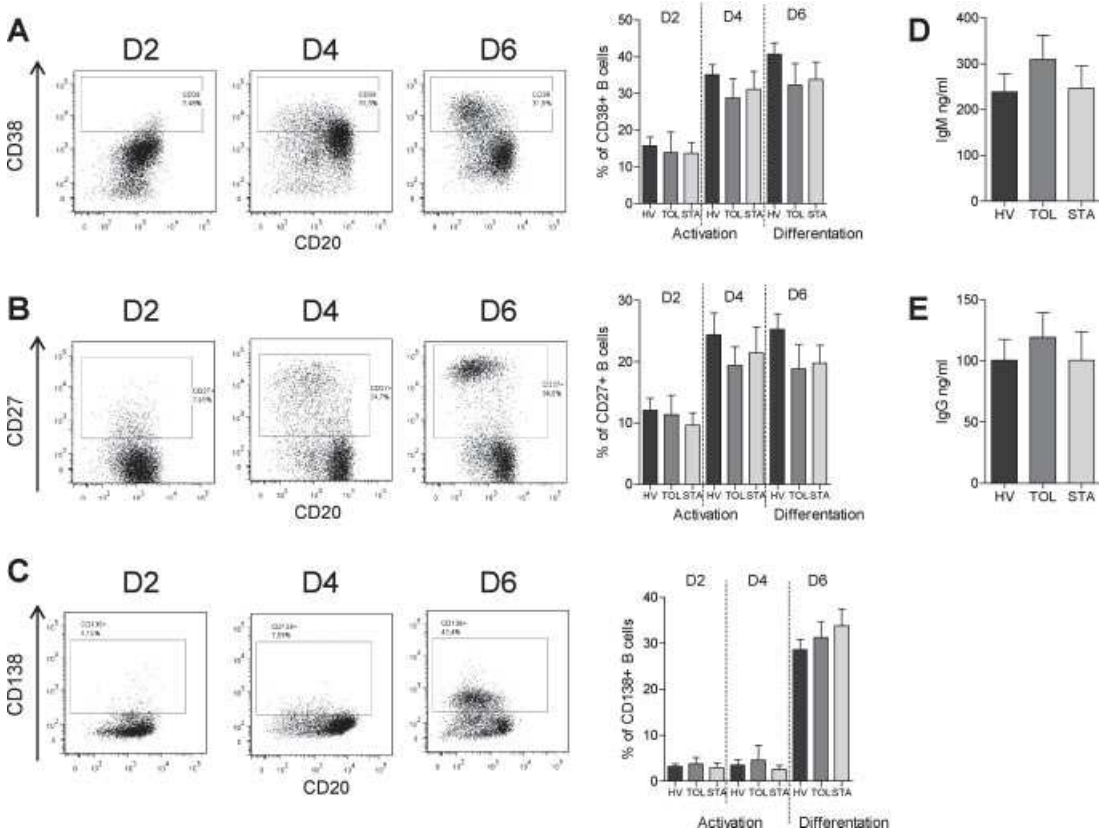

Figure 3: Activation and differentiation into plasma cells. Dot plot and histograms for the generation of $CD20^{+}CD38^{+}$ B cells (A), $CD20^{+}CD27^{+}$ B cells (B) and plasma $CD20^{lo}CD138^{+}$ cells (C) analyzed by flow cytometry on total B cells on days 2, 4 and 6 of the culture. IgM (D) and 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

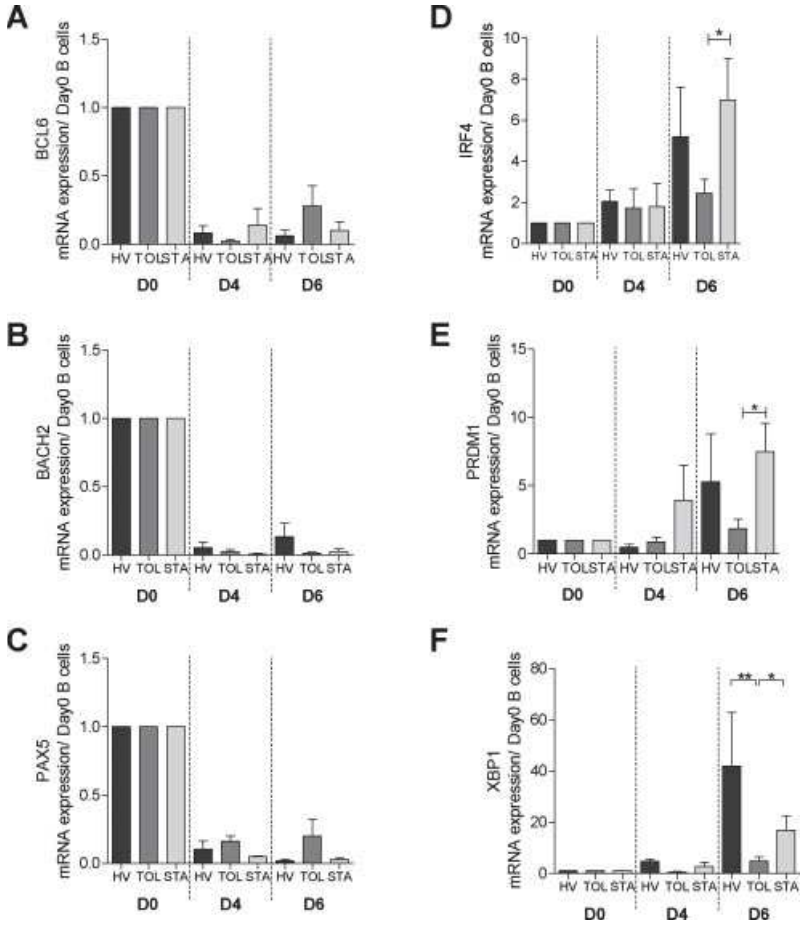

Figure 4: B cell differentiation. *BCL6* (A), *BACH2* (B), *PAX5* (C), *IRF4* (D), *PRDM1* (E) and *XBP1* (F) transcriptional levels in B cells are shown relative to the day of experiment. Results of quantitative polymerase chain reaction are expressed relative to gene expression at day 0. Bars represent 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

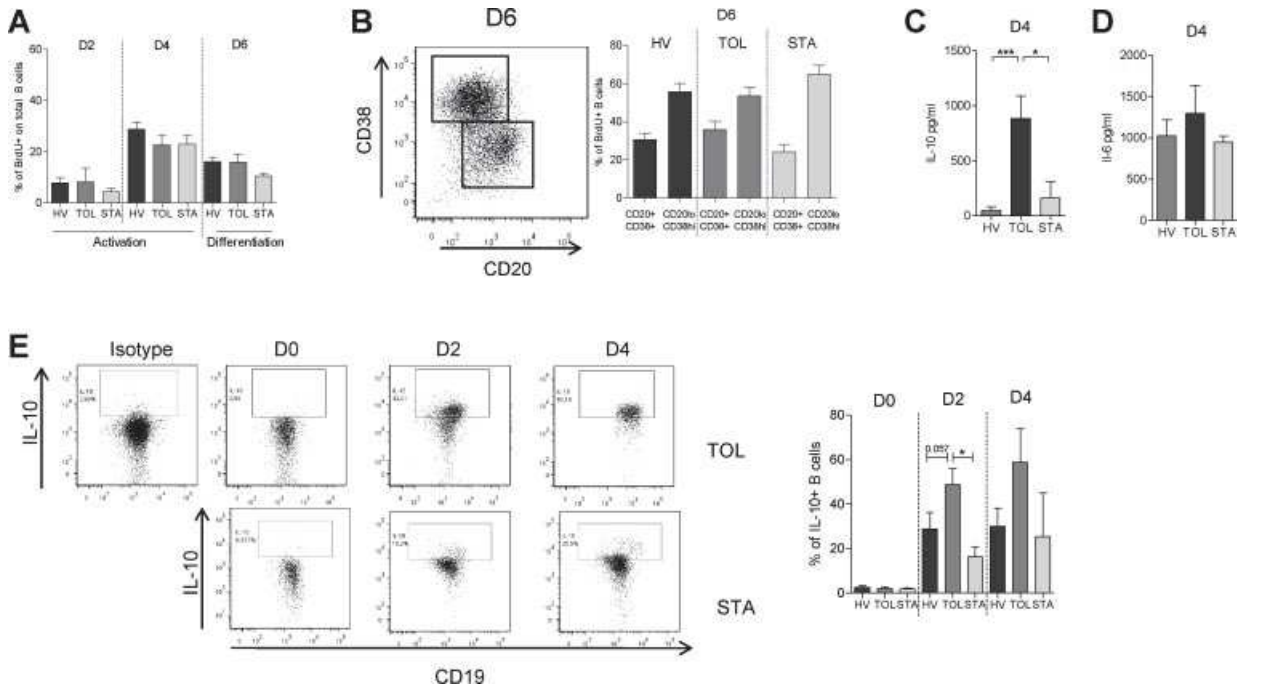

Figure 5: Proliferation and cytokines secretion. Proliferation was analyzed by intracellular staining of BrdU in total B cells at days 2, 4 and 6 (A). Proliferation of B cell subset at day 6: $CD20^{+}CD38^{+}$ B cells and $CD20^{lo}CD38^{hi}$ B cells with the representative dot plot (B). IL-10 (C) and 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

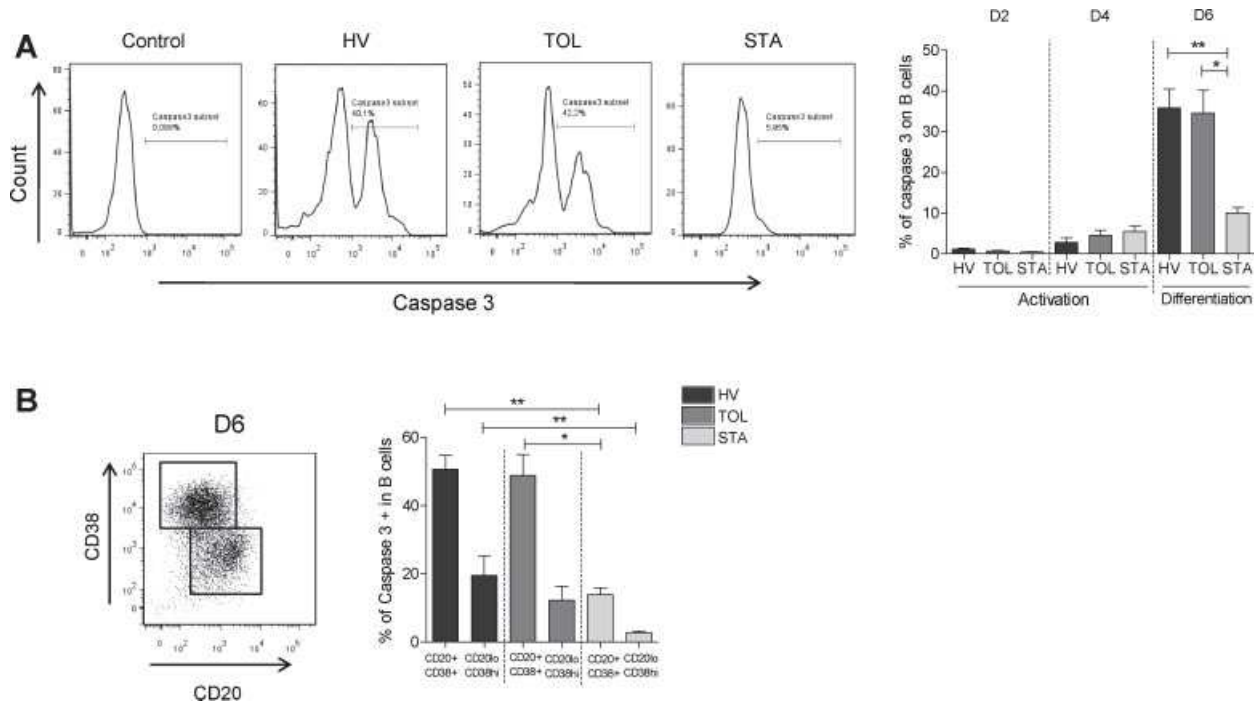

Figure 6: Apoptosis signaling. Apoptosis was analyzed by flow cytometry by measuring caspase-3 staining in total B cells, caspase-3 representative histograms and percentage of caspase-3+ on B cells at days 2, 4 and 6 (A) and in $CD20^{+}CD38^{+}$ and $CD20^{lo}CD38^{hi}$ B cell subsets at day 6 (B) (*$p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

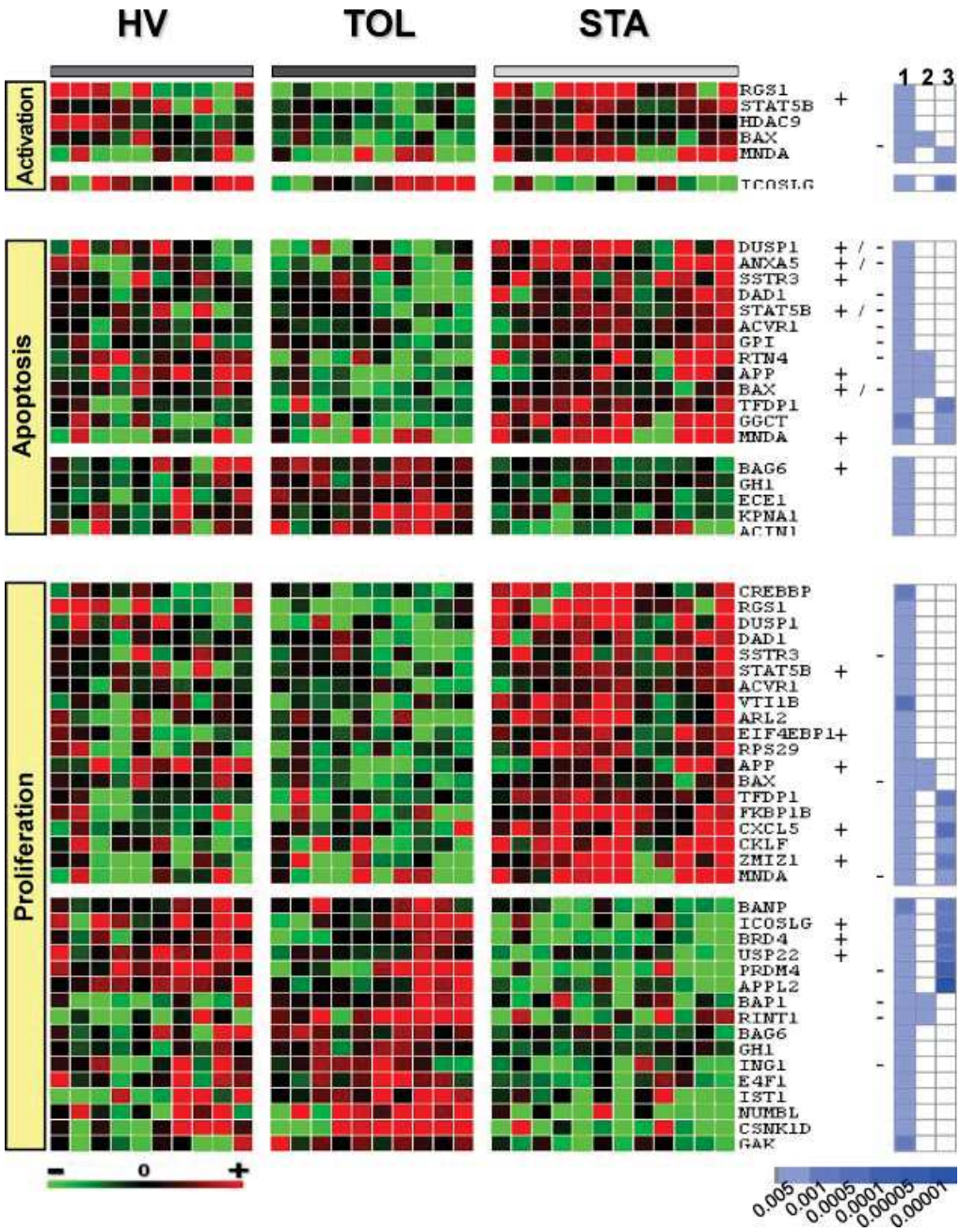

Figure 7: Gene expression of the genes related to B cell activation, apoptosis and proliferation from the tolerant gene signature. Expression values across HV, TOL and STA are presented in heatmaps. Each cell in the matrix corresponds to the expression level of a gene in a sample, with red for over-expression, 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

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*.

References

1. Jovanovic V, Lair D, Soulillou J-P, Brouard S. Transfer of tolerance to heart and kidney allografts in the rat model. Transpl Int 2008; 21: 199–206.
2. Kawai T, Cosimi AB, Spitzer TR, et al. HLA-mismatched renal transplantation without maintenance immunosuppression. N Engl J Med 2008; 358: 353–361.
3. Poirier N, Blancho G, Vanhove B. Alternatives to calcineurin inhibition in renal transplantation: Belatacept, the first co-stimulation blocker. Immunotherapy 2010; 2: 625–636.
4. Leonard DA, Cetrulo CL Jr, McGrouther DA, Sachs DH. Induction of tolerance of vascularized composite allografts. Transplantation 2013; 95: 403–409.

5. Leventhal J, Abecassis M, Miller J, et al. Tolerance induction in HLA disparate living donor kidney transplantation by donor stem cell infusion: Durable chimerism predicts outcome. Transplantation 2013; 95: 169–176.
6. Roussey-Kesler G, Giral M, Moreau A, et al. Clinical operational tolerance after kidney transplantation. Am J Transplant 2006; 6: 736–746.
7. Brouard S, Pallier A, Renaudin K, et al. The natural history of clinical operational tolerance after kidney transplantation through twenty-seven cases. Am J Transplant 2012; 12: 3296–3307.
8. Louis S, Braudeau C, Giral M, et al. Contrasting $CD25^{hi}CD4^{+}$ T cells/FOXP3 patterns in chronic rejection and operational drug-free tolerance. Transplantation 2006; 81: 398–407.
9. Brouard S, Mansfield E, Braud C, et al. Identification of a peripheral blood transcriptional biomarker panel associated with operational renal allograft tolerance. Proc Natl Acad Sci USA 2007; 104: 15448–15453.
10. Pallier A, Hillion S, Danger R, et al. Patients with drug-free long-term graft function display increased numbers of peripheral B cells with a memory and inhibitory phenotype. Kidney Int 2010; 78: 503–513.
11. Newell KA, Asare A, Kirk AD, et al. Identification of a B cell signature associated with renal transplant tolerance in humans. J Clin Invest 2010; 120: 1836–1847.
12. Sagoo P, Perucha E, Sawitzki B, et al. Development of a cross-platform biomarker signature to detect renal transplant tolerance in humans. J Clin Invest 2010; 120: 1848–1861.
13. Gallou SL, Caron G, Delaloy C, Rossille D, Tarte K, Fest T. IL-2 requirement for human plasma cell generation: Coupling differentiation and proliferation by enhancing MAPK–ERK signaling. J Immunol 2012; 189: 161–173.
14. Perfetto SP, Chattopadhyay PK, Roederer M. Seventeen-colour flow cytometry: Unravelling the immune system. Nat Rev Immunol 2004; 4: 648–655.
15. Lamirault G, Meur NL, Roussel J-C, et al. Molecular risk stratification in advanced heart failure patients. J Cell Mol Med 2010; 14: 1443–1452.
16. Baron D, Bihouée A, Teusan R, et al. MADGene: Retrieval and processing of gene identifier lists for the analysis of heterogeneous microarray datasets. Bioinformatics 2011; 27: 725–726.
17. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA 1998; 95: 14863–14868.
18. Zeeberg BR, Feng W, Wang G, et al. GoMiner: A resource for biological interpretation of genomic and proteomic data. Genome Biol 2003; 4: R28.
19. Lozano JJ, Pallier A, Martinez-Llordella M, et al. Comparison of transcriptional and blood cell-phenotypic markers between operationally tolerant liver and kidney recipients. Am J Transplant 2011; 11: 1916–1926.
20. Eynon EE, Parker DC. Small B cells as antigen-presenting cells in the induction of tolerance to soluble protein antigens. J Exp Med 1992; 175: 131–138.
21. Chung JB, Wells AD, Adler S, Jacob A, Turka LA, Monroe JG. Incomplete activation of CD4 T cells by antigen-presenting transitional immature B cells: Implications for peripheral B and T cell responsiveness. J Immunol 2003; 171: 1758–1767.
22. Chen X, Jensen PE. Cutting edge: Primary B lymphocytes preferentially expand allogeneic FoxP3+ CD4 T cells. J Immunol 2007; 179: 2046–2050.
23. Reichardt P, Dornbach B, Rong S, et al. Naive B cells generate regulatory T cells in the presence of a mature immunologic synapse. Blood 2007; 110: 1519–1529.
24. Fuchs EJ, Matzinger P. B cells turn off virgin but not memory T cells. Science 1992; 258: 1156–1159.
25. Blair PA, Chavez-Rueda KA, Evans JG, et al. Selective targeting of B cells with agonistic anti-CD40 is an efficacious strategy for the generation of induced regulatory T2-like B cells and for the suppression of lupus in MRL/lpr mice. J Immunol 2009; 182: 3492–3502.
26. Lemoine S, Morva A, Youinou P, Jamin C. Human T cells induce their own regulation through activation of B cells. J Autoimmun 2011; 36: 228–238.
27. Jourdan M, Caraux A, Caron G, et al. Characterization of a transitional preplasmablast population in the process of human B cell to plasma cell differentiation. J Immunol 2011; 187: 3931–3941.
28. Huggins J, Pellegrin T, Felgar RE, et al. CpG DNA activation and plasma-cell differentiation of CD27− naive human B cells. Blood 2007; 109: 1611–1619.
29. Choe J, Kim HS, Zhang X, Armitage RJ, Choi YS. Cellular and molecular factors that regulate the differentiation and apoptosis of germinal center B cells. Anti-Ig down-regulates Fas expression of CD40 ligand-stimulated germinal center B cells and inhibits Fas-mediated apoptosis. J Immunol 1996; 157: 1006–1016.
30. Heidt S, Hester J, Shankar S, Friend PJ, Wood KJ. B cell repopulation after alemtuzumab induction-transient increase in transitional B cells and long-term dominance of naïve B cells. Am J Transplant 2012; 12: 1784–1792.
31. Knechtle SJ, Pascual J, Bloom DD, et al. Early and limited use of tacrolimus to avoid rejection in an alemtuzumab and sirolimus regimen for kidney transplantation: Clinical results and immune monitoring. Am J Transplant 2009; 9: 1087–1098.
32. Pasare C, Medzhitov R. Control of B-cell responses by toll-like receptors. Nature 2005; 438: 364–368.
33. Barr TA, Shen P, Brown S, et al. B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells. J Exp Med 2012; 209: 1001–1010.
34. Lampropoulou V, Hoehlig K, Roch T, et al. TLR-activated B cells suppress T cell-mediated autoimmunity. J Immunol 2008; 180: 4763–4773.
35. Kaminski DA, Wei C, Qian Y, Rosenberg AF, Sanz I. Advances in human B cell phenotypic profiling. Front Immunol 2012; 3: 302.
36. Matz M, Lehnert M, Lorkowski C, et al. Effects of sotrastaurin, mycophenolic acid and everolimus on human B-lymphocyte function and activation. Transpl Int 2012; 25: 1106–1116.
37. Fuleihan R, Ramesh N, Horner A, et al. Cyclosporin A inhibits CD40 ligand expression in T lymphocytes. J Clin Invest 1994; 93: 1315–1320.
38. Liu C, Noorchashm H, Sutter JA, et al. B lymphocyte-directed immunotherapy promotes long-term islet allograft survival in nonhuman primates. Nat Med 2007; 13: 1295–1298.
39. Haynes LD, Jankowska-Gan E, Sheka A, et al. Donor-specific indirect pathway analysis reveals a B-cell-independent signature which reflects outcomes in kidney transplant recipients. Am J Transplant 2012; 12: 640–648.
40. Baeten D, Louis S, Braud C, et al. Phenotypically and functionally distinct CD8+ lymphocyte populations in long-term drug-free tolerance and chronic rejection in human kidney graft recipients. J Am Soc Nephrol 2006; 17: 294–304.
41. Coelho V, Saitovitch D, Kalil J, Silva HM. Rethinking the multiple roles of B cells in organ transplantation. Curr Opin Organ Transplant 2013; 18: 13–21.
42. Silva HM, Takenaka MCS, Moraes-Vieira PMM, et al. Preserving the B-cell compartment favors operational tolerance in human renal transplantation. Mol Med 2012; 18: 733–743.

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 ($^*p < 0.05$, $^{**}p < 0.01$).

Bibliographie

Acosta-Rodriguez, E.V., G. Napolitani, A. Lanzavecchia, and F. Sallusto. 2007. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 8:942-949.

Akdis, M., J. Verhagen, A. Taylor, F. Karamloo, C. Karagiannidis, R. Crameri, S. Thunberg, G. Deniz, R. Valenta, H. Fiebig, C. Kegel, R. Disch, C.B. Schmidt-Weber, K. Blaser, and C.A. Akdis. 2004. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med* 199:1567-1575.

Al-Ramli, W., D. Prefontaine, F. Chouiali, J.G. Martin, R. Olivenstein, C. Lemiere, and Q. Hamid. 2009. T(H)17-associated cytokines (IL-17A and IL-17F) in severe asthma. *J Allergy Clin Immunol* 123:1185-1187.

Amu, S., S.P. Saunders, M. Kronenberg, N.E. Mangan, A. Atzberger, and P.G. Fallon. 2010. Regulatory B cells prevent and reverse allergic airway inflammation via FoxP3-positive T regulatory cells in a murine model. *J Allergy Clin Immunol* 125:1114-1124 e1118.

Arnon, T.I., R.M. Horton, I.L. Grigorova, and J.G. Cyster. 2013. Visualization of splenic marginal zone B-cell shuttling and follicular B-cell egress. *Nature* 493:684-688.

Baay-Guzman, G.J., S. Huerta-Yepez, M.I. Vega, D. Aguilar-Leon, M. Campillos, J. Blake, V. Benes, R. Hernandez-Pando, and L.M. Teran. 2012. Role of CXCL13 in asthma: novel therapeutic target. *Chest* 141:886-894.

Baeten, D., X. Baraliakos, J. Braun, J. Sieper, P. Emery, D. van der Heijde, I. McInnes, J.M. van Laar, R. Landewe, P. Wordsworth, J. Wollenhaupt, H. Kellner, J. Paramarta, J. Wei, A. Brachat, S. Bek, D. Laurent, Y. Li, Y.A. Wang, A.P. Bertolino, S. Gsteiger, A.M. Wright, and W. Hueber. 2013. Anti-interleukin-17A monoclonal antibody secukinumab in treatment of ankylosing spondylitis: a randomised, double-blind, placebo-controlled trial. *Lancet* 382:1705-1713.

Baeten, D.L., and V.K. Kuchroo. 2013. How Cytokine networks fuel inflammation: Interleukin-17 and a tale of two autoimmune diseases. *Nat Med* 19:824-825.

Baines, K.J., J.L. Simpson, N.A. Bowden, R.J. Scott, and P.G. Gibson. 2010. Differential gene expression and cytokine production from neutrophils in asthma phenotypes. *Eur Respir J* 35:522-531.

Baines, K.J., J.L. Simpson, L.G. Wood, R.J. Scott, N.L. Fibbens, H. Powell, D.C. Cowan, D.R. Taylor, J.O. Cowan, and P.G. Gibson. 2014. Sputum gene expression signature of 6 biomarkers discriminates asthma inflammatory phenotypes. *J Allergy Clin Immunol* 133:997-1007.

Bao, Y., and X. Cao. 2014. The immune potential and immunopathology of cytokine-producing B cell subsets: A comprehensive review. *J Autoimmun* 1-14.

Bao, Y., X. Liu, C. Han, S. Xu, B. Xie, Q. Zhang, Y. Gu, J. Hou, L. Qian, C. Qian, H. Han, and X. Cao. 2014. Identification of IFN-gamma-producing innate B cells. *Cell Res* 24:161-176.

Bar-Or, A., L. Fawaz, B. Fan, P.J. Darlington, A. Rieger, C. Ghorayeb, P.A. Calabresi, E. Waubant, S.L. Hauser, J. Zhang, and C.H. Smith. 2010. Abnormal B-cell cytokine responses a trigger of T-cell-mediated disease in MS? *Ann Neurol* 67:452-461.

Barr, T.A., P. Shen, S. Brown, V. Lampropoulou, T. Roch, S. Lawrie, B. Fan, R.A. O'Connor, S.M. Anderton, A. Bar-Or, S. Fillatreau, and D. Gray. 2012. B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells. *J Exp Med* 209:1001-1010.

Batten, M., J. Groom, T.G. Cachero, F. Qian, P. Schneider, J. Tschopp, J.L. Browning, and F. Mackay. 2000. BAFF mediates survival of peripheral immature B lymphocytes. *J Exp Med* 192:1453-1466.

Baumgarth, N. 2011. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol* 11:34-46.

Bisgaard, H., K. Bonnelykke, P.M. Sleiman, M. Brasholt, B. Chawes, E. Kreiner-Moller, M. Stage, C. Kim, R. Tavendale, F. Baty, C.B. Pipper, C.N. Palmer, and H. Hakonarsson. 2009. Chromosome 17q21 gene variants are associated with asthma and exacerbations but not atopy in early childhood. *Am J Respir Crit Care Med* 179:179-185.

Blair, P.A., L.Y. Norena, F. Flores-Borja, D.J. Rawlings, D.A. Isenberg, M.R. Ehrenstein, and C. Mauri. 2010. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. *Immunity* 32:129-140.

Bracke, K.R., F.M. Verhamme, L.J. Seys, C. Bantsimba-Malanda, D.M. Cunoosamy, R. Herbst, H. Hammad, B.N. Lambrecht, G.F. Joos, and G.G. Brusselle. 2013. Role of CXCL13 in cigarette smoke-induced lymphoid follicle formation and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 188:343-355.

Brandt, E.B., M.B. Kovacic, G.B. Lee, A.M. Gibson, T.H. Acciani, T.D. Le Cras, P.H. Ryan, A.L. Budelsky, and G.K. Khurana Hershey. 2013. Diesel exhaust particle induction of IL-17A contributes to severe asthma. *J Allergy Clin Immunol* 132:1194-1204 e1192.

Braza, F., J. Chesne, S. Castagnet, A. Magnan, and S. Brouard. 2014. Regulatory functions of B cells in allergic diseases. *Allergy*

Bullens, D.M., E. Truyen, L. Coteur, E. Dilissen, P.W. Hellings, L.J. Dupont, and J.L. Ceuppens. 2006. IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? *Respir Res* 7:135.

Burgler, S., N. Ouaked, C. Bassin, T.M. Basinski, P.Y. Mantel, K. Siegmund, N. Meyer, C.A. Akdis, and C.B. Schmidt-Weber. 2009. Differentiation and functional analysis of human T(H)17 cells. *J Allergy Clin Immunol* 123:588-595, 595 e581-587.

Candando, K.M., J.M. Lykken, and T.F. Tedder. 2014. B10 cell regulation of health and disease. *Immunol Rev* 259:259-272.

Carter, N.A., R. Vasconcellos, E.C. Rosser, C. Tulone, A. Munoz-Suano, M. Kamanaka, M.R. Ehrenstein, R.A. Flavell, and C. Mauri. 2011. Mice lacking endogenous IL-10-producing regulatory B cells develop exacerbated disease and present with an increased frequency of Th1/Th17 but a decrease in regulatory T cells. *Journal of immunology* 186:5569-5579.

Cherukuri, A., D.M. Rothstein, B. Clark, C.R. Carter, A. Davison, M. Hernandez-Fuentes, E. Hewitt, A.D. Salama, and R.J. Baker. 2014. Immunologic Human Renal Allograft Injury Associates with an Altered IL-10/TNF-alpha Expression Ratio in Regulatory B Cells. *Journal of the American Society of Nephrology : JASN*

Chesne, J., F. Braza, G. Mahay, S. Brouard, M. Aronica, and A. Magnan. 2014. IL-17 in Severe Asthma: Where Do We Stand? *Am J Respir Crit Care Med*

Chesneau, M., L. Michel, N. Degauque, and S. Brouard. 2013. Regulatory B cells and tolerance in transplantation: from animal models to human. *Front Immunol* 4:497.

Chung, J.B., M. Silverman, and J.G. Monroe. 2003. Transitional B cells: step by step towards immune competence. *Trends Immunol* 24:343-349.

Correale, J., M. Farez, and G. Razzitte. 2008. Helminth infections associated with multiple sclerosis induce regulatory B cells. *Ann Neurol* 64:187-199.

Corren, J., R.F. Lemanske, N.A. Hanania, P.E. Korenblat, M.V. Parsey, J.R. Arron, J.M. Harris, H. Scheerens, L.C. Wu, Z. Su, S. Mosesova, M.D. Eisner, S.P. Bohen, and J.G. Matthews. 2011. Lebrikizumab treatment in adults with asthma. *N Engl J Med* 365:1088-1098.

Cosmi, L., L. Maggi, V. Santarlasci, M. Capone, E. Cardilicchia, F. Frosali, V. Querci, R. Angeli, A. Matucci, M. Fambrini, F. Liotta, P. Parronchi, E. Maggi, S. Romagnani, and F. Annunziato. 2010. Identification of a novel subset of human circulating memory CD4(+) T cells that produce both IL-17A and IL-4. *J Allergy Clin Immunol* 125:222-230 e221-224.

Dalwadi, H., B. Wei, M. Schrage, K. Spicher, T.T. Su, L. Birnbaumer, D.J. Rawlings, and J. Braun. 2003. B cell developmental requirement for the G alpha i2 gene. *Journal of immunology* 170:1707-1715.

Danger, R., F. Braza, M. Giral, J.P. Soulillou, and S. Brouard. 2014. MicroRNAs, Major Players in B Cells Homeostasis and Function. *Front Immunol* 5:98.

Das, A., G. Ellis, C. Pallant, A.R. Lopes, P. Khanna, D. Peppa, A. Chen, P. Blair, G. Dusheiko, U. Gill, P.T. Kennedy, M. Brunetto, P. Lampertico, C. Mauri, and M.K. Maini. 2012. IL-10-producing regulatory B cells in the pathogenesis of chronic hepatitis B virus infection. *Journal of immunology* 189:3925-3935.

Dass, S., E.M. Vital, and P. Emery. 2007. Development of psoriasis after B cell depletion with rituximab. *Arthritis Rheum* 56:2715-2718.

De Togni, P., J. Goellner, N.H. Ruddle, P.R. Streeter, A. Fick, S. Mariathasan, S.C. Smith, R. Carlson, L.P. Shornick, J. Strauss-Schoenberger, and et al. 1994. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science* 264:703-707.

DeFuria, J., A.C. Belkina, M. Jagannathan-Bogdan, J. Snyder-Cappione, J.D. Carr, Y.R. Nersesova, D. Markham, K.J. Strissel, A.A. Watkins, M. Zhu, J. Allen, J. Bouchard, G. Toraldo, R. Jasuja, M.S. Obin, M.E. McDonnell, C. Apovian, G.V. Denis, and B.S. Nikolajczyk. 2013. B cells promote inflammation in obesity and type 2 diabetes through regulation of T-cell function and an inflammatory cytokine profile. *Proc Natl Acad Sci U S A* 110:5133-5138.

DiLillo, D.J., J.B. Weinberg, A. Yoshizaki, M. Horikawa, J.M. Bryant, Y. Iwata, T. Matsushita, K.M. Matta, Y. Chen, G.M. Venturi, G. Russo, J.P. Gockerman, J.O. Moore, L.F. Diehl, A.D. Volkheimer, D.R. Friedman, M.C. Lanasa, R.P. Hall, and T.F. Tedder. 2013. Chronic lymphocytic leukemia and regulatory B cells share IL-10 competence and immunosuppressive function. *Leukemia* 27:170-182.

Ding, Q., M. Yeung, G. Camirand, Q. Zeng, H. Akiba, H. Yagita, G. Chalasani, M.H. Sayegh, N. Najafian, and D.M. Rothstein. 2011. Regulatory B cells are identified by expression of TIM-1 and can be induced through TIM-1 ligation to promote tolerance in mice. *The Journal of clinical investigation* 121:3645-3656.

Doe, C., M. Bafadhel, S. Siddiqui, D. Desai, V. Mistry, P. Rugman, M. McCormick, J. Woods, R. May, M.A. Sleeman, I.K. Anderson, and C.E. Brightling. 2010. Expression of the T helper 17-associated cytokines IL-17A and IL-17F in asthma and COPD. *Chest* 138:1140-1147.

Duddy, M., M. Niino, F. Adatia, S. Hebert, M. Freedman, H. Atkins, H.J. Kim, and A. Bar-Or. 2007. Distinct effector cytokine profiles of memory and naive human B cell subsets and implication in multiple sclerosis. *Journal of immunology* 178:6092-6099.

Erle, D.J., and D. Sheppard. 2014. The cell biology of asthma. *J Cell Biol* 205:621-631.

Evans, J.G., K.A. Chavez-Rueda, A. Eddaoudi, A. Meyer-Bahlburg, D.J. Rawlings, M.R. Ehrenstein, and C. Mauri. 2007. Novel suppressive function of transitional 2 B cells in experimental arthritis. *Journal of immunology* 178:7868-7878.

Fanta, C.H. 2009. Asthma. *N Engl J Med* 360:1002-1014.

Fillatreau, S., C.H. Sweenie, M.J. McGeachy, D. Gray, and S.M. Anderton. 2002. B cells regulate autoimmunity by provision of IL-10. *Nat Immunol* 3:944-950.

Fleige, H., S. Ravens, G.L. Moschovakis, J. Bolter, S. Willenzon, G. Sutter, S. Haussler, U. Kalinke, I. Prinz, and R. Forster. 2014. IL-17-induced CXCL12 recruits B cells and induces follicle formation in BALT in the absence of differentiated FDCs. *J Exp Med* 211:643-651.

Flood-Page, P., C. Swenson, I. Faiferman, J. Matthews, M. Williams, L. Brannick, D. Robinson, S. Wenzel, W. Busse, T.T. Hansel, N.C. Barnes, and G. International Mepolizumab Study. 2007. A study to evaluate safety and efficacy of mepolizumab in patients with moderate persistent asthma. *Am J Respir Crit Care Med* 176:1062-1071.

Flores-Borja, F., A. Bosma, D. Ng, V. Reddy, M.R. Ehrenstein, D.A. Isenberg, and C. Mauri. 2013. CD19+CD24hiCD38hi B cells maintain regulatory T cells while limiting TH1 and TH17 differentiation. *Sci Transl Med* 5:173ra123.

Fu, Y.X., G. Huang, Y. Wang, and D.D. Chaplin. 1998. B lymphocytes induce the formation of follicular dendritic cell clusters in a lymphotoxin alpha-dependent fashion. *J Exp Med* 187:1009-1018.

Fuchs, E.J., and P. Matzinger. 1992. B cells turn off virgin but not memory T cells. *Science* 258:1156-1159.

Galli, S.J., and M. Tsai. 2012. IgE and mast cells in allergic disease. *Nat Med* 18:693-704.

Gao, N., J. Dresel, V. Eckstein, R. Gellert, H. Storch, R.K. Venigalla, V. Schwenger, R. Max, N. Blank, H.M. Lorenz, and T. Tretter. 2014. Impaired suppressive capacity of activation-induced regulatory B cells in Systemic Lupus Erythematosus. *Arthritis Rheumatol* n/a-n/a.

GeurtsvanKessel, C.H., M.A. Willart, I.M. Bergen, L.S. van Rijt, F. Muskens, D. Elewaut, A.D. Osterhaus, R. Hendriks, G.F. Rimmelzwaan, and B.N. Lambrecht. 2009. Dendritic cells are crucial for maintenance of tertiary lymphoid structures in the lung of influenza virus-infected mice. *J Exp Med* 206:2339-2349.

Godin, I.E., J.A. Garcia-Porrero, A. Coutinho, F. Dieterlen-Lievre, and M.A. Marcos. 1993. Para-aortic splanchnopleura from early mouse embryos contains B1a cell progenitors. *Nature* 364:67-70.

Goetz, M., R. Atreya, M. Ghalibafian, P.R. Galle, and M.F. Neurath. 2007. Exacerbation of ulcerative colitis after rituximab salvage therapy. *Inflamm Bowel Dis* 13:1365-1368.

Green, R.H., C.E. Brightling, G. Woltmann, D. Parker, A.J. Wardlaw, and I.D. Pavord. 2002. Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. *Thorax* 57:875-879.

Grunig, G., M. Warnock, A.E. Wakil, R. Venkayya, F. Brombacher, D.M. Rennick, D. Sheppard, M. Mohrs, D.D. Donaldson, R.M. Locksley, and D.B. Corry. 1998. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 282:2261-2263.

Gupta, A., S. Dimeloe, D.F. Richards, E.S. Chambers, C. Black, Z. Urry, K. Ryanna, E. Xystrakis, A. Bush, S. Saglani, and C.M. Hawrylowicz. 2014. Defective IL-10 expression and in vitro steroid-induced IL-17A in paediatric severe therapy-resistant asthma. *Thorax* 69:508-515.

Haldar, P., C.E. Brightling, B. Hargadon, S. Gupta, W. Monteiro, A. Sousa, R.P. Marshall, P. Bradding, R.H. Green, A.J. Wardlaw, and I.D. Pavord. 2009. Mepolizumab and exacerbations of refractory eosinophilic asthma. *N Engl J Med* 360:973-984.

Haldar, P., I.D. Pavord, D.E. Shaw, M.A. Berry, M. Thomas, C.E. Brightling, A.J. Wardlaw, and R.H. Green. 2008. Cluster analysis and clinical asthma phenotypes. *Am J Respir Crit Care Med* 178:218-224.

Halim, T.Y., C.A. Steer, L. Matha, M.J. Gold, I. Martinez-Gonzalez, K.M. McNagny, A.N. McKenzie, and F. Takei. 2014. Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. *Immunity* 40:425-435.

Halle, S., H.C. Dujardin, N. Bakocevic, H. Fleige, H. Danzer, S. Willenzon, Y. Suezer, G. Hammerling, N. Garbi, G. Sutter, T. Worbs, and R. Forster. 2009. Induced bronchus-associated lymphoid tissue serves as a general priming site for T cells and is maintained by dendritic cells. *J Exp Med* 206:2593-2601.

Hamilton, J.A. 2008. Colony-stimulating factors in inflammation and autoimmunity. *Nat Rev Immunol* 8:533-544.

Hardy, R.R., and K. Hayakawa. 1991. A developmental switch in B lymphopoiesis. *Proc Natl Acad Sci U S A* 88:11550-11554.

Harris, D.P., S. Goodrich, A.J. Gerth, S.L. Peng, and F.E. Lund. 2005a. Regulation of IFN-gamma production by B effector 1 cells: essential roles for T-bet and the IFN-gamma receptor. *Journal of immunology* 174:6781-6790.

Harris, D.P., S. Goodrich, K. Mohrs, M. Mohrs, and F.E. Lund. 2005b. Cutting edge: the development of IL-4-producing B cells (B effector 2 cells) is controlled by IL-4, IL-4 receptor alpha, and Th2 cells. *Journal of immunology* 175:7103-7107.

Harris, D.P., L. Haynes, P.C. Sayles, D.K. Duso, S.M. Eaton, N.M. Lepak, L.L. Johnson, S.L. Swain, and F.E. Lund. 2000. Reciprocal regulation of polarized cytokine production by effector B and T cells. *Nat Immunol* 1:475-482.

Hauser, S.L., E. Waubant, D.L. Arnold, T. Vollmer, J. Antel, R.J. Fox, A. Bar-Or, M. Panzara, N. Sarkar, S. Agarwal, A. Langer-Gould, C.H. Smith, and H.T. Group. 2008. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. *N Engl J Med* 358:676-688.

Haynes, L.D., E. Jankowska-Gan, A. Sheka, M.R. Keller, M.P. Hernandez-Fuentes, R.I. Lechler, V. Seyfert-Margolis, L.A. Turka, K.A. Newell, and W.J. Burlingham. 2012. Donor-specific indirect pathway analysis reveals a B-cell-independent signature which reflects outcomes in kidney transplant recipients. *Am J Transplant* 12:640-648.

He, R., H.Y. Kim, J. Yoon, M.K. Oyoshi, A. MacGinnitie, S. Goya, E.J. Freyschmidt, P. Bryce, A.N. McKenzie, D.T. Umetsu, H.C. Oettgen, and R.S. Geha. 2009. Exaggerated IL-17 response to epicutaneous sensitization mediates airway inflammation in the absence of IL-4 and IL-13. *J Allergy Clin Immunol* 124:761-770 e761.

He, R., M.K. Oyoshi, H. Jin, and R.S. Geha. 2007. Epicutaneous antigen exposure induces a Th17 response that drives airway inflammation after inhalation challenge. *Proc Natl Acad Sci U S A* 104:15817-15822.

Holan, V., A. Zajicova, E. Javorkova, P. Trosan, M. Chudickova, M. Pavlikova, and M. Krulova. 2014. Distinct cytokines balance the development of regulatory T cells and interleukin-10-producing regulatory B cells. *Immunology* 141:577-586.

Holt, P.G., D.H. Strickland, M.E. Wikstrom, and F.L. Jahnsen. 2008. Regulation of immunological homeostasis in the respiratory tract. *Nat Rev Immunol* 8:142-152.

Irvin, C., I. Zafar, J. Good, D. Rollins, C. Christianson, M.M. Gorska, R.J. Martin, and R. Alam. 2014. Increased frequency of dual-positive T2/T17 cells in bronchoalveolar lavage fluid characterizes a population of patients with severe asthma. *J Allergy Clin Immunol*

Isenberg, D., C. Gordon, D. Licu, S. Copt, C.P. Rossi, and D. Wofsy. 2014. Efficacy and safety of atacicept for prevention of flares in patients with moderate-to-severe systemic lupus erythematosus (SLE): 52-week data (APRIL-SLE randomised trial). *Ann Rheum Dis* annrheumdis-2013-205067.

Iwata, Y., T. Matsushita, M. Horikawa, D.J. Dilillo, K. Yanaba, G.M. Venturi, P.M. Szabolcs, S.H. Bernstein, C.M. Magro, A.D. Williams, R.P. Hall, E.W. St Clair, and T.F. Tedder. 2011. Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. *Blood* 117:530-541.

Jia, G., R.W. Erickson, D.F. Choy, S. Mosesova, L.C. Wu, O.D. Solberg, A. Shikotra, R. Carter, S. Audusseau, Q. Hamid, P. Bradding, J.V. Fahy, P.G. Woodruff, J.M. Harris, J.R. Arron, and G. Bronchoscopic Exploratory Research Study of Biomarkers in Corticosteroid-refractory Asthma Study. 2012. Periostin is a systemic biomarker of eosinophilic airway inflammation in asthmatic patients. *J Allergy Clin Immunol* 130:647-654 e610.

Jin, G., Y. Hamaguchi, T. Matsushita, M. Hasegawa, D. Le Huu, N. Ishiura, K. Naka, A. Hirao, K. Takehara, and M. Fujimoto. 2013. B-cell linker protein expression contributes to controlling allergic and autoimmune diseases by mediating IL-10 production in regulatory B cells. *J Allergy Clin Immunol* 131:1674-1682.

Kappos, L., H.P. Hartung, M.S. Freedman, A. Boyko, E.W. Radu, D.D. Mikol, M. Lamarine, Y. Hyvert, U. Freudensprung, T. Plitz, J. van Beek, and A.S. Group. 2014. Atacicept in multiple sclerosis (ATAMS): a randomised, placebo-controlled, double-blind, phase 2 trial. *Lancet Neurol* 13:353-363.

Kappos, L., D. Li, P.A. Calabresi, P. O'Connor, A. Bar-Or, F. Barkhof, M. Yin, D. Leppert, R. Glanzman, J. Tinbergen, and S.L. Hauser. 2011. Ocrelizumab in relapsing-remitting multiple sclerosis: a phase 2, randomised, placebo-controlled, multicentre trial. *Lancet* 378:1779-1787.

Kato, A., K.E. Hulse, B.K. Tan, and R.P. Schleimer. 2013. B-lymphocyte lineage cells and the respiratory system. *J Allergy Clin Immunol* 131:933-957; quiz 958.

Katz, S.I., D. Parker, and J.L. Turk. 1974. B-cell suppression of delayed hypersensitivity reactions. *Nature* 251:550-551.

Kim, B.S., M.C. Siracusa, S.A. Saenz, M. Noti, L.A. Monticelli, G.F. Sonnenberg, M.R. Hepworth, A.S. Van Voorhees, M.R. Comeau, and D. Artis. 2013. TSLP elicits IL-33-independent innate lymphoid cell responses to promote skin inflammation. *Sci Transl Med* 5:170ra116.

Kim, H.Y., H.J. Lee, Y.J. Chang, M. Pichavant, S.A. Shore, K.A. Fitzgerald, Y. Iwakura, E. Israel, K. Bolger, J. Faul, R.H. DeKruyff, and D.T. Umetsu. 2014. Interleukin-17-producing innate lymphoid cells and the NLRP3 inflammasome facilitate obesity-associated airway hyperreactivity. *Nat Med* 20:54-61.

Kudo, M., S.M. Khalifeh Soltani, S.A. Sakuma, W. McKleroy, T.H. Lee, P.G. Woodruff, J.W. Lee, K. Huang, C. Chen, M. Arjomandi, X. Huang, and K. Atabai. 2013. Mfge8 suppresses airway hyperresponsiveness in asthma by regulating smooth muscle contraction. *Proc Natl Acad Sci U S A* 110:660-665.

Kuperman, D.A., X. Huang, L.L. Koth, G.H. Chang, G.M. Dolganov, Z. Zhu, J.A. Elias, D. Sheppard, and D.J. Erle. 2002. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat Med* 8:885-889.

Lambrecht, B.N., and H. Hammad. 2012. The airway epithelium in asthma. *Nat Med* 18:684-692.

Lampropoulou, V., K. Hoehlig, T. Roch, P. Neves, E. Calderon Gomez, C.H. Sweenie, Y. Hao, A.A. Freitas, U. Steinhoff, S.M. Anderton, and S. Fillatreau. 2008. TLR-activated B cells suppress T cell-mediated autoimmunity. *Journal of immunology* 180:4763-4773.

Lassila, O., O. Vainio, and P. Matzinger. 1988. Can B cells turn on virgin T cells? *Nature* 334:253-255.

Leckie, M.J., A. ten Brinke, J. Khan, Z. Diamant, B.J. O'Connor, C.M. Walls, A.K. Mathur, H.C. Cowley, K.F. Chung, R. Djukanovic, T.T. Hansel, S.T. Holgate, P.J. Sterk, and P.J. Barnes.

2000. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* 356:2144-2148.
Lemoine, S., A. Morva, P. Youinou, and C. Jamin. 2011. Human T cells induce their own regulation through activation of B cells. *J Autoimmun* 36:228-238.
Leon, B., A. Ballesteros-Tato, J.L. Browning, R. Dunn, T.D. Randall, and F.E. Lund. 2012. Regulation of T(H)2 development by CXCR5+ dendritic cells and lymphotoxin-expressing B cells. *Nat Immunol* 13:681-690.
Leon, B., A. Ballesteros-Tato, and F.E. Lund. 2014. Dendritic Cells and B Cells: Unexpected Partners in Th2 Development. *Journal of immunology* 193:1531-1537.
Li, X., H. Zhong, W. Bao, N. Boulad, J. Evangelista, M.A. Haider, J. Bussel, and K. Yazdanbakhsh. 2012. Defective regulatory B-cell compartment in patients with immune thrombocytopenia. *Blood* 120:3318-3325.
Lin, W., L. Jin, H. Chen, Q. Wu, Y. Fei, W. Zheng, Q. Wang, P. Li, Y. Li, W. Zhang, Y. Zhao, X. Zeng, and F. Zhang. 2014. B cell subsets and dysfunction of regulatory B cells in IgG4-related diseases and primary Sjogren's syndrome: the similarities and differences. *Arthritis Res Ther* 16:R118.
Lindell, D.M., A.A. Berlin, M.A. Schaller, and N.W. Lukacs. 2008. B cell antigen presentation promotes Th2 responses and immunopathology during chronic allergic lung disease. *PLoS One* 3:e3129.
Lindner, S., K. Dahlke, K. Sontheimer, M. Hagn, C. Kaltenmeier, T.F. Barth, T. Beyer, F. Reister, D. Fabricius, R. Lotfi, O. Lunov, G.U. Nienhaus, T. Simmet, R. Kreienberg, P. Moller, H. Schrezenmeier, and B. Jahrsdorfer. 2013. Interleukin 21-induced granzyme B-expressing B cells infiltrate tumors and regulate T cells. *Cancer Res* 73:2468-2479.
Linton, P.J., B. Bautista, E. Biederman, E.S. Bradley, J. Harbertson, R.M. Kondrack, R.C. Padrick, and L.M. Bradley. 2003. Costimulation via OX40L expressed by B cells is sufficient to determine the extent of primary CD4 cell expansion and Th2 cytokine secretion in vivo. *J Exp Med* 197:875-883.
Litsiou, E., M. Semitekolou, I.E. Galani, I. Morianos, A. Tsoutsa, P. Kara, D. Rontogianni, I. Bellenis, M. Konstantinou, K. Potaris, E. Andreakos, P. Sideras, S. Zakynthinos, and M. Tsoumakidou. 2013. CXCL13 production in B cells via Toll-like receptor/lymphotoxin receptor signaling is involved in lymphoid neogenesis in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 187:1194-1202.
Liu, Z.Q., Y. Wu, J.P. Song, X. Liu, Z. Liu, P.Y. Zheng, and P.C. Yang. 2013. Tolerogenic CX3CR1+ B cells suppress food allergy-induced intestinal inflammation in mice. *Allergy* 68:1241-1248.
Lloyd, C.M., and C.M. Hawrylowicz. 2009. Regulatory T cells in asthma. *Immunity* 31:438-449.
Lund, F.E., and T.D. Randall. 2010. Effector and regulatory B cells: modulators of CD4+ T cell immunity. *Nat Rev Immunol* 10:236-247.
Lundy, S.K., and D.L. Boros. 2002. Fas ligand-expressing B-1a lymphocytes mediate CD4(+)-T-cell apoptosis during schistosomal infection: induction by interleukin 4 (IL-4) and IL-10. *Infection and immunity* 70:812-819.
Madan, R., F. Demircik, S. Surianarayanan, J.L. Allen, S. Divanovic, A. Trompette, N. Yogev, Y. Gu, M. Khodoun, D. Hildeman, N. Boespflug, M.B. Fogolin, L. Grobe, M. Greweling, F.D. Finkelman, R. Cardin, M. Mohrs, W. Muller, A. Waisman, A. Roers, and C.L. Karp. 2009. Nonredundant roles for B cell-derived IL-10 in immune counter-regulation. *Journal of immunology* 183:2312-2320.
Martin, F., and A.C. Chan. 2006. B cell immunobiology in disease: evolving concepts from the clinic. *Annu Rev Immunol* 24:467-496.
Martin, R.A., S.R. Hodgkins, A.E. Dixon, and M.E. Poynter. 2014. Aligning mouse models of asthma to human endotypes of disease. *Respirology* 19:823-833.
Maseda, D., S.H. Smith, D.J. DiLillo, J.M. Bryant, K.M. Candando, C.T. Weaver, and T.F. Tedder. 2012. Regulatory B10 cells differentiate into antibody-secreting cells after transient IL-10 production in vivo. *Journal of immunology* 188:1036-1048.
Matsumoto, M., Y. Fujii, A. Baba, M. Hikida, T. Kurosaki, and Y. Baba. 2011. The calcium sensors STIM1 and STIM2 control B cell regulatory function through interleukin-10 production. *Immunity* 34:703-714.

Matsushita, T., M. Horikawa, Y. Iwata, and T.F. Tedder. 2010. Regulatory B cells (B10 cells) and regulatory T cells have independent roles in controlling experimental autoimmune encephalomyelitis initiation and late-phase immunopathogenesis. *Journal of immunology* 185:2240-2252.

Mauri, C., and P.A. Blair. 2014. Regulatory B cells: Are we really ready to manipulate them for the benefit of patients with autoimmune diseases? *Arthritis Rheumatol* n/a-n/a.

Mauri, C., D. Gray, N. Mushtaq, and M. Londei. 2003. Prevention of arthritis by interleukin 10-producing B cells. *J Exp Med* 197:489-501.

McKinley, L., J.F. Alcorn, A. Peterson, R.B. Dupont, S. Kapadia, A. Logar, A. Henry, C.G. Irvin, J.D. Piganelli, A. Ray, and J.K. Kolls. 2008. TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. *Journal of immunology* 181:4089-4097.

Miyara, M., Y. Yoshioka, A. Kitoh, T. Shima, K. Wing, A. Niwa, C. Parizot, C. Taflin, T. Heike, D. Valeyre, A. Mathian, T. Nakahata, T. Yamaguchi, T. Nomura, M. Ono, Z. Amoura, G. Gorochov, and S. Sakaguchi. 2009. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity* 30:899-911.

Mizoguchi, A., E. Mizoguchi, H. Takedatsu, R.S. Blumberg, and A.K. Bhan. 2002. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. *Immunity* 16:219-230.

Mjosberg, J.M., S. Trifari, N.K. Crellin, C.P. Peters, C.M. van Drunen, B. Piet, W.J. Fokkens, T. Cupedo, and H. Spits. 2011. Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. *Nat Immunol* 12:1055-1062.

Molnarfi, N., U. Schulze-Topphoff, M.S. Weber, J.C. Patarroyo, T. Prod'homme, M. Varrin-Doyer, A. Shetty, C. Linington, A.J. Slavin, J. Hidalgo, D.E. Jenne, H. Wekerle, R.A. Sobel, C.C. Bernard, M.J. Shlomchik, and S.S. Zamvil. 2013. MHC class II-dependent B cell APC function is required for induction of CNS autoimmunity independent of myelin-specific antibodies. *J Exp Med* 210:2921-2937.

Montandon, R., S. Korniotis, E. Layseca-Espinosa, C. Gras, J. Megret, S. Ezine, M. Dy, and F. Zavala. 2013. Innate pro-B-cell progenitors protect against type 1 diabetes by regulating autoimmune effector T cells. *Proc Natl Acad Sci U S A* 110:E2199-2208.

Moore, W.C., A.T. Hastie, X. Li, H. Li, W.W. Busse, N.N. Jarjour, S.E. Wenzel, S.P. Peters, D.A. Meyers, E.R. Bleecker, L. National Heart, and P. Blood Institute's Severe Asthma Research. 2014. Sputum neutrophil counts are associated with more severe asthma phenotypes using cluster analysis. *J Allergy Clin Immunol* 133:1557-1563 e1555.

Moore, W.C., D.A. Meyers, S.E. Wenzel, W.G. Teague, H. Li, X. Li, R. D'Agostino, Jr., M. Castro, D. Curran-Everett, A.M. Fitzpatrick, B. Gaston, N.N. Jarjour, R. Sorkness, W.J. Calhoun, K.F. Chung, S.A. Comhair, R.A. Dweik, E. Israel, S.P. Peters, W.W. Busse, S.C. Erzurum, E.R. Bleecker, L. National Heart, and P. Blood Institute's Severe Asthma Research. 2010. Identification of asthma phenotypes using cluster analysis in the Severe Asthma Research Program. *Am J Respir Crit Care Med* 181:315-323.

Moyron-Quiroz, J.E., J. Rangel-Moreno, L. Hartson, K. Kusser, M.P. Tighe, K.D. Klonowski, L. Lefrancois, L.S. Cauley, A.G. Harmsen, F.E. Lund, and T.D. Randall. 2006. Persistence and responsiveness of immunologic memory in the absence of secondary lymphoid organs. *Immunity* 25:643-654.

Muranski, P., and N.P. Restifo. 2013. Essentials of Th17 cell commitment and plasticity. *Blood* 121:2402-2414.

Nagasaki, T., H. Matsumoto, Y. Kanemitsu, K. Izuhara, Y. Tohda, H. Kita, T. Horiguchi, K. Kuwabara, K. Tomii, K. Otsuka, M. Fujimura, N. Ohkura, K. Tomita, A. Yokoyama, H. Ohnishi, Y. Nakano, T. Oguma, S. Hozawa, I. Ito, T. Oguma, H. Inoue, T. Tajiri, T. Iwata, Y. Izuhara, J. Ono, S. Ohta, T. Yokoyama, A. Niimi, and M. Mishima. 2014. Integrating longitudinal information on pulmonary function and inflammation using asthma phenotypes. *J Allergy Clin Immunol* 133:1474-1477, 1477 e1471-1472.

Nair, P., M. Gaga, E. Zervas, K. Alagha, F.E. Hargreave, P.M. O'Byrne, P. Stryszak, L. Gann, J. Sadeh, P. Chanez, and I. Study. 2012. Safety and efficacy of a CXCR2 antagonist in patients

with severe asthma and sputum neutrophils: a randomized, placebo-controlled clinical trial. *Clin Exp Allergy* 42:1097-1103.

Nanzer, A.M., E.S. Chambers, K. Ryanna, D.F. Richards, C. Black, P.M. Timms, A.R. Martineau, C.J. Griffiths, C.J. Corrigan, and C.M. Hawrylowicz. 2013. Enhanced production of IL-17A in patients with severe asthma is inhibited by 1alpha,25-dihydroxyvitamin D3 in a glucocorticoid-independent fashion. *J Allergy Clin Immunol* 132:297-304 e293.

Natarajan, P., A. Singh, J.T. McNamara, E.R. Secor, Jr., L.A. Guernsey, R.S. Thrall, and C.M. Schramm. 2012. Regulatory B cells from hilar lymph nodes of tolerant mice in a murine model of allergic airway disease are CD5+, express TGF-beta, and co-localize with CD4+Foxp3+ T cells. *Mucosal Immunol* 5:691-701.

Neill, D.R., S.H. Wong, A. Bellosi, R.J. Flynn, M. Daly, T.K. Langford, C. Bucks, C.M. Kane, P.G. Fallon, R. Pannell, H.E. Jolin, and A.N. McKenzie. 2010. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* 464:1367-1370.

Neves, P., V. Lampropoulou, E. Calderon-Gomez, T. Roch, U. Stervbo, P. Shen, A.A. Kuhl, C. Loddenkemper, M. Haury, S.A. Nedospasov, S.H. Kaufmann, U. Steinhoff, D.P. Calado, and S. Fillatreau. 2010. Signaling via the MyD88 adaptor protein in B cells suppresses protective immunity during Salmonella typhimurium infection. *Immunity* 33:777-790.

Nishimura, S., I. Manabe, S. Takaki, M. Nagasaki, M. Otsu, H. Yamashita, J. Sugita, K. Yoshimura, K. Eto, I. Komuro, T. Kadowaki, and R. Nagai. 2013. Adipose Natural Regulatory B Cells Negatively Control Adipose Tissue Inflammation. *Cell metabolism*

Noh, J., J.H. Lee, G. Noh, S.Y. Bang, H.S. Kim, W.S. Choi, S. Cho, and S.S. Lee. 2010. Characterisation of allergen-specific responses of IL-10-producing regulatory B cells (Br1) in Cow Milk Allergy. *Cellular immunology* 264:143-149.

Noh, J., G. Noh, S.J. Lee, J.H. Lee, A. Kim, H.S. Kim, and W.S. Choi. 2012. Tolerogenic effects of interferon-gamma with induction of allergen-specific interleukin-10-producing regulatory B cell (Br1) changes in non-IgE-mediated food allergy. *Cellular immunology* 273:140-149.

Noster, R., R. Riedel, M.F. Mashreghi, H. Radbruch, L. Harms, C. Haftmann, H.D. Chang, A. Radbruch, and C.E. Zielinski. 2014. IL-17 and GM-CSF Expression Are Antagonistically Regulated by Human T Helper Cells. *Sci Transl Med* 6:241ra280.

Oliphant, C.J., Y.Y. Hwang, J.A. Walker, M. Salimi, S.H. Wong, J.M. Brewer, A. Englezakis, J.L. Barlow, E. Hams, S.T. Scanlon, G.S. Ogg, P.G. Fallon, and A.N. McKenzie. 2014. MHCII-Mediated Dialog between Group 2 Innate Lymphoid Cells and CD4 T Cells Potentiates Type 2 Immunity and Promotes Parasitic Helminth Expulsion. *Immunity*

Olkhanud, P.B., B. Damdinsuren, M. Bodogai, R.E. Gress, R. Sen, K. Wejksza, E. Malchinkhuu, R.P. Wersto, and A. Biragyn. 2011. Tumor-evoked regulatory B cells promote breast cancer metastasis by converting resting CD4(+) T cells to T-regulatory cells. *Cancer Res* 71:3505-3515.

Papp, K.A., C. Leonardi, A. Menter, J.P. Ortonne, J.G. Krueger, G. Kricorian, G. Aras, J. Li, C.B. Russell, E.H. Thompson, and S. Baumgartner. 2012. Brodalumab, an anti-interleukin-17-receptor antibody for psoriasis. *N Engl J Med* 366:1181-1189.

Pappu, R., A.M. Cheng, B. Li, Q. Gong, C. Chiu, N. Griffin, M. White, B.P. Sleckman, and A.C. Chan. 1999. Requirement for B cell linker protein (BLNK) in B cell development. *Science* 286:1949-1954.

Pavord, I.D., S. Korn, P. Howarth, E.R. Bleecker, R. Buhl, O.N. Keene, H. Ortega, and P. Chanez. 2012. Mepolizumab for severe eosinophilic asthma (DREAM): a multicentre, double-blind, placebo-controlled trial. *Lancet* 380:651-659.

Perkins, C., N. Yanase, G. Smulian, L. Gildea, T. Orekov, C. Potter, F. Brombacher, B. Aronow, M. Wills-Karp, and F.D. Finkelman. 2011. Selective stimulation of IL-4 receptor on smooth muscle induces airway hyperresponsiveness in mice. *J Exp Med* 208:853-867.

Perros, F., P. Dorfmuller, D. Montani, H. Hammad, W. Waelput, B. Girerd, N. Raymond, O. Mercier, S. Mussot, S. Cohen-Kaminsky, M. Humbert, and B.N. Lambrecht. 2012. Pulmonary lymphoid neogenesis in idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med* 185:311-321.

Pillai, S., and A. Cariappa. 2009. The follicular versus marginal zone B lymphocyte cell fate decision. *Nat Rev Immunol* 9:767-777.

Pipet, A., K. Botturi, D. Pinot, D. Vervloet, and A. Magnan. 2009. Allergen-specific immunotherapy in allergic rhinitis and asthma. Mechanisms and proof of efficacy. *Respir Med* 103:800-812.

Ramesh, R., L. Kozhaya, K. McKevitt, I.M. Djuretic, T.J. Carlson, M.A. Quintero, J.L. McCauley, M.T. Abreu, D. Unutmaz, and M.S. Sundrud. 2014. Pro-inflammatory human Th17 cells selectively express P-glycoprotein and are refractory to glucocorticoids. *J Exp Med* 211:89-104.

Randall, T.D. 2010. Bronchus-associated lymphoid tissue (BALT) structure and function. *Adv Immunol* 107:187-241.

Randall, T.D., and R.E. Mebius. 2014. The development and function of mucosal lymphoid tissues: a balancing act with micro-organisms. *Mucosal Immunol* 7:455-466.

Rangel-Moreno, J., D.M. Carragher, M. de la Luz Garcia-Hernandez, J.Y. Hwang, K. Kusser, L. Hartson, J.K. Kolls, S.A. Khader, and T.D. Randall. 2011. The development of inducible bronchus-associated lymphoid tissue depends on IL-17. *Nat Immunol* 12:639-646.

Rangel-Moreno, J., J.E. Moyron-Quiroz, L. Hartson, K. Kusser, and T.D. Randall. 2007. Pulmonary expression of CXC chemokine ligand 13, CC chemokine ligand 19, and CC chemokine ligand 21 is essential for local immunity to influenza. *Proc Natl Acad Sci U S A* 104:10577-10582.

Rauch, P.J., A. Chudnovskiy, C.S. Robbins, G.F. Weber, M. Etzrodt, I. Hilgendorf, E. Tiglao, J.L. Figueiredo, Y. Iwamoto, I. Theurl, R. Gorbatov, M.T. Waring, A.T. Chicoine, M. Mouded, M.J. Pittet, M. Nahrendorf, R. Weissleder, and F.K. Swirski. 2012. Innate response activator B cells protect against microbial sepsis. *Science* 335:597-601.

Rawlings, D.J., M.A. Schwartz, S.W. Jackson, and A. Meyer-Bahlburg. 2012. Integration of B cell responses through Toll-like receptors and antigen receptors. *Nat Rev Immunol* 12:282-294.

Risse, P.A., T. Jo, F. Suarez, N. Hirota, B. Tolloczko, P. Ferraro, P. Grutter, and J.G. Martin. 2011. Interleukin-13 inhibits proliferation and enhances contractility of human airway smooth muscle cells without change in contractile phenotype. *Am J Physiol Lung Cell Mol Physiol* 300:L958-966.

Rosser, E.C., P.A. Blair, and C. Mauri. 2014. Cellular targets of regulatory B cell-mediated suppression. *Mol Immunol*

Rubtsov, Y.P., J.P. Rasmussen, E.Y. Chi, J. Fontenot, L. Castelli, X. Ye, P. Treuting, L. Siewe, A. Roers, W.R. Henderson, Jr., W. Muller, and A.Y. Rudensky. 2008. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* 28:546-558.

Salinas, G.F., F. Braza, S. Brouard, P.P. Tak, and D. Baeten. 2013. The role of B lymphocytes in the progression from autoimmunity to autoimmune disease. *Clin Immunol* 146:34-45.

Sanchez Rodriguez, R., M.L. Pauli, I.M. Neuhaus, S.S. Yu, S.T. Arron, H.W. Harris, S.H. Yang, B.A. Anthony, F.M. Sverdrup, E. Krow-Lucal, T.C. MacKenzie, D.S. Johnson, E.H. Meyer, A. Lohr, A. Hsu, J. Koo, W. Liao, R. Gupta, M.G. Debbaneh, D. Butler, M. Huynh, E.C. Levin, A. Leon, W.Y. Hoffman, M.H. McGrath, M.D. Alvarado, C.H. Ludwig, H.A. Truong, M.M. Maurano, I.K. Gratz, A.K. Abbas, and M.D. Rosenblum. 2014. Memory regulatory T cells reside in human skin. *The Journal of clinical investigation* 124:1027-1036.

Sattler, S., G.S. Ling, D. Xu, L. Hussaarts, A. Romaine, H. Zhao, L. Fossati-Jimack, T. Malik, H.T. Cook, M. Botto, Y.L. Lau, H.H. Smits, F.Y. Liew, and F.P. Huang. 2014. IL-10-producing regulatory B cells induced by IL-33 (Breg(IL-33)) effectively attenuate mucosal inflammatory responses in the gut. *J Autoimmun* 50:107-122.

Scapini, P., C. Lamagna, Y. Hu, K. Lee, Q. Tang, A.L. DeFranco, and C.A. Lowell. 2011. B cell-derived IL-10 suppresses inflammatory disease in Lyn-deficient mice. *Proc Natl Acad Sci U S A* 108:E823-832.

Schmidl, C., L. Hansmann, T. Lassmann, P.J. Balwierz, H. Kawaji, M. Itoh, J. Kawai, S. Nagao-Sato, H. Suzuki, R. Andreesen, Y. Hayashizaki, A.R. Forrest, P. Carninci, P. Hoffmann, M. Edinger, M. Rehli, and F. consortium. 2014. The enhancer and promoter landscape of human regulatory and conventional T-cell subpopulations. *Blood* 123:e68-78.

Shen, P., T. Roch, V. Lampropoulou, R.A. O'Connor, U. Stervbo, E. Hilgenberg, S. Ries, V.D. Dang, Y. Jaimes, C. Daridon, R. Li, L. Jouneau, P. Boudinot, S. Wilantri, I. Sakwa, Y. Miyazaki, M.D. Leech, R.C. McPherson, S. Wirtz, M. Neurath, K. Hoehlig, E. Meinl, A. Grutzkau, J.R. Grun, K. Horn, A.A. Kuhl, T. Dorner, A. Bar-Or, S.H. Kaufmann, S.M. Anderton, and S.

Fillatreau. 2014. IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases. *Nature* 507:366-370.
Shin, J.I. 2013. Induction of severe systemic lupus erythematosus by TNF blockade and response to an anti-IL-6 strategy: role of IL-17? *J Allergy Clin Immunol* 132:1257.
Siewe, B., J.T. Stapleton, J. Martinson, A. Keshavarzian, N. Kazmi, P.M. Demarais, A.L. French, and A. Landay. 2013. Regulatory B cell frequency correlates with markers of HIV disease progression and attenuates anti-HIV CD8(+) T cell function in vitro. *Journal of leukocyte biology* 93:811-818.
Smith, K.G., R.B. Jones, S.M. Burns, and D.R. Jayne. 2006. Long-term comparison of rituximab treatment for refractory systemic lupus erythematosus and vasculitis: Remission, relapse, and re-treatment. *Arthritis Rheum* 54:2970-2982.
Su, T.T., and D.J. Rawlings. 2002. Transitional B lymphocyte subsets operate as distinct checkpoints in murine splenic B cell development. *Journal of immunology* 168:2101-2110.
Sung, S.S., L.K. Jung, J.A. Walters, E.W. Jeffes, 3rd, G.A. Granger, and S.M. Fu. 1989. Production of lymphotoxin by isolated human tonsillar B lymphocytes and B lymphocyte cell lines. *The Journal of clinical investigation* 84:236-243.
Tliba, O., D. Deshpande, H. Chen, C. Van Besien, M. Kannan, R.A. Panettieri, Jr., and Y. Amrani. 2003. IL-13 enhances agonist-evoked calcium signals and contractile responses in airway smooth muscle. *Br J Pharmacol* 140:1159-1162.
Tokunaga, M., K. Saito, D. Kawabata, Y. Imura, T. Fujii, S. Nakayamada, S. Tsujimura, M. Nawata, S. Iwata, T. Azuma, T. Mimori, and Y. Tanaka. 2007. Efficacy of rituximab (anti-CD20) for refractory systemic lupus erythematosus involving the central nervous system. *Ann Rheum Dis* 66:470-475.
Turner, M., A. Galloway, and E. Vigorito. 2014. Noncoding RNA and its associated proteins as regulatory elements of the immune system. *Nat Immunol* 15:484-491.
Vallerskog, T., I. Gunnarsson, M. Widhe, A. Risselada, L. Klareskog, R. van Vollenhoven, V. Malmstrom, and C. Trollmo. 2007. Treatment with rituximab affects both the cellular and the humoral arm of the immune system in patients with SLE. *Clin Immunol* 122:62-74.
Valmori, D., A. Merlo, N.E. Souleimanian, C.S. Hesdorffer, and M. Ayyoub. 2005. A peripheral circulating compartment of natural naive CD4 Tregs. *The Journal of clinical investigation* 115:1953-1962.
van de Veen, W., B. Stanic, G. Yaman, M. Wawrzyniak, S. Sollner, D.G. Akdis, B. Ruckert, C.A. Akdis, and M. Akdis. 2013. IgG4 production is confined to human IL-10-producing regulatory B cells that suppress antigen-specific immune responses. *J Allergy Clin Immunol* 131:1204-1212.
van der Vlugt, L.E., E. Mlejnek, A. Ozir-Fazalalikhan, M. Janssen Bonas, T.R. Dijksman, L.A. Labuda, R. Schot, B. Guigas, G.M. Moller, P.S. Hiemstra, M. Yazdanbakhsh, and H.H. Smits. 2014. CD24(hi)CD27(+) B cells from patients with allergic asthma have impaired regulatory activity in response to lipopolysaccharide. *Clin Exp Allergy* 44:517-528.
van Lochem, E.G., V.H. van der Velden, H.K. Wind, J.G. te Marvelde, N.A. Westerdaal, and J.J. van Dongen. 2004. Immunophenotypic differentiation patterns of normal hematopoiesis in human bone marrow: reference patterns for age-related changes and disease-induced shifts. *Cytometry B Clin Cytom* 60:1-13.
Victora, G.D., and M.C. Nussenzweig. 2012. Germinal centers. *Annu Rev Immunol* 30:429-457.
Walker, J.A., J.L. Barlow, and A.N. McKenzie. 2013. Innate lymphoid cells--how did we miss them? *Nat Rev Immunol* 13:75-87.
Walker, J.A., and A. McKenzie. 2012. Innate lymphoid cells in the airways. *Eur J Immunol* 42:1368-1374.
Wang, R.X., C.R. Yu, I.M. Dambuza, R.M. Mahdi, M.B. Dolinska, Y.V. Sergeev, P.T. Wingfield, S.H. Kim, and C.E. Egwuagu. 2014. Interleukin-35 induces regulatory B cells that suppress autoimmune disease. *Nat Med* 20:633-641.
Wasserstrom, H., J. Bussel, L.C. Lim, and C. Cunningham-Rundles. 2008. Memory B cells and pneumococcal antibody after splenectomy. *Journal of immunology* 181:3684-3689.
Weber, G.F., B.G. Chousterman, I. Hilgendorf, C.S. Robbins, I. Theurl, L.M. Gerhardt, Y. Iwamoto, T.D. Quach, M. Ali, J.W. Chen, T.L. Rothstein, M. Nahrendorf, R. Weissleder, and F.K.

Swirski. 2014. Pleural innate response activator B cells protect against pneumonia via a GM-CSF-IgM axis. *J Exp Med* 211:1243-1256.

Wener, R.R., and E.H. Bel. 2013. Severe refractory asthma: an update. *Eur Respir Rev* 22:227-235.

Wenzel, S., L. Ford, D. Pearlman, S. Spector, L. Sher, F. Skobieranda, L. Wang, S. Kirkesseli, R. Rocklin, B. Bock, J. Hamilton, J.E. Ming, A. Radin, N. Stahl, G.D. Yancopoulos, N. Graham, and G. Pirozzi. 2013. Dupilumab in persistent asthma with elevated eosinophil levels. *N Engl J Med* 368:2455-2466.

Wenzel, S.E. 2012. Asthma phenotypes: the evolution from clinical to molecular approaches. *Nat Med* 18:716-725.

Wenzel, S.E., L.B. Schwartz, E.L. Langmack, J.L. Halliday, J.B. Trudeau, R.L. Gibbs, and H.W. Chu. 1999. Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. *Am J Respir Crit Care Med* 160:1001-1008.

Wills-Karp, M., J. Luyimbazi, X. Xu, B. Schofield, T.Y. Neben, C.L. Karp, and D.D. Donaldson. 1998. Interleukin-13: central mediator of allergic asthma. *Science (New York, N.Y.)* 282:2258-2261.

Wilson, R.H., G.S. Whitehead, H. Nakano, M.E. Free, J.K. Kolls, and D.N. Cook. 2009. Allergic sensitization through the airway primes Th17-dependent neutrophilia and airway hyperresponsiveness. *Am J Respir Crit Care Med* 180:720-730.

Wojciechowski, W., D.P. Harris, F. Sprague, B. Mousseau, M. Makris, K. Kusser, T. Honjo, K. Mohrs, M. Mohrs, T. Randall, and F.E. Lund. 2009. Cytokine-producing effector B cells regulate type 2 immunity to H. polygyrus. *Immunity* 30:421-433.

Wolf, S.D., B.N. Dittel, F. Hardardottir, and C.A. Janeway, Jr. 1996. Experimental autoimmune encephalomyelitis induction in genetically B cell-deficient mice. *J Exp Med* 184:2271-2278.

Wood, L.G., K.J. Baines, J. Fu, H.A. Scott, and P.G. Gibson. 2012. The neutrophilic inflammatory phenotype is associated with systemic inflammation in asthma. *Chest* 142:86-93.

Woodruff, P.G., H.A. Boushey, G.M. Dolganov, C.S. Barker, Y.H. Yang, S. Donnelly, A. Ellwanger, S.S. Sidhu, T.P. Dao-Pick, C. Pantoja, D.J. Erle, K.R. Yamamoto, and J.V. Fahy. 2007. Genome-wide profiling identifies epithelial cell genes associated with asthma and with treatment response to corticosteroids. *Proc Natl Acad Sci U S A* 104:15858-15863.

Woodruff, P.G., B. Modrek, D.F. Choy, G. Jia, A.R. Abbas, A. Ellwanger, L.L. Koth, J.R. Arron, and J.V. Fahy. 2009. T-helper type 2-driven inflammation defines major subphenotypes of asthma. *Am J Respir Crit Care Med* 180:388-395.

Worm, M., and R.S. Geha. 1994. CD40 ligation induces lymphotoxin alpha gene expression in human B cells. *Int Immunol* 6:1883-1890.

Wynn, T.A., and T.R. Ramalingam. 2012. Mechanisms of fibrosis: therapeutic translation for fibrotic disease. *Nat Med* 18:1028-1040.

Xiao, S., C.R. Brooks, C. Zhu, C. Wu, J.M. Sweere, S. Petecka, A. Yeste, F.J. Quintana, T. Ichimura, R.A. Sobel, J.V. Bonventre, and V.K. Kuchroo. 2012. Defect in regulatory B-cell function and development of systemic autoimmunity in T-cell Ig mucin 1 (Tim-1) mucin domain-mutant mice. *Proc Natl Acad Sci U S A* 109:12105-12110.

Xu, S., J.E. Tan, E.P. Wong, A. Manickam, S. Ponniah, and K.P. Lam. 2000. B cell development and activation defects resulting in xid-like immunodeficiency in BLNK/SLP-65-deficient mice. *Int Immunol* 12:397-404.

Yanaba, K., J.D. Bouaziz, K.M. Haas, J.C. Poe, M. Fujimoto, and T.F. Tedder. 2008. A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses. *Immunity* 28:639-650.

Yanaba, K., J.D. Bouaziz, T. Matsushita, T. Tsubata, and T.F. Tedder. 2009. The development and function of regulatory B cells expressing IL-10 (B10 cells) requires antigen receptor diversity and TLR signals. *Journal of immunology* 182:7459-7472.

Yanaba, K., M. Kamata, N. Ishiura, S. Shibata, Y. Asano, Y. Tada, M. Sugaya, T. Kadono, T.F. Tedder, and S. Sato. 2013. Regulatory B cells suppress imiquimod-induced, psoriasis-like skin inflammation. *Journal of leukocyte biology* 94:563-573.

Yang, M., J. Deng, Y. Liu, K.H. Ko, X. Wang, Z. Jiao, S. Wang, Z. Hua, L. Sun, G. Srivastava, C.S. Lau, X. Cao, and L. Lu. 2012. IL-10-producing regulatory B10 cells ameliorate collagen-induced arthritis via suppressing Th17 cell generation. *Am J Pathol* 180:2375-2385.

Yoshizaki, A., T. Miyagaki, D.J. DiLillo, T. Matsushita, M. Horikawa, E.I. Kountikov, R. Spolski, J.C. Poe, W.J. Leonard, and T.F. Tedder. 2012. Regulatory B cells control T-cell autoimmunity through IL-21-dependent cognate interactions. *Nature* 491:264-268.

Youinou, P., and C. Jamin. 2009. The weight of interleukin-6 in B cell-related autoimmune disorders. *J Autoimmun* 32:206-210.

Zaba, L.C., M. Suarez-Farinas, J. Fuentes-Duculan, K.E. Nograles, E. Guttman-Yassky, I. Cardinale, M.A. Lowes, and J.G. Krueger. 2009. Effective treatment of psoriasis with etanercept is linked to suppression of IL-17 signaling, not immediate response TNF genes. *J Allergy Clin Immunol* 124:1022-1010 e1021-1395.

Zhang, D.H., L. Yang, L. Cohn, L. Parkyn, R. Homer, P. Ray, and A. Ray. 1999. Inhibition of allergic inflammation in a murine model of asthma by expression of a dominant-negative mutant of GATA-3. *Immunity* 11:473-482.

Zhao, J., C.M. Lloyd, and A. Noble. 2013. Th17 responses in chronic allergic airway inflammation abrogate regulatory T-cell-mediated tolerance and contribute to airway remodeling. *Mucosal Immunol* 6:335-346.

Zhu, H.Q., R.C. Xu, Y.Y. Chen, H.J. Yuan, H. Cao, X.Q. Zhao, J. Zheng, Y. Wang, and M. Pan. 2014. Impaired Function of CD19 CD24 CD38 Regulatory B Cells in Pemphigus Patients. *Br J Dermatol* n/a-n/a.

Zouggari, Y., H. Ait-Oufella, P. Bonnin, T. Simon, A.P. Sage, C. Guerin, J. Vilar, G. Caligiuri, D. Tsiantoulas, L. Laurans, E. Dumeau, S. Kotti, P. Bruneval, I.F. Charo, C.J. Binder, N. Danchin, A. Tedgui, T.F. Tedder, J.S. Silvestre, and Z. Mallat. 2013. B lymphocytes trigger monocyte mobilization and impair heart function after acute myocardial infarction. *Nat Med* 19:1273-1280.

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COMMUNAUTÉ D'UNIVERSITÉS ET ÉTABLISSEMENTS

UNIVERSITÉ DE NANTES

Thèse de Doctorat

Faouzi Braza

Étude d'une population B régulatrice dans un modèle murin d'asthme allergique aux acariens

Study of regulatory B cells in a mouse model of house dust mite asthma

Résumé

Décrits essentiellement pour leur rôle dans l'immunité humorale, les lymphocytes B sont de plus en plus décrits pour leur capacité à réguler les réponses inflammatoires. On distingue ainsi des lymphocytes B inflammatoires et régulateurs capables de réguler l'activation des cellules du système immunitaire.
Les cellules B régulatrices représentent aujourd'hui un intérêt particulier. Plusieurs études décrivent ainsi leur intérêt thérapeutique en transplantation et en autoimmunité. Ces cellules caractérisées par la production d'IL-10 sont capables d'inhiber de nombreux acteur du système immunitaire et de maintenir la tolérance immunologique.
L'asthme allergique est typiquement une situation ou il existe une rupture de tolérance face à un allergène respiratoire. Dans cette pathologie, les mécanismes régulateurs étaient essentiellement réduits à la simple description des cellules T régulatrices. Or les cellules B régulatrices représentent un intérêt potentiel dans cette pathologie.
Les travaux de cette thèse portent ainsi sur la caractérisation phénotypique et fonctionnelle de ces cellules dans un modèle d'asthme allergique aux acariens

Mots clefs : Lymphocytes B, Asthme, IL-10, Tolérance

Abstract

Essentially described for their role in humoral immunity, B cells are increasingly described for their ability to regulate inflammatory responses. We can so distinguish inflammatory and regulatory B cells able to regulate the activation of immune cells.
Regulatory B cells are of special interest today. Several studies described their capacity to control inflammation in transplantation and autoimmunity. These cells are mainly characterized by the production of IL-10. They are able to inhibit many and control many components of the immune system in order to maintain immunological tolerance.
Allergic asthma is typically a situation where immune tolerance is broken in response to respiratory allergen. In this pathology, regulatory mechanisms were essentially reduced to the simple description of the regulatory T cells. Given the importance of regulatory B cells in immune regulation, these cells represent a potential interest in asthma
Then the work of this thesis focus on the phenotypic and functional characterization of these cells in a model of allergic asthma due to mites

Mots clefs : Lymphocytes B, Asthme, IL-10, Tolérance