Les sélectines inhibent la fonction des lymphocytes T régulateurs et contribuent à la pathogénie du lupus érythémateux systémique

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Année 2020 **Université de Bordeaux**

Thèse

Pour l'obtention du

Doctorat de Sciences

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École doctorale

Sciences de la Vie et de la Santé

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Né le 20/01/1989 à Mulhouse

Soutenue publiquement le 11.09.2020 à Bordeaux

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REMERCIEMENTS

Madame Le Docteur Dumortier,

Je vous remercie d'avoir accepté d'être rapporteuse de mon travail de doctorat et membre de mon Jury. J'espère que nous aurons l'occasion de travailler ensemble sur la thématique du lupus lors de mon retour à Strasbourg.

Monsieur le Professeur Martin,

Je vous remercie d'avoir accepté d'être rapporteur de mon travail de doctorat et membre de mon Jury. Le dynamisme médical et scientifique de l'équipe strasbourgeoise du CNR RESO a confirmé mon souhait de poursuivre la voie d'une carrière de clinicien-chercheur. Je serais honoré de pouvoir rejoindre vos équipes à l'issue de mon stage post-doctoral.

Madame le Docteur Boniface,

Je vous remercie d'avoir accepté d'être membre de mon Jury de thèse.

Monsieur le Professeur Gottenberg,

Je vous remercie de m'avoir accueilli dans votre service à Strasbourg durant ce riche semestre. L'Immunologie et la Médecine sont deux disciplines passionnantes mais difficiles. J'espère me montrer à la hauteur dans chacune de ces disciplines, comme vous avez su le faire avec vos collègues à Strasbourg.

Monsieur le Docteur Miyara,

Je vous remercie d'avoir accepté d'être rapporteur de mon travail de doctorat et membre de mon Jury. Vos travaux sur les lymphocytes T régulateurs m'ont grandement aidé à appréhender cette thématique complexe.

Patrick,

Merci pour ton accueil dans la team lupus. Ces trois années m'ont apporté bien plus que je ne l'aurais imaginé au départ. Merci de m'avoir enseigné ta persévérance, ton bon sens de clinicien-chercheur et ta rigueur. Enfin, merci de m'avoir recommandé chez George Tsokos afin que je continue ma formation de chercheur à Boston.

Christophe,

Merci d'avoir dirigé cette thèse et bien plus encore depuis mon arrivée à Bordeaux il y a six ans. Que de chemin parcouru. Durant ces six années, tu as su te montrer disponible, à l'écoute, et toujours prêt à donner des conseils bienveillants. J'ai pris beaucoup de plaisir à grandir au sein de la famille de la Rhumatologie bordelaise et espère continuer à collaborer avec vous à l'avenir !

Un remerciement tout particulier à **Isabelle** qui m'a guidé tout au long de ma thèse.

A mes collègues de l'équipe Blanco**, Vivien**, **Nathalie M., Clément, Angela, Paoline, Thomas.**

A mes co-thésards : **Hannah, Damien, Gaël, Nathalie Y., Andréa, Elena, Adrien, Corentin, Charlotte, Gabriel.**

A la plateforme de cytométrie d'Immunoconcept pour leur précieuse aide : **Vincent** et **Atika.**

L'ensemble des cliniciens : **Estibaliz, Pierre, Emmanuel, Lionel Couzi, Marie-Élise, Manon, Agathe.**

A **Julie Dechanet-Merville** pour son accueil à Immunoconcept.

Aux chercheurs d'Immunoconcept : **Vanja Sisirak, Nicolas Larmonier, Nathalie Schmitt**, **Maya Saleh**, **Jean-François, Thomas, Maël.**

Aux chercheurs de l'IGBC qui ont déménagé à l'IBMC à Strasbourg : **Benoit et Louise**.

À **l'École de l'Inserm Liliane Bettencourt** sans quoi cette thèse n'aurait pas eu lieu, ainsi qu'à l'ensemble de mes collègues et amis des doubles cursus, en particulier **Thomas**.

I) INTRODUCTION

1) LE LUPUS ÉRYTHEMATEUX SYSTEMIQUE : GENERALITES

Le lupus érythémateux systémique est une maladie auto-immune systémique rare caractérisée par une perte de tolérance vis-à-vis des auto-antigènes nucléaire.

Sa prévalence en France est estimée à 49/100.000, la classant donc dans la catégorie des maladies rares *(1)*. Le lupus touche essentiellement la femme (sexe-ratio à 9 femmes pour un homme), jeunes entre 20 et 55 ans. Les formes juvéniles sont rares et témoignent de la présence de prédispositions génétiques, voire dans certains cas rares, de mutations isolées responsable de lupus dit « monogéniques ».

La présentation de la maladie est extrêmement polymorphe avec toutefois le plus souvent une atteinte cutanée et articulaire. Tous les organes peuvent être atteints, ce qui peut rendre difficile le diagnostic et surtout la prise en charge. Le LES est responsable d'une morbi-mortalité importante liée aux atteintes d'organes (e.g., rénale, neurologique) ou aux complications de la maladie et des traitements. En effet, malgré les progrès réalisés dans la prise en charge du LES, les principales causes de mortalité restent les infections et les complications cardiovasculaires, pour lesquelles les glucocorticoïdes et autres immunosuppresseurs portent une importante part de responsabilité *(2, 3)*. Outre ces complications, le LES est également responsable d'une altération de la qualité de vie et d'un handicap significatifs pour les patients *(4)*.

À l'heure actuelle, les traitements prescrits ont un rôle purement suspensif sur la maladie, qui évolue par périodes de poussée et de rémission. C'est notamment le cas des immunosuppresseurs dont le rôle est de limiter la dérégulation du système immunitaire, mais qui aggravent aussi le risque infectieux et cardiovasculaire. Ainsi, la découverte de traitements permettant de rétablir l'homéostasie du

système immunitaire sans promouvoir le risque infectieux et/ou cardiovasculaire sont particulièrement attendus.

Depuis l'avènement de corticoïdes vers la fin des années 40 puis de l'hydroxychloroquine dans les années 60, seul un traitement a reçu une autorisation de mise sur le marché pour la prise en charge du LES : le belimumab. Le belimumab est une biothérapie ciblant le lymphocyte B en bloquant BlyS (B lymphocyte Stimulator ; ou BAFF), une cytokine essentielle à leur développement. De nombreuses autres voies thérapeutiques ont été testé dans le LES : blocage des molécules de co-stimulation (eg, CTLA-4 ligand), blocage des interféron des interférons de type I ou le blocage du récepteur à l'interleukine-6, avec des résultats contrastés *(5)*. Dernièrement, un anticorps bloquant le recepteur des interférons de type I, l'anifrolumab, a montré son efficacité dans un essai de phase de phase 3, ouvrant donc la voie à de nouveaux traitements ciblés du LES *(6)*.

Dans tous les cas, une meilleure compréhension des mécanismes immuno-physiopathologiques impliqués dans le LES sera indispensable pour découvrir de nouvelles voies thérapeutiques, et mieux caractériser l'hétérogénéité clinique de cette maladie.

2) APERÇU DE LA PHYSIOPATHOLOGIE DU LES

La pathogénèse du LES est multifactorielle et complexe, impliquant de multiples facteurs génétiques, hormonaux et environnementaux.

Sur le plan génétique, le fait que la concordance entre deux jumeaux monozygotes se situe entre 24 et 57%, plaide en faveur d'une influence de celle-ci, tout en n'étant cependant pas un facteur suffisant au déclenchement du LES *(7, 8)*. Des études de type Genome-Wide Association Study (GWAS) ont permis d'identifier de nombreux polymorphismes (ou mutation) prédisposant au développement d'un LES *(9)*.

En dehors des syndromes lupus-like monogéniques, les facteurs génétiques les plus associés au développement du LES sont un déficit du C1q du C2/C4 ou du gène codant l'endonucléase TREX1 *(10)*. De manière intéressante, le rôle principal actuellement attribué au C1q est la clairance des corps apoptotiques, tandis que celui de TREX1 est la dégradation de l'ADN circulant. Ces observations ont fait évoquer l'hypothèse que le *primum movens* du LES était un déficit de clairance des corps apoptotiques (et de leur contenu en auto-antigènes) responsable d'une accumulation de ceux-ci dans la circulation.

Les facteurs hormonaux, notamment les œstrogènes, jouent un rôle certain et participent à l'inégalité du sex-ratio dans le LES. Les mécanismes sont multiples tels qu'une action activatrice des œstrogènes sur les lymphocytes, une majoration de la libération de cytokines pro-inflammatoires ou l'augmentation de molécules d'adhésion sur les cellules endothéliales *(11)*. De manière notable, plusieurs arguments font rapport d'un défaut d'inactivation du deuxième chromosome X par les ARN non codant du complexe Xist *(12)*. En effet, le chromosome X porte de nombreux gènes impliqués dans la physiopathogénie du LES tels que le *TLR7* ou le *CD40L*, et un défaut d'inactivation de celui-ci pourrait expliquer les différences liées au sexe. Cette hypothèse est soutenue par une prévalence du lupus similaire entre les femmes et les hommes atteints du syndrome de Klinefelter (caryotype 47, XXY) *(13)*, et une prévalence diminué du LES parmi les femmes atteintes du syndrome de Turner (45, X0) *(14)*.

De multiples facteurs environnementaux tels que les rayonnements ultraviolets (via une majoration de l'immunogénicité des kératinocytes et de leur apoptose), les infections virales (par la sécrétion d'interférons ou par mimétisme moléculaire), le tabac (par des mécanismes incomplètement compris) et certains médicaments ont été associés avec le développement ou une poussée de LES *(15–17)* .

La reconnaissance de ces facteurs a fait émerger une théorie de la genèse du LES. Un défaut de clairance de corps apoptotiques serait responsable d'une majoration des auto-antigènes circulants. Ces auto-antigènes seraient ensuite pris en charge par les cellules présentatrices d'antigènes, qui les présenteraient aux lymphocytes T et induiraient ainsi une réponse (auto-)immune permettant

l'émergence de clones B/T autoréactifs responsables de la production d'auto-anticorps. Ces auto-anticorps en reconnaissant les auto-antigènes circulants formeraient alors des complexes immuns capables de se déposer dans les tissus et provoquer des lésions d'organes.

La physiopathologie du LES ne se limite cependant pas aux auto-anticorps et aux complexes immuns. L'ensemble du système immunitaire inné et adaptatif participe à la genèse de la maladie. Dans la complexité de la réponse auto-immune du LES, les mécanismes régulateurs du système immunitaire sont dépassés et ne permettent plus de réguler la réponse inflammatoire.

Dans la 1ère partie de ce travail, nous présenterons le rôle des lymphocytes T régulateurs, cellule clé de la régulation de la réponse immunitaire, et plus particulièrement dans le LES. Nous poursuivrons ensuite avec le rôle d'un composant particulier du sang, la plaquette, dont les propriétés immunitaires ne cessent d'être mises en avant (revue de la littérature en article 1). Nous présenterons enfin nos travaux concernant l'interaction potentielle entre les lymphocytes T régulateurs et les plaquettes, et l'intérêt de cette interaction dans le LES.

3) LYMPHOCYTES T REGULATEURS ET ROLE DANS LE LUPUS SYSTEMIQUE

L'existence de lymphocytes thymiques régulateurs (Tregs) a été évoquée dès le début des années 1970 lorsque l'adjonction de thymocytes à des souris irradiées et thymectomisées entrainait une diminution de la réponse à un stimulus antigénique *(18)*. Ce n'est que dans le milieu des années 90 que Sagakuchi et al. décriront une sous population de lymphocytes T CD4+ exprimant fortement la chaine alpha du recepteur à l'IL2 (CD25), capable d'induire une tolérance dans plusieurs modèles murins d'autoimmunité *(19)*. En effet, la déplétion des cellules $CD25^+$ par un anticorps spécifique aggravait le phénotype de la maladie, tandis que l'injection de ces cellules induisait une amélioration des symptômes. Cette population sera ensuite également retrouvée chez l'homme *(20)*.

Depuis leur découverte, les Tregs ont été la source de nombreuses recherches visant à mettre en évidence leur origine, leurs mécanismes d'actions et leurs rôles dans les maladies auto-immunes et inflammatoires. Nous résumerons ici l'état actuel des connaissances concernant ces différents aspects, ce qui nous permettra ensuite de mieux aborder le rôle du Treg dans la physiopathologie du LES.

a) Généralité sur les lymphocytes T régulateurs (Tregs)

i. Définition et rôle de FoxP3

Les Tregs constituent une sous-population de lymphocyte T CD4+. Ils sont caractérisés chez l'homme comme chez la souris par la co-expression du CD25 et du facteur de transcription FoxP3 *(21)*, et chez l'homme, par une faible expression du CD127 (récepteur de l'IL7).

La protéine FoxP3 est un facteur de transcription responsable de la différentiation du Treg, de sa stabilité et de ses fonctions. Historiquement, la protéine FoxP3 a été identifié en étudiant un modèle de souris « scurfy » présentant un phénotype d'autoimmunité létale de transmission récessive lié à l'X *(22)*. Chez l'homme, la mutation du gène FoxP3 est responsable d'un syndrome associant dysimmunité (Immune-dysrégulation), une polyendocrinopathie et une entéropathie, liés à l'X (syndrome IPEX) *(22)*. FoxP3 est une protéine « master Controller » de l'expression des gènes liés à la différentiation et à la fonction des lymphocytes T régulateurs. Il s'agit d'une protéine multi-domaine avec une portion en

doigt de zinc et une portion N-terminale en fourchette permettant la liaison à l'ADN. FoxP3 contrôle l'expression d'un nombre important de transcrits spécifiques des Tregs tels que le CD25, CTLA4, GITR et FoxP3 lui-même tandis qu'il réprime l'expression de l'IL-2, d'IFNγ et d'IL-4 *(21)*. Ainsi, la transfection de FoxP3 dans un lymphocyte CD4+ naïfs les transforme en cellules $CD25^{high}$ ayant des fonctions régulatrices semblables au Tregs isolés *ex vivo (21)*.

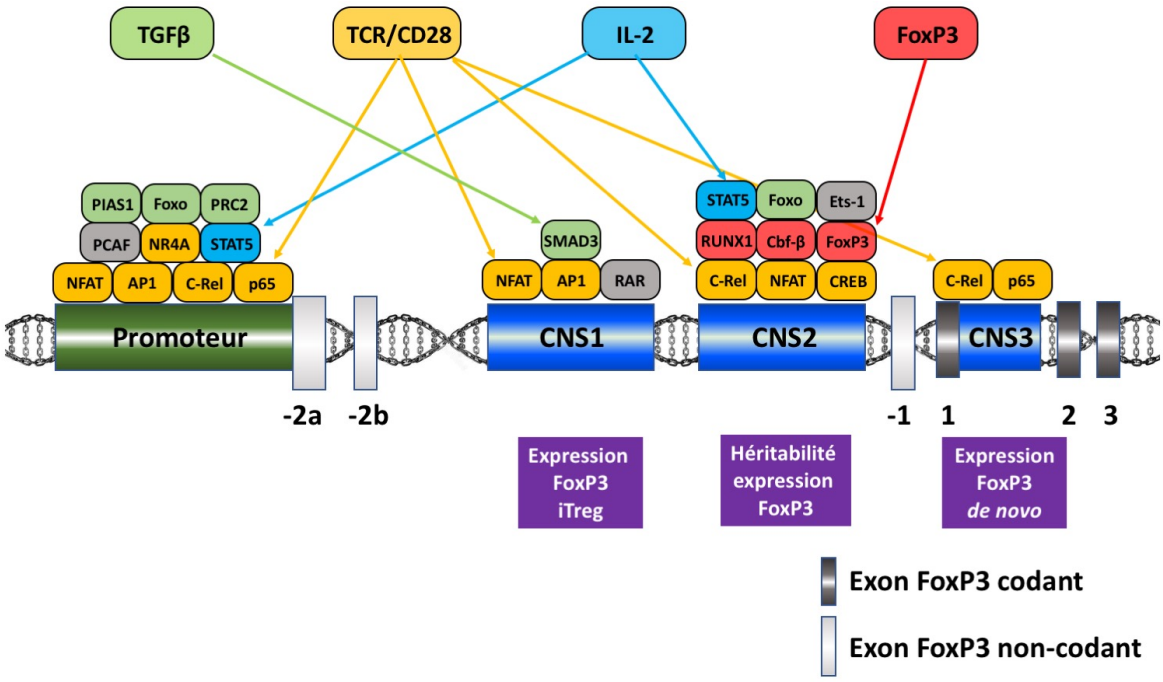


Figure 1 : Contrôle de l'expression de FoxP3. Adapté à partir de Huehn et al.*(23)*

De multiples facteurs de transcription et/ou complexes protéiques influent sur l'expression et la stabilité de l'expression de FoxP3. Ces facteurs se fixent à l'ADN au niveau du promoteur de FoxP3 ou au niveau des séquences non codantes conservées (conserved non-coding sequences, CNS 1-3). La fixation de ces facteurs est influencée par des stimulus extérieurs (TGF-β, IL-2 ...), l'expression de FoxP3 lui-même et l'état de méthylation des CNS eux-même (non montré ici). Le CNS-1 est responsable de l'expression *de novo* de FoxP3 dans des iTregs ; le CNS-2 est responsable de l'héritabilité de l'expression de FoxP3 aux cellules filles ; CNS-3 est également responsable de l'expression *de novo* de FoxP3 (y compris dans les tTregs).

La régulation de l'expression de FoxP3 est un processus complexe et contrôlé à plusieurs niveaux. L'essentiel de ce contrôle a lieu au niveau épigénétique par le biais de la méthylation de trois séquences hautement conservées, (CNS, Conserved Non-coding Sequence), riches en ilôts CpG et situées à proximité du promoteur de celui-ci (**figure 1**). Les ilôts CpG des CNS sont fortement déméthylés (permettant l'expression de FoxP3) dans les Tregs isolés *ex vivo* tandis qu'ils sont fortement méthylés parmis les lymphocytes T CD4+ non régulateurs *(24, 25)*. Les CNS ont des rôles distincts : le CNS-1 joue un rôle pour l'expression de FoxP3 dans les cellules en périphérie (non thymiques), le CNS-2 pour l'héritabilité de l'expression de FoxP3 après division cellulaire et le CNS-3 pour l'induction *de novo* de l'expression de FoxP3 *(24)*. De nombreux facteurs tel que SMAD3, NFAT, AP1, STAT5, CREL, CREB et FoxP3 lui-même interagissent avec ces CNS et contrôlent la méthylation de ceux-ci (**figure 1**). De manière notable, le niveau de méthylation des Tregs différentiés *in vitro* à partir de CD4+ naïfs diminue fortement pour atteindre un niveau proche de celui des Tregs thymiques, sauf pour le CNS-2 qui reste méthylé et explique la moindre stabilité des Tregs induits en périphérie par rapport aux Tregs thymiques *(24)*. L'hypométhylation des CNS est un facteur indispensable au phénotype et aux fonctions des Tregs *(26)*. Des modifications post-traductionnelle de FoxP3 ont été décrites tel que l'acétylation, la phosphorylation ou l'ubiquitinylation qui modulent la liaison de FoxP3 à l'ADN et le recyclage de celui-ci par le protéasome *(27)*.

D'autres marqueurs de Tregs ont été décrits tels que le facteur de transcription Helios *(28)*. Néanmoins il n'existe pas de marqueurs de surface spécifique aux Tregs ce qui complexifie leur étude et leur isolation notamment chez l'homme.

ii. Hétérogénéité des Tregs

Les Tregs représentent une population cellulaire extrêmement hétérogène sur le plan phénotypique, transcriptomique et fonctionnel. Les Tregs sont historiquement classés selon leur origine : ceux d'origine thymiques (tTregs), ceux d'origine périphériques dans les tissus (pTregs) et ceux induits *in vitro* (iTregs). De manière intéressante, il semble que les tTregs possèdent des régions CNS plus déméthylées que les iTregs (et pTreg), leur conférant donc une meilleure stabilité (voir ci-dessous) *(26, 29)*.

Miyara *et al.* ont décrit phénotypiquement et fonctionnellement les différentes sous-populations de Tregs humains (**figure 2** extraite de la figure 1 de *(30)*). Les Tregs circulants peuvent être catégorisés en 3 populations selon leur expression de CD25 et du CD45RA. Les resting (r-) ou naive (n-) Treg (CD45RA+ CD25+, population I) ; les effector (e-) ou activated (a-) Tregs (population II) caractérisés par la perte du CD45RA et la très forte expression de CD25 (et de FoxP3) ; les « Tregs » <u>non suppressifs *in vitro*</u> qui sécrètent des cytokines (population III) caractérisés par la perte du CD45RA et une expression intermédiaire du CD25 (et de FoxP3). Cette dernière population correspond vraisemblablement à une population de lymphocytes CD4+ activés exprimant de manière transitoire le CD25 (marqueur d'activation) et le FoxP3.

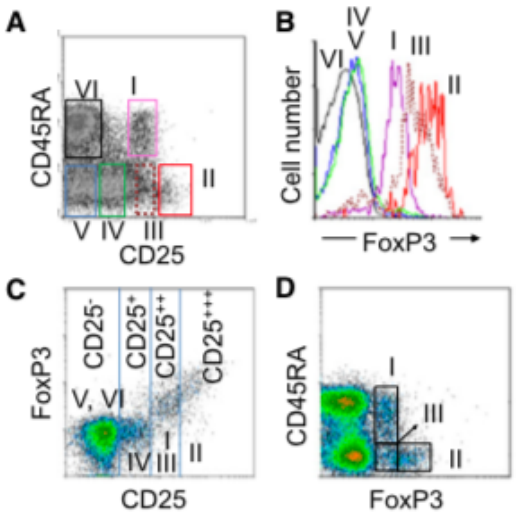


Figure 2 extraite de la figure 1 de Miyara *et al.* *(30)* **:** Évaluation en cytométrie de flux des différentes sous-population de Tregs (I, II et III) ou de T effecteurs (Teff, IV, V et VI) à partir de PBMC d'un donneur sain. Les panels A, B, C et D sont fenêtrés sur les PBMCs $CD3^+$ $CD4^+$ d'un donneur sain.

Il a été mis en évidence que les nTregs sont moins immunosuppressifs *in vitro* mais qu'ils possèdent des capacités de proliférations contrairement aux eTregs qui sont fortement immunosuppressifs mais rapidement sénescents. *In vivo* chez la souris et chez l'homme, les nTregs prolifèrent et constituent la source principale des eTregs *(30)*.

Le même groupe a démontré que les nTreg et surtout les eTreg exprimaient de manière importante le Sialyl Lewis X (sLe^X ou CD15s) qui correspond à la forme fucolysée du P-Selectin Glycoprotein Ligand 1 (PSGL-1) *(31)*. L'isolement et l'étude de la sous-population de Tregs humains exprimant le CD15s a ainsi permis de démontrer qu'il s'agissait d'une population fortement immunosuppressive par rapport aux Tregs $CD15^-$. De plus, les lymphocytes non suppresseurs $FoxP3^{mid}$ (population III) n'expriment pas le CD15s ce qui renforce la notion qu'il s'agit d'une population distincte des Tregs *(31)*. Cette fucosylation (modfication post-traductionnelle) permet au PSGL-1 de se lier à son récepteur la P- ou la E-selectine *(32)*. Cet aspect revêtira un intérêt majeur dans travail que nous présenterons.

D'autres marqueurs membranaires tel que ICOS (inducible T-cell Costimulator), ont été décrit, scindant les Tregs en deux populations fonctionnellement distinctes : les Tregs ICOS+ correspondant aux eTregs dont l'activité immunorégulatrice serait liée à l'IL-10 ; et les Tregs ICOS- correspondant aux nTregs dont l'activité immunorégulatrice serait liée au TGF-β *(33)*.

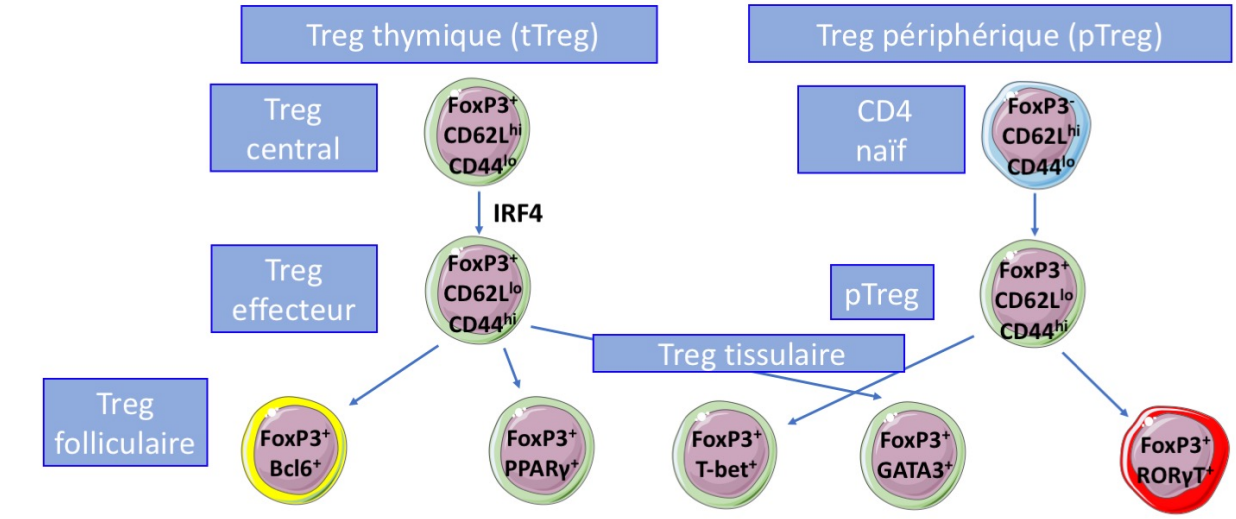
Les Tregs peuvent également être différentiés selon l'expression de facteurs de transcription de T helper chez la souris et chez l'homme (**figure 3**). Il existe par conséquent des Tregs Th1 (exprimant le facteur de transcription TBET), des Tregs Th2 (exprimant GATA3) et les Tregs Th17 (exprimant RORγC), ayant tous une activité immunorégulatrice *in vitro (34)*.

Les lymphocytes T folliculaires helper (Tfh) expriment le marqueur de surface CXCR5 (permettant la migration dans les structures lymphoïdes où est exprimé son ligand le CXCL13) et le facteur de transcription Bcl6. Ils sont responsables de la réponse B T-dépendante par leur action dans le centre germinatif des ganglions notamment via l'IL-21 et le CD40-ligand *(35)*. Les Tfh possèdent aussi leur *alter ego* régulateur, les lymphocytes T folliculaire régulateurs (Tfr). Les Tfr coexpriment FoxP3, CXCR5

et Bcl6 et possèdent une signature transcriptomique bien distincte des Tregs renforçant l'identité de cette sous-population de Tregs *(36)*. Ces Tfr inhibent la réponse B dans le centre germinatif par plusieurs mécanismes qui seront détaillés plus tard. Le transfert dans une souris TCR-KO de $CD4^+$ naïfs $CD45.1^+$ et de Tregs $CD45.2^+$ a permis de montrer que la quasi-intégralité des Tfr dérivaient de Tregs pré-existant suggérant une plasticité des Tregs *(37)*.

Enfin, des Tregs résidant dans les tissus tels que la peau, le tissus adipeux, le muscle et l'os ont été décrits (**figure 3**) *(38)*. Ces Tregs tissulaires participent aux mécanismes d'homéostasie et de réparation des tissus, et interviennent dans des processus pathologiques tels que la résistance à l'insuline et les désordres métaboliques liés à l'obésité *(38)*.

Deux populations lymphocytaires régulatrices qui ne seront pas discutée en détail dans la suite de ce travail sont les $CD8^+$ régulateurs et les cellules Tr1 (T regulatory type 1). Les cellules Tr1 qui sont des cellules T régulatrices dites non-conventionnelles car elles n'expriment ni le CD25 ni le FoxP3 *(39)*. Il s'agit d'une sous-population de lymphocytes T présents chez la souris et chez l'homme, différentiée en périphérie à partir de lymphocytes T naïfs en réponse à une stimulation antigénique en présence d'IL-10. Leurs mécanismes immunosuppressifs sont assez proches de ceux des Tregs conventionnels (expression de CTLA-4, IL-10 et TGF-β). Les lymphocytes $CD8^+$ régulateurs sont une sous-population de lymphocytes CD8+ exprimant le CD122 (IL-2Rβ) et exprimant faiblement le CD49 *(40)*. Cette sous-population régulatrice agit de façon antigène-dépendante, principalement via une cytotoxicité médiée par FAS/FASL *(40)*.



Localisation	Centre germinatif	Tissu adipeux	Autres tissus	Peau	Intestin
Cibles	Tfh	Métabolisme TA	Th1	Th2	Th17/Th2
Fracteur de transcription	C-Maf TCF1/LEF1	IRF4 BATF	STAT1	IRF4 BATF	C-Maf STAT3
Recepteur membranaire	CXCR5	CCR1	CX3CR1	CCR4 CCR6	CCR6

Figure 3 : Hétérogénéité des Tregs (murins) et facteurs de transcriptions associés. adapté à partir de Koizumi et al. *(41)*

iii. Instabilité et plasticité des Tregs

L'étude de modèles murins possédant un gène rapporteur (tel que la Green Fluorescent Protein, GFP) à proximité de FoxP3 a permis d'étudier de manière dynamique les Tregs et leur devenir. Pour étudier la stabilité des Tregs *in vivo* et *ex vivo*, Zhou et al. ont généré un modèle murin en croisant des souris FoxP3-GFP-Cre (exprimant le recombinase Cre) à des souris ayant un potentiel d'exprimer la YFP sous réserve d'excision du codon stop par l'expression de la recombinase Cre (Rosa26-loxP-Stop-loxP-YFP) *(42)*. Ainsi dans ce modèle, les Tregs sont verts (GFP+) mais également jaune car exprimant la Cre recombinase qui excise le codon stop et permet l'expression de YFP. Les cellules ayant secondairement perdu l'expression de FoxP3 (nommées ExTregs) sont jaunes seulement. Dans cette étude, les auteurs démontrent qu'une faible proportion de Tregs perdent l'expression de FoxP3 et acquièrent un phénotype effecteur mémoire avec la production de cytokines pro-inflammatoire (IFNγ, IL-17) *(42)*. De plus, le transfert passif d'ExTregs à des souris NOD induisait un phénotype d'autoimmunité confirmant leur potentiel pathogène *(42)*. L'étude de la clonalité des TCR des ExTreg, des Tconv et des Treg suggère que les ExTreg dérivent en partie des Tregs mais également de Tconv ayant exprimé de manière transitoire FoxP3 durant leur activation *(42)*. L'utilisation d'un modèle où la recombinase est contrôlée par le Tamoxifène a permis d'exclure les Teff exprimant de manière transitoire FoxP3 des « ExTregs » et a toutefois confirmé que même si la majorité des Tregs sont stables, une portion d'entre eux perdent l'expression de FoxP3 surtout en cas d'inhibition de l'IL-2 dont la présence est indispensable à la stabilité des Tregs *(43)*. Même si de telles expérimentations sont impossibles chez l'homme, il est vraisemblable qu'il existe également une instabilité des Tregs humain, comme l'atteste la diminution des niveaux de FoxP3 chez les patients atteints de plusieurs maladies auto-immunes dont le lupus systémique *(44)*, bien que d'autres mécanismes puissent également être en jeu (production moindre de Tregs ou apoptose de ceux-ci).

L'instabilité de l'expression de FoxP3 parmi les Tregs démontre une plasticité dans le programme de différentiation des Tregs. Cette plasticité est retrouvée chez la majorité des cellules du système

immunitaire et lui permet de réagir de manière dynamique à un environnement et à des agressions diverses (agents infectieux, toxiques, cancers ...) *(45)*.

Comme nous l'avons décrit précédemment, les Tregs peuvent exprimer des facteurs de différentiation de CD4 T-helper 1 (T-bet), Th2 (GATA3), Th17 (RORγC) ou Tfh (Bcl6), que nous dénommerons Th1/Th2 ou Th17-like Tregs. Il semble que les Tregs ayant acquis l'expression d'un facteur de transcription de Teff régulent de manière plus efficace la réponse T helper associée avec celui-ci, comme démontré chez la souris pour la réponse Th1 *(46)*, ou Th2 *(47)*, ou Th17 *(48)*. Cependant, une inflammation locale en cas de greffe d'organe *(49)*, ou de maladie auto-immune *(50)*, peut entrainer une majoration de la proportion de Th-like Tregs perdant l'expression de FoxP3 et acquérant une capacité de sécrétion de cytokines, leur conférant un rôle effecteur et pathogénique. Le rôle de certaines cytokines pro-inflammatoires semble majeur dans la plasticité des Tregs. En effet, l'interleukine 23 et l'interleukine 6 ont montré leur rôle dans la conversion de Tregs en Ex-Treg sécrétant de l'IL-17 et responsable de l'aggravation de la réponse inflammatoire *in vivo (49, 50)*. Dans le cadre de l'infection cornéenne à HSV1, une conversion des Tregs en ex-Tregs Th1 a été mise en évidence, par un mécanisme dépendant de l'IL-12 *(51)*. En dehors des cytokines inflammatoires, d'autres stimulus peuvent être responsables de la plasticité des Tregs. Dans l'athérosclerose (la maladie inflammatoire la plus répandue dans les pays développés) au sein de laquelle le rôle protecteur des Tregs a été maintes fois démontré *(52)*, les lipoprotéines de faible densité circulantes oxydées (LDL-ox) induisent une conversion des Tregs en Tfh pro-athérogénique *(53)*. Mécanistiquement, il existe une diminution de l'expression du récepteur de haute affinité à l'IL-2 (CD25) par l'augmentation du cholestérol intra-cellulaire induite par les LDL-ox *(53)*.

Il semble donc exister une balance T régulatrice / T effectrice dont l'équilibre est altéré dans des conditions pathologiques tel que dans les maladies auto-immunes et les infections (à la faveur de la population effectrice) ou dans le cancer (à la faveur de la population régulatrice).

Après avoir défini les Tregs et leur plasticité, il semble important de décrire leur mode d'action en physiologie et en pathologie.

b) Mécanismes d'immunosuppression des lymphocytes T régulateurs

Plusieurs mécanismes ont été décrits pour expliquer les fonctions immunosuppressives des Tregs (**figure 4**). La limite principale des connaissances acquises dans ce champ est que celles-ci ont été majoritairement acquises *in vitro* et qu'il est actuellement peu clair quels mécanismes suppresseurs sont prépondérants *in vivo,* notamment en pathologie humaine.

Nous décrirons dans un premier temps les techniques d'étude des Tregs *in vitro* ayant permis de caractériser leur fonctions. Nous discuterons ensuite des différents mécanismes d'immunosuppressions connus puis de l'importance de la spécificité antigénique des Tregs, parfois encore débattue.

i. Expérimentations permettant l'étude des Tregs : l'importance du recul

Les premières expérimentations permettant d'étudier *in vitro* les capacités régulatrices des Tregs ont été décrite par les équipes de Sakaguchi et de Shevach dans la fin des années 90 *(54, 55)*. Celles-ci consistent en une co-culture de Tregs et de cellules effectrices (Teff). Pour stimuler la prolifération des cellules effectrices, les cellules étaient cultivées en présence d'anti-CD3 soluble (fournissant le 1er signal) et de cellules présentatrices d'antigènes d'origine splénique irradiées exprimant le CD80/86 (cellule présentatrice d'antigène, CPA ; fournissant le deuxième signal). La majorité des études réalisées ensuite chez l'Homme utilisaient un anti-CD28 à la place des CPA, responsable d'une plus forte stimulation des Teff et imposant par conséquent de majorer le rapport Treg/Teff par rapport aux expérimentation murines. D'autre part, l'utilisation d'une plaque de culture « coaté » par un anti-CD3 induisait également un signal plus fort (et possiblement différent) de celui induit par un anti-CD3 soluble, imposant également de majorer le rapport Treg/Teff *(56)*. Notablement, l'utilisation de CPA ne permet pas de différencier si l'effet immunosuppresseur des Tregs est dirigé vers le Teff ou la CPA (ou vraisemblablement les deux). Enfin, ces modèles d'étude *in vitro* ne reconstituent pas la complexité de l'environnement immunologique retrouvé *in vivo* et toutes les conclusions tirées de ces

modèles doivent être interprétées avec du recul. En effet, l'utilisation de chambre de Boyden (transwell) a montré que le contact cellulaire était indispensable à l'effet immunosuppresseur des Tregs *in vitro*, limitant donc potentiellement le rôle des facteurs solubles tels que les interleukines ou certains métabolites *(54, 55)*. Cependant, et comme nous le verrons, des facteurs solubles ont été identifié comme jouant un rôle majeur dans des modèles *in vivo* de maladies inflammatoires remettant en question ces premières conclusions.

Avant de décrire les différents mécanismes proposés pour expliquer les fonctions immunosuppressives des Tregs, il convient de rappeler que ceux-ci ne sont probablement pas exclusifs les uns des autres mais probablement intriqués de manière complexe et mis en jeu selon le contexte local/systémique et la cellule cible en jeu.

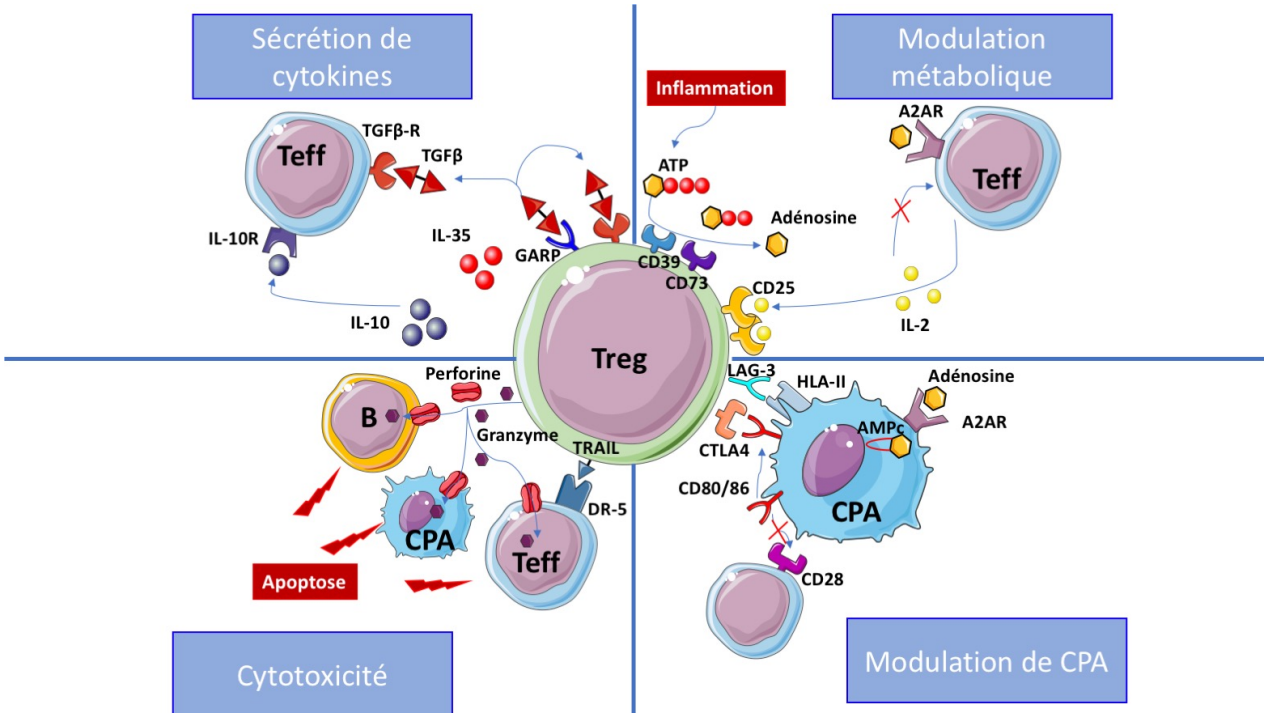


Figure 4 : Principaux mécanismes immunosuppressifs des Tregs.

Légende : 1/**Sécrétion de cytokines**. Le Treg exprime plusieurs cytokines tels que l'IL-10, l'IL-35 mais également le TGF-b via une molécule chaperone GARP. Ces cytokines auront un effet sur les Teff et/ou sur les CPA. 2/ **Modulation métabolique**. Les Tregs transforment enzymatiquement l'ATP extracellulaire en ADP puis en adénosine qui se fixe à son recepteur sur les Teff et les CPA. D'autre part, les Tregs consomment de manière préferentielle l'IL-2 extracellulaire, privant les Teff de ce signal de survie et de prolifération indispensable. 3/**Cytotoxicité**. Les Tregs peuvent induire une cytotoxicité direct envers les lymphocytes B, les CPA ou les Teff via plusieurs mécanismes (perforine/granzyme, TRAIL). 4/ **Modulation de CPA**. Les Tregs modulent les fonctions des CPA via l'expression de CTLA4 et de LAG-3.

Abréviations : A2AR, récepteur A2 à l'adénosine ; ATP, Adenosine triphosphate ; CPA, cellule présentatrice d'antigènes ; CTLA4, cytotoxic T lymphocyte antigen 4 ; DR-5, death receptor 5 ; HLA-II, human leucocyte antigen class II ; LAG-3, lymphocyte activation gene 3 ; Teff, lymphocyte T effecteur ; TRAIL, TNF-related apoptosis-inducing ligand ; Treg, lymphocyte T régulateur.

ii. Sécrétion de cytokines immunorégulatrices

Interleukine 10

L'interleukine 10 (IL-10) est une cytokine initialement décrite à la fin des années 80 comme un facteur soluble produit par les CD4 de phénotype Th2, ayant la capacité d'inhiber la production de cytokine Th1 tel que l'IL-2, le TNF et l'IFNγ *(57)*. L'IL-10 est une puissant inhibiteur de l'activation des macrophages *in vitro*, limitant leurs fonction de cellule accessoire de manière indépendante à la présentation d'antigène *(58)*. Toutefois, l'IL-10 a également un effet stimulant sur les lymphocytes B, en amplifiant la réponse humorale, en stimulant la commutation isotypique et la production d'anticorps *(59)*.

Les modèles murins déficient en IL-10 ont permis d'évaluer son rôle immunologique *in vivo (60)*. Ces souris ne présentent pas de déficit quantitatif en lymphocytes B/T et développent une réponse humorale normale en réponse à des antigènes T-dépendants. Cependant, ces souris développent une réponse Th1 exagérée, menant au développement d'une colite inflammatoire spontanée.

Rubtsov et al. ont développé un modèle murin caractérisé par des Treg KO pour l'IL-10 par un système d'expression de la recombinase Cre *(61)*. Sans présenter d'altération du nombre de Tregs, ces souris présentaient une aggravation des réaction d'hypersensibilité cutanée, une majoration de l'inflammation allergique pulmonaire à l'ovalbumine et le développement d'une inflammation colique spontanée. Ces résultats suggèrent un rôle de l'IL-10 dans les fonctions immunosuppressives des Tregs *in vivo (61)*.

D'autres équipes ont montré qu'un KO conditionnel de l'IL-10 dans les Tregs n'abrogeait pas les fonctions immunosuppressive des Tregs *(62)*. Cependant, l'IL-10 agirait directement sur les Tregs pour renforcer l'expression de FoxP3 et leurs fonction immunosuppressives *(62)*.

En conclusion, l'IL-10 produite par les Tregs ou par d'autres cellules immunitaires possède un effet immunorégulateur direct sur les cellules effectrices (lymphocytes T, CPA, macrophages) et indirect en renforçant le phénotype et la stabilité des Tregs.

Transforming Growth Factor beta

Le TGF-β est un homodimère synthétisé sous la forme d'une pré-protéine nécessitant une maturation post-traductionnelle avant être relargué dans le milieu extra-cellulaire sous forme active *(63)*. Le TGF-β interagit avec l'un de ses trois récepteurs (TGFBRI, TGFBRII, TGFBRIII), qui sont exprimés par un large spectre de cellules immunes et non immunes.

Cuende *et al* ont souligné le rôle de la protéine GARP (codée par le gène lrrc32) exprimée à la surface des Tregs *(64)*. GARP est une protéine chaperone qui fixe le TGF-β latent à la surface du Treg et dont la présence est nécessaire à sa transformation en TGF-β actif. L'inhibition du complexe TGF-β/GARP par un anticorps neutralisant inhibe les fonctions suppressives des Tregs *in vitro* et *in vivo* dans un modèle murin de Graft versus Host (GvH) *(64)*.

Le rôle du TGF-β est triple (**figure 5**). Le TGF-β produit par les Tregs a un effet autocrine qui renforce l'expression de FoxP3 et donc la stabilité du Treg en condition inflammatoire via l'activation de la voie SMAD3 *(65)*. Deuxièmement, le TGF-β a la capacité d'induire un phenotype Treg à partir de lymphocytes T $CD4^+$ $CD25^-$ et donc de renforcer la balance Tregs/Teff *(66)*. Enfin, production de TGF-β par les Tregs a un effet suppressif direct sur les cellules immunitaires et ce de manière indépendantes aux autres fonctions des Tregs comme montré dans un modèle croisant le phénotype *scurfy* ($FoxP3^{-/-}$) à un modèle de diabète auto-immun *(67)*.

L'importance *in vivo* de la sécrétion de TGF-β par les Tregs a été confirmée par Li et al. en utilisant un modèle de co-transfert adoptif de Tregs WT ou TGF-β-KO et des lymphocytes T CD4 chez des souris lymphopénique ($RAG\text{-}1^{-/-}$). Les souris injectées avec des lymphocytes CD4 développaient une colite, et le co-transfert de Tregs wild-type et non de Tregs TGF-β-KO permettait d'en améliorer le phénotype *(68)*.

En conclusion, le TGF-β semble avoir un rôle majeur dans la physiologie du Treg, responsable de sa différentiation, de sa stabilité mais également de ses fonctions immunosuppressives.

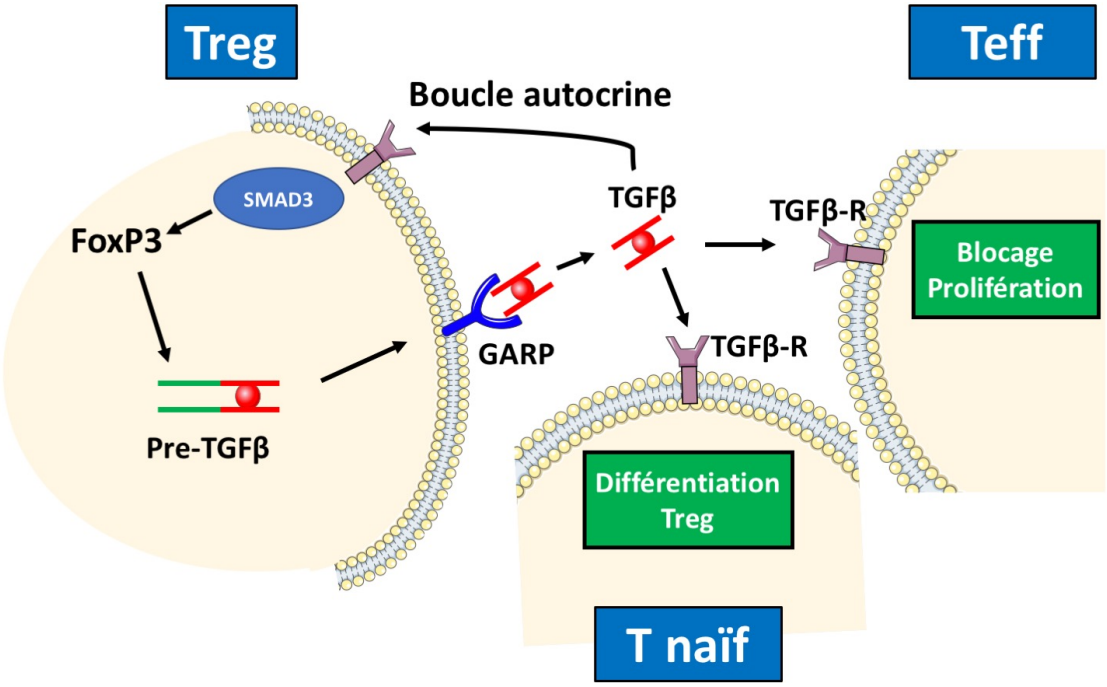


Figure 5 : Origine et modes d'action immunologiques du TGF-β produit par le Treg.

Le TGF-β est produit par les Treg et relargué sous forme mature à l'aide de la protéine chaperone GARP. Une fois dans le milieu extra-cellulaire, le TGF-β a trois actions immunologiques principales. 1/ Il renforce le phénotype régulateur des Treg via l'activation de SMAD3 par une boucle autocrine. 2/ Le TGF inhibe la proliferation des Teff via le TGFβR. 3/ Le TGFβ peut induire une différentiation de Tnaïfs en Treg hors condition inflammatoire (si présence d'IL-6, une différentiation Th17 sera obtenue).

Interleukine 35

L'interleukine 35 est une interleukine hétérodimérique de la famille de l'IL-12 produite principalement par les Tregs et lymphocytes B régulateurs. Chez la souris, les Tregs KO pour l'IL-35 perdent leur potentiel immunosuppresseur *in vivo*, et l'IL-35 recombinant permet de bloquer la prolifération des Teff *in vitro (69)*. Au cours de l'infection à Salmonella ou de l'encéphalite aigue expérimentale (EAE), les plasmocytes CD138+ produisent de l'IL-35 et modulent la réponse inflammatoire *(70)*. Les mécanismes d'action de l'IL-35 sont incomplètement élucidé mais il semble que cette cytokine induise la tolérance via une exhaustion du lymphocyte T par la stimulation de la voie BLIMP1 *(71)*. Cependant, les preuves expérimentales du rôle de l'IL-35 dans les fonctions immunosuppressives des Tregs humains sont plus tenues, le seul article (Chaturvedi et al.) soutenant cette hypothèse ayant été rétracté *(72)*.

iii. Disruption métabolique des cellules effectrices

Consommation préférentielle de l'interleukine 2 par les Tregs

Les Tregs sont caractérisés par une forte expression du CD25 correspondant à la sous-unité alpha du récepteur de haute affinité à l'interleukine 2. Sa trimérisation avec les sous-unités béta (CD122) et gamma (CD132) permet de former le récepteur de haute affinité à l'IL-2.

Ainsi, cette forte expression du récepteur de haut affinité à l'IL-2 est responsable d'une consommation préférentielle de l'IL-2 par les Tregs, privant donc les Teff de l'IL-2 indispensables à leur survie et leur prolifération (**figure 4**). Cette théorie initiée par Palacios et Möller aux début des années 1980 *(73)* a été étayée par plusieurs équipes. Pandiyan *et al.* ont montré chez la souris*, in vitro* et *in vivo*, que les Tregs induisaient une apoptose des Teff par une consommation préférentielle de l'IL-2 *(74)*. Ce manque d'IL-2 est responsable d'un défaut d'activation de la voie Akt, responsable d'une apoptose dépendante de BIM *(74)*. Plus récemment, le concept d'espace ou de « niche » immunologique est apparu. La réponse immunitaire se fait dans un tissu périphérique ou un organe lymphoïde contenant des cellules effectrices, des cellules productrices d'IL-2 et des Tregs. L'équipe de Oyler-Yaniv a

démontré, par le biais de modélisation mathématique confirmées par des expérimentations *in vitro* que des cellules consommant l'IL-2 (les Tregs) pouvaient être responsable d'une forte diminution de la concentration d'IL-2 intra-tissulaire, confirmant donc l'hypothèse évoquée par Palacios près de 40 ans plus tôt *(75)*.

Il faut cependant souligner que certaines équipes ont apporté des arguments allant à l'encontre de ce mécanisme comme médiateur de l'activité immunorégulatrice des Tregs *(56)*. Chinen et coauteurs ont utilisé un modèle de souris de Tregs KO pour le CD25 (*IL2RB^{flox}-FoxP3^{Cre}*) couplé à une souris possédant un transgène de STAT5b portant une mutation gain de fonction activatrice exprimée dans les Tregs uniquement (via l'expression de la recombinase Cre) *(76)*. Ce modèle permet ainsi d'assurer une activation de la voie STAT5 chez des Tregs KO pour le CD122, ces derniers ne pouvant être fonctionnels sans cette l'activation de cette voie de signalisation. Ce modèle a permis de montrer que le recepteur de haute affinité à l'IL-2 était indispensable aux fonctions immunosuppressives des lymphocytes T CD8+ mais non des CD4+, arguant une fois de plus que les Tregs utilisent des mécanismes immunosuppresseurs différents selon la situation immunologique et la cellule inhibée *(76)*.

Production de métabolites immunosuppresseurs

La production de métabolites par le Treg peut altérer la composition du microenvironnement immunologique et par ce biais altérer la réponse des Teff.

ATP/Adénosine. La production d'adénosine extracellulaire par le Treg en est un exemple bien documenté. L'adénosine Tri-Phosphate (ATP) est une molécule organique dont la fonction essentielle est de constituer une réserve en énergie rapidement utilisable par la cellule pour réaliser des réactions chimiques notamment enzymatiques (ATPases). L'ATP peut également être retrouvé dans le compartiment extra-cellulaire dans des conditions physiologiques ou pathologiques (nécrose, inflammation) *(77)*. En effet, à forte concentration extracellulaire, l'ATP est considéré comme un marqueur de souffrance cellulaire ou Damage associated molecular pattern (DAMP), capable de potentialiser la réponse inflammatoire *(77)*. D'un point de vue mécanistique, l'ATP se fixe à un de ses

récepteur membranaire (P2X 1 à 7) et induit un signal calcique intracellulaire menant à l'activation de la voie NFAT *(78)*.

Les Tregs murins et humains sont caractérisés par l'expression membranaire de deux protéines impliquées dans le métabolisme des purines, le CD39 et le CD73 *(79)*. Le CD39 est une glycoprotéine membranaire de 510 acides aminés qui catalyse la dégradation de l'ATP en Adénosine Di-Phosphate (ADP), ainsi que la dégradation de l'ADP en Adénosine Mono-Phosphate (AMP). L'AMP extracellulaire est ensuite métabolisé en adénosine par le CD73, une enzyme homo-dimérique attachée à la membrane par une ancre glycosylphosphatidyl inositol (GPI).

L'adénosine extra-cellulaire est reconnue par les récepteurs P1 (A1R, A2AR, A2BR et A3R) exprimés par la majorité des cellules immunitaires (**figure 4**). La fixation de l'adénosine à un de ses récepteur induit dans la plupart des cas la production d'AMP cyclique (AMPc) intracellulaire, connue pour ses effets anti-inflammatoire *(77)*. L'importance de la production d'adénosine pour l'activité immunosuppressive des Tregs a été suggérée dans des études chez l'homme *(80)*, et confirmé dans des études *in vitro* et *in vivo* chez la souris *(81)*. L'effet de l'adénosine est direct sur les cellules effectrices, mais également sur le Treg lui-même exprimant également le A2AR, renforçant son phénotype régulateur *(82)*. L'adénosine impacte également la CPA humaine en diminuant son expression d'HLA de type II, l'expression de molécules de co-stimulation et de cytokines dans des conditions inflammatoires *(83)*. D'un point de vue mécanistique, l'engagement de l'adénosine sur l'A2AR induit une production intra-cytoplasmique d'AMP cyclique (AMPc) ayant un rôle anti-inflammatoire via l'activation de l'élément de réponse à l'AMPc (CREB) *(84)*. Cette voie de signalisation anti-inflammatoire est d'ailleurs utilisée en clinique avec un inhibiteur de la phosphodiestérase 4 (aprémilast), qui inhibe la dégradation de l'AMPc et qui a montré son efficacité dans plusieurs maladies inflammatoires. Le Treg agirait donc dans les zones inflammatoires en convertissant l'ATP pro-inflammatoire en adénosine anti-inflammatoire. L'importance de ce mécanisme dans les pathologie auto-immune a été évoquée par plusieurs auteurs, retrouvant une diminution de l'expression de CD39

sur les Tregs de patients atteints de LES, et un effet protecteur du récepteur à l'adénosine A2AR dans le modèle MRL/Lpr *(85, 86)*.

iv. Effet sur les cellules présentatrices d'antigènes

Expression de l'inhibiteur de costimulation CTLA-4. Le Cytotoxic T lymphocyte antigen 4 (CTLA-4) est une protéine membranaire de « point de contrôle immunitaire » (immune checkpoint en anglais). Il s'agit d'un homologue du CD28, ayant par conséquent les mêmes ligands (CD80 ou B7-1 et CD86 ou B7-2) mais avec une plus haute affinité *(87)*. Le Treg exprime de manière constitutionnelle le CTLA-4, contrairement aux lymphocytes Teff qui l'expriment seulement en cas d'activation *(88)*. Il a été initialement montré par l'équipe de Sakaguchi que le blocage du CTLA-4 par un anticorps bloquant induisait un phénotype auto-immun chez la souris *(88)*. D'autre part, des expériences d'immunosuppression *in vitro* montraient que le blocage du CTLA-4 inhibaient les fonctions immunosuppressives des Tregs *(88)*. L'importance du CTLA-4 exprimé par les Tregs a été confirmé par la même équipe quelques années plus tard par le biais d'une modèle murin avec un KO conditionnel de CTLA-4 dans les Tregs *(89)*. Toutefois, les souris ayant un KO complet de CTLA-4 présentaient un phénotype auto-immune et un pronostic plus sévère que les souris présentant un KO conditionnel au Treg, plaidant en faveur de l'importance d'un rôle régulateur du CTLA-4 non strictement limité au Treg *(89)*.

Le rôle immunomodulateur du CTLA-4 exprimé par le Treg est médié par plusieurs mécanismes (**figure 6**). Après stimulation du TCR par son ligand, le lymphocyte T effecteur doit recevoir un second signal activateur, habituellement fourni par la CPA présentant le CD80/CD86 au CD28 du Teff *(90)*. Ainsi, l'expression de CTLA-4 par les Tregs pourrait agir en compétiteur direct au Teff exprimant le CD28 pour leur ligand commun, le CD80/CD86 exprimé par la CPA *(87)*. D'autre part, il a été montré en utilisant des lymphocytes humains (*in vitro* et *in vivo* via un modèle de transfert utilisant des souris $RAG2^{-/-}$) que l'expression de CTLA-4 était responsable d'une trans-endocytose du CD80/CD86 dans la cellules exprimant le CTLA-4, diminuant par conséquent l'expression de CD80/CD86 par la CPA et donc son

potentiel activateur de lymphocyte T *(91)*. Outre l'effet de compétition du CTLA-4 pour son ligand et la déplétion de CD80/CD86 des CPA, la littérature suggérant une signalisation intracellulaire inhibitrice dans la cellule portant le CTLA-4 est contradictoire *(92)*. Cette signalisation ne semble pas être une voie majeure du mécanisme inhibiteur de CTLA-4 pour plusieurs raisons. Tout d'abord, l'expression cellulaire d'un CTLA-4 déficient de sa portion cytoplasmique inhibe la réponse T avec la même efficacité que le CTLA-4 sauvage *(93)*. D'autre part, l'utilisation de CTLA-4-Ig recombinant (eg, abatacept) chez des patients atteints de pathologies auto-immunes ou inflammatoire a montré un effet clinique dans plusieurs indications.

Lymphocyte activation gene-3 (LAG-3). LAG-3 est une protéine transmembranaire associée au CD4 reconnaissant le HLA de classe II. Il a été montré que LAG-3 était exprimée par les Tregs et que sa présence était associée à leur activité immunosuppressive *in vitro* et *in vivo (94)*. D'un point de vue mécanistique, LAG-3 exprimée par le Treg se fixe au HLA-II des CPA en leur conférant un phénotype inhibiteur via une signalisation mettant en jeu le FcγRγ et la kinase ERK *(95)*. Cette signalisation induit notamment une baisse d'expression des molécules de costimulation (CD80/CD86) *(95)*.

Indoléamine 2,3 dioxygénase/tryptophane. Les Tregs induisent l'expression d'indoléamine 2,3 dioxygenase (IDO) par la cellule dendritique, via un mécanisme dépendant *(96)*, ou indépendant du CTLA-4 *(80)*. L'IDO est responsable du catabolisme du tryptophane et nécessaire à la synthèse de plusieurs dérivés (les kynurénines) ayant une activité biologique. Les CPA exprimant l'IDO après activation par du LPS acquièrent un profil tolérogène avec une forte expression d'IL-10 et une diminution d'IL-12 *(98)*. L'activité de l'IDO a un rôle dans la génération périphérique des Tregs *(99)* et dans le renforcement de leur phénotype immunsuppresseur *(100)*.

D'autres mécanismes discutés plus haut ont également un rôle régulateur sur les CPA, telle la sécrétion de cytokines régulatrices ou la disruption métabolique (production de dérivés de l'adénosine).

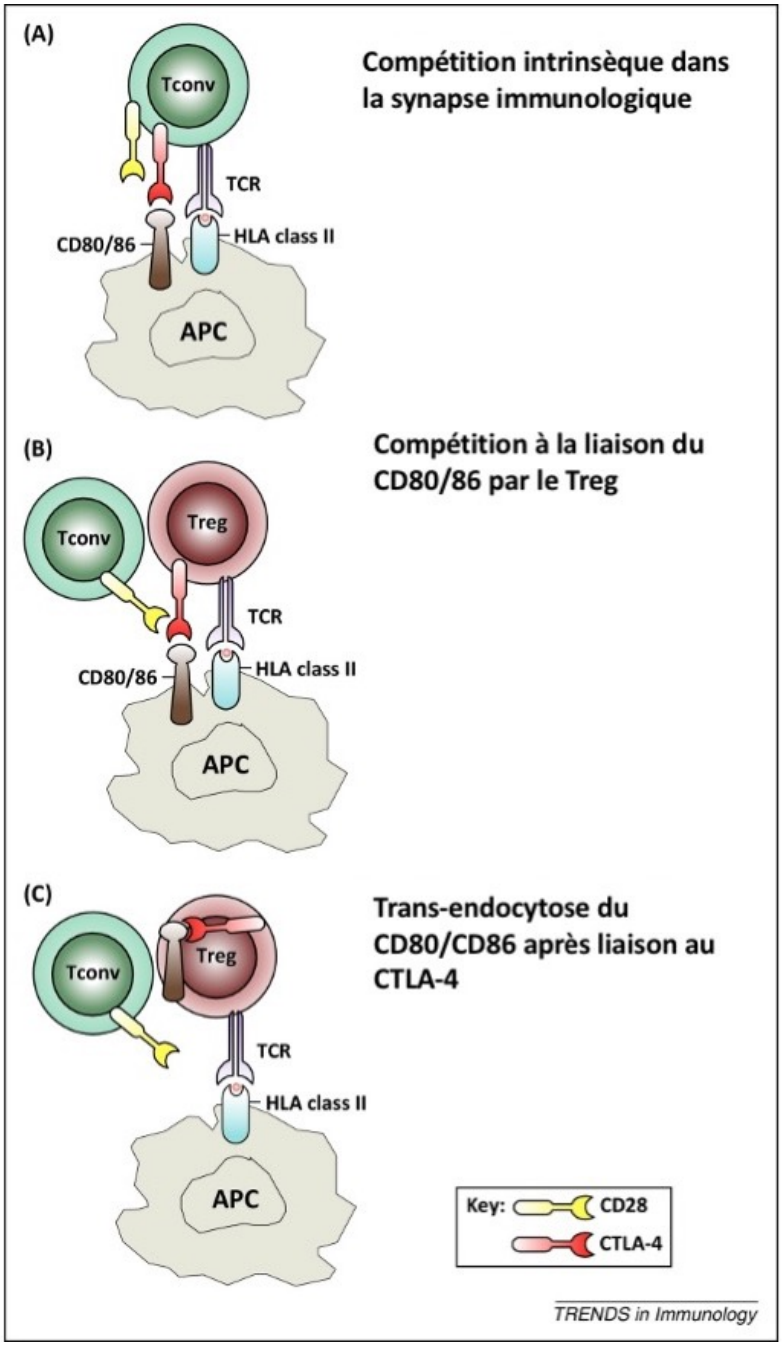


Figure 6 : Mode d'action du CTLA-4. Adapté à partir de Walker *et al.* *(92)*

(A) Le lymphocyte T conventionnel (Teff) exprime le CTLA-4 après activation. Le CTLA-4 ayant une plus forte affinité au CD80/86 par rapport au CD28, une compétition a lieu dans la synapse immunologique, responsable de ce phénomène de rétrocontrole.

(B) Le CTLA-4 exprimé par le Treg va se fixer de manière préférentielle au CD80/86 exprimé par le Teff et par conséquent bloquer le second signal normalement fourni par la cellule présentatrice d'antigène (CPA).

(C) La fixation du CD80/86 au CTLA-4 exprimé par le Treg va être responsable d'une trans-endocytose du CD80/86, limitant la capacité de la CPA de fournir le second signal.

v. *Action cytolytique sur les cellules effectrices*

Cytolyse directe via Granzyme.

Le couple Granzyme/Perforine est une voie bien connue induisant l'apoptose cellulaire principalement par les cellules CD8+ cytotoxique ou par les cellules NK. L'équipe de Timothy Ley a montré que des Tregs humains ($CD4^{+}CD25^{high}$) expriment fortement granzyme A après stimulation par CD3/CD46/IL-2, et à moindre degré après stimulation par CD3/CD28/IL-2 *(101)*. Ces Tregs activés induisent *in vitro* une apoptose de cellules effectrices (Teff, T CD8, CPA) via un mécanisme indépendant du FAS-Ligand et, contact et perforine-dépendant *(101)*. Il a également été montré que les Tregs peuvent inhiber directement les lymphocytes B via une cytolyse dépendante de la perforine *(102)*. De manière intéressante, la littérature étudiant la cytolyse médiée par le Treg chez la souris retrouve un rôle prépondérant de granzyme B et peu de rôle de granzyme A, soulignant les différences entre l'immunologie du Treg humain et murin *(103)*.

Cytolyse directe via TRAIL. Ren et coauteurs ont montré que chez la souris, les Tregs activés (CD3/CD28/IL-2) induisaient une apoptose des Teff *in vitro* et ce de manière contact-dépendante (via une expérience de transwell) *(104)*. Cet effet était indépendant de FAS-Ligand, mais dépendante de l'expression de Tumor necrosis factor-Related Apoptosis Inducing Ligand (TRAIL) par les Tregs activés, et du Death Receptor 5 (DR5) par les Teff activés *(104)*. Pour confirmer ces résultats *in vivo,* les auteurs ont réalisé une expérience élégante visant à injecter à des souris nude/nude (n'ayant pas de thymus, et par conséquent de lymphocytes T), $3x10^{7}$ lymphocytes Teff marqués fortement au CFSE et des $3x10^{7}$ lymphocytes Teff marqués faiblement au CFSE avec des Tregs activés. Dix-huit heures après l'injection, les rates étaient récupérées, et le ratio Teff $CFSE^{low}$ et $CFSE^{high}$ était évalué. Le transfert de Tregs activés induisait une diminution des deux populations mais avec un rapport strictement égal à 1. Cependant, lorsque la population de Teff $CFSE^{low}$ était pré-traitée avec un anticorps bloquant DR5, le ratio s'inversait nettement avec une augmentation du ratio $CFSE^{low}/CFSE^{high}$ aux alentours de 3 après 18

heures, indiquant une protection des cellules pré-traitées par l'anti-DR5 *(104)*. L'importance de la voie TRAIL dans la physiologie du Treg a été confirmée par une autre équipe qui a montré que des souris KO pour l'IL-10 et l'IL-35 présentaient des Tregs avec des fonctions immunosuppressive conservées mais avec une augmentation de l'expression de TRAIL *(105)*. Cette surexpression de TRAIL était responsable de la conservation des fonctions immunosuppressives des Tregs IL-10/IL-35 KO car l'ajout d'un anticorps bloquant DR5 inhibait les fonctions des Tregs KO mais pas des Tregs sauvages *(105)*.

Ces résultats nombreux et parfois discordants entre eux illustrent les nombreux mécanismes mis en jeu par les Tregs pour contrôler les différents acteurs du système immunitaire. L'hypothèse la plus vraisemblable est que ces mécanismes soient non exclusifs les uns des autres et qu'ils interviennent de manière préférentielle selon le type cellulaire engagé, la localisation (tissulaire versus organes lymphoïdes), la cinétique et l'intensité de la réponse immunitaire. Cependant, les techniques actuelles ne permettant pas de suivre de manière aisée les lymphocytes *in vivo*, il est difficile d'étudier l'importance relative de chaque mécanisme dans telle ou telle situation.

vi. Réponse Treg antigène-spécifique versus polyclonale

Les possibilités de thérapies cellulaires utilisant le Tregs dans le cadre de pathologies auto-immunes ou de greffes nécessitent de comprendre les déterminants des fonctions immunosuppressives des Tregs. D'autre part, il semble particulièrement important de comprendre s'il est nécessaire d'amplifier des Tregs spécifiques de l'(ou des) antigène(s) contre lequel la réponse immunitaire est dirigée, ou si des Tregs polyclonaux ont la même efficacité.

De premières expériences menées par l'équipe de Shevach suggèrent qu'une fois le Treg activé, il peut inhiber *in vitro* les lymphocytes T, et ce, quelque-soit leur cible antigénique *(55)*. Les expérimentations *in vivo* de plusieurs équipes ont toutefois démontré que l'utilisation de Tregs spécifiques d'antigènes était plus efficace pour inhiber une réponse auto-immune ou de rejet immunologique *in vivo (106, 107)*. Ce surcroit d'efficacité provient probablement de la capacité du Treg spécifique d'antigène à s'activer dans l'organe lymphoïde secondaire ou dans le tissu siège de la réponse immune, et par conséquent d'y induire localement la réponse immunosuppressive.

Des techniques novatrices permettent une expansion *in vitro* de Tregs spécifiques d'allo-antigènes, dont l'efficacité est significativement supérieure aux Tregs polyclonaux. D'autre part, l'avènement des récepteur chimérique (chimeric antigen receptor, CAR) T cell ouvrent la voie à la production de CAR Treg, spécifique d'un antigène *(108)*.

c) Régulation de la fonction des lymphocytes T régulateurs

Nous avons exposé plus tôt la plasticité du Treg en réponse aux stimulus rencontrés. Outre cette plasticité, l'environnement peut moduler l'état d'activation et les fonctions des Tregs.

i. La stimulation via le TCR

Le rôle du signal TCR a été débattu durant de nombreuses années, certains auteur suggérant que la réponse TCR n'était pas fonctionnelle dans les Tregs, participant au maintien de leur fonction immunosuppressives *(109)*.

Plusieurs travaux ont depuis démontré l'importance du signal TCR dans l'homéostasie du Treg. Tout d'abord, une technologie récemment décrite dénomée Timer of Cell Kinetics and Activity (TOCKY ; toki signifiant « temps » en japonais) permet de suivre dans le temps une stimulation antigénique par le biais d'un système rapporteur bi-couleur temps- et intensité-dépendant *(110)*. Les auteurs ont ainsi démontré que durant leur maturation thymique, les tTreg bénéficiaient d'une stimulation continue du TCR soutenant leur programme transcriptionnel. En périphérie, les Tregs continuent à recevoir un signal du TCR, plus important par rapport aux T conventionnels *(111)*. Enfin, ce signal TCR persistant en périphérie est un déterminant de la fonction des Tregs, les Tregs périphériques possédant un KO-conditionnel du TCRα perdant leur capacités immunosuppressives *in vitro* et *in vivo (112)*. Il est important de noter que la perte du signal TCR induit une modulation de la voie mTOR, directement impliquée dans le contrôle métabolique de la cellule *(112)*.

ii. Immunométabolisme

Généralités sur le métabolisme. Le métabolisme cellulaire permet de produire de l'énergie (ATP) ainsi que des acides aminés, des acides gras ou des intermédiaires métaboliques nécessaires aux fonctions, à la croissance et à la division cellulaire. Ces voies métaboliques sont modulées par des « interrupteurs » (switch) métaboliques contrôlant notamment l'expression des enzymes limitantes de chaque voie. Les technologies récentes permettant d'étudier presque en direct les voies

métaboliques intracellulaires ont mis en évidence les liens bidirectionnels existants entre métabolisme et fonctions des cellules immunitaires *(113)*.

Les principales voies métaboliques utilisées dans la cellule sont (**figure 7**):

- la glycolyse, qui mène à la production de pyruvate et de 6 ATP. Selon le statut métabolique de la cellule, le pyruvate sera transformé en acétyl-CoA (ACoa) si la cellule utilise le métabolisme oxydatif (ou aérobie), ou en lactate (au prix de 4 ATP, menant donc une rentabilité plus faible de la glycolyse) en cas de métabolisme anaérobie.
- Le catabolisme des acides gras est quant à lui entièrement oxydatif, et relativement lent dans sa production d'énergie, par conséquent favorisé dans les cellules ayant une demande énergétique modérée.
- Enfin, les voies du pentose phosphate et les voies du métabolismes des acides aminés (notamment de la glutamine) fournissent des intermédiaires métaboliques aux cellules en croissance/division *(113)*.

Dualité métabolique Treg/Th17. Nous avons décrit plus tôt la dualité identitaire existant entre les Tregs et les Th17, avec l'existence de plasticité entre ces deux états de différentiation. Cette dualité est également retrouvée au niveau métabolique. Gerriets et coauteurs ont montré que la différentiation, l'expansion et les fonctions des Tregs reposaient sur le métabolisme oxydatif, notamment le catabolisme des acides gras *(114)*. Cette programmation métabolique était diamétralement opposée à celle des Th17 qui utilisaient préférentiellement la glycolyse, notamment anaérobie, pour soutenir leur prolifération et leur fonction. La pyruvate deshydrogénate kinase 1 (PDHK1) contrôle l'orientation métabolique vers la glycolyse anaérobie ou le métabolisme oxydatif. Influencer le métabolisme cellulaire en modulant PDHK1 permet de d'inhiber la différentiation et les fonctions des Th17 et de favoriser les Treg *in vitro* et *in vivo (114)*. De la même manière, l'inhibition pharmacologique de l'acetyl-Coa carboxylase 1 (ACC1) contrôlant la voie de synthèse des acides gras inhibe la différentiation Th17 et favorise le développement de Tregs *in vitro* et *in vivo (115)*.

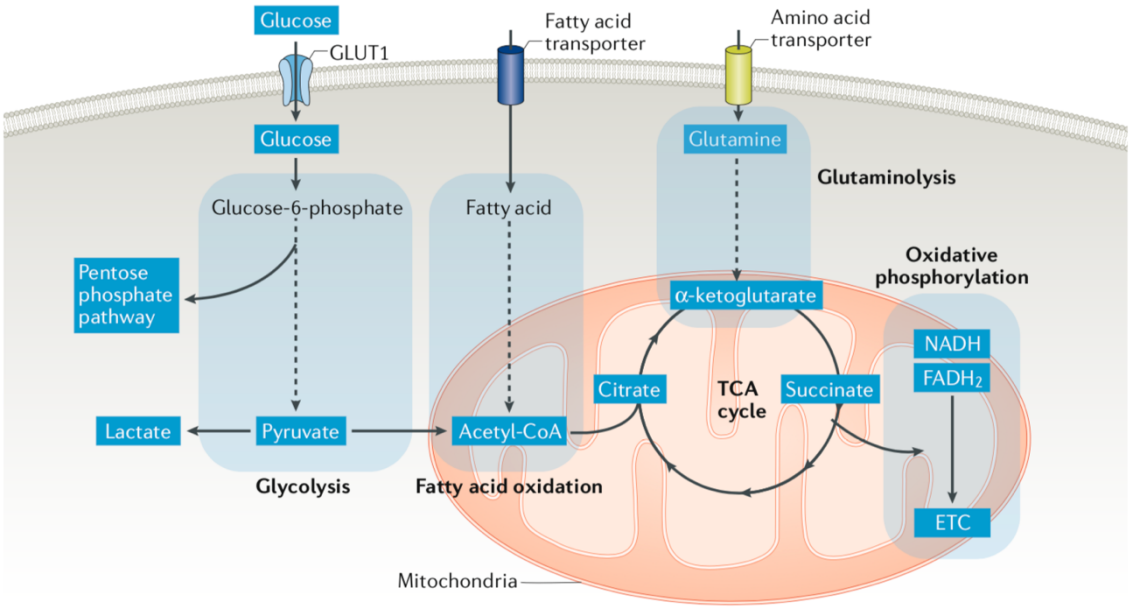


Figure 7 (extraite de Sharabi et al. *(116)***) : voies métaboliques mises en jeu dans les cellules immuniotaires.**

On denombre 3 voies principales. 1/ **La glycolyse**, qui se décompose en glycolyse **anaérobie** (produisant du lactate et « seulement » 2 molécule d'ATP par molécule de glucose) et en glycolyse **aérobie** générant de l'acetyl-Coa qui une fois entrée de la cycle de Krebs génèrera 30-34 molécules d'ATP par molécule de glucose. Une voie alternative de la glycolyse est la voie du pentose phosphate qui permettra de produire des intermédiaires métaboliques nécessaire à l'anabolisme cellulaire. 2/ **L'oxydation des acides gras** qui est un phénomène oxydatif uniquement. 3/ **Le catabolisme des acides aminés** (y compris glutaminolyse) qui permettra également la production d'intermédiaire métabolismes indispensable à l'anabolisme cellulaire.

Les principales interrupteurs « switch » immunométaboliques et leur modulation pharmacologique.

- La voie mTOR (mammalian Target Of Rapamycine) est composée de deux enzymes portant une activité sérine/thréonine kinase régulatrice clée du cycle cellulaire. Il s'agit de deux complexes multi-protéiques, mTORC1 et mTORC2. La voie mTOR peut être activée par de nombreux stimulus, tels que la stimulation TCR/CD28, des cytokines, des chimiokines, des facteurs de croissances et des métabolites *(117)*. L'activation de la voie mTOR induit une reprogrammation rapide du métabolisme cellulaire par activation de la glycolyse (via mTORC1 et mTORC2) *(118)*, et de la glutaminolyse (via mTORC1 et Myc) *(119)*, favorisant la différentiation Teff y compris Th17. L'inhibition de mTOR par la rapamycine ou le sirolimus inhibe la différentiation Th17 et favorise le développement et la fonction des Tregs chez l'homme, *in vitro (120)*, et *in vivo* (voir plus bas).
- La voie de l'AMP kinase (AMPK) est une voie métabolique activée en cas de privation énergétique. L'AMPK active le métabolisme oxydatif cellulaire, notamment le catabolisme des acides gras, la biogénèse mitochondriale, et l'autophagie *(121)*. D'autre part, l'activation de l'AMPK inhibe la voie mTOR par la disruption du complexe mTORC1 et une phosphorylation inhibitrice du cofacteur Raptor *(122)*. La voie AMPK est fortement activée dans les tTreg et les iTreg, et l'activation pharmacologique (par la metformine) de celle-ci promeut la différentiation Treg *in vitro* et *in vivo (123)*.

Ces voies métaboliques peuvent être influencées pharmacologiquement (voir plus loin), mais également par des métabolites intra- ou extracellulaire tels que les acides gras libres (*e.g.*, palmitate) qui activent la voie AMPK et favorisent la différentiation et l'activité immunosuppressive des Tregs *(123, 124)*.

iii. L'environnement inflammatoire

L'environnement dans lequel évolue le Treg joue un rôle indéniable sur l'homéostasie et les fonctions de celui-ci.

Les molécules de co-stimulation. Outre la stimulation du TCR décrite plus haut, les molécules de co-stimulation ont un rôle certain sur le développement et le maintien des fonctions immunosuppressives des Tregs. Nous ne reviendrons pas sur la stimulation du CD28 induisant le second signal indispensable à la génération et aux fonctions des Tregs, déjà décrites plus haut. La liaison du CTLA-4 à un de ses ligands favorise l'expression de FoxP3 par le Treg l'exprimant, ceci de manière indépendante des cellules présentatrice d'antigène. En effet, l'interaction entre un Treg et un anticorps agoniste ciblant le CTLA-4 induit une augmentation de l'expression de FoxP3 *in vitro (125)*. D'autre part, après transfert à ratio 1:1 de lymphocytes CD4+ naïfs provenant de souris sauvages ou CTLA-4-KO dans des souris RAG, la majorité des Tregs induits *in vivo* provenaient de la souris sauvage, suggérant que le CTLA-4 a un également un effet intrinsèque sur le Treg *(125)*. Notre équipe a montré qu'une autre molécule de co-stimulation avait un effet dans l'homéostasie des Tregs. L'OX40, une molécule de la famille des récepteurs au TNF est exprimé de manière basale par le Treg. Sa liaison à son ligand, l'OX40L, exprimé par les monocytes en conditions inflammatoires notamment dans le lupus systémique induit une perte des fonctions immunosuppressive des Tregs humain *ex vivo* (voir **article 3**, page 98).

Les cytokines. Comme nous l'avons précédemment décrit, de fortes concentrations d'IL-6 présentes dans l'environnement inflammatoire peuvent provoquer une transdifférentiation des Tregs en Teff de type Th17. Le tumor necrosis alpha (TNF-α) est une cytokine inflammatoire produite en excès dans de nombreuses situations inflammatoires, tel que les rhumatismes inflammatoires chroniques (*e.g.,* polyarthrite rhumatoïde). Les Tregs expriment les récepteurs au TNF-α, le récepteur de type I (TNFR1) étant responsable de l'effet pro-inflammatoire du TNF-α, et le recepteur TNFR2 étant responsable d'un effet anti-inflammatoire. Il a été montré que les Tregs isolés de patients atteints de polyarthrite rhumatoïde sont faiblement suppressifs, et que le traitements des patients avec un anti-TNF

(infliximab) améliore les fonctions immunosuppressives des Tregs humains *ex vivo (126)*. Mécanistiquement, le TNF-α induit une diminution de l'expression de FoxP3 par le Treg *(126)*. Néanmoins, les Tregs expriment également le TNFR2 et il a été montré que les Tregs TNFR2-KO perdaient une partie de leurs fonction immunosuppressives, *in vitro* et *in vivo* dans un modèle de colite *(127)*. Ces résultats répliqués par d'autres équipes *(128)*, suggèrent l'intérêt du développement d'anticorps monoclonaux ciblant le TNFR2 plutôt que la cytokine elle-même, dans l'objectif de conserver les effets protecteurs liés à la signalisation via TNFR2. D'autres cytokines inflammatoires, tels que les interférons de type I *(129)* et l'IL-1 *(130)*, ont montré un rôle inhibiteur de la fonction des Tregs, bien qu'il existe des données contradictoires *(131)*.

Le déficit en interleukine-2. L'IL-2 est une cytokine indispensable à la différentiation, la survie et les fonctions des Tregs. L'IL-2 est principalement produit par les Teff tandis que les Treg n'ont pas de capacité à le produire. Un déficit de production en IL-2, comme montré dans le LES favorise la différentiation des lymphocytes T CD4 vers un profil Th1/Th17 pro-inflammatoire *(132)*. Plusieurs mécanismes ont été évoqués pour expliquer le déficit de production en IL-2. L'équipe de George Tsokos a publié de nombreux travaux impliquant l'activation augmentée calcium/calmodulin-dependent kinase IV (Camk4) chez les patients atteints de LES *(133, 134)*. L'inhibition de cette kinase, ou la supplémentation en IL-2 permet de restaurer les fonction immunosuppressive des Tregs dans des modèles murins *(134)* , mais également chez l'homme (voir plus bas).

iv. Le microbiome

Le microbiome notamment digestif joue un rôle majeur dans l'homéostasie de l'immunité mucosale mais également systémique. Les souris axénique (dénuées de microbiome digestif) présentent significativement moins de pTregs dans les tissus lymphoïdes associés aux muqueuses (MALT) digestifs *(135)*, mais également au niveau hépatique. L'effet du microbiote sur la génération de pTreg est direct (stimulation antigénique via le TCR) mais également indirect par la production de métabolites favorisant leur apparition (butyrate, propionate) *(136)*.

Comme nous l'avons vu, de nombreux facteurs locaux ou systémiques peuvent altérer le développement et/ou les fonctions des Tregs. Ces facteurs ont été retrouvés dans de nombreuses pathologies, y compris dans le lupus systémique, participant de manière significative à la physiopathologie de la maladie.

d) Les lymphocytes T régulateurs dans le lupus systémique

Il est communément admis que les Tregs jouent un rôle important dans la physiopathologie du LES. En effet, il existe des données (parfois contradictoires comme nous le verrons) en faveur d'un déficit quantitatif et/ou fonctionnel des Tregs dans le LES. D'autre part, le transfert passifs de Tregs, ou l'injection d'IL-2 à faible dose (stimulant les Tregs) permettent d'améliorer la maladie *in vivo* dans des modèles murins *(137)*, mais aussi chez l'homme *(138–140)*.

Nous détaillerons dans un premier temps les arguments en faveur d'un défaut de Tregs dans le LES, puis les thérapies visant à restaurer les capacités des Tregs actuellement discutées ou en cours d'essai thérapeutique chez l'homme.

i. Aspect quantitatif des Tregs dans le lupus érythémateux systémique

Tandis que le déficit quantitatif en Treg est évident dans certaines maladies auto-immunes monogéniques tel que l'IPEX syndrome, les données publiées concernant les patients atteints de LES sont discordantes et ce pour au moins trois raisons : 1/ la pluralité des définitions des Tregs, 2/ l'hétérogénicité des patients atteints de LES et 3/ des considération techniques.

La première raison vient de la pluralité des définitions utilisées pour identifier les Tregs. En effet, de nombreuses définitions ont été utilisés par les auteurs, utilisant au minimum le CD4 et le CD25 mais associant parfois le CD127, le CD15s, le CD45RA et des facteurs de transcription spécifiques (FoxP3 ou Hélios). Une étude menée par Lin et coauteurs, définissant les Tregs comme CD4+CD25+FoxP3+ montrait une augmentation de leur proportion chez les patients atteints de LES, avec une corrélation positive à l'activité de la maladie *(141)*. Dans l'étude de Miyara et coauteurs, il était montré que la fraction de eTreg ($CD45RA^{-}FoxP3^{high}$), définie comme la plus immunosuppressive, était significativement diminuée chez les patients atteints de lupus systémique par rapport aux donneurs sains et aux patients atteints de sarcoïdose (une maladie inflammatoire granulomateuse) *(30)*. En revanche, la proportion de rTreg ($CD45RA^{+}FoxP3^{+}$) définie comme précurseurs des eTreg était significativement majorée, pouvant suggérer un déficit de maturation de rTreg en eTreg. La dichotomie selon le CD45RA pourrait permettre de réconcilier les résultats discordants entre Lin et Miyara.

La deuxième explication provient de l'hétérogénéité des patients atteints de LES. Cette hétérogénéité se décline par les différence phénotypiques et immunologique entre les patients, par l'activité de la maladie qui évolue dans le temps chez un même maladie, et enfin par les traitements utilisés. Ainsi, il existe une forte hétérogénéité inter-individuelle mais également intra-individuelle selon si le patient est en poussée et si ses traitements (*e.g.,* glucocorticoïdes) ont été modifiés. Très peu d'études longitudinales suivant la dynamique temporelle des Tregs chez des patients atteints de LES ont été publiées. Deux études montraient qu'il existait une diminution significative des Tregs (en valeur absolue et en pourcentage) chez les mêmes patients passant d'un statut faible activité/rémission à actif *(142, 143)*.

Le troisième point nécessitant discussion est purement technique. En effet, la grande majorité des auteurs étudie le pourcentage de Tregs par rapport aux CD4+ isolés à partir des PBMC. Cette méthodologie pose deux problèmes principaux. 1/ Les patients atteints de LES présentent souvent une lymphopénie prédominante en cellules CD4+, d'autant plus chez les patients ayant une maladie active *(144)*. Ainsi, l'augmentation du pourcentage de Tregs parfois mise en évidence dans certaines publications est contrebalancée par la lymphopénie CD4 faisant qu'en valeur absolue, les Tregs circulants sont bien diminués. Cette observation a été faite par Miyara concernant les Tregs $CD15s^+$ *(31)*, et confirmée dans une méta-analyse d'articles publiés *(145)*. 2/ Les Tregs circulants constituent un reflet très imparfait de leur nombre tissulaire et dans les organes lymphoïdes secondaires. Dans une étude incluant des patients avec un lupus cutané, des biopsie cutanées révélaient une infiltration $CD4^+$ identiques à d'autres maladies inflammatoires (psoriasis, lichen plan) mais une plus faible proportion de Tregs caractérisés par l'expression de FoxP3 *(146)*. De la même manière, une étude portant sur des biopsies rénales montrait que celles de patients atteints de lupus systémique (notamment les biopsies de grade histologique III et IV) avaient un rapport $Fox3^+/CD3^+$ significativement plus faible à des biopsie de patients atteints de pathologies non auto-immune (néphroangiosclérose ou nephrite tubulo-interstitielle) *(147)*. Enfin, Miyara et coauteurs ont trouvé des résultats identiques dans des ganglions lymphatiques de patients ayant un lupus actif *(142)*.

ii. Aspects fonctionnels des Tregs dans le lupus érythémateux systémique

Une fois de plus la littérature est discordante en ce qui concerne les fonctions immunosuppressives des Tregs humains *ex vivo* provenant de patients atteints de LES. Miyara et Yan ne retrouvaient pas de différences en terme de fonction immunosuppressives de Tregs ($CD4^{+}CD25^{high}$) triés à partir de patients porteurs de LES par rapport à des Tregs de donneurs sains *(142, 148)*. En revanche, deux autres groupes ont décrit une diminution significative des capacités immunosuppressives des Tregs isolés de patients LES *(149, 150)*. Ces différences peuvent en partie s'expliquer par une tri différents des Tregs (CD4+CD25highCD127- pour Chowdary et al.) ou d'autres différences techniques dans les expérimentations d'étude fonctionnelle des Tregs (cf paragraphe I,3,b,i). Nous ne discuterons pas ici les mécanismes impliqués dans cette potentielle dysfonction Tregs que nous avons détaillé plus haut.

iii. Thérapies visant à renforcer le compartiment Tregs dans le lupus érythémateux systémique

Les nombreux arguments décrits en faveur d'un déficit quantitatif et/ou fonctionnel des Tregs dans le LES souligne l'intérêt potentiel de thérapies visant à rétablir l'homéostasie immunitaire en manipulant le compartiment Tregs. Plusieurs stratégies sont à l'étude dans cette optique.

Interleukine-2 faible dose. Initialement, l'IL-2 a été utilisée par les oncologues à fortes doses dans l'objectif d'induire une réponse anti-tumorale via la stimulation des Teff. Les résultats étaient inconstants, avec environ 15% des patients ayant une réponse dans le cas de cancer rénale ou mélanome métastatique, mais avec une tolérance médiocre (jusqu'à 4% de décès par infection, syndrome de fuite capillaire ou tempête cytokinique *(151)*). Rapidement, une expansion du compartiment Treg en réponse à l'IL-2 a été mis en évidence, y compris à faible doses. L'utilisation de l'IL-2 à faible dose permettant de stimuler spécifiquement le compartiment Treg (portant le récepteur de haute affinité à l'IL-2) a été initialement proposé dans la vascularite cryoglobulinémique secondaire au virus de l'hépatite C *(152)*. Dans les suites, plusieurs études dans le lupus ont démontré l'efficacité biologique (expansion du compartiment Treg) mais également clinique de l'IL-2 à faible dose *(138, 153)*. L'avenir des thérapies à base d'IL-2 à faible dose passera probablement vers le développement

d'IL-2 fusionnée à une protéine porteuse tel qu'un fragment Fc permettant une plus longue demi-vie (IL-2 native ayant une demi-vie inférieure à une heure) et/ou le développement d'IL-2 modifiée stimulant plus spécifiquement les récepteurs à l'IL-2 des Tregs (portant le CD25) et moins ceux des cellules NK responsable de la cytotoxicité *(151)*.

Altération du métabolisme. La voie mTOR discutée plus tôt joue un rôle important dans le contrôle du métabolisme cellulaire y compris du Treg. La modulation de cette voie par des inhibiteurs spécifiques (rapamycine/sirolimus) a montré des résultats prometteurs dans le LES. En 2018, une étude ouverte de phase I/II a rapportait que chez des patients atteints de LES réfractaire, l'utilisation de sirolimus permettait une expansion du compartiment Treg et une amélioration clinique avec diminution significative des doses de corticoïdes chez les patients ayant terminé l'essai (11/40 exclus de l'analyse finale) *(154)*. La Metformine est une molécule de la famille des biguanides connues pour activer la voie de l'AMPK tout en inhibant la phosphorylation oxydative. Il a été montré dans un modèle *in vitro* et dans des modèles murins de LES que la Metformine pouvait reverser le phénotype auto-immun et améliorer la pathologie *(155, 156)*. Une très récente étude de phase II en double aveugle a étudié l'intérêt de la metformine à 1,5g/j chez 140 patients atteints de LES. La survenue du critère de jugement principal (poussée de la maladie) était diminué de 32% sans atteinte la significativité (p=0,078) du fait du nombre relativement faible de patient inclus *(157)*. Néanmoins, plusieurs critères de jugement « exploratoires » tels que l'activité de la maladie et la survenu de poussées sévères suggéraient une efficacité de la metformine qui compte-tenu de son excellente tolérance pourrait devenir un traitement adjuvant intéressant dans le LES.

Autogreffe de Treg. L'autogreffe de Treg après différentiation et/ou expansion à partir de T naïfs *in vitro* pourrait être une option thérapeutique. Cette stratégie a été utilisé chez l'Homme dans le cadre du diabète de type 1 avec des prémices d'efficacité dans un essai preuve de concept *(158)*. Cependant, les difficultés techniques liées à l'expansion des cellules tout en garantissant une pureté importante en Tregs limitent fortement la faisabilité de ce genre d'étude (et de traitements). Un article preuve de

concept chez un patient atteint de LES avec une atteinte cutané a été publié, montrant une migration des Treg dans les lésions cutanées *(140)*. De manière intéressante, cette autogreffe de Tregs était aussi associée à une augmentation des $CD4^+$ exprimant l'IL17 dans la peau, apportant des arguments pour la possibilité d'une trans-différentiation de Tregs en Th17 *in vivo*. Une étude enregistrée sur la plateforme clinicaltrials.gov dans l'indication lupus a été arrêtée pour raison de faisabilité (NCT02428309). Il n'y a pas d'autre étude de ce type en cours pour le LES. Il est probable que des traitements moins onéreux et plus simples à mettre en œuvre (IL-2 faible dose, modulation pharmacologique des Tregs) aient plus de chance d'aboutir à une utilisation clinique.

4) ROLE IMMUNOLOGIQUE DES PLAQUETTES DANS LE LES

Bien au-delà de leur rôle bien connu dans l'hémostase, leur rôle dans la réponse immunitaire a été fortement mise en avant durant les vingt dernières années. Les mécanismes immunologiques

impliquant les plaquettes sont multiples, impliquant la coagulation (immuno-thrombose), la présentation de micro-organisme au système immunitaire ou l'activation du système immunitaire inné ou adaptatif.

Le LES est caractérisé par une activation du système plaquettaire (**figure 8**), ceci en réponse à plusieurs stimulus tel que les complexes immuns (via le FcγRII *(159)*), certains auto-anticorps (anti-ADN natifs *(160)*, les anti-phospholipides *(161)*) et les stimulus viraux *(162)*. Une fois activées, les plaquettes vont exprimer un certain nombre de protéines membranaires ayant un rôle immunologique (CD40L, P-selectine) et sécréter des molécules immunoactives (*eg,* sérotonine) ou des composants cellulaires antigéniques (*eg,* mitochondries et ADN mitochondrial). Les plaquettes activées auront ainsi un impact sur de nombreuses cellules du système immunitaire inné et adaptatif, participant à la physiopathogénie du LES (**figure 8**).

Ces différents aspects ainsi que leur rôle dans le lupus érythémateux systémique ont été résumés dans une revue récemment publiée (**article 1**).

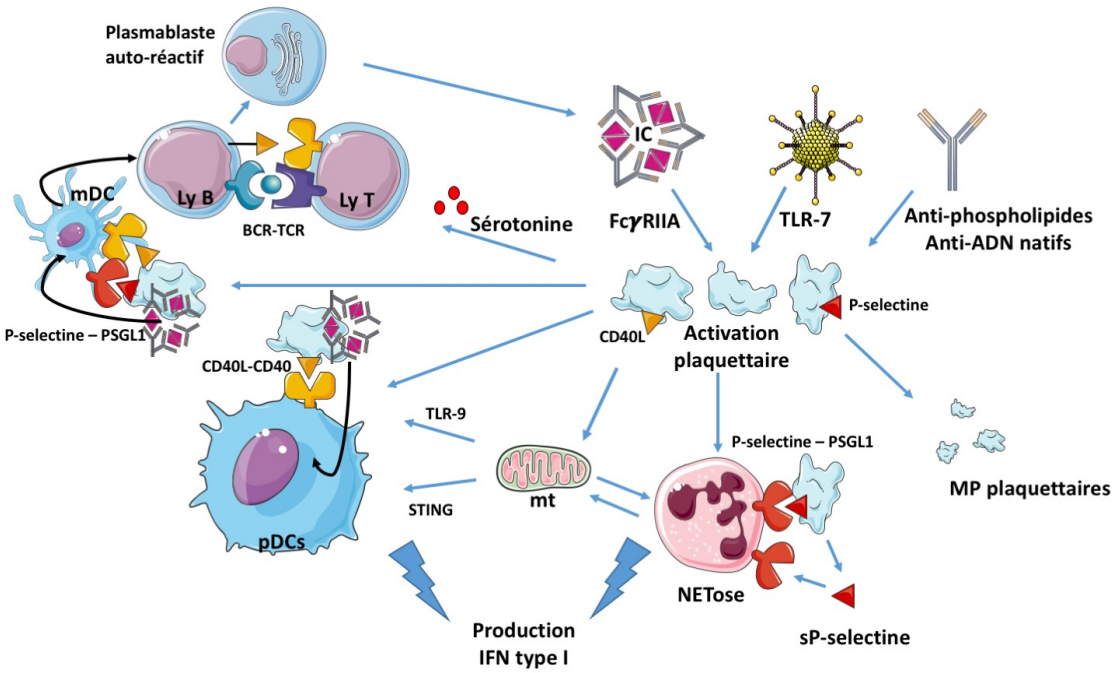


Figure 8 : aperçu du rôle immunologique des plaquettes dans le LES (adapté de Scherlinger *et al.* *(163)*).

Plusieurs facteurs concourent à l'activation plaquettaire dans le LES tel que les complexes immuns (IC), les virus (via le TLR-7) et certains auto-anticorps. L'activation plaquettaire induit l'expression membranaire de CD40L et de P-sélectine ainsi que la production de microparticules plaquettaires qui auront un impact sur plusieurs cellules immunitaires innées (neutrophile, pDC) ou adaptative (cellule présentatrice d'antigène, mDC).

Abréviations : BCR-TCR, B-cell receptor / T-cell receptor ; IC, complexes immuns ; Ly B/T, lymphocyte B/T ; MP plaquettaires, microparticules plaquettaires ; mDC, cellule dendritique myéloïde ; mt, mitochondrie ; pDC, cellule dendritique plasmacytoïde ; sP-selectine, P-selectine soluble ; STING, stimulator of interferon genes ; TLR-7/9, toll-like receptor 7/9.

Cette revue de la littérature indique qu'il co-existe au cours du lupus systémique 1/ une dysfonction des Tregs participant à la physiopathogénie du LES et 2/ une activation du compartiment plaquettaire, dont les conséquences dans le LES sont encore mal connues.

L'objectif de notre travail était d'évaluer les mécanismes de la dysfonction des Tregs dans le lupus systémique et les liens existant entre cette dysfonction et le système plaquettaire. Nous avons identifié les sélectines, et notamment la sélectine plaquettaire (P-sélectine) comme un facteur régulant les fonctions immunosuppressives des Tregs dans le LES (**article 2**). Une fois activée (*via* par exemple les complexes immuns circulant), les plaquettes relocalisent la P-sélectine à leur surface. Cette P-sélectine permet à la plaquette activée d'interagir physiquement avec le Treg via son ligand, la forme sialylée du PSGL-1, exprimée en forte quantité spécifiquement sur les Tregs. Cette interaction est responsable d'une signalisation calcique intra-cellulaire via la phosphorylation de la kinase Syk, induisant une diminution de l'expression des molécules de la voie du TGF-β et de FoxP3 lui-même. Ces modifications phénotypiques du Tregs expliquent le blocage de leurs fonctions immunosuppressives mis en évidence *in vitro*. Chez les patients atteints de LES, les quantités de P-sélectines solubles et microparticulaires sont augmentées, et nous avons identifié une corrélation positive avec l'activité de la maladie. Enfin, le blocage de la P-sélectine plaquettaire par un anticorps monoclonal permet une amélioration du phénotype de souris lupiques (DNA1L3-KO), en particulier des taux d'auto-anticorps circulant et de l'atteinte rénale. Ces résultats suggèrent que la P-sélectine, via l'activation plaquettaire, constitue un axe important dans la pathogénie du LES. Le blocage de la P-sélectine pourrait être une voie thérapeutique innovante dans le LES, permettant de restaurer les fonctions immunosuppressives des Tregs sans pour autant d'induire d'immunosuppression.

II) ARTICLES

1) ROLE DES PLAQUETTES DANS LE LUPUS SYSTEMIQUE ET LA SCLERODERMIE SYSTEMIQUE

Autoimmunity Reviews 17 (2018) 625–635

Contents lists available at ScienceDirect

Autoimmunity Reviews

journal homepage: www.elsevier.com/locate/autrev

Review

Systemic lupus erythematosus and systemic sclerosis: All roads lead to platelets

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ARTICLE INFO

Article history:
Received 13 January 2018
Accepted 18 January 2018
Available online 7 April 2018

Keywords:
Systemic lupus erythematosus
Systemic sclerosis
Platelets
Microparticles
Auto-immunity

ABSTRACT

Systemic lupus erythematosus (SLE) and systemic sclerosis (SSc) are two phenotypically distincts inflammatory systemic diseases. However, SLE and SSc share pathogenic features such as interferon signature, loss of tolerance against self-nuclear antigens and increased tissue damage such as fibrosis. Recently, platelets have emerged as a major actor in immunity including auto-immune diseases. Both SLE and SSc are characterized by strong platelet system activation, which is likely to be both the witness and culprit in their pathogenesis. Platelet activation pathways are multiple and sometimes redundant. They include immune complexes, Toll-like receptors activation, antiphospholipid antibodies and ischemia-reperfusion associated with Raynaud phenomenon. Once activated, platelet promote immune dysregulation by priming interferon production by immune cells, providing CD40L supporting B lymphocyte functions and providing a source of autoantigens. Platelets are actively implicated in SLE and SSc end-organ damage such as cardiovascular and renal disease and in the promotion of tissue fibrosis. Finally, after understanding the main pathogenic implications of platelet activation in both diseases, we discuss potential therapeutics targeting platelets.

Contents

Abbreviations: ADP/ATP, adenosine di/tri phosphate; ApL, antiphospholipid; BTG, beta-thrombomodulin; COX, cyclo oxygenase; CVD, cardiovascular disease; DAMP, damage-associated molecular patterns; EC, endothelial cells; ECM, extracellular matrix; ET-1, endothelin-1; HCQ, hydroxychloroquine; HIT, heparin-induced thrombocytopenia; HMGB-1, high-mobility group box-1; IC, immune complex; ICAM, intercellular adhesion molecule; LN, lupus nephritis; MCP-1, monocyte chemoattractant protein-1; MP, microparticle; MPV, mean platelet volume; MMP-9, matrix metalloprotease-9; mtDNA, mitochondrial DNA; NET, neutrophil extracellular trap; PGI2, prostacyclin; PMP, platelet-derived microparticles; PSGL-1, p-selectin glycoprotein-1; RAGE, receptor of advanced glycation endproduct; RP, Raynaud phenomenon; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; Tfh, T follicular helper; TIMP-1, tissue inhibitor of metallopeptidase-1; TLR, toll-like receptor; TSLP, Thymic stromal lymphopoietin; TXA2, thromboxane A2; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor.

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https://doi.org/10.1016/j.autrev.2018.01.012
1568-9972/

1. Introduction

Initially thought to be merely circulating cell fragments with a relatively fixed repertoire of functional properties, platelets have emerged to be versatile effector cells that bridge thrombotic, inflammatory, and immune continuums.

Systemic lupus erythematosus (SLE) and systemic sclerosis (SSc) are inflammatory systemic disorders clinically characterized by vasculopathy and dysimmunity associated with different levels of renal involvement and fibrosis. SLE may present with life-threatening manifestations such as renal or neurologic involvement whereas SSc presents usually with progressive skin fibrosis, digital ulcers secondary to severe micro-vasculopathy and sometime life-threatening organ involvement such as renal crisis (a thrombotic microangiopathy), lung fibrosis or pulmonary arterial hypertension. While clearly distinct from a phenotypic point of view, SSc and SLE share multiple pathogenic features including genetic factors of susceptibility, loss of tolerance against nuclear-components, platelet activation, and type I interferon signature.

The purpose of this review is to highlight direct implications of platelets and/or platelet-derived microparticles (PMPs) in these phenotypically distinct autoimmune disorders.

2. Platelets: roles and physiology

2.1. Origins

Platelets are small enucleated subcellular particles originating from megakaryocytes. They represent the second most prevalent circulating blood component after red blood cells and their number far outweigh the need for coagulation purposes suggesting their extra-hemostatic roles [1]. Their shape in their inactivated state consists of a disk measuring from 2 to 5 μm in diameter, 0.5 μm thick with a mean corpuscular volume of 6 to 10 μm^3 [2]. Since their life span is short (8–10 days), roughly 10^{11} new platelets are daily produced in order to maintain the homeostatic platelet count ($150–450 \times 10^9$/L) [3].

Platelet biogenesis is a unique physiologic process where megakaryocyte progenitors deriving from hematopoietic stem cells undertake endomitosis (nuclear material replication without cell division) in order to increase their ploidy, achieving 16 N to 128 N [4]. This process continues toward the terminal differentiation of megakaryocytes and is under the control of several cytokines: mainly thrombopoietin but also interleukine-6 (IL-6), IL-3, IL-11 and stem cell factor [5]. Once mature, megakaryocytes migrate to the bone marrow's "vascular niche" which represents a vascular scaffold supporting platelet production by extension of megakaryocytes' membrane through blood sinuses. Each megakaryocytes gives birth to 10–20 proplatelets which mature in the circulation to further release 1000 to 3000 platelets [6]. A recent report indicate that lungs might play a substantial role in platelets genesis since it hosts megakaryocytes and proplatelets fragmentation as well as hematopoietic progenitors with the capacity to repopulate bone marrow if necessary [7].

2.2. Platelet content & hemostatic role

Alpha granules are the most abundant platelet's organelle containing multiple molecules responsible for hemostasis (e.g. von Willebrand Factor (vWF), factor V), angiogenesis (e.g. vascular endothelium growth factor, VEGF), inflammation (e.g. P-selectin, complement C3 and C4) and antimicrobial activity (e.g. thymosin-beta4, thrombocidins) [2]. Dense granules are less frequent in platelets and contain high concentrations of adenosine (ADP and ATP) and calcium which act as promoter of the coagulation cascade. Platelets are able to scavenge and concentrate circulating bioactive molecule such as 5-HT (serotonin) [8]. Platelets contain mitochondria, lysosomes and nucleic acids like mRNA [9] but also miRNA together with the machinery necessary for their function (DICER and Argonaute 2) [10,11].

Platelets are the guardian and sentinel of blood vessels' integrity by patrolling, and being activated upon specific stimuli encounter. Platelet role in hemostasis has already been reviewed extensively [2,12]. Briefly, collagen bound with vWF is exposed from the sub-endothelial matrix of injured endothelium providing a strong activating stimulus for platelets. Platelet-bound glycoprotein VI (GP VI) and integrin α2β1 bind endothelial collagen [13,14] and platelet-bound GP Ib-IX-V binds vWF [15]. These stimuli lead to intense platelet adhesion to the damaged endothelium and the formation of the primary platelet aggregate. Upon this primary aggregation, platelet's integrin αIIbβ3 (previously termed GPIIb/IIIa) is upregulated and transformed from a low to a high-affinity receptor to fibrinogen and vWF thereby strengthening platelet aggregates [16]. Several molecules or proteins further promote platelet aggregation and primary and secondary hemostasis. They are brought by platelets themselves in an autocrine and paracrine enhancing loop (e.g. ADP, calcium) or externally from the coagulation cascade (e.g. thrombin, fibrinogen) [17,18].

Platelet cytoplasm contains a strong actin/myosin cytoskeleton allowing quick and durable shape remodeling in case of activation. Recent work from Gaertner et al. show that platelets are polarized and have the potential to migrate in vivo [19]. Once activated, platelet can migrate against blood flow toward endothelial injury and actively reorganize themselves in the clot [19]. Platelet activation induces many processes such as the production of platelet-derived microparticles (PMPs).

2.3. Microparticles

Microparticles (MPs) are subcellular circulating particles measuring from 0.1 to 1 μm. To note, exosomes are a class of small MPs (from 30 to 100 nm) deriving from multi-vesicular bodies that will not be discussed here. MPs are produced after activation or apoptosis from most eukaryotic cells. They can be differentiated depending on phosphatidylserin

(PS) exposure suggesting a membrane loss of symmetry (apoptosis or outward membrane budding). They harbor surface markers (e.g. glycoproteins) from their cells of origin allowing their characterization using flow cytometry. Platelets also inherit proteins, active molecules, mRNA and miRNA. MPs are important players in intercellular communication through different mechanisms: 1) the transfer of genetic materials (e.g. miRNA, mRNA) [11,20,21]; 2) the transfer of surface glycoproteins (e.g. receptors) [22]; 3) the transfer of active molecules or proteins [23]. Because of their small size and high number, MPs represent efficient and powerful messengers able to diffuse the effect of an activated parental cells.

In human, PMPs are consistently found as the most prevalent circulating MPs [24,25], and their roles in health and disease have been reviewed elsewhere [26]. For example, during primary hemostasis, activated platelets release high numbers of MPs harboring negatively charged PS and tissue factor (TF) [27]. During the hemostasis cascade, coagulation factor VII interacts with TF in order to product prothrombinase, an essential enzymatic complex responsible for thrombin production. This reaction must be conducted on a negatively charged surface, which is provided by PS-positive PMPs [28]. PMPs are now described to act in numerous physiological or pathological conditions such as anti-bacterial and anti-viral immunity [29,30], thrombosis [26], and, last but not least, auto-immunity [31].

3. Platelets activation in SLE and SSc: the chicken or the egg?

Many reports show an activation of the platelet system during SLE [32,33] as well as SSc [34,35]. Platelet activation is a marker of disease activity and participates to immune dysregulation associated with both diseases. In addition, immune dysfunctions in SLE and SSc patients cause platelet activation, thus questioning their position as the cause or the consequence of the disease the otherwise chicken or the egg dilemma.

3.1. Platelet activation and its association to SLE and SSc pathogenesis

Thrombocytopenia is a common feature of SLE and a platelet counts $<100 \times 10^6$/mL represent one of the American College of Rheumatology and the SLICC 2012 SLE classification criteria [36,37]. SLE patients presenting thrombocytopenia have higher disease activity [38] and worse prognosis in terms of organ damage [39] and mortality [40,41]. In SSc, thrombocytopenia is not a classical manifestation of the disease except in case of overlap syndrome (e.g. immune thrombocytopenic purpura) or renal complication (e.g. renal crisis) [42]. However, relatively high incidence of anti-platelets antibodies have been reported in SSc, responsible for platelet activation [43]. Activated platelets are more likely to be removed from the circulation, thereby placing thrombocytopenia as an indirect potential marker of platelet activation.

Mean Platelet Volume (MPV) is an easily accessible parameter available from the complete blood count. Upon platelet activation, phospholipase C activation induces a cytoplasmic calcium release leading to the activation of the actin cytoskeleton [44]. This phenomenom induces transformation of the platelet from a discoid shape to a sphere, thus increasing its overall volume [45]. In SLE, MPV has been found to be positively correlated with disease activity (SLEDAI) in pediatric [46] and adult cohorts [47]. However, a decrease in platelet volume was reported to be associated with an increased disease activity by another group, correlating with PMPs production [48]. It is easy to assume that upon platelet activation, MPV increase with platelet reshaping but that PMPs shedding induces a secondary decrease in platelet volume. In SSc, MPV has been shown to be more elevated than in healthy controls and that this MPV elevation predicted vascular (e.g. digital ulcer) and cardiac involvement [49]. The fact that some publication led to opposite results are most likely due to pre-analytical discrepancies, the presence of antiphospholipid antibodies and/or concurrent medications [48,50]. In consequence, further studies are needed before MPV starts being used as a marker of SLE or SSc disease activity in the daily clinical practice.

Platelet selectin (P-selectin, CD62P) and CD154 (CD40L) are two transmembrane glycoproteins located in platelets' alpha granules [2,51]. P-selectin is an adhesion molecule and CD154 is a member of the TNF superfamily with numerous roles in adaptive immunity (see Paragraph 2.2). Once platelets become activated, P-selectin and CD154 are translocated on their outer membrane and partly secreted as a soluble form upon cleavage [32,52]. Consequently, increased soluble and platelet-bound P-selectin and CD154 are hallmarks of platelet activation, and are commonly found in SLE [53,54] and SSc patients [55,56].

Platelet-derived microparticles (PMPs) production is strongly stimulated upon platelet activation [57] and multiple reports suggested PMPs potential use as a marker of platelet activation [58,59]. In SLE, platelet activation is a cornerstone finding correlating with elevated amount of circulating PMPs [25,48]. In fact, PMPs levels have been shown to positively correlate with disease activity (SLEDAI) in large SLE patients' cohorts [60,61] while it was not the case in a much smaller cohort [25]. Recently, *McCarthy* et al. sought to investigate the use of PMPs measurement to evaluate endothelial dysfunction [62]. Interestingly, they found that PMPs levels significantly correlated with large vessels endothelial dysfunction measured by flow-mediated vasodilatation using ultrasonography. They also found that in SSc, PMPs levels were increased and correlated with peripheral microvascular function. Surprisingly, higher PMPs levels were associated with better microcirculation in SSc. These data underline the potential use of PMPs as biomarkers of disease activity and organ damage but additional studies are required to validate their use.

Altogether, these observations suggest that platelets are activated in SLE and SSc with a direct correlation to disease activity and long-term outcomes. Thus, understanding the mechanisms implicated in platelets activation would provide a better understanding of SSc and SLE pathogenesis.

3.2. Mechanisms of platelets activation in SLE and SSc

Immune complexes (ICs) are found in high quantities in SLE patients' serum [63] and also in a fraction of SSc patients presenting a more severe phenotype (e.g. diffuse form, lung fibrosis) [64]. They consist of large aggregates of antigens (e.g. self-nuclear antigens) and antibodies and are the witness and culprits in SLE pathogenesis [65]. Immune complexes can activate platelets through their recognition by the Ig receptor FcγRIIA (CD32), and this process was described as main driver of platelet activation in SLE patients [53,66]. Immune complexes also activate platelets through Toll-like receptors 7 which have been shown to be expressed and functional in platelets [30]. Platelet-bound IgG levels correlate with platelet activation and P-selectin expression thus supporting immune dysregulation (see Paragraph 2.3) and demonstrating a vicious circle promoting auto-immunity [67]. Finally, in SLE patients, circulating MPs and especially PMPs might act as high order ICs since they were found to exhibit increased amount of bound IgGs identified as auto-antibodies on their surface [61,68].

Toll-like receptors (TLRs) are highly evolutionary-conserved innate immune receptors able to recognize pathogen-associated molecular patterns (PAMPs). Platelets at least express functional TLRs 1, 2, 4 (recognizing extracellular PAMPs found mostly in bacteria) and TLRs 3, 7 and 9 (recognizing respectively dsRNA, ssRNA and DNA found mostly in viruses) [69]. Therefore, a wide range of pathogens can induce platelet activation such as viruses, which role in SLE [70] and SSc [71] pathogenesis have long been discussed. Interestingly, *Koupenova* et al. showed that upon viral infection, platelet were activated via TLR-7 leading to P-selectin and CD154 (CD40L) exposure. P-selectin exposure mediated a physical platelet-neutrophil interaction through its binding with P-selectin glycoprotein ligand-1 (PSGL-1) as well as interaction with other leukocytes [30]. The clinical significance of TLR-7-mediated platelet activation in immunity is supported by the fact that TLR-7-KO

mice had a better survival to viral infection after transfusion of normal platelets expressing TLR-7 [30]. Thus TLRs including TLR-7 are potent activation pathway in SLE and SSc.

Antiphospholipid antibodies (ApL) are found in roughly one third of SLE [72], but also in 14 to 24% of SSc patients [73,74], and their presence is an indicator of poor clinical prognosis. ApL implication in platelet activation is now clearly established and is mediated by their binding to platelets receptors (e.g. GP-Ibα) [75], by direct interaction with platelet's membrane [76], and through the promotion of complement deposition on platelets [77].

Collagen I is exposed from damaged endothelium and activates platelets through GP-VI and integrin α2β1. The non-integrin 65 kDa collagen I receptor is another glycoprotein expressed on platelet [78]. Interestingly, it has been described to be upregulated in platelets from SSc but not from SLE patients and therefore to be an additional indication for the abnormal platelet activation in SSc [79].

Ischemia-reperfusion associated with Raynaud phenomenon (RP) might be another significant stimulus involved in platelet activation. RP is found in virtually all SSc and in 18 to 49% of SLE patients [80,81]. Studies that evaluated patients with primary and/or secondary RP showed increased markers of platelet activation (MPV and P-selectin surface expression) [55,82]. Several mechanisms might contribute in ischemia-reperfusion-associated platelet activation such as the release of radical oxygen species, of damage associated molecular patterns (DAMPs), of DNA, and the activation of complement [83,84].

In summary, SLE and SSc platelets are activated through several but sometime redundant mechanisms (Table 1) and the understanding of these activation pathways might offer potent therapeutic opportunities (Fig. 1). Understanding platelet's role in immune system activation is the next step to understand the close relationship between platelet activation and immune dysregulation.

3.3. Mechanisms of platelet-induced immune dysregulation

Neutrophils extracellular trap (NET) is a specialized programmed cellular death where neutrophils release in the extracellular milieu their nuclear material including DNA, proteins (e.g. histones) and other cytoplasmic components (e.g. myeloperoxidase) [85]. Initially, NETs containing DNA and protein were described to control pathogen dissemination in the context of sepsis [85–87]. Numerous reports have also implicated NETs' in the exacerbation of autoimmunity, mostly in SLE [88,89] and recently in SSc [90]. In fact, the release of cryptic antigens, mostly DNA and mitochondrial DNA (mtDNA) induces interferon (IFN) production by plasmacytoid DCs (pDCs) [91,92], which in turn participate in the overall exacerbation of SLE or SSc pathogenesis. Interestingly, mtDNA promotes NETosis by itself, underlying another vicious circle of auto-immune activation. Platelets promote NETosis through several mechanisms. *Boudreau* et al. demonstrated that upon activation, platelets release mitochondria in PMPs as well as in the extracellular milieu [93]. Extracellular mitochondria are providers of damage associated molecular patterns (DAMPs) such as bacterial-like formyl-peptide and mitochondrial DNA (mtDNA) [94]. Platelet-derived mtDNA, and particularly oxidized mtDNA are powerful inducers of NETs [93], as well as IFN production by pDCs through the stimulation of TLR-9 and STING [94–96]. Activated platelets further support NETosis through P-selectin – PSGL-1 interaction (PSGL-1 is expressed on neutrophils) and by an interaction involving platelet GPIb and neutrophil CD18 [97,98].

CD154 (also named CD40L) is a co-stimulator protein belonging to the tumor necrosis factor alpha family. CD154 is a key factor by which T cell help B-cell responses, allowing germinal center reaction involved in Ig class switching, Ig affinity maturation and the generation of a pool of memory B-cells [99,100]. Therefore, CD154-CD40 interaction is at the center of the pathogenesis of diseases where the Th2 response is prominent, such as SLE and SSc [53,101]. CD154 can be membrane-bound (historically described on T follicular helper cells (Tfh)) or in a soluble bioactive conformation. Activated platelet are the major source of soluble and platelet-associated CD154 [102]. Our team previously demonstrated that activated platelet act as adjuvant of pDCs, increasing their sensitivity to interferogenic stimuli (e.g. ICs) through CD154-CD40 axis [53]. In a lupus-prone mice models, blocking platelets activation or platelet depletion could decrease anti-dsDNA antibody levels and increased the overall survival. Finally, through CD154, platelets may modulate the adaptive immune response by theirs effect on myeloid DCs (mDCs) maturation, by acting as Tfh cell promoting isotype switching and by enhancing cytotoxic T cells functions [103].

Platelets provide a source of auto-antigens. Immune thrombocytopenia (ITP) is an auto-immune condition where antibodies targeting platelets are produced and responsible for their premature destruction. The sustained platelet activation seen in SLE and SSc together with their close interaction with immune cells leading to mDCs-platelets aggregates could promote the uptake of platelet antigens [104]. This hypothesis is supported by the fact that ITP is a common manifestation of SLE, that antibodies targeting platelets and/or GPIIbIIIa are not uncommon in SSc [43], and that platelet-derived CD154 can directly activate B lymphocyte to promote auto-antibodies production [105]. Additionally, Sisirak et al. recently demonstrated that apoptotic cell-derived MPs carried DNA protected from digestion by most circulating DNAse (except DNA1L3) and that this DNA was antigenic and implicated in the development of autoimmunity [106]. These data suggest that MPs including PMPs can serve as antigen cargo assuring a protected antigen delivery to the antigen presenting cells. A recent study by *Gaertner* et al. describe how platelet behave as mechano-scavengers able to bind and accumulate bacteria on their surface in order to prime neutrophils for phagocytosis, therefore acting as an antigen-presenting cell (however missing costimulation signals) [19].

Damage-associated molecular patterns (DAMPs) are danger signals released by cells upon stress inducing conditions. Protein S100A8/A9 (also called calprotectin) is a heterodimeric activator of TLR-4 found in higher quantity in serum and platelets from SLE patients [107] and in serum and broncho-alveolar fluid from SSc patients [108,109]. Interestingly, S100A8/A9 is an interferon-regulated gene and its platelet concentration is up-regulated in SLE and strongly correlates with cardiovascular complications [107]. Activated platelets can express on their surface and release S100A8/A9 thus supporting sterile inflammation [107]. High Mobility Group Box-1 (HMGB-1) is a nuclear protein capable of activating innate and adaptive immune system [110]. In SLE, increased HMGB1 levels were reported to facilitate self-DNA induced macrophage activation [111]. In SSc, HMGB-1 is exposed on activated platelets and secreted on PMPs consequently promoting SSc vascular disease (detailed in Section 4.2.2).

The transfer of pro-inflammatory nuclear material is an original mechanism of platelet-induced immune dysregulation. Platelets contain miRNAs and the molecular machinery for their processing [10]. Laffont et al. demonstrated that activated platelets could use PMPs to deliver miRNA to macrophage thereby increasing the transcription pro-inflammatory cytokines and promoting their activation [21]. Another study reveals the mechanisms of delivery of functional transcription factors such as NF-κB from PMPs to neutrophils, thus promoting pro-inflammatory neutrophils phenotype [112]. Although there is no evidence

Table 1
Platelet activation stimuli involved in SLE and SSc.

Stimulus	Pathway involved	References
Immune complex	FcγRIIA (CD32), TLR-7	[53,66], [30]
Nucleic acids	TLRs 3, 7, 9	[30,69]
Antiphospholipid antibody	GP-Ibα, direct activation, complement	[75], [76], [77]
Collagen I	GPVI, non-integrin 65 kDa collagen I receptor	[13], [78,79]
Raynaud phenomenon	ROS, DAMPs, complement	[83,84]

Abbreviations: DAMP, damage associated molecular patterns; ROS, reactive oxygen species TLR, toll-like receptor.

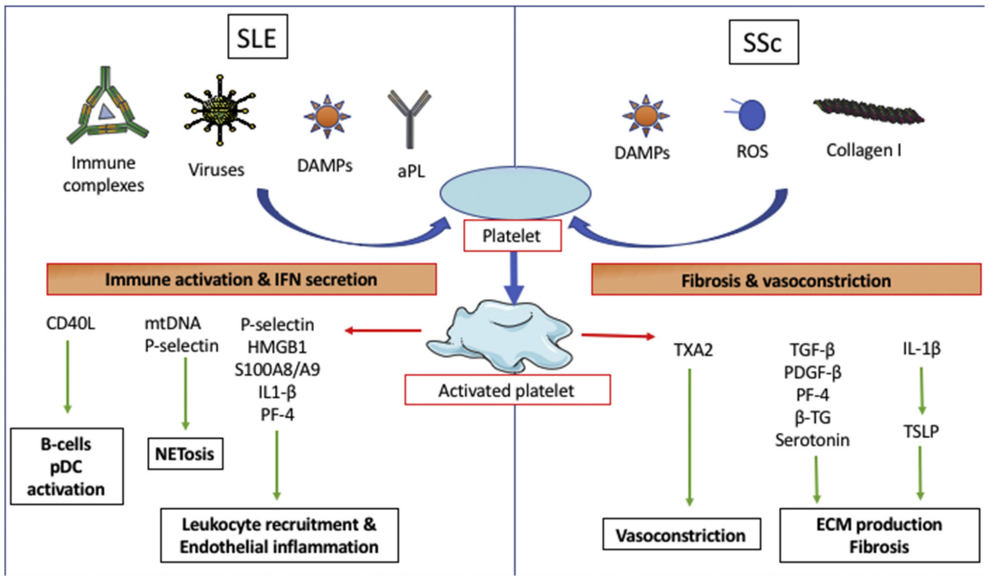

Fig. 1. Putative role of platelets in the pathogenesis of systemic lupus erythematosus and systemic sclerosis. Platelet-derived mediators are involved in immune activation, endothelial damage, fibrosis and vasoconstriction. Abbreviations: aPL, anti-phospholipid antibody; B-cells, B lymphocytes; CD, cluster of differentiation; CD40L, CD40 ligand; mtDNA, mitochondrial DNA; DAMPS, damage associated molecular patterns; ECM, extra cellular matrix; HMGB1, high mobility group box 1; IL1-β, interleukin 1-β; pDC, plasmacytoid dendritic cell; PDGF-β, platelet-derived growth factor-β; PF-4, platelet-factor 4 (also called CXCL4); P-selectin, platelet-selectin; ROS, reactive oxygen species; S100A8/A9, calprotectin; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; TGF-β, transforming growth factor-β; β-TG, β-thromboglobulin; TSLP, Thymic Stromal Lymphopoietin; TXA2, thromboxan A2.

suggesting that these mechanisms apply in SLE or SSc, the extent of platelets-immune cells interactions makes it likely that genetic material is exchanged during the process.

Serotonin (5-HT) is a tryptophan-derived neurotransmitter implicated in numerous physiologic processes. Many observations report that platelet's serotonin content is decreased in SLE [113] and SSc [114]. Decreased platelet serotonin correlates with serotonin release during platelet activation and is associated with pathologic characteristics of disease (e.g. fibrosis) [113,114]. Serotonin might have pro-inflammatory effect by promoting proliferation and activation of T-lymphocytes via 5-HT7 receptor [115]. Other reports suggest that serotonin could act through the alteration of the Th17/Treg balance or the activation and maturation of mDCs [116,117].

We have summarized here how platelets promote autoimmunity (Table 2). However, organ damage including vascular damage are a major concern in SLE and SSc, and platelets play their full part in their development.

4. Platelet role in the promotion of vascular disease

4.1. Burden of vascular disease in SLE and SSc

Most autoimmune disorders, including SLE and SSc, are associated with an increased risk of cardiovascular disease (CVD) such as coronary heart disease or stroke [118,119]. Among SLE patients, those with lupus nephritis, auto-antibodies or neurological disorders are at higher risk of CVD [120]. While SLE and SSc patient's mortality has been significantly reduced with better monitoring and immunosuppressive treatments, CVD remains a leading cause of death [121,122]. Besides clinical manifestation, subclinical atherosclerosis is shown to be more frequent in SLE and SSc patients [123,124]. Interestingly, up-regulation of type I IFN-regulated proteins, a major player in SLE and SSc pathogenesis, was reported as strongly associated with vascular disease [125]. Taken together with the well-established increased relative risk of venous thrombosis [126], these data provide strong arguments for vascular damage as a central component of SLE and SSc pathogenesis.

4.2. Evidences of platelets contribution to vascular damage

4.2.1. Platelets induce inflammatory phenotype in endothelial cells

Promotion of endothelial inflammation by platelets implicate a tripartite network involving platelets, endothelial cells (ECs) and leukocytes [127]. From a mechanistic point of view, platelets and endothelial cells communicate on multiple levels. Cross-talk may occur over distance (e.g. soluble factors), via transient interactions or through receptor-mediated cell-cell interaction such as P-selectin/PSGL1 [128].

Table 2
Mechanisms of platelet-induced immune dysregulation in SLE and SSc.

Mechanisms	Pathway involved	References
NET formation	Mitochondria and mtDNA, P-selectin/PSGL-1, GPIb/CD18	[93,94], [97], [98]
Increased IFN production by pDC	CD40/CD154, mtDNA	[53], [91,92]
B lymphocyte stimulation	CD40/CD154, mDC maturation	[53], [103]
T lymphocytes stimulation	Serotonin, mDC maturation	[115], [103]
Source of autoantigens	Direct antigen exposition	[19,104]
DAMPs	S100A8/A9, HMGB-1	[107], [111,156,157]
Transfer of pro-inflammatory nuclear material	PMP-mediated transfer of miRNA	[21,111]

Abbreviations: DAMP, damage associated molecular patterns; HMGB-1, high mobility group box-1; IFN, interferon; mDC, myeloid dendritic cell; mtDNA, mitochondrial DNA; pDC, plasmacytoid dendritic cell; PMP, platelet-derived microparticles TLR, toll-like receptor.

Platelet factor 4 (PF4) (also known as CLCX4) has been described as a robust biomarker in SSc [129]. Produced by platelets, it exerts his activity via promoting the expression of thrombospondin-1, by repressing vascular endothelial growth factor (VEGF) effects and by inducing endothelin-1 (ET-1) secretion in ECs leading to vascular damage [129].

Interleukin-1β, a pro inflammatory cytokine which belong to IL-1 family, is currently targeted in CVD because of its involvement in formation of atheroma plaque [130]. In fact, canakinumab, an anti-IL-1β monoclonal antibody has shown efficacy in reducing CVD in high risk populations [130,131]. Activated platelets and PMPs release IL-1β that alter endothelial cells phenotype, leading to the expression of several pro inflammatory genes, intercellular adhesion molecule 1 (ICAM-1) and promoting endothelial dysfunction [33]. Interestingly, two studies showed a protective role of PSGL-1 (P-selectin glycoprotein ligand-1) deficiency against prothrombotic effect of IL-1β [132,133]. These studies suggest that IL-1β may promote atherosclerosis by favoring platelets and endothelial cells interaction through P-selectin/PSGL1.

Complement activation can be sustained by platelets. Complement system participate to pathogenesis of both SSc and SLE [134,135]. C4d deposition on platelets (P-C4d) is found in roughly 20% of SLE patients, and it may be a marker of worse prognosis [136]. P-C4d in SLE is associated with vascular events, but there are still discrepancies in the literature with regard to which type of vascular event, venous or arterial [136,137]. Platelet complement deposition is explained by increased exposition of C1q binding epitope on activated platelet membrane and is supported by the presence of aPL antibodies [77,138]. Additionally, platelets are able to bind directly properdin and activate the alternative complement pathway [139], contributing to adversely influence ECs function [140]. In turn, complement activation products such as the membrane attack complex (C5b-9) can directly act on ECs and adversely influence their function by inducing secretion of multimers of endothelial vWF [141], stimulating endothelial prothrombinase and tissue factor activity [142], and activating platelets and fibrin deposition, thus generating a pro-thrombotic endothelial cell surface [143]. Furthermore, C5b-9 upregulates expression of leucocyte adhesion molecules on ECs which might also contribute to platelet adhesion and thrombotic events [144].

Altogether these data suggest that activated platelets may induce phenotype change in ECs via PF-4 and IL-1β production as well as complement activation (Fig. 1).

4.2.2. Platelets promote leukocyte recruitment and local inflammation

Platelets enable leukocyte recruitment on ECs by exposing P-selectin. Platelets can adhere to stimulated endothelial cells which in turn express the chemokine fractalkine (CX3CL1) and trigger P-selectin exposure on adherent platelets. P-selectin exposure initiates the local accumulation of leukocytes, illustrating the collaboration between ECs, platelets and leukocytes [145]. In addition, platelets can form heterotypic aggregates with leukocytes in the circulation favoring endothelial damage and activation in a CD40L dependent manner [146,147].

Damage Associated Molecular Patterns (DAMPS) & complement exposure on platelets drive local vascular inflammation. As explained previously, HMGB1 and S100A8/A9 are two DAMPs exposed by activated platelets and PMPs [148]. These DAMPs have the potency to promote systemic and local vascular inflammation. HMGB1 was shown to induce vascular remodeling [149], promote angiogenesis [150], promote vascular lesion via TLR-9 [151], and inflammatory cells recruitment to injured tissues by forming a heterocomplex with the chemokine CXCL12 [152]. In SLE vasculitis, ICs-induced ECs inflammation occurs through HMGB1-Receptor of advanced glycation endproduct (RAGE) axis [153]. In SSc, HMGB1 and soluble RAGE levels are correlated with clinical vasculopathy signs such as pitting scars and digital ulcers [154]. In SLE and SSc, the S100A8/A9 proteins level was shown to be higher than in healthy individuals, and to strongly correlate with the occurrence of cardiovascular disease such as myocardial infarction [107,108,155]. These DAMPs may act by binding ECs allowing an increase permeability via RAGE and TLR-4 [156] signaling favoring leukocyte trafficking across vessel wall [157].

4.2.3. Vasomotor imbalance

Dysregulation of vasomotor balance. Thromboxane A2 (TXA2), the predominant cyclo oxygenase product of arachidonic acid in platelets, is a major potent vasoconstrictor involved in CVD. It constitutes the main rational for the use of low-dose aspirin as anti-thrombotic primary prevention of CVD [158]. Conversely, prostacyclin (PGI2) is a relaxing factor produced by ECs [159]. On one hand, elevation of TXA2 is well described in SSc patients [160]. On the other hand, defect of PGI2 has been shown to be one of the phenotypic alterations found in lung ECs in case of severe pulmonary hypertension, thus causing diffuse microvascular dysfunction [161]. Moreover, PGI2 deficit facilitates platelet aggregation and promotes TXA2 secretion secondary to platelet activation in a vicious circle way [127]. Additionally, platelets promote vasoconstriction through release of other vasoactive molecules such as serotonin, platelet-derived growth factor (PDGF) or dinucleoside polyphosphates [127]. This vasomotor imbalance contributes to the vasoconstrictive and pro-thrombotic vascular state found in SLE and SSc.

In conclusion, by releasing bioactive molecules, activating complement cascade or exposing P-selectin and DAMPs, activated platelet participate to vascular damage in both SLE and SSc. They promote endothelial inflammation, leukocyte recruitment and vasoconstriction. In addition, several studies suggest a relevant pro-fibrotic role of platelet.

5. Platelet role in fibrosis

5.1. Skin and lung fibrosis

Platelets are a major source of TGF-β, which is secreted from alpha granules upon activation, and plays an important role in multiple fibrotic diseases [162,163]. In SSc, several arguments support TGF-β contribution in fibrosis such as its ability to potentiate pro-fibrotic genes expression in the skin and lungs of these patients [164,165]. Collagen 1 (COL1A1 and COL1A2) and fibrillin are examples of such pro-fibrotic genes involved in the generation of extracellular matrix (ECM). TGF-β also represses ECM degradation, promotes fibroblast proliferation and myofibroblast phenotype [166–168] which altogether favoring fibrosis. Its effects are dependent on the SMAD signaling pathway which has been shown to be up-regulated in SSc patients and to correlate to the extent of skin fibrosis [167]. The pro-fibrotic role of TGF-β is less documented in SLE given the fact that fibrosis is a less prominent feature of the disease. However, Solé et al. showed increased TGF-β signature in skin biopsies from SLE patients presenting discoid lupus erythematosus, characterized by skin fibrosis [169]. TGF-β seems to be more extensively engaged in lupus nephritis, which will be discussed later.

Beta-thromboglobulin (BTG) and CXCL4 (PF4) are both implicated in lung fibrosis. It is now clearly demonstrated that BTG is elevated in SSc patients [34] and that it could be a discriminating factor in the differential diagnosis of primary Raynaud's Phenomenon [170]. BTG and CXCL4, two platelet proteins, have long been described as platelet's activation markers [171]. Even if their biological effects have long been known, pro-fibrotic impact of BTG and CXCL4 in SSc was reaffirmed by *Kowal-Bielecka* et al. They showed that BTG and CXCL4 were found exclusively in bronchoalveolar lavage fluid of patients with SSc lung disease suggesting their active role in pulmonary fibrosis [172]. CXCL4-KO mice displayed significantly reduced liver fibrosis, and this outcome was associated with a different expression pattern of fibrosis-related genes [173]. Pro-fibrotic genes such as TGF-β and TIMP-1 (tissue inhibitor of metalloproteinase 1) were significantly reduced when in the same time expression of anti-fibrotic genes such as matrix metallopeptidase 9 (MMP9) and interleukin-10 (IL-10) were increased [173].

Platelet serotonin and platelet-induced production of thymic stromal lymphopoetin (TSLP) promote extracellular matrix production. As

described earlier, platelets are the main source of serotonin (5-HT) in circulation. Dees and colleagues have demonstrated that platelet-derived serotonin stimulates ECM production by stimulating the TGF-β/SMAD pathway in fibroblasts through the 5HT-2B receptor [114]. To support the relevance of these findings, 5-HT2B receptor was shown to be overexpressed in SSc patients' skin, and 5-HT2B-KO mice presented a reduction of fibrosis scores in a model of bleomycin-induced fibrosis [114]. Finally, inhibition of platelet activation using the $P2Y_{12}$-receptor inhibitor (clopidogrel) or the use of a model of serotonin-depleted platelet both showed a decrease in skin fibrosis scores in mice [114]. TSLP is a IL-7 cytokine family member highly expressed in the skin of SSc patients and in lung fibrotic specimen, but not in healthy control [174]. The amount of TSLP correlates with the skin fibrosis score (Rodnan) in patients with SSc [175]. In skin samples from SSc patients, TSLP co-localized with epidermal keratinocytes, dermal cells (including fibroblasts, mast cells and mononuclear cells), and ECs [175,176]. In a recent study, our group showed that, in vitro activated platelets induced TSLP production by human dermal microvascular endothelial cells (HDMECs) in an interleukin-1β-dependent manner and thus may contribute to overall fibrosis [175].

Altogether, these data suggest a new physiopathological loop linking platelet activation, vasculopathy and fibrosis during SSc (Fig. 1).

5.2. Renal disease

Evidence for platelet role in lupus and SSc related-kidney involvements is suggested by the presence of platelets within lupus nephritis (LN) biopsies as constituent of micro thrombi [177,178], as well as in scleroderma renal crisis where they participate in the formation of thrombi and fibrin deposition [179]. In LN, the presence of microthrombi correlates with macrophage kidney infiltration suggesting a chemo-attractant role of platelets. *Jin* et al. demonstrated that lower levels of circulating TGF-β in SLE patient was associated with higher disease activity and LN [180]. Conversely, urine from patient with LN is enriched TGF-β but also in P-selectin, a previously described marker of platelet activation [181]. In addition, *Lu* et al. showed higher levels of circulating PMPs in LN, emphasizing the importance of platelet activation in LN [182].

Platelet promote mesangial proliferation and glomerular remodeling. A tight collaboration between platelets and mesangial cells have been identified in LN. A recent study showed that plasma from SLE patients induced mesangial cells proliferation [183]. Mesangial proliferation was mediated by PDGF [183], a bioactive molecule released by platelets and PMPs. Moreover, PDGF levels correlated with proteinuria in LN patients [183]. Local production of TGF-β in patients with LN is suggested by studies demonstrating decreased circulating levels and increased urinary levels of TGF-β [180,184]. Mechanistically, platelets promote TGF-β production from mesangial cells in a CD40/CD154-dependent manner driving mesangial remodeling and fibrosis [185]. In this context, urinary TGF-β was associated with a worse prognosis and correlated to symptomatic LN [180,186]. Finally, platelets are able to induce secretion of monocyte chemoattractant protein 1 (MCP-1) by mesangial cells through CD40-CD154 signaling, leading to the recruitment of inflammatory cell in the mesangium and the promotion of local inflammation [187].

In summary, platelets drive mesangial proliferation and fibrosis as well as monocyte recruitment in LN through direct cell-to-cell interaction or cytokine and PMP production. In scleroderma renal crisis, platelets role is obvious in microthrombi formation and fibrin deposition leading to microangiopathic kidney disease.

6. Targeting platelets as a therapeutic strategy for SLE and SSc patients

Hydroxychloroquine (HCQ) is an antimalarial compound universally used in SLE [188]. In SSc, HCQ could also be clinically efficient, at least for articular involvement [189]. Platelet sensitivity to activation stimuli as well as the release of alpha-granules content are decreased after HCQ treatment [190,191]. The relevance of these observations are supported by the fact that HCQ decreases the rate of thrombotic events in SLE patients. Furthermore, a recent proof of concept study added more data in favor of the use of HCQ as antiplatelet therapy. When given to healthy subjects alone or in combination with aspirin, HCQ decreased platelet activation from various agonists, with the best results in the combination group [192].

Aspirin is an irreversible cyclooxygenase 1 (COX-1) inhibitor recommended in patients presenting high risk of CVD. Thromboxane A2 is a metabolite produced by activated-platelet's COX-1 and its level is increased in SLE and SSc [160,193]. Aspirin decreases thromboxane A2 production but this effect is dampened in SLE patients [194], suggesting that platelet activation in auto-immune disease is multifactorial and that multimodal platelet modulation (e.g. HCQ and aspirin) might provide benefit in these patients.

Clopidogrel is a $P2Y_{12}$ antagonist, a G-protein receptor recognizing ADP responsible for platelet activation in thrombosis, and is recommended in patients with severe atherosclerotic disease. Our group showed that treatment of lupus-prone mice with clopidogrel could increase survival and improve several disease activity markers (dsDNA, proteinuria) [53]. As stated before, in SSc mice models, clopidogrel has been shown to decrease fibrosis scores in a mice model of experimental fibrosis [114]. These observations mandated the evaluation of clopidogrel in SLE and SSc with two clinical trials (CLOPUS, NCT02320357; PSSIT, registration ongoing).

Iloprost is a synthetic analogue of prostacycline (PGI_2) used in refractory digital ulcers in SSc or in case of pulmonary arterial hypertension. Many authors report that iloprost decrease platelet activation explaining some of its benefit in its indications [195]. Heparin-induced thrombopenia (HIT) is a condition characterized by the formation of immune-complex between self-produced IgG and CXCL4, inducing major platelet activation through FcγRIIa and diffuse thrombosis. Interestingly, a report found that iloprost could prevent platelet activation in HIT [196]. Considering the physiopathological proximity between HIT and SLE or SSc IC-induced platelet activation, it is attractive to consider iloprost as an anti-platelet therapy in both diseases.

Metformin is a drug used in type 2 diabetes as insulin sensitizer. A recent report found that metformin was able to reduce platelet activation and platelet release of mtDNA, both previously discussed as implicated in SLE and SSc pathogenesis [197]. The relevance of these data is supported by a proof-of-concept human trial of metformin in SLE showing promising results [198]. Metformin decreased NETosis, mtDNA release and IFN production through pDC-mtDNA axis in vitro. Moreover, they showed that in SLE patients, metformin could reduce flares incidence as well as prednisone exposure, two major clinical endpoints, thus mandating larger clinical trials for this very safe and cheap drug.

Dapirolizumab is a PEGylated Fab′ targeting CD154 and its interaction with CD40. The exploratory human phase I trial showed an excellent tolerance profile and some interesting preliminary data suggesting its clinical efficacy assessed by usual SLE clinical endpoints (SRI-4, BICLA) [199]. Importantly, no case of thrombotic adverse event was reported contrary to the study testing BG9588. In this study, the Fc fragment of the therapeutic IgG activated platelet and was responsible for severe thrombotic adverse events (e.g. myocardial infarctions) [200].

To conclude, we demonstrated the close interaction between platelet and the immune system in SLE and SSc, two phenotypically distinct autoimmune systemic diseases. Platelet system activation may be seen as a marker of disease activity, as an adjuvant of immune dysregulation as well as an effector of tissue damage. The better understanding of these interactions will allow a better understanding of these complex systemic disease together with the potential discovery of new potent therapeutic pathways.

7. Disclosure of potential conflict of interest

This article contains no conflicts of interest.

8. Ethical statement

This article contain no clinical data.

Disclosure of interest

None.

Acknowledgements

Servier Medical for providing free art for the figure.

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2) IMPACT DES SELECTINES SUR LA FONCTION DES LYMPHOCYTES T REGULATEURS (EN COURS DE REVISION)

Selectins impair regulatory T cell function and contribute to systemic lupus erythematosus pathogenesis

Short title : Selectins impair Tregs in systemic lupus

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Summary:

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by a loss of tolerance toward self-nucleic acids, autoantibody production, an interferon signature, and a defect in the T regulatory cells (Tregs) compartment. In this work, we identified that platelets from active SLE patients preferentially interacted with Tregs *via* the P-selectin/PSGL-1 axis. Selectin interaction with PSGL-1 blocked the regulatory/suppressive properties of Tregs and follicular Tregs by triggering Syk phosphorylation and an increase in intracytosolic calcium. Mechanistically, P-selectin engagement on Tregs induced a downregulation of the TGF-beta axis, altering Tregs phenotype and limiting their immunosuppressive response. In patients, we found a significant upregulation of P- and E-selectin levels both expressed by microparticles and in their soluble forms that correlated with SLE disease activity. Finally, blocking P-selectin in a mouse model of SLE improved cardinal features of the disease. Overall, our results identify a selectin-dependent pathway active in SLE patients and validate it as a potential therapeutic avenue.

Keywords: systemic lupus erythematosus; selectin; PSGL-1; T regulatory lymphocytes; platelets; autoimmune disease

Introduction:

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by a loss of tolerance against self-nucleic acids and type I interferon production *(1)*. Self-reactive B cells primed by interferons are responsible for auto-antibody production, immune complex formation and deposition in tissues, leading to end-organ disease. Previously most targeted therapies were directed against B cells or interferons *(2)*. However, the vast majority of these drugs did not reach significant efficacy endpoints in human clinical trials, indicating that alternative therapeutic strategies should be considered. Along these lines, the role of effector T cells including follicular helper T cells, known for their ability to induce germinal center responses, and regulatory T cells (Tregs), which dampen immune responses, have been highlighted and could represent therapeutic opportunities *(3)*.

Foxp3$^+$ Tregs are important for the maintenance of immunological tolerance, and their absence or deregulation results in fatal autoimmune diseases in humans and mice *(3)*. In SLE, defects in the Treg compartment have been observed both in mouse models and patients *(3)*. Importantly, failure of Tregs to control exaggerated T and B cell activation in SLE may depend on cell-extrinsic factors, including the interplay of Tregs with other immune cells in the inflammatory milieu. As an example, we, and others, have recently identified the ability of OX40L-expressing monocytes, activated by immune complexes (ICs), to induce the differentiation of naïve and memory T cells into B-helper cells *(4, 5)*, while inhibiting the immunosuppressive properties of Tfr and Tregs *(6)*. ICs also influence the function of several cells of the innate immune system, including neutrophils and platelets. Blood platelets, typically involved in primary hemostasis, are activated in several autoimmune disorders and constitute an adjuvant factor in autoimmunity through their ability to tightly interact with other cells by the formation of platelet/cell aggregates *(7, 8)*. In SLE patients, platelets express high levels of P-selectin, are activated by circulating ICs, form aggregates with pDCs and promote the secretion of type I interferon by pDCs in a CD40/CD40L-dependent mechanism ultimately contributing to SLE pathogenesis *(9)*.

Selectins play an important role in the migration of circulating leukocytes to the site of inflammation. During inflammation, P- and E-selectins are upregulated on platelets and endothelial cells and mediate cell adhesion under flow, therefore playing a crucial role in the immune response *(10)*. Accordingly, selectins are elevated at both the tissue and circulating levels in many inflammatory conditions, including sepsis and atherosclerosis *(11–13)*. Selectins bind to P-selectin glycoprotein ligand-1 (PSGL-1), which is an adhesion molecule expressed by many types of hematopoietic cells including T cells. The binding of selectins to PSGL-1 depends on its post-translational modifications such as fucosylation that inserts a sialyl-lewis X motif (sLe^x). sLe^x, designated as cluster of differentiation 15s (CD15s), is a tetrasaccharide carbohydrate usually fused to O-glycans *(14)*. Although PSGL-1 has long been studied as an adhesion molecule involved in immune cell trafficking, it is now increasingly recognized as a regulator of many aspects of the immune response *(15, 16)*. Strikingly, we confirmed that among lymphoid cells, Tregs and Tfr express the highest levels of PSGL-1/CD15s, potentially conferring a specific platelet/selectin interaction *(17)*. Since SLE is characterized by platelet activation that induces P-selectin exposure and secretion, we asked whether Treg function is affected by platelets in SLE patients.

Here, we found that P-selectin impairs the immunosuppressive functions of Tregs and Tfr cells. Mechanistically, through binding to CD15s-associated PSGL-1,

selectins induced a Syk-dependent calcium increase, decreased GARP expression, TGF-beta release and Foxp3 expression. We show that soluble P-selectin, P-selectin-associated microparticles, and platelet-Treg aggregates have the strongest association with disease severity in SLE patients. In a mouse model of SLE, P-selectin blockade attenuated disease severity. Collectively, our results provide clinical and functional evidence of the role of P-selectin in SLE pathogenesis and point to P-selectin as a valuable therapeutic target in SLE patients.

Results:

Tregs and Tfr express high level of fucosylated (CD15s) PSGL-1

In SLE patients, platelets have an activated phenotype in relation to disease activity and severity *(8)*, and the relocation of P-selectin on the platelet membrane is a hallmark of platelet activation *(10)*. To investigate which immune cell populations interacted with P-selectin-expressing platelets, we first analyzed PSGL-1 expression using flow cytometry on fresh blood samples. In healthy donors (HD), PSGL-1 expression was highest on plasmacytoid dendritic cells (pDCs), moderate on myeloid dendritic cells (mDCs), monocytes, granulocytes and T cells, and nearly absent on B lymphocytes (**figure 1A**; gating strategy see **figure S1A**). Among T lymphocyte subpopulations, $CD4^+$ $CD25^{high}$ $CD127^{low}$ T regulatory cells (Tregs) showed the highest expression of PSGL-1 compared to other subpopulations ($p < 0.0001$, **figure 1B**; gating strategy see **figure S1B**). Since PSGL-1 fucosylation is necessary for its interaction with its receptor (e.g., selectins), we next monitored CD15s expression at the plasma membrane of circulating leukocytes in HD. We observed that among T cells, Tregs (specifically $CD45RA^-$ $FoxP3^+$) expressed higher levels of CD15s compared to Teff cells ($p < 0.001$, **figures S1D and S1E**). Interestingly, the Tfr population $CD3^+$ $CD45RA^-$ $CXCR5^+$ $Foxp3^+$ $CD25^+$ also expressed high levels of CD15s (**figures S1D and S1E**). In SLE patients, both resting ($CD45RA^+$) and effector ($CD45RA^-$) $FoxP3^{high}$ Tregs expressed significantly higher PSGL-1 (**figure 1C,** gating shown in **figure S1C**) and CD15s (**figure S1F**) levels than T effector lymphocytes. Tregs from SLE and HD expressed similar levels of PSGL-1 and CD15s (**figure S1G**).

We next explored whether CD15s-expressing Tregs interacted with platelets in HD and SLE patients. Using flow cytometry, circulatory platelet/Treg aggregates (defined as $CD61^+$[platelet]/$CD4^+CD127^-CD25^+$[Treg]) were significantly higher in SLE patients, notably those with active disease, than in HD (**Figures 1D and 1E**). Further, the frequency of such $CD61^+$ Treg aggregates significantly correlated with SLE disease activity, as assessed by the SLE disease activity index (SLEDAI) (**figure 1F**). Of note, the frequency of platelet/T-effector aggregates was not significantly different between SLE and HD (**figure 1G**) and did not correlate with the SLEDAI (data not shown). Together, these results suggest that in active SLE patients, activated (P-selectin-expressing) platelets are responsible for a specific platelet/Treg interaction most likely *via* P-selectin/PSGL1 interaction.

Platelets block Tregs and Tfr suppressive functions through P-selectin/PSGL-1 interaction

To investigate the consequences of platelet-Treg interaction, we conducted Treg suppressive assays in the presence or absence of autologous platelets. While the platelets did not directly impact Teff cell proliferation, they prevented the suppressive function of Tregs (**figure 2A and 2B**) in a dose-dependent manner (**figure 2C**), and at a platelet-Treg ratio as low as 5:1. To distinguish whether platelet-mediated Treg dysfunction was mediated through a soluble or membrane-bound factor, we separated secreted (supernatant) and membrane-bound factors (pellet) from thrombin-activated platelets by ultracentrifugation and added each fraction to the Treg-Teff co-culture. Only the pellet from activated platelets inhibited Treg suppressive functions (**figure S2A**). Interestingly, preincubating Tregs with an anti-PSGL1 blocking antibody prevented platelet-induced Treg dysfunction (**figure 2D**), suggesting that the PSGL-

1/P-selectin interaction mediated the platelet-driven inhibition of Treg suppressive function.

To assess whether this effect was specific to the PSGL-1 axis, we repeated the Treg suppressive assays, replacing platelets with recombinant forms of the known PSGL-1 receptors, i.e. Platelet- (P-), Endothelial- (E-) or Leukocyte (L-) selectin. Platelet-induced Treg dysfunction was observed following culture with each of the recombinant selectins (**figure 2E**), with treatment with P-Selectin showing the strongest inhibition. P-selectin inhibited the suppressive function of Tregs in a dose-dependent manner (**figure 2F**), and this effect was prevented when Tregs were pre-treated with anti-PSGL-1 blocking antibody (**figure 2G)**. Importantly, Treg viability was not affected by P-selectin (**figure S2B**). Together, these results indicate that platelet-induced Treg dysfunction was mediated by the interaction between P-selectin expressed on platelets and PSGL-1 expressed on Tregs.

To investigate if selectins had a similar effect on Tfr cells, we cocultured autologous memory B cells, Tfh and Tfr cells (or Tregs) sorted from HD, in the presence of Staphylococcal Enterotoxin B (SEB). While no impact on B cells was observed in the absence of regulatory cells, P-selectin inhibited Tfr and Treg immunosuppressive functions with respect to B cell differentiation into $CD38^{high}$ plasmablasts (**figures 2H and I**) and IgG production (**Figure 2J**). These results suggest that P-selectin is also able to block the suppressive properties of Tfr cells. These data identify a novel function of selectins in disrupting the suppressive activities of Tregs and Tfr cells.

P-selectin induces Syk kinase phosphorylation and intracellular calcium release in Tregs

PSGL-1 engagement has previously been described to induce Erk phosphorylation in neutrophils *(18)* and Syk phosphorylation in T cells *(19)*. To address the intracellular signaling pathways activated by selectins in Tregs, we used Phosflow® on freshly sorted Tregs from a HD. We found that P-selectin induced Syk phosphorylation in a dose-dependent manner (**figures 3A and 3B**), and this was inhibited by pre-incubation of Tregs with an anti-PSGL1 blocking antibody or with a Syk-specific inhibitor used as a negative control (**figure 3B**). Consistent with a role for Syk in regulating Treg function, Syk inhibition prevented the P-selectin-induced Treg dysfunction in immunosuppressive assays (**figure 3C**). In fresh blood samples from active SLE patients, $FOXP3^+$ Tregs aggregating with platelets ($CD61^+$ $FoxP3^+$ Tregs) had increased pSYK levels compared to $CD61^-$ Tregs (**figures 3D and 3E**). In contrast, we did not detect differences in pSYK levels between $FoxP3^-$ Teff cells alone or those that aggregate with platelets (**figure 3F**), likely due to the absence of CD15s. No significant difference was observed in Erk phosphorylation in Tregs in the presence of P-selectin stimulation (**figure S3A**). In addition, pre-incubating Tregs with different levels of P-selectin did not alter STAT5 phosphorylation in response to IL-2, suggesting that P-selectin did not affect the IL-2 axis in Tregs (**figure S3B**).

Phosphorylation-driven Syk activation leads to its interaction with and activation of phospholipase C-γ1 (PLC-γ1), triggering a cytosolic calcium ($[Ca^{2+}]_{cyt}$) signal *(20)*. To measure $[Ca^{2+}]_{cyt}$ in Tregs, we conducted single-cell cytosolic calcium imaging on freshly isolated HD Tregs. Upon P-selectin exposure, cytosolic calcium concentration dramatically increased in Tregs (**figures 3G and 3H**), but not in B lymphocytes devoid of PSGL1 expression, or in Teff cells expressing low levels of fucosylated PSGL-1 (**figures S3C and S3D**). The pre-incubation of Tregs with anti-PSGL1 blocking antibody or Syk inhibitor prevented the P-selectin-mediated $[Ca^{2+}]_{cyt}$ increases

(**figures 3G and 3H**). Collectively, these data identified the Syk/PLC-γ1 as key downstream mediators of selectin-induced Treg dysfunction, and that this pathway is activated in Tregs that aggregate with platelets in SLE patients.

Selectins trigger Treg dysfunction through inhibition of the TGF-beta pathway

To investigate the pathways altered downstream of Syk/PLC-γ1/calcium signaling in Tregs upon P-selectin exposure, we cultured freshly sorted human Tregs from HD for 8 hours with P-selectin and then investigated the transcriptional changes in the cells by microarray analysis. 3408 transcripts were significantly downregulated and 2229 were upregulated (|fold change| > 1.5 and p-value < 0.05) in response to P-selectin exposure (**Supplementary file 4 for the list of transcripts**). Interestingly, Treg-specific transcripts were downregulated including *FOXP3*, *IKZF2* (Helios), and *BCL11B* (**figure 4A**), while there was a moderate increase in transcripts of pro-inflammatory genes such as *IL17A*, *CCL4* and *IL18*. Gene set enrichment analysis identified that the TGF-beta signaling pathway was enriched in untreated versus P-selectin-treated Tregs, suggesting a selectin-dependent impairment of the TGF-beta axis in Tregs (**figure 4B**). Indeed, the transcripts of TGF-beta (*TGFB1*), its chaperone protein GARP (*LRRC32*) and the downstream transcription factor *SMAD3* were significantly downregulated in Tregs upon exposure to P-selectin (**figure 4A**).

Using qRT-PCR, we confirmed the downregulation of *TGFB1* and *LRRC32* mRNA in Tregs exposed to P-selectin ($p<0.01$, **figure 4C**). Next, to evaluate the impact of P-selectin treatment on Tregs at the protein level, we cultured HD Tregs for 48 hours with or without P-selectin and assessed GARP surface expression on Tregs using flow cytometry. We observed that P-selectin treatment was associated with decreased GARP expression on Tregs in a dose-dependent manner (**figures 4D and 4E**). Pre-incubation of Tregs with anti-PSGL-1 or Syk inhibitor restored GARP expression (**figure 4E**). GARP is a chaperone protein essential for the processing and release of active (free) TGF-beta by Tregs *(21)*. Therefore, we measured free TGF-beta levels in Tregs culture supernatants and found that P-selectin significantly decreased TGF-beta secretion by Tregs (**figure 4F),** without significantly altering the levels of IL-4, IL-10 or IL-17A (data not shown).

In accordance with our microarray analysis, the Treg-specific transcription factors FoxP3 and Helios were significantly downregulated at the mRNA (**figure 4C**) and protein (**figures 4G-I**) levels after P-selectin exposure. FoxP3 and Helios downregulation was blocked by treating Tregs with the either anti-PSGL1 blocking antibody or Syk inhibitor (**figures 4H and 4I**). Importantly, upon P-selectin exposure, supplementing Tregs with TGF-beta rescued FoxP3 and Helios expression, supporting the importance of the TGF-beta pathway in Treg function and maintenance (**figures S4A, and S4B**). In contrast, Treg surface expression of CD25, CTLA4 and GITR were not modified by P-selectin exposure (**figure S4C**). Moreover, P-selectin did not induce Th17 gene set expression, and Tregs cultured with P-selectin for 48 hours did not upregulate RORγt or IL17 at the protein level (**figure S4D**). Exposure to P-selectin did not affect the expression of Tbet, GATA3 or RORγt in primary Th1, Th2 or Th17 cells respectively (**figure S4E**), further demonstrating the specificity of the selectin/PSGL-1 axis in Tregs. Collectively, these data suggest that P-selectin leads to specific Treg dysfunction by inhibiting the TGF-beta pathway and decreasing both FoxP3 and Helios expression.

P- and E- selectins are upregulated in SLE patients

To confirm the role of selectins in human SLE pathogenesis, we evaluated soluble selectin concentrations and selectin-bound microparticles in patients with SLE. Platelet-free plasma was isolated from fresh blood samples through a rigorous preanalytical protocol to avoid non-specific platelet activation as described in the *methods* section.

We subsequently measured levels of soluble selectins by ELISA. Consistent with previous findings, we found that soluble selectins (roughly the size of albumin) are lost in the urine in case of proteinuria seen in active glomerulonephritis *(22)*. Urinary P-selectin levels correlated with albuminuria (figure S5A), while blood P-selectin concentrations inversely correlated with albuminemia (reflecting urinary protein loss; figure S5B), suggesting urinary loss of P-selectin during active glomerulonephritis. For these reasons, we excluded patients with active renal disease (n = 6) from soluble selectin analysis. P- and E-selectin levels were increased in patients with active SLE compared to HD and quiescent SLE patients (**figure 5A**), and they significantly correlated with SLE disease activity (P-selectin: $r = 0.35$, $p = 0.005$; E-selectin: $r = 0.41$, $p < 0.001$; **figure 5B**), while this was not observed for L-selectin.

Next, we studied circulating levels of platelet- (PMP), endothelial- (EMP) and granulocyte-derived microparticles (GMP) in patient platelet-free plasma, as well as the expression of P-, E- or L-selectin, respectively by flow cytometry **(figure S5C and S5D**). In line with a recent study (Mobarrez et al., Sci Rep 2016), we found that patients with active SLE had a moderate but significant increase in PMPs when compared to HD (**figure 5C**). Interestingly, while MP counts did not correlate with disease activity (**figure 5E**), selectin-positive MPs were markedly higher in active SLE patients (**figure 5D**) and correlated with disease activity as assessed by the SLEDAI (**figure 5F**). Precisely, we found a significant correlation between P-selectin$^+$ PMPs/E-selectin$^+$ EMPs and the SLEDAI (Psel$^+$ PMPs : $r = 0.52$, $p < 0.0001$; Esel$^+$ EMPs : $r = 0.42$, $p < 0.001$; **figure 5F**). In contrast to soluble P-selectin levels (**figure S5E**, left panel), P-selectin+ PMPs were significantly elevated in SLE with active renal disease compared to HD (**figure S5E,** right panel). Altogether, these data establish that active SLE patients are characterized by increased expression of selectins both in their soluble and microparticle-bound forms.

P-selectin blockade ameliorates SLE pathogenesis in lupus-prone mice

Finally, we investigated whether blocking P-selectin in a lupus-prone mouse model could alleviate cardinal features of the disease. To address this question, we first confirmed whether PSGL-1 expression in mice recapitulates that found in humans. We analyzed PSGL-1 expression on Tregs and Teff cells from C57BL/6 wild-type (WT) mice and observed that it was higher on FoxP3$^+$ Tregs compared to effector T cells. (**figure 6A-B**). Then, we explored whether P-selectin induced a calcium response in Tregs (CD4$^+$CD25$^+$ T cells) and Teff cells (CD4$^+$CD25$^-$ T cells) sorted from the spleen of WT mice. As with human Tregs, recombinant mouse P-selectin induced a strong cytosolic calcium increase in mouse Tregs but not in Teff cells (**figures 6C and 6D**). Finally, we investigated whether P-selectin alters mouse Treg phenotype and function. We purified naïve CD4+ T cells from the spleen of WT mice, labelled them with CFSE and cultured them under different polarizing conditions to induce differentiation towards Th1, Th2, Th17 or Treg lineages. P-selectin treatment was associated with a specific decrease in FoxP3 expression in Tregs, with no impact on the lineage-specific expression of Tbet, GATA3, or RORgt in other subpopulations or overall proliferation

of cells detected (**figures 6E and 6F**). Importantly, supplement of the cultures with increasing levels of TGF-beta was able to rescue FoxP3 expression in Tregs exposed to P-selectin (**figure S6A**). Together, these results confirmed that PSGL1 is highly expressed in murine Tregs and that selectins negatively impact murine Tregs and the TGF-beta pathway in the same manner as in humans. We further studied the relevance of selectins in a murine model of SLE by using mice deficient for DNase1L3. These mice develop all the major clinical features of human SLE including DNA autoreactivity and glomerulonephritis *(23)*. Furthermore, SLE pathogenesis in this model was shown to depend on the aberrant accumulation of self-DNA on MPs *(23)*. Therefore, we measured the circulatory levels of PMPs and their expression of P-selectin. While the total number of PMPs did not change **(figure S6B left panel**), the levels of P-selectin^{+} PMPs were significantly higher in the plasma of *Dnase1l3*$^{-/-}$ knockout (KO) mice compared to WT ones **(figure S6B right panel**).

Together, these observations prompted us to test the therapeutic potential of P-selectin blocking antibody in *Dnase1l3* KO, lupus-prone mice. We treated 30 week-old KO mice with an anti-P-selectin monoclonal antibody (mAb), or with a control mAb during 10 consecutive weeks with 3 treatments/week. As expected, the anti-P-selectin antibody blocked P-selectin-induced calcium signaling in *ex-vivo* treated Tregs (**Figure S6C**) and reduced circulatory levels of P-selectin+ PMPs (**Figure S6D**). 30 week-old KO mice display elevated levels of anti-dsDNA autoantibodies compared to WT animals, and while these titers continued to increase over time in animals treated with control antibody, anti-P-selectin antibody prevented this increase (**figure 7A**). SLE development in KO animals is accompanied by a loss of marginal zone B cells (MZB), which was also partially rescued by anti-P-selectin antibody (**figures S7A and S7B),** while other B cell subpopulations were not significantly modified (**figures S7C and S7D**). The accumulation of autoreactive antibodies together with C3 complement causes their deposition in the kidney glomeruli of KO mice (**figure 7B, lower panels**) and ultimately leads to the development of glomerulonephritis manifested by enlarged glomeruli, mesangial proliferation and sometimes glomerular thrombosis (**figure 7D middle panel**). Finally, after 10 weeks of anti-P-selectin treatment IgG/C3 deposition in the kidney was reduced (**figure 7C),** and the overall pathology was significantly improved (**figure 7D, right panel and figure 7E)**. These results show that P-selectin blockade significantly ameliorates pathogenesis in a murine model of SLE.

Discussion:

In this study we identified selectins, in particular P-selectin, as a new potential therapeutic target in SLE. Indeed, we observed that selectins produced by activated platelets (P-selectin), granulocytes (L-selectin), and endothelial cells (E-selectin) specifically block the immunosuppressive properties of Tregs, without affecting effector T cells. Selectins induced a Syk-dependent calcium increase, which inhibited TGF-beta and led to reduced expression of Foxp3. Assessment of circulating levels of selectins, selectin-bound microparticles, and platelet/Treg aggregates revealed that this P-selectin pathogenic pathway was active in SLE patients. Finally, we observed a similar effect of P-selectin on murine Tregs, and targeting P-selectin by a blocking mAb in a mouse model of SLE improved cardinal disease parameters, including anti-DNA antibody levels and kidney pathology.

Tregs are vital to the preservation of immune tolerance and prevention of exacerbated immune responses that lead to autoimmunity. Foxp3$^+$ Tregs are characterized by their constitutive and high expression of CD25 (IL-2 receptor a chain) and the co-inhibitory molecule CTLA-4. Tregs quickly sense and consume IL-2 produced by effector T cells to prevent their activation *(24)*. Foxp3$^+$ Tregs use additional suppressive molecules including IL-10, TGF-beta, IL-35, TIGIT, CD39, and CD73 to mediate their inhibitory functions. In the context of SLE, studies have led to contradictory results regarding Treg numbers *(25)* and/or function *(26)*. Nevertheless, an impairment in Treg function is likely since treatment with low-dose IL-2, which restores the Treg compartment, improves the disease in humans *(27, 28)*. However, the exact mechanisms involved in Treg dysfunction in SLE remained largely unknown. Our data established selectins as inhibitory factors that selectively target Tregs and Tfr cells. Binding of the selectin family on PSGL-1 depends on post-translational modifications such as fucosylation, which inserts the Sialyl Lewis X motif (sLe^x). We observed that CD15s, which is specifically expressed by Tregs and Tfr cells, was associated with the ability of PSGL-1 to induce intracellular signals in response to selectin, leading to alterated Treg functions. Interestingly, CD15s^{high} Tregs have been described as terminally differentiated and mostly immunosuppressive Tregs *(17)*.

Selectins are upregulated in a wide range of inflammatory disorders, and their function was believed to be limited to chemotaxis *(10)*. Several SLE mouse models show elevated levels of P-selectin in the serum and affected tissues *(22, 29)*. The role of the selectin/PSGL-1 pathway in the context of immune tolerance is poorly understood. Tinoco *et al.* demonstrated that the deletion of PSGL-1 in mice was associated with an increased virus-specific or tumor-specific CD8$^+$ T cell response and induced an uncontrolled autoimmune response that quickly became fatal *(16)*. However, as this effect was mainly driven by CD8$^+$ T cells that do not express fucosylated PSGL-1 and treatment with P-, E- or L-selectin did not have an impact on the antiviral response, PSGL-1 likely mediated its effects through a selectin-independent mechanism in this model. These results were recently explained by Johnston et al who showed that PSGL-1 is a ligand of a checkpoint molecule, the V-domain Immunoglobulin Suppressor of T cell Activation (VISTA) in acidic condition such as the tumor microenvironment *(15)*. Another work suggested that PSGL-1 engagement with P-selectin induced a tolerogenic phenotype in immature DCs *in vitro (30)*. Therefore, it appears that PSGL-1 exerts multiple functions in inflammation in a cell- and environment-specific manner, beyond its initial role described in selectin-mediated rolling and diapedesis. In addition to blocking Treg function, P-selectin was also shown to both trigger NETosis in mouse neutrophils *(31)* and activate monocytes *(32)*, two important features of SLE pathogenesis. Whether these mechanisms (e.g. platelet-dependent activation of monocytes and neutrophils) contribute to SLE remains unclear. However, it is tempting to speculate that P-selectin-targeting therapies could impact multiple immune pathways involved in SLE pathogenesis.

Platelets are one of the main sources of circulating P-selectin, and besides their well-established role in hemostatic functions, they are increasingly recognized as important players in inflammation. Pioneering studies from Boilard *et al.* and our group have established a role for platelets in the amplification of inflammation by microparticle production in rheumatoid arthritis and aggregation to plasmacytoid dendritic cells in SLE *(9, 33)*. Furthermore, platelets have been clearly implicated in many inflammatory disorders including multiple sclerosis, systemic sclerosis, or

Crohn's disease *(34, 35)*. While our results establish a new role for platelets in SLE, it is likely that the described P-selectin-mediated pathogenic pathway operates in other autoimmune diseases. In the context of SLE, epidemiologic studies revealed that platelet activation correlated with disease severity, occurrence of thrombosis, premature atherosclerosis, and long-term mortality *(8)*.

Premature atherosclerosis represents the first cause of death in SLE patients, and current immunosuppressive treatments fail to control its progression *(36)*. While traditional cardiovascular risk factors may partially be involved, the precise mechanisms leading to this accelerated atherosclerosis in SLE patients remain poorly understood *(37)*. The protective role of Tregs in atherosclerosis was previously characterized *(38)*, and elevated P-selectin levels have been widely associated with atherosclerosis progression and myocardial infarction in humans *(11)*, with causative links demonstrated in mouse models *(39)*. Together with our study these observations further indicate that P-selectin blockade could be an innovative treatment in SLE, targeting both the immune system dysregulation and the accelerated atherosclerosis. Notably, crizanlizumab, a human anti-P-selectin antibody has been developed and tested in sickle-cell disease, a haemoglobin disease characterized by red blood cell/platelet aggregation and small vessel thrombosis. This treatment has shown to be efficient in terms of vaso-occlusive crisis without safety warnings, paving the way for trials in other indications such as SLE *(40)*.

In conclusion, we describe a new pathologic pathway in SLE, where activated platelets and endothelium interact with Tregs to block their immunosuppressive functions. Blocking P-selectin improved a murine model of SLE, supporting it as a potential therapeutic target for future clinical trials.

Materials and methods:

Human Samples. Healthy donor samples were obtained from the French National Blood bank (Etablissement Français du Sang). SLE patients were recruited in the rheumatology, nephrology and internal medicine departments of Bordeaux University Hospital. Patients were diagnosed with systemic lupus erythematosus using the 2012 SLICC SLE diagnosis criteria (Petri et al., arthritis, 2012). Oral and written consent were obtained from patients before samples (blood and/or urine) were retrieved. Our research protocol (MICROLUPS) conforms to French and European Ethics standards and was reviewed and approved by an independent ethical committee (authorization number 2018-A00599-46). The MICROLUPS research protocol was registered on *clinicaltrials.gov* (NCT03575156).

Phenotyping of blood cells. 200µL of fresh whole blood was stained with antibodies recognizing lymphocyte markers (see supplementary table 1). After 15 minutes of incubation at room temperature, red blood cells were lysed using 3mL of ACK buffer (150mM NH4Cl, 10mM KHCO3, 1mM EDTA2Na) for 25 minutes then washed with PBS. In order to study rare subpopulations (e.g., Tfr), PBMCs were isolated using Ficoll-Paque, and 10^7 PBMCs were stained for 15 minutes before washing and cytometer analysis.

Blood cell isolation. PBMCs were retrieved from healthy donors or patients using Ficoll-Paque separation medium (Eurobio, France®). To sort Tregs and Teff, PBMCs were pre-sorted using CD4-microbeads with Magnetic-Activated Cell Sorting (Miltenyi©) following the manufacturer's instructions. CD4-sorted cells were then stained with CD4-PE-Cy7 (Beckman-Coulter©), CD25-PE-Cy5 (Beckman-Coulter©) and CD127-PE (Beckman-Coulter©). The $CD4^+$ Cells were subsequently sorted using FACS Aria 2-Blue 6-Violet 3-Red 5-YelGr, 2 UV laser configuration (BD Biosciences©) in flow cabinet. T regulatory lymphocytes (Tregs) were identified as $CD4^+/CD25^{high}/CD127^{dim}$ and T-effector (Teff) lymphocytes as $CD4^+/CD25^-/CD127^+$. Subset purity was 95% or higher. T follicular regulatory cells (Tfr) were sorted as $CD4^+/CD25^{high}/CD127^{dim}/CXCR5^+$ cells and T follicular helper (Tfh) cells as $CD4^+/CD25^-/CD127^+/CXCR5^+$.
Magnetically enriched $CD19^+$ cells were sorted using ARIA FACS. Memory B cells were sorted as $CD19^+CD27^+IgD^-$ and naïve B cells were $CD19^+CD27^-IgD^+$.
Platelets were isolated from platelet-rich plasma (PRP), which was generated from freshly drawn whole blood samples by 20 minutes centrifugation 180 x g, with no brake applied. Prostaglandin E1 (1µM). was added to the PRP to prevent excessive platelet activation during sample preparation. Platelets were pelleted by centrifugation at 890 x g, without brake, for 10 minutes, and re-solubilized in Tyrode's buffer (NaCl 0.134M, KcL 2.9mM, NaH2PO4 0.34mM, NaHCO 12m, HEPES 20mM, MgCl2 1mM, Glucose 5mM, BSA 0.5%). Before use, platelets were counted using a Beckman Coulter© ACT diff analyser.

Treg immunosuppressive assay. After sorting, Teffs were stained with carboxyfluorescein succinimidyl ester (CFSE) 2mM to monitor proliferation. 50,000 Tregs / well were plated in a 96-well plate coated with anti-CD3 (UHT1, Beckman Coulter©, 1 µg/mL). When used, anti-PSGL1 antibody 1/200 (clone KPL1) or Syk inhibitor 1µM (Cayman Chemicals©, Ref 622387-85-3) were added to the Tregs and

incubated at 37°C for 30 minutes. Platelets or selectins (200pg/mL) were subsequently added and incubated at 37°C for 45 minutes. In experiments involving selectins, the cells were subsequently washed and supernatant containing inhibitor and selectin removed after centrifugation. After washing, Teffs were added at a 1:1 ratio with Tregs and an anti-CD28 antibody was added (clone CD28.2, Beckman coulter©, 3 µg/mL). As a control, Teff cells were also cultured alone with or without platelets and/or selectin to assess the potential effect on proliferation. Co-cultures were incubated 4 to 6 days then stained with DAPI and CD4 to assess CFSE fluorescence dilution in viable cells using a BD CantoII® cytometer.

Tfr immunosuppressive assays. In a 96-well plate, 30,000 freshly-sorted B memory cells were cultured at a 1:1:1 ratio with autologous Tfh cells and Tfr or Treg cells. Cells were cultured with SEB (1µg/mL) for 7 days at 37°C, with or without P-selectin (200pg/mL). Supernatants were subsequently frozen, and the cell pellets analyzed by flow cytometry (see supplementary table 1 for antibodies). Plasmablasts were defined as live $CD4^{-}CD19^{+}CD27^{high}CD38^{high}$ cells. IgG measurements from supernatants were conducted using the ELISA Human IgG quantification kit (Bethyl laboratories©, cat. E80-104) per the manufacturer's instruction.

Calcium signaling assay. Single-cell cytosolic calcium imaging was performed using the fluorescent calcium dye cali-520 (AAT Bioquest, CA, USA). Glass coverslips were mounted in a Attofluor cell chamber (Thermo Fisher Scientific, Saint Herblain, France) positioned on the stage of an inverted epifluorescence microscope (IX70, Olympus) equipped with an ×40 UApo/340- 1.15W objective. $5x10^5$ Tregs or Teff were loaded with cali-520 (1µM) at room temperature (20–25 °C) in Hank's Balanced Salt Solution (HBSS, 2mM CaCl2, pH 7.25) for 30 minutes. Cali-520 exhibits limited compartmentalization in intracellular stores and is resistant to leakage. The cells were rinsed with HBSS and incubated in the absence of the Ca^{2+} probe for 15min to complete de-esterification of the dye. Cali-520 was excited at 485+/− 22nm, and images were captured at 530+/− 30nm at constant 10-s intervals, at 12-bit resolution, by a fast-scan camera (CoolSNAP fx Monochrome, Photometrics). All images were background-subtracted. Regions of interest corresponding to cells recorded were drawn to analyze the fluorescence signal. Imaging was controlled by Universal Imaging software, including Metafluor and Metamorph. Fluorescence intensity changes were normalized to the initial fluorescence value F0 and expressed as F/F0 (relative $[Ca^{2+}]_{cyt}$). One field was acquired from each coverslip, and the data pooled from six independent coverslips on three different days. The calcium traces were quantified by determining the area under the curve (AUC) using OriginPro 7.5 software (Origin Lab).

Microarray analysis and RT-qPCR. $5x10^5$ Tregs were incubated at 37°C on a CD3-coated plate (1µg/mL) during 8 hours with or without recombinant human P-selectine (R&D systems) at 200pg/mL. After incubation, cells were washed with PBS and lysed using QIAGEN RNeasy micro kit plus®. RNA was subsequently extracted using the manufacturer's instructions. RNA purity was assessed by Agilent Total RNA nano Series II asay with RIN > 8.0. Transcriptomic assay was performed using a Nugen Chip at the GENOM'IC platform, Institut Cochin, Paris. Analysis was conducted using R software (R project©). Differential expression of transcripts was computed on paired samples (each donors' Tregs), untreated or treated with P-selectin, using limma paired sample analysis. Reverse transcriptase reactions were conducted on 200ng of RNA using Promega© GOSCRIPT RT protocol®. qPCR reaction (Promega© SYBR

GoTaq®) was conducted using a CFX384 C1000Touch® thermal cycler (Bio-Rad©) on 3ng of complementary DNA using specific primers from Sigma-Aldrich© (suppl table XX). Stable housekeeping genes was selected (EEF1A1). Differential expressions were calculated using the threshold cycle (Ct) and the comparative Ct method (ΔΔCt) for relative quantification.

Study of phosphorylated proteins. 10^5 purified Tregs were plated on a non-coated 96-well plate. After 4 hours of culture, 37°C pre-warmed medium containing the stimulus (P-selectin or IL-2) or control medium was added to the selected wells, and the plate was incubated at 37°C for 5 to 30 minutes. After incubation, BD Cytofix® was added for fixation. The BD Perm III buffer® was used for permeabilization following the manufacturer's instructions (BD phosflow®). Staining was conducted on ice for 1 hour (see supplementary table for antibodies and dilution).

Culture of Tregs and FoxP3 phenotyping. 10^5 freshly purified Tregs were plated on a CD3-coated 96-well plate. Tregs were incubated with anti-PSGL1 antibody (1/200) or Syk inhibitor (1 µM) during 30 minutes before adding the P-selectin. After 48 hours of incubation, the cells were fixed and permeabilized using EBioscience© FoxP3 staining kit following the manufacturer's instructions.

Platelet-free plasma isolation. Preanalytics was standardized for all samples to prevent non-specific platelet activation and microparticle production after blood puncture. Venipuncture was performed on resting patients, without tourniquet, using a 21- or 19-gauge needle. Samples were collected in 7mL EDTA-coated tubes (BD Vacutainer®) after a 3mL blood purge. Immediately after collection the tube was inverted once to prevent coagulation. The samples were kept vertical on a tube holder and were transferred to the lab for processing in less than 2 hours after blood puncture. Platelet-free plasma (PFP) was produced by two sequential 15-minute centrifugations at 3500 x g without brake. After each centrifugation, two-thirds of the supernatant was retrieved and placed in a new tube. PFP samples were subsequently aliquoted and stored at -80°C until further analysis.

ELISA and cytokine measurement. Human P-, E- and L-selectins were measured using ELISA (R&D systems, DuoSet ELISA) on PFP samples following the manufacturer's instructions. Free active TGF-beta was measured from Treg culture supernatants using cytometric bead assay (CustomPlex, Biolegends©) according to the manufacturer's protocol.

Microparticle analysis by flow cytometry. Absolute counts of subset-specific, circulating MPs were characterized by flow cytometry using the BD LSRFortessa™ X-20 cell analyzer. PFP aliquots were thawed at room temperature and 10 µl of PFP was used for MP staining. MPs were stained in a total volume of 100µL of 0.22µm filtered PBS-BSA 0.5%, in BD Falcon Polystyrene round Bottom tubes for 2 hours in the dark at room temperature with titrated antibodies specific for different MP subsets (see supplementary table 1 for clones and dilutions). MPs derived from platelets (PMPs) were identified using anti-CD61-viogreen, MPs from endothelial cells (EMPs) by anti-CD31-APC and MPs from granulocytes (GMPs) by anti-CD66b-PE-Cy7. Selectin expression status on PMPs, EMPs and GMPs were identified by using respectively anti-CD62P-PE, anti-CD62E-PEvio770 and anti-CD62L-PE (see supplementary table 1 for dilution). After staining, MPs were diluted by adding 400 µL of staining buffer. 50 µl of labeled and diluted MP

suspensions were transferred into BD Trucount™ Tubes (BD Bioscience®) for absolute quantification (MP counts/mL) of PMPs, EMPs and GMPs. The overall number of MPs was defined as the result of the sum of the absolute count of each MP subset (PMPs and EMPs). Forward scatter (FSC) and side scatter (SSC) were adjusted to logarithmic gain. A standardized method for determining the MP gate was established using fluorescent beads of different sizes (Megamix-Plus SSC: 0.16, 0.20, 0,24 and 0,5 µm (BioCytex, Marseille®)). To reduce background noise, we adopted a fluorescent-triggering strategy as described by Arraud et al *(41)*. Briefly, fluorescence signal from Viogreen, APC or PC-7 were used to trigger detection of MPs labeled with anti-CD61-viogreen (PMPs), anti-CD31-APC (EMPs) or anti-CD66b-PE-Cy7, respectively. As a negative control for labeled-MPs, PFP samples were prepared as above and then treated with 0.5% Triton X-100 solution in order to disrupt vesicles (**figure S5D**). PMPs were considered as $CD61^+$ events in the MP gate, which were removed by Triton treatment. Similar gates were used for EMPs and GMPs using CD31 and CD66b, respectively.
For measurement of mouse PMPs, the PFP was isolated following the same centrifugation protocol as in human. Before staining, 100µL of PFP diluted with 400µL of filtered PBS-BSA 0.5% was concentrated by centrifugation for 45 minutes at 21,000xg. After discarding 450µL of supernatant, 30µL of concentrated PFP was stained with CD61-FITC and CD62P-PE (see supplementary table 1). Staining was conducted in the dark for 2 hours before analysis. We used the same acquisition and gating strategy for mouse MPs as for human MPs. MPs were quantified using BD Trucount™ Tubes following the manufacturer's instructions.

Mice. CD57/BL6 mice WT and *Dnase1l3*-KO were generously provided by V. Sisirak. 30 week-old mice were treated with anti-P-selectin antibody (clone RMP-1, Biolegend©) or its isotype control (MOPC-173, Biolegend©), 0.1mg intraperitoneally, 3 times per week for 10 weeks. The treatment protocol was reviewed and approved by an external animal ethics committee (authorization number APAFiS #19915). At the end of treatment, mice were euthanized and peripheral blood, kidneys and spleen were harvested. PFP was made from a terminal blood sample in an Eppendorf containing 50µL of EDTA 0.5M. Sections from frozen or paraffin-embedded kidneys were analyzed by immunofluorescence or light microscopy, respectively. Kidney sections were stained with hematoxylin-eosin for morphological study by a blinded kidney pathologist (AV). A glomerular injury score was calculated based on glomerular enlargement (0-1), interstitial infiltration (0-1 scale), mesangial proliferation (0-4 scale), glomerular thrombosis (0-2) and fibrosis (0-2 scale). Immunofluorescence staining was conducted on 10µm kidney section using Goat F(ab') anti-mouse IgG-PE (e-Bioscience©, ref 12-4010-82, 1/250) and a rat monoclonal anti-C3 IgG (clone 11H9, Abcam© ref 11862, 1/200) with a secondary Goat anti-rat IgG-AF488 (Invitrogen©, ref A11006, 1/1000). The mean fluorescence intensity of each fluorochrome was evaluated in 40 glomeruli for each mouse using ImageJ software.

Anti-dsDNA ELISA. Anti-dsDNA antibodies were measured using ELISA as previously described *(23)*. Briefly, 96-wells plates were pre-coated with poly-L-lysine (0.05 mg/mL) for 2 hours at room temperature and then coated with 0.1 mg/mL of calf thymus DNA (Sigma-Aldrich) overnight. Coated plates were then incubated with sera samples for 2 hours. After washing, the amount of bound IgG was measured with an alkaline phosphatase-conjugated goat anti-mouse IgG antibody (1/5000, Jackson

Immunoresearch). Anti-dsDNA IgG titers were determined using serial dilution of the serum from a positive animal as a standard, and expressed as units per volume.

Spleen phenotyping. Mouse spleens were mechanically crushed through a filter, and red blood cells were removed using ACK buffer *(23)*. The absolute count of live cells was obtained using BD Accuri® flow cytometer, and 10^7 cells were subjected to FcR-blocking followed by staining for phenotypic analysis (see supplementary table 1).

Mouse T lymphocyte differentiation. Naïve $CD62L^+$ $CD4^+$ T lymphocytes were purified using MACS (Miltenyi Biotec©; 130-104-453) from PBMCs isolated from spleens of CD57BL/6 wild type mice. 10^5 cells were plated in 96-well plate coated with anti-CD3 antibody (1µg/mL) with sCD28 (3 µg/mL) under polarizing conditions. For Tregs: TGF-beta (5ng/mL) and IL2 (20ng/mL); Th1: IL12 (10ng/mL) and IL2 (10ng/mL); Th2: IL4 (20ng/mL); Th17: IL6 (40ng/mL) and TGF-b (0.5ng/mL). After 6 days of culture, viable cells were stained with CD4 and their respective differentiation markers: CD25 and FoxP3 for Tregs, Tbet for Th1, GATA3 for Th2 and RORgt for Th17.

Statistics. Data are shown as individual values with the mean and standard deviation (except when specified otherwise). Quantitative data were compared using non-parametric Mann-Whitney test or non-parametric Kruskal-Wallis test with Dunn's correction for multiple testing of more than 2 groups (except when specified otherwise). Correlations were assessed using the non-parametric Spearman's correlation. Statistical significance was defined as p-value < 0.05. All statistical tests were conducted using GraphPad Prism® V7.
Data and code availability. Microarray results are available in the ArrayExpress repository (https://www.ebi.ac.uk/arrayexpress/).

Acknowledgements:
We would like to thank all sources of funding including the Fondation pour la Recherche Médicale (FRM), the Société Française de Rhumatologie (SFR), the Centre national de reference maladie auto-immune et systémique rares Est/Sud-ouest (RESO), the Bordeaux University Hospital and the Centre National de Recherche Scientifique (CNRS). V.S is supported by the IdEx program of the university of Bordeaux and the CLIP program from the cancer research institute. We thank all the patients involved in this study, as well as all the clinicians involved in their recruitment. We would like to thank Thomas Paz Del Soccoro for his help. Very warm thanks to Katie Sawai for her help concerning English editing.

Funding: All funding was provided by academic source or scientific foundations. [This work was not supported by any pharmaceutical companies.] → The authors declare no competing financial interests.

Ethical statement: All patients included in this study gave oral and written consent to participate. The study's protocol is in accordance with French and European Ethics standards and was approved by an independent ethical committee (number 2018-A00599-46). Healthy donors consented to take part in the study and were recruited through the French National Blood bank (Etablissement Français du Sang). All animal experiments were approved by an external animal ethics committee (authorisation number APAFiS #19915

Author contributions:

MSc performed most of the experiments, conducted the statistics analysis and designed the figures. V.G performed microparticle measurements from patients and HD. ID, IR and EM performed immunosuppressive assay experiments. PV conducted calcium measurement assays. JPG conducted the microarray analysis. NM and MSc conducted the mouse experiments with help from AG and AB. AV is a trained kidney pathologist who evaluated kidney histology. EL, LC, PD, JLP, JFV, TS, MET and CR were involved in the active recruitment of SLE patients. TB helped for the writing of the study protocol and submission to the ethics committee. VS provided mice and assistance with the related experiments and analysis of results. MSa, CCB and PL helped to design the experiments. PB and CR designed the study, oversaw all experiments and analysis of results. All authors critically reviewed and approved the manuscript before submission.

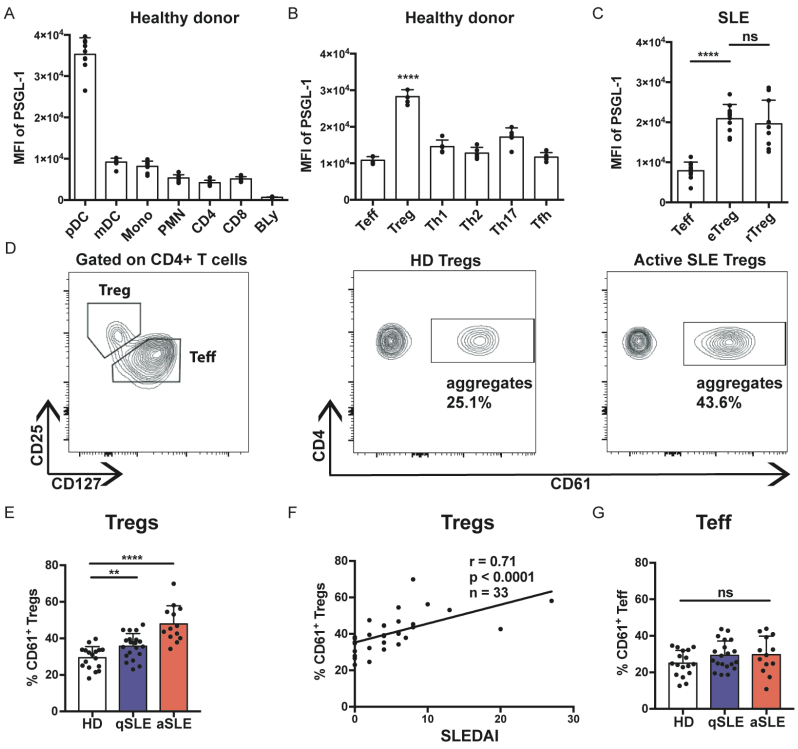

Figure 1: Tregs express high levels of PSGL-1 and interact with platelets in SLE patients

(A) Mean Fluorescence intensity (MFI) of PSGL-1 on circulating immune cells from healthy donors (n = 10).

(B) PSGL-1 MFI on T cell subsets from healthy donors (n = 5). ****, $p < 0.0001$ vs all other subpopulations using one-sided ANOVA with Holm-Sidak's correction for multiple tests.

(C) PSGL-1 MFI of $CD4^+CD25^-FoxP3^-$ T effector cells, resting ($CD45RA^+$) or effector ($CD45RA^-$) $CD4^+CD25^+FoxP3^+$ Tregs in patients with SLE (n=10).

(D) Representative gating of Treg-Teff/platelet aggregates from healthy donors and SLE patients.

(E) Cumulative results of Treg/platelets from HD and patients with quiescent SLE (qSLE, SLE disease activity index (SLEDAI) < 6, n = 20) or active SLE (aSLE, SLEDAI ≥6, n = 13) patients as shown in (D).

(F) Spearman's correlation between the percentage of Treg/platelet aggregates and the SLEDAI.

(G) Cumulative results of Teff/platelets in HD and qSLE and aSLE patients as show in (D). Dots represent the value of individual donors, columns represent the mean and error bars indicate S.D. **, $p < 0.01$; ****, $p < 0.0001$ using non-parametric Kruskall-Wallis test with Dunn's correction.

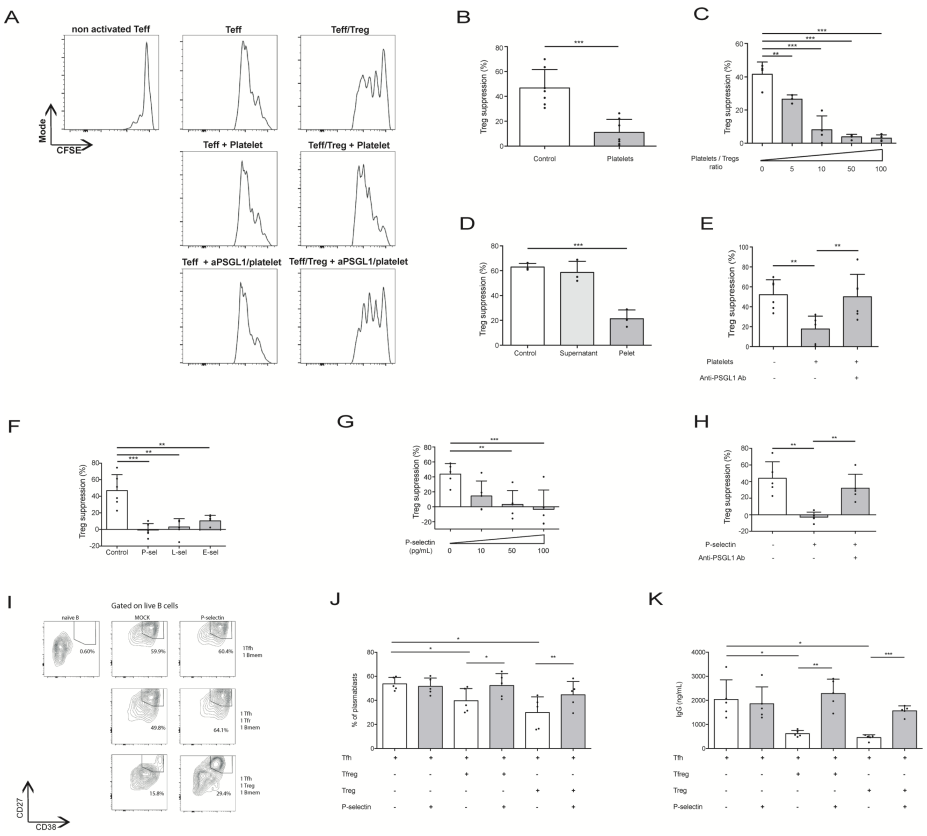

Figure 2: Platelets block Tregs suppressive capacities through the P-selectin/PSGL-1 axis

(A) Representative results of CFSE dilution in a Treg/Teff suppression assay with or without platelet (ratio Treg/platelet 1:100).

(B) Cumulative results of Treg/Teff suppression assay with or without platelets (ratio Treg/platelets 1:100; n = 7 independent experiments).

(C) Dose-response effect of platelets in the Treg/Teff suppression assays (n = 4).

(D) Effect of PSGL-1 blockade (clone KPL-1, dilution 1/200) on platelet-induced Treg dysfunction in suppression assay (n = 6).

(E) Effect of P- E- and L-selectin (200pg/mL) on Treg suppressive capacities in suppression assays (n = 6).

(F) Dose-response effect of P-selectin in the Treg/Teff suppression assay (n = 5).

(G) Effect of PSGL-1 blockade on P-selectin-induced Treg dysfunction in suppression assays (n = 5).

(H) Representative results of a B response suppression assay showing plasmablast ($CD27^{hi}CD38^{hi}$ B cells) differentiation with or without Tregs/Tfr and P-selectin (200pg/mL).

(I) Cumulative results of B response suppressions assays with or without P-selectin 200pg/mL (n = 5).

(J) IgG production measured by ELISA from the supernatant of the B response suppression assays (n = 5).

Dots represent the result of an independent experiment, the histograms represent the mean and the bars the S.D. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ using one-sided ANOVA with Holm-Sidak's correction.

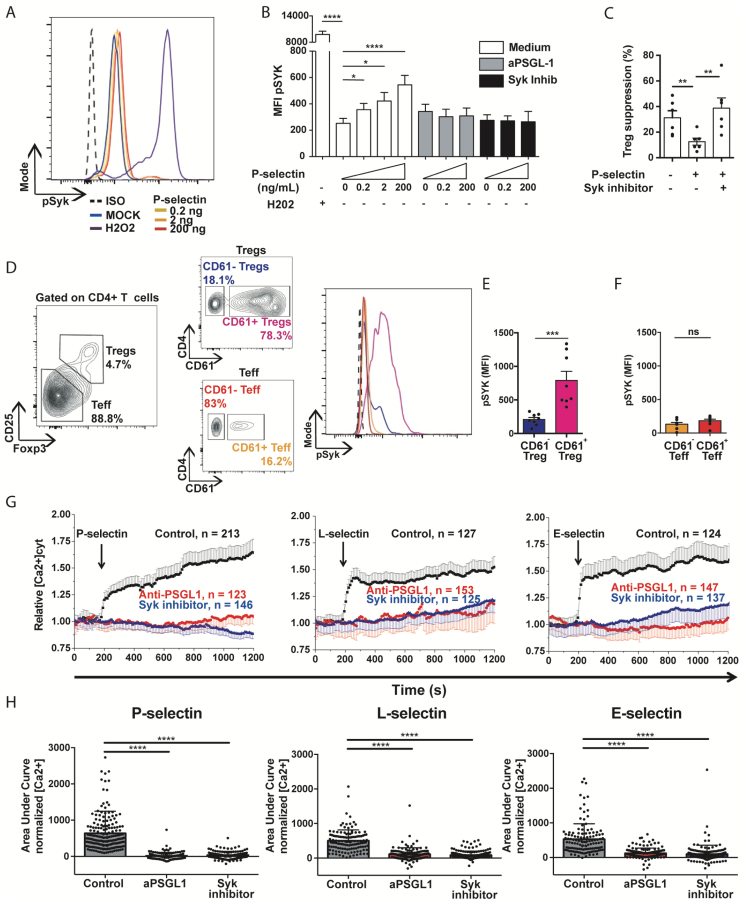

Figure 3: Selectins induce Syk/calcium signaling in Tregs *in vitro* and *ex vivo*

(A) Syk phosphorylation in Tregs exposed to P-selectin for 5 minutes using cytometry (Phosflow® protocole): representative results.

(B) Cumulative data of Syk phosphorylation in Tregs exposed to P-selectin for 5 minutes (n = 13 independent experiments, with or without 30 minutes preincubation with anti-PSGL1 antibody (1/200, n = 10) or a Syk inhibitor (1 µM, n = 4).

(C) Effect of Syk inhibitor (1 µM, 30 minutes) preincubation on Treg immunosuppressive functions by suppression assays (n = 6).

(D) Gating and representative pSYK expression of $CD25^{+}FoxP3^{+}$ Tregs and $CD25^{-}FoxP3^{-}$ Teff aggregating or not with platelets (CD61 expression) in fresh blood samples of active SLE patients (n = 6).

(E) Cumulative results of pSYK mean fluorescence intensity of $CD61^{+}$ and $CD61^{-}$ Tregs (n = 6). ***, $p < 0.001$ using bilateral unpaired Student t-test.

(F) Cumulative results of pSYK mean fluorescence intensity of $CD61^{+}$ and $CD61^{-}$ Teff (n = 6). NS, non-significant using bilateral unpaired Student t-test.

(G) Effects of P-selectin (left panel), L-selectin (middle panel), E-selectin (right panel) 200ng/mL on the intracytosolic calcium concentration ($[Ca^{2+}]_{cyt}$ using cal-520 as fluorescent calcium probe) of HD Tregs in control conditions (black), anti-PSGL1

antibody (1/200)-treated cells (red) and syk inhibitor (1µM)-treated cells (blue). Shown are representative results of 3 independent experiments from different HD, lines represent the mean value, bars the S.D. and n corresponds to the number of studied cells.

(H) Quantification of calcium responses using the determination of the area under curves (AUC) of the recordings shown in G. Each dots show the AUC of an individual cell.

Dots represent the result of an independent experiment; the histograms represent the mean and the bars the S.D. **, $p < 0.01$; ****, $p < 0.0001$ using Kruskall-Wallis test with Dunn's correction.

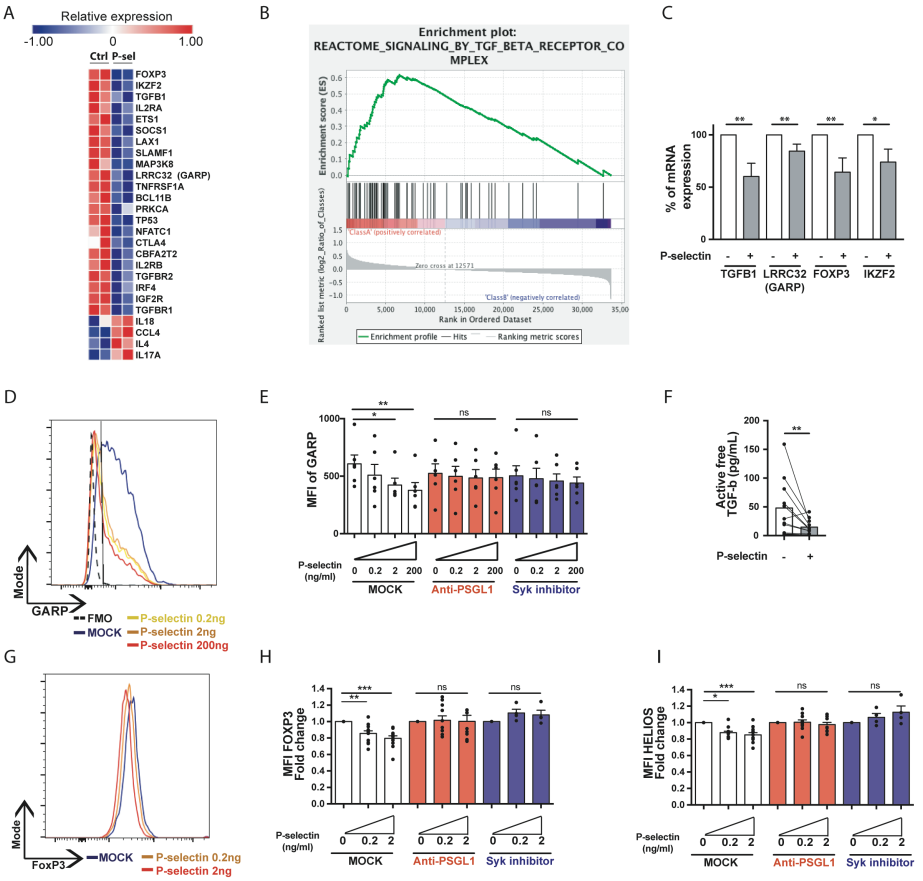

Figure 4: Selectins alter Treg phenotype through TGF-beta pathway modulation

(A) Heat map of selected gene showing a downregulation of Treg specific gene and upregulation of pro-inflammatory genes. Transcriptomics was conducted on a Affymetrix Nugen microarray using Tregs from 2 healthy donors treated for 8 hours with P-selectin 200pg/mL.

(B) GSEA analysis showing enrichment for TGF-beta signaling pathway in control Tregs compared with P-selectin treated Tregs.

(C) RT-qPCR of genes involved in the TGF-beta axis and FoxP3 signature. Tregs from 5 individual donors were cultured with or without P-selectin in the same conditions than transcriptomics. The results are given as a percentage of expression (ΔΔCT using a housekeeping gene) compared to the control. *, $p < 0.05$ and **, $p < 0.01$ using non parametric Mann-Whitney test.

(D) Tregs from HD were cultured during 48 hours with different dose of P-selectin with or without anti-PSGL1 (1/200) or Syk inhibitor (1 µM). Representative staining of GARP on viable Tregs.

(E) Cumulative data showing GARP MFI in Tregs cultured with P-selectin (n = 6 experiments).

(F) Free active TGF-beta was measured in the supernatant of Tregs culture with or without P-selectin (200pg/mL) for 48 hours (n = 11 independent experiments). **, $p < 0.01$ using the paired non-parametric Wilcoxon test.
(G) Tregs from HD were cultured during 48 hours with different dose of P-selectin with or without anti-PSGL1 (1/200) or Syk inhibitor (1 μM). Representative staining of FoxP3 on viable Tregs.
(H) Cumulative data showing FoxP3 MFI fold change in Tregs cultured with P-selectin under control condition (n = 12) of with anti-PSGL-1 antibody (1/200; n = 12) or Syk inhibitor (1μM, n = 4).
(I) Cumulative data showing Helios MFI fold change in Tregs cultured with P-selectin under control condition (n = 12) of with anti-PSGL-1 antibody (1/200; n = 12) or Syk inhibitor (1μM, n = 4).

Each dots represent the mean value of an independent experiment, histograms and bars represent the mean and the s.e.m. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ using non-parametric paired Friedman, test with Dunn's correction for multiple testing.

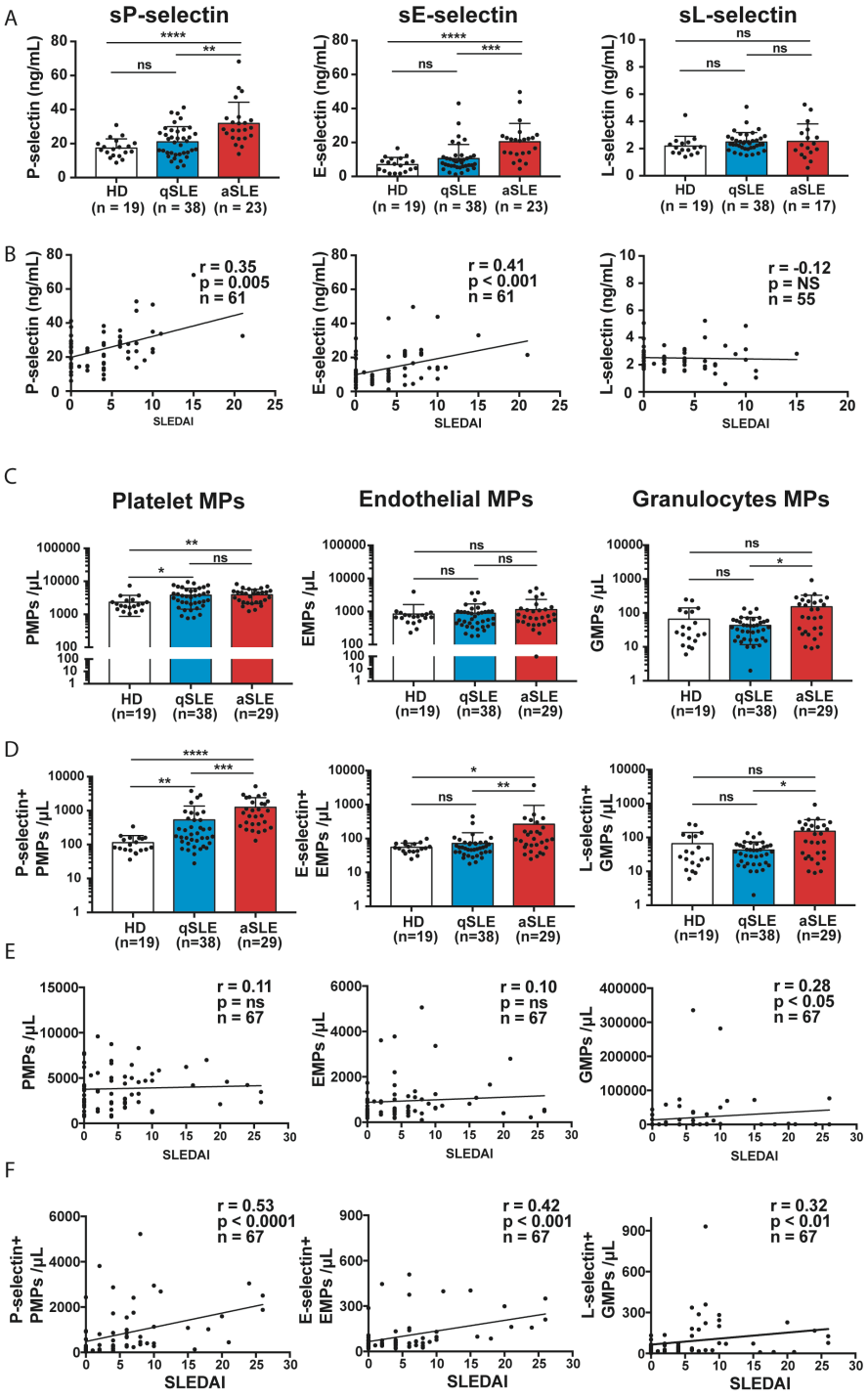
A
sP-selectin
sE-selectin
sL-selectin
P-selectin (ng/mL)
E-selectin (ng/mL)
L-selectin (ng/mL)
HD (n = 19)
qSLE (n = 38)
aSLE (n = 23)
aSLE (n = 17)
B
r = 0.35
p = 0.005
n = 61
r = 0.41
p < 0.001
n = 61
r = -0.12
p = NS
n = 55
SLEDAI
C
Platelet MPs
Endothelial MPs
Granulocytes MPs
PMPs /µL
EMPs /µL
GMPs /µL
HD (n=19)
qSLE (n=38)
aSLE (n=29)
D
P-selectin+ PMPs /µL
E-selectin+ EMPs /µL
L-selectin+ GMPs /µL
E
r = 0.11
p = ns
n = 67
r = 0.10
p = ns
n = 67
r = 0.28
p < 0.05
n = 67
F
r = 0.53
p < 0.0001
n = 67
r = 0.42
p < 0.001
n = 67
r = 0.32
p < 0.01
n = 67

Figure 5: Soluble and microparticle-bound selectin are upregulated in active SLE patients

(A) Soluble selectins were measured from the platelet-free plasma of age- and sex-matched healthy donors, quiescent SLE (qSLE, SLEDAI < 6) and active SLE (aSLE, SLEDAI ≥6). Patients with active glomerulonephritis were excluded from this analysis (n = 6).

(B) Spearman correlation between selectin levels and SLE disease activity as assessed by the SLEDAI.

(C) Platelet-(CD61+, left panel), Endothelial- (CD31+, middle panel) or granulocyte-(CD66b+, right panel) derived Microparticles (PMP, EMP and GMP) were measured using cytometry from platelet-free plasma of HD and patients with quiescent or active SLE. The absolute count of microparticles was measured using Trucount Beads®.

(D) P-selectin+ PMPs, E-selectin+ EMPs and L-selectin+ GMPs were measured by cytometry from HD and SLE patients.

(E) Spearman correlation between PMPs (left panel), EMPs (middle panel) or GMPs (right panel) and SLE disease activity (SLEDAI).

(F) Spearman correlation between selectin^{+} PMPs (left panel), EMPs (middle panel) or GMPs (right panel) and SLE disease activity (SLEDAI).

Each dots represent the value of an individual, histograms and bars represent the mean and the S.D. $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ using non-parametric Kruskal-Wallis test with Dunn's correction for multiple testing.

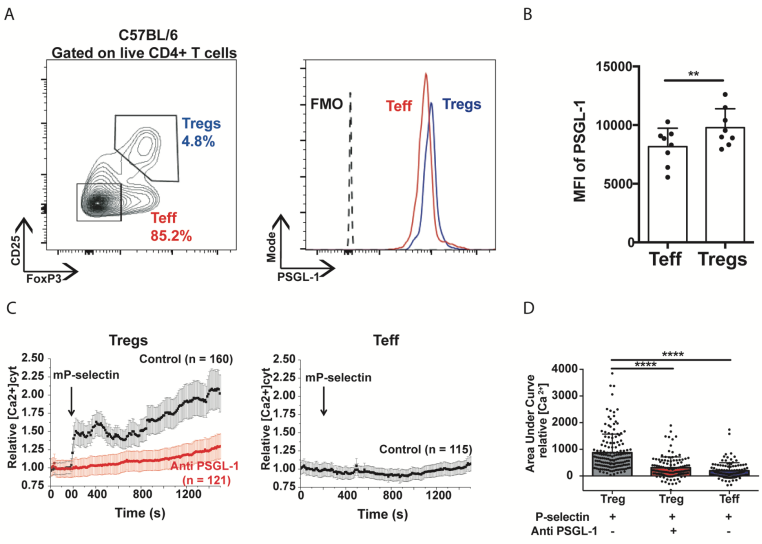
A
C57BL/6
Gated on live CD4+ T cells
Tregs
4.8%
Teff
85.2%
CD25
FoxP3
FMO
Teff
Tregs
Mode
PSGL-1
B
MFI of PSGL-1
15000
10000
5000
0
**
Teff
Tregs
C
Tregs
Control (n = 160)
mP-selectin
Anti PSGL-1
(n = 121)
Relative [Ca2+]cyt
Time (s)
Teff
mP-selectin
Control (n = 115)
Relative [Ca2+]cyt
Time (s)
D
Area Under Curve
relative [Ca2+]
****
****
Treg
Treg
Teff
P-selectin + + +
Anti PSGL-1 - + -
E
Tregs
Control
P-selectin
74.8%
71.8%
FoxP3
CFSE
Th1
Control
P-selectin
66%
69%
Tbet
CFSE
Th2
Control
P-selectin
73%
70%
GATA3
CFSE
Th17
Control
P-selectin
66%
69%
RORγT
CFSE
F
Tregs
**
MFI FoxP3
P-selectin (pg/ml)
0
50
200
2000
Th1
ns
MFI Tbet
P-selectin (pg/ml)
0
50
200
2000
Th2
ns
MFI GATA3
P-selectin (pg/ml)
0
50
200
2000
Th17
ns
MFI RORgt
P-selectin (pg/ml)
0
50
200
2000

Figure 6: WT and dnase1l3-KO with CD57B/6 background recapitulate human P-selectin findings

(A) Gating strategy of FoxP3$^+$ Tregs and Teff extracted from the spleen of C57/B6 WT mice (n = 8).

(B) Cumulative results of PSGL-1 expression on Teff and Tregs (n = 8). **, $p < 0.01$ using matched non-parametric Wilcoxon test.

(C) CD25+CD4+ Tregs (left panel) or CD25-CD4+ Teff (right panel) were incubated with recombinant mouse P-selectin with or without pre-treatment with mouse anti-PSGL1 antibody. Single-cell normalized intracytosolic calcium concentration ($[Ca^{2+}]_{cyt}$ using cal-520 as fluorescent calcium probe). The n shows the number of cells evaluated in each conditions. The data were obtained from three independent experiments.

(D) Area under curve of calcium levels in cells exposed to P-selectin. Each dots represent the AUC of a single cells.

(E) Naïve CD62L$^+$ CD4$^+$ mouse T cells were stained with CFSE and cultured under polarizing conditions for Treg, Th1, Th2 and Th17 differentiation, with or without P-selectin. Representative staining of CFSE and differentiation transcription factor after 7 days of incubation. The percentage indicates the proportion of proliferating cells.

(F) Mean fluorescence intensity of the transcription factor of differentiation for each polarizing conditions.

Each dot represent the value of one mouse or one independent experiment with means and S.D.**, $p < 0.01$; ****, $p < 0.0001$ using non-parametric Kruskal-Wallis test with Dunn's correction for multiple testing.

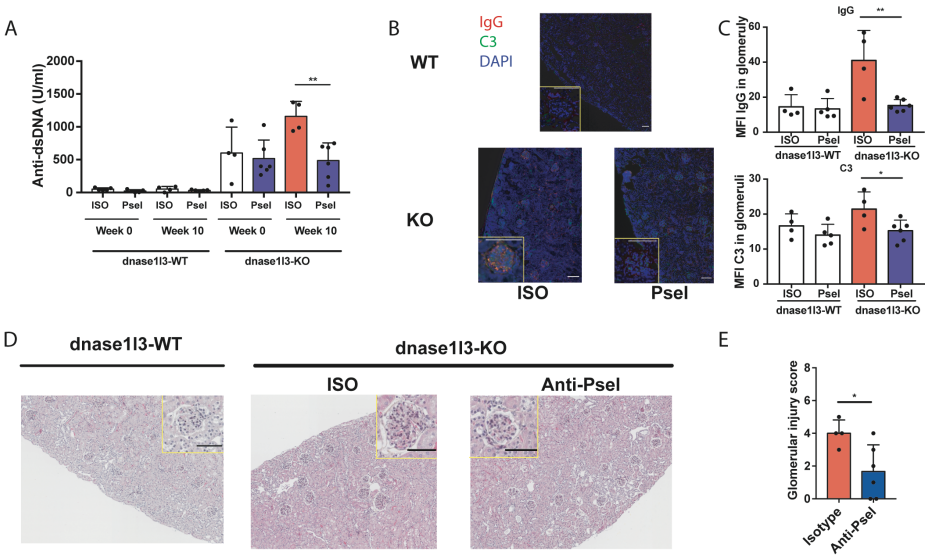

Figure 7: P-selectin blockade improves SLE pathogenesis in *dnase1l3-KO* lupus-prone mice.

(A) Levels of anti-dsDNA antibodies in dnase1l3-KO mice measured by ELISA (WT mice, n = 4 in ISO group and n = 5 in anti-Psel group; for KO mice, n = 4 in the ISO group and n = 6 in anti-Psel group).

(B) Representative Immunofluorescence staining of kidney of dnase1l3-WT (upper panel) and dnase1l3-KO mouse treated with the isotype (lower left panel) or the anti-P-selectin (lower right panel) antibody for 10 weeks. White bars indicate 100 µm.

(C) Cumulative results of the MFI of IgG (upper panel) and C3 (lower panel) in the glomeruli of dnase1l3-WT and dnase1l3-KO mice treated with the isotype or the anti-P-selectin antibody.

(D) Representative HES staining of kidney of dnase1l3-WT and dnase1l3-KO mice treated with the isotype of the anti-P-selectin antibody for 10 weeks. Black bars indicate 50 µm.

(E) Glomerular injury score evaluated in a blinded manner by a kidney pathologist, as described in the *methods*.

Each dot represents the value of one mouse with means and S.D. *, $p < 0.05$; **, $p < 0.01$ using unpaired Student's t-test.

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Supplementary figures and legends :

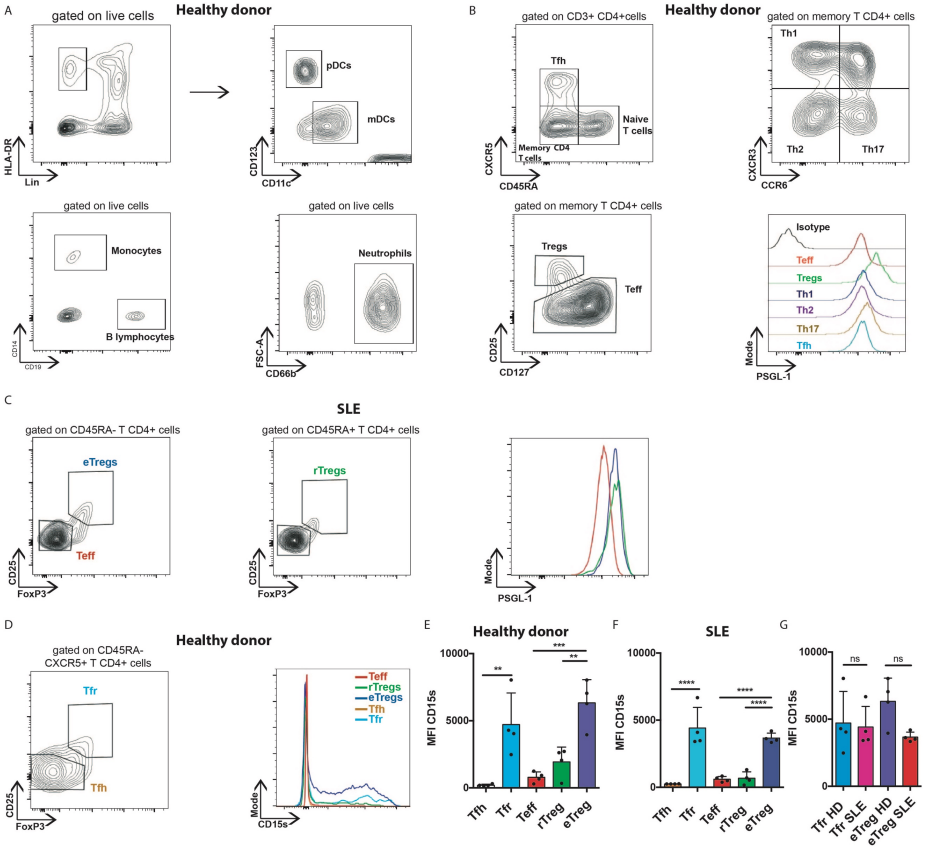

Figure S1:

(A) Representative gating strategy of the population used in PSGL-1 expression study in healthy donors (HD).

(B) Representative gating strategy of T cell subsets in HD, and histogram overlay shows the corresponding levels of PSGL-1 expression .

(C) Representative gating strategy of FoxP3^{+} Tregs and used in PSGL-1 and C15s expression study in SLE patients.

(D) Representative gating of Tregs and representative CD15s expression in Tregs, Tfr and Teff of HD.

(E) Cumulative results of CD15s expression in Tregs, Tfr, Teff and Tfh of HD (n = 4).

(F) Cumulative results of CD15s expression in Tregs, Tfr, Teff and Tfh of SLE patients (n = 4).

(G) Comparison of CD15s expression in Tregs and Tfr between HD and SLE patients.

Dots represent the value of each individual donor, the histograms represent the mean and the bars the S.D. **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ using one-sided ANOVA with Holm-Sidak's correction for multiple tests.

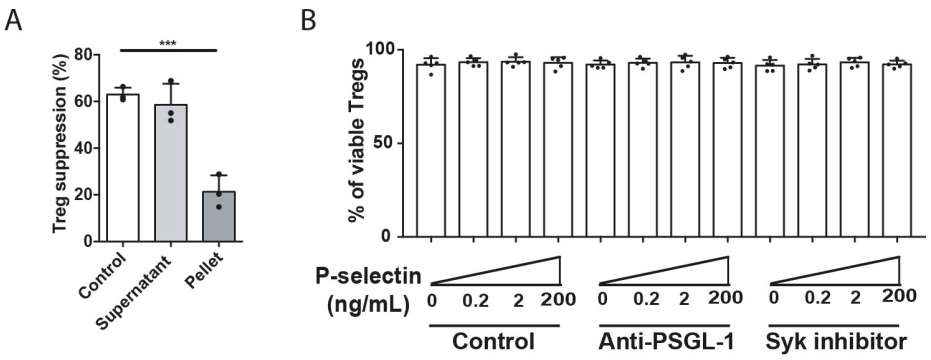

Figure S2 :

(A) Platelets were activated with thrombin (1UI during 30 minutes) and ultracentrifugated at 19.000xg for 1 hour. Effect of the platelet supernatant or pellet on Treg/Teff suppression assays (n = 3 experiments).

(B) Tregs viability after 48 hours of culture with P-selectin with or without anti-PSGL1 or a Syk inhibitor (n = 4 experiments).

Dots represent the result of an independent experiment; the histograms represent the mean and the bars the S.D. ***, $p < 0.001$ using unpaired Student's test.

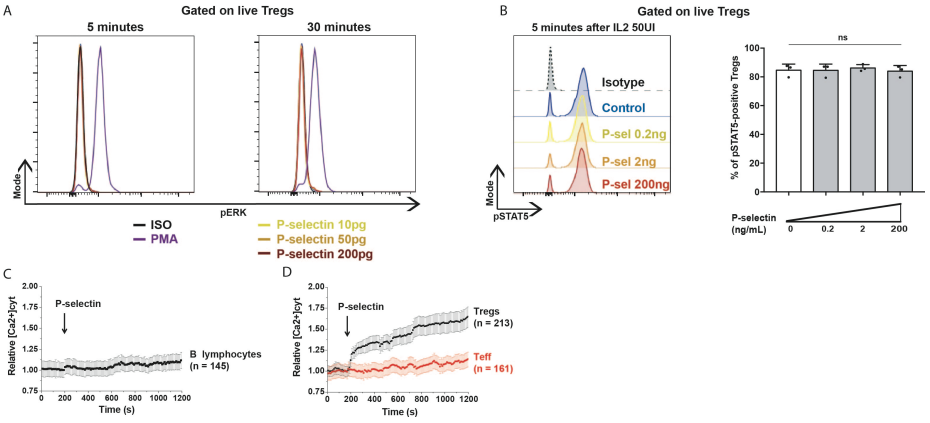

Figure S3 :

(A) ERK phosphorylation in Tregs exposed to P-selectin for 15 and 30 minutes using Phosflow® : representative experiment.

(B) STAT5 phosphorylation in Tregs exposed to IL2 50UI for 15 minutes after a two day incubation with P-selectin using Phosflow® : representative experiment.

(C) Representative results of intracellular calcium assay of B lymphocytes exposed to P-selectin (200ng/mL), n corresponds to the number of studied cells from at least 3 independent experiments.

(D) Representative results of intracellular calcium assay of Treg and Teff exposed to P-selectin (200 ng/mL), n corresponds to the number of studied cells from at least 3 independent experiments.

Dots represent the result of an independent experiment, the histograms represent the mean and the bars the S.D. Ns, non-significant using Kruskal-Wallis test with Dunn's correction.

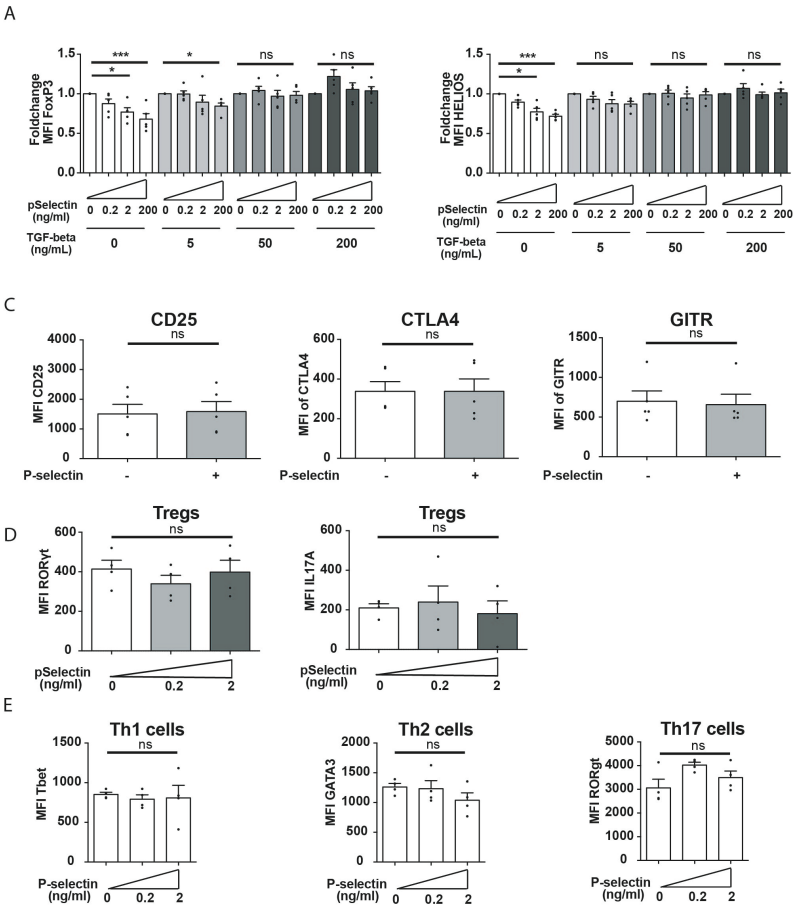

Figure S4:

(A) Tregs from HD were cultured during 48 hours with different doses of P-selectin and different dose of TGF-beta. Cumulative data of FoxP3 expression among viable Tregs (n = 4).

(B) Tregs from HD were cultured during 48 hours with different doses of P-selectin and different dose of TGF-beta. Cumulative data of Helios expression among live Tregs (n = 4).

(C) Cumulative data of CD25, CTLA4 and GITR expression among viable Tregs after 48h of incubation with P-selectin (200pg/mL), (n = 5). ns, non-significant using non parametric Mann-Whitney test.

(D) Tregs from HD were cultured during 48 hours with different doses of P-selectin. Cumulative data of RORγT and IL17 expression (after PMA/ionomycin stimulation) among live Tregs (n = 4).

(E) Th1, Th2 and Th17 were sorted from HD and cultured for 48 hours with different doses of P-selectin. Cumulative data of Tbet, GATA3 and RORγT expression (n = 4).

Each dots represent the mean value of an independent experiment, histograms and bars represent the mean and the s.e.m. ns, non-significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ using non-parametric Kruskal-Wallis test with Dunn's correction for multiple testing.

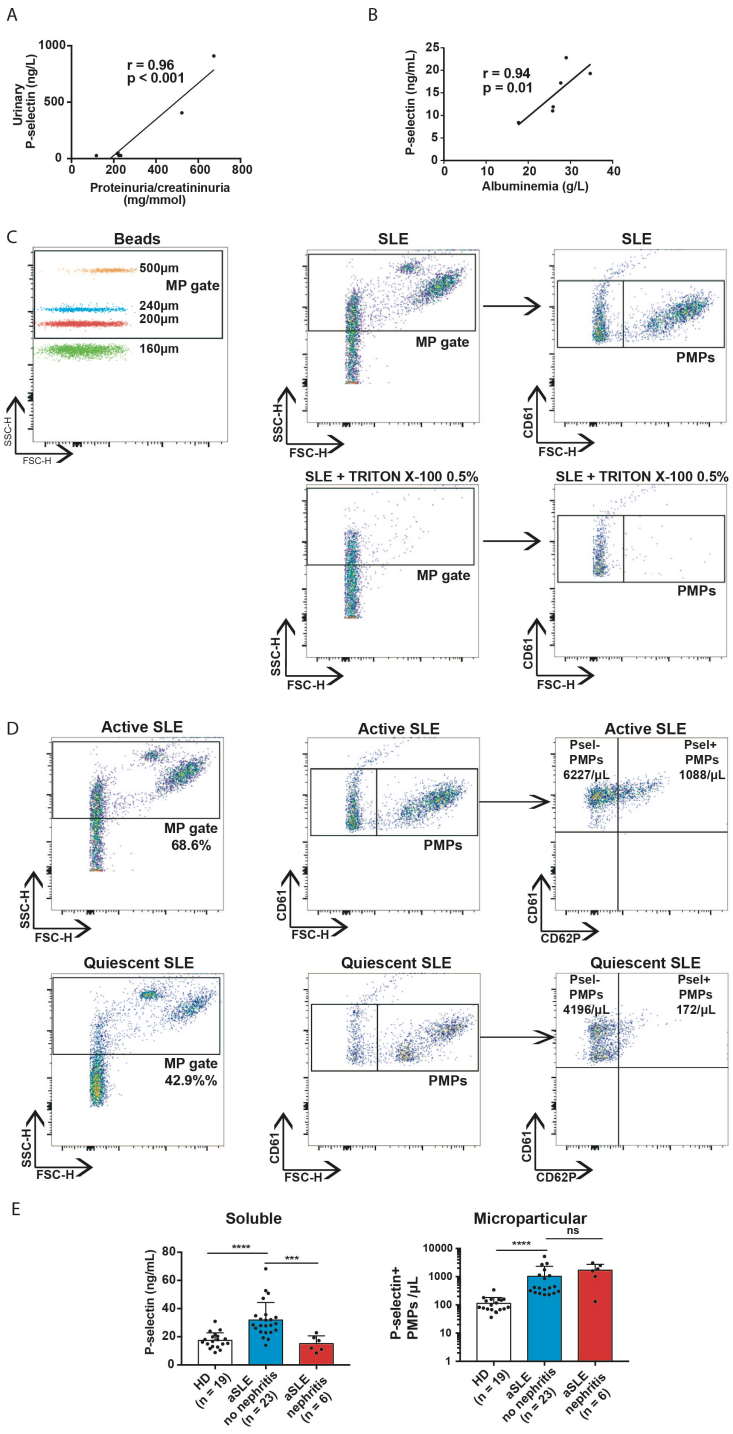
A
Urinary P-selectin (ng/L)
r = 0.96
p < 0.001
Proteinuria/creatininuria (mg/mmol)
B
P-selectin (ng/mL)
r = 0.94
p = 0.01
Albuminemia (g/L)
C
Beads
500µm
MP gate
240µm
200µm
160µm
SSC-H
FSC-H
SLE
MP gate
PMPs
CD61
SLE + TRITON X-100 0.5%
D
Active SLE
MP gate
68.6%
PMPs
Psel-
PMPs
6227/µL
Psel+
PMPs
1088/µL
CD62P
Quiescent SLE
MP gate
42.9%%
4196/µL
172/µL
E
Soluble
Microparticular
ns
P-selectin (ng/mL)
P-selectin+ PMPs /µL
HD (n = 19)
aSLE no nephritis (n = 23)
aSLE nephritis (n = 6)

Figure S5 :

(A) Spearman correlation between urinary P-selectin and proteinuria (assessed by the albumin/creatinine ratio) in 6 patients with active lupus nephritis.

(B) Spearman correlation between serum P-selectin and albuminemia in 6 patients with active lupus nephritis.

(C) Representative gating of microparticles (MP) analysis. The MP gate was established using Megamix-Plus SSC® (upper left panel). $CD61^+$ particles were identified in the MP gate using a fluorescence threshold of 2000 for the associated fluorochrome (upper middle panel). Platelet-derived MPs (PMPs) were identified as the population which is disposed by 0.5% of TRITON-X100 treatment (upper right panel and lower panels).

(D) Representative results of PMPs and P-$selectin^+$ PMPs in a quiescent and an active SLE patient. The absolute number of PMPs was measured using Trucount Beads®.

(E) Comparisons of soluble (left panel) and microparticular (right panel) P-selectin in patients with or without active renal disease.

Each dots represent the value of an individual, histograms and bars represent the mean and the S.D. $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ using non-parametric Kruskal-Wallis test with Dunn's correction for multiple testing.

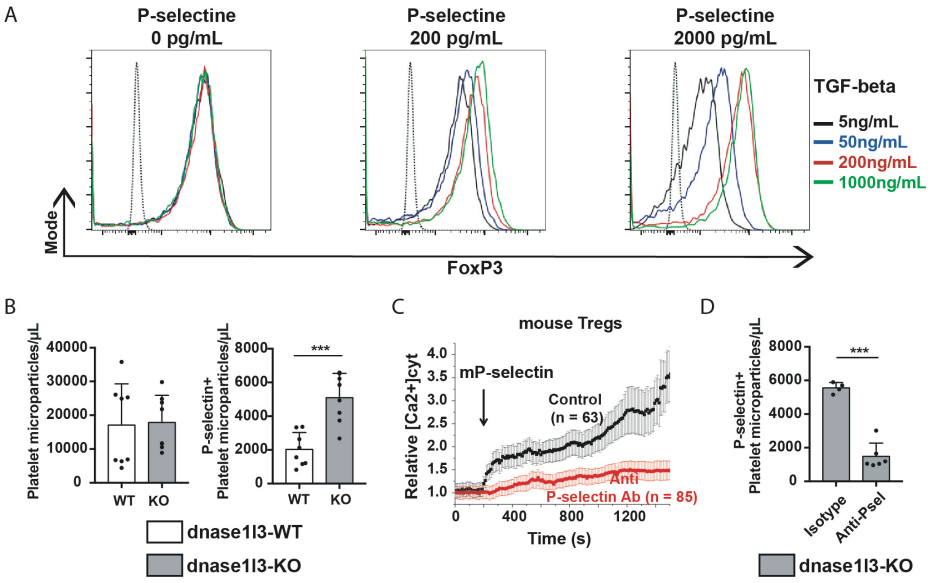

Figure S6 :

(A) Naïve CD62L^{+} CD4^{+} mouse T cells were cultured under polarizing conditions for Treg differentiation using different concentration of TGF-beta, with or without P-selectin. Representative FoxP3 staining among live CD4^{+} T cells after 7 days of culture.

(B) PMPs (left panel) and P-selectin+ PMPs (right panel) were measured using cytometry from dnase1l3-WT and dnase1l3-KO mice from the C57/B6 background (n = 8 for each group).

(C) Area under curve of calcium levels in Tregs exposed to P-selectin with or without pre-incubation with anti-P-selectin antibody (clone RMP-1). Each dots represent the AUC of a single cells.

(D) PMPs and P-selectin+ PMPs were measured using cytometry from dnase1l3-KO mice from the C57/B6 background treated with anti-P-selectin antibody (n = 6) or isotype control (n = 4).

Each dot represent the value of one mouse with means and S.D ***, $p < 0.001$ using unpaired Student's t-test.

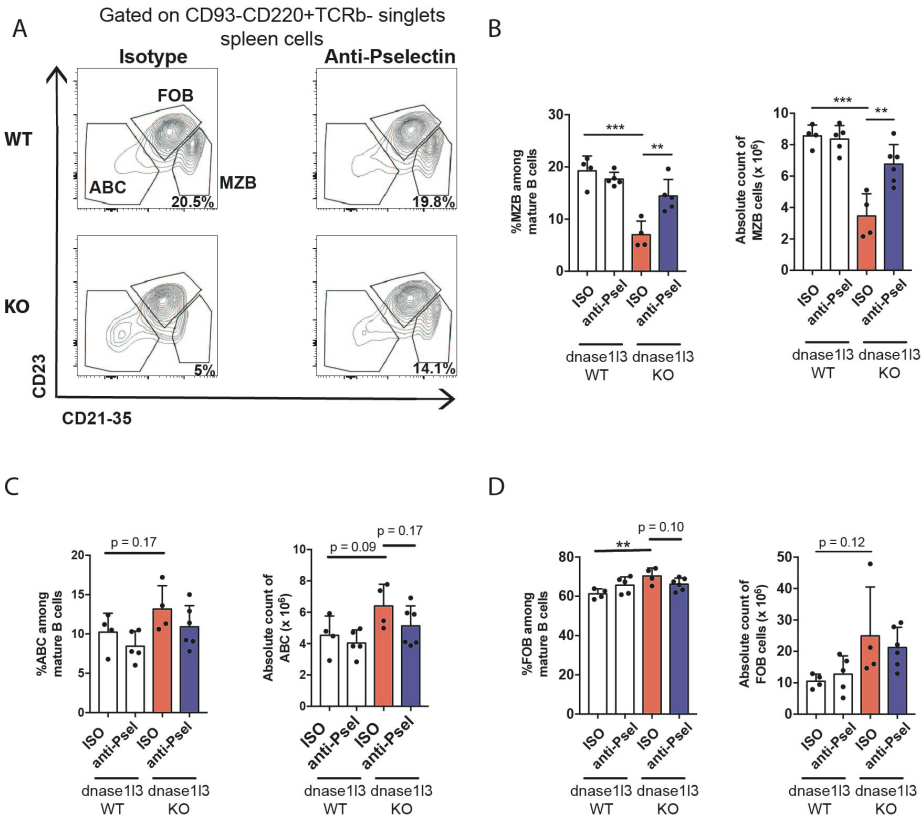

Figure S7 :

(A) Representative gating of marginal zone B (MZB) cells from the spleen of dnase1l3-WT and dnase1l3-KO mice.

(B) Percentage (left panel) and absolute number (right panel) of marginal zone B cells among total mature B cells in the spleen of DNAse1L3 WT and KO mice, treated with the isotype or the anti-P-selectin antibody.

(C) Percentage (left panel) and absolute number (right panel) of age-related B cells (ABC) among total mature B cells in the spleen of DNAse1L3 WT and KO mice, treated with the isotype or the anti-P-selectin antibody.

(D) Percentage (left panel) and absolute number (right panel) of follicular B cells (FOB) among total mature B cells in the spleen of DNAse1L3 WT and KO mice, treated with the isotype or the anti-P-selectin antibody.

Each dot represents the value of one mouse with means and S.D. **, $p < 0.01$; ***, $p < 0.001$ using unpaired Student's t-test. Abbreviations : ABC, Age-related B cells; FOB, Follicular B cells.

JCI insight

RESEARCH ARTICLE

OX40L/OX40 axis impairs follicular and natural Treg function in human SLE

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Tregs are impaired in human systemic lupus erythematosus (SLE) and contribute to effector T cell activation. However, the mechanisms responsible for the Treg deficiency in SLE remain unclear. We hypothesized that the OX40L/OX40 axis is implicated in Treg and regulatory follicular helper T (Tfr) cell dysfunction in human SLE. OX40L/OX40 axis engagement on Tregs and Tfr cells not only specifically impaired their ability to regulate effector T cell proliferation, but also their ability to suppress T follicular helper (Tfh) cell–dependent B cell activation and immunoglobulin secretion. Antigen-presenting cells from patients with active SLE mediated Treg dysfunction in an OX40L-dependent manner, and OX40L-expressing cells colocalized with Foxp3$^+$ cells in active SLE skin lesions. Engagement of the OX40L/OX40 axis resulted in Foxp3 downregulation in Tregs, and expression in SLE Tregs correlated with the proportion of circulating OX40L-expressing myeloid DCs. These data support that OX40L/OX40 signals are implicated in Treg dysfunction in human SLE. Thus, blocking the OX40L/OX40 axis appears to be a promising therapeutic strategy.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by a loss of tolerance toward nuclear components, leading to autoantibody production, immune complex formation, and organ/tissue damages (1). There is a strong need for a better understanding of human SLE pathogenesis, as only a few drugs are effective, and only one has been approved for lupus treatment over the past 50 years (2). The precise immunological events that trigger the onset of SLE remain unknown, but the established chronic innate activation of the DC system plays a critical role in the activation of autoreactive T and B lymphocytes while overwhelming natural regulatory mechanisms (3, 4).

Foxp3$^+$ Tregs are important for the maintenance of immunological tolerance, and their absence results in fatal autoimmune diseases in humans and mice (5). In SLE, defects in the Treg compartment have been recorded in both mouse models and in patients. Treg depletion 3 days after birth in the NZB×NZW F1 SLE mouse model is associated with the development of nephritis and an increased production of anti-DNA antibodies, whereas adoptive transfer of purified and ex vivo–expanded Tregs slows the progression of renal disease when administered after the onset of proteinuria (6). In SLE patients, studies assessing Tregs have generated contradictory results regarding quantitative and qualitative aspects of these cells in this disease (7). Certain studies have reported reduced numbers or impaired function of circulating Tregs (8, 9), while other groups have not observed any impairment (10). Moreover, some groups have even found increased levels of Tregs in comparison to levels found in healthy subjects (11, 12). Finally, the failure of Tregs to control exaggerated T cell and B cell activation in SLE may depend on cell-extrinsic factors, including the interplay of Tregs with other immune cells in an inflammatory milieu. Moreover, two groups described follicular regulatory T (Tfr) cells that express (Foxp3) and B cell lymphoma 6 (Bcl-6) and that have high expression of CXCR5, ICOS, and programmed cell death 1 (PD1). This population migrates to the B cell follicles and inhibits antibody production (13, 14). As SLE patients with active disease exhibit abnormalities in the peripheral B cell compartment, including intensive germinal center (GC) activity (15),

Authorship note: CJ and JFA are co–first authors.

Conflict of interest: The authors have declared that no conflict of interest exists.

Submitted: May 11, 2018
Accepted: November 14, 2018
Published: December 20, 2018

Reference information:
JCI Insight. 2018;3(24):e122167. https://doi.org/10.1172/jci.insight.122167.

studying the consequences of this population of cells and its mixed regulatory and follicular features is of particular interest in SLE.

Our group recently reported that OX40L was expressed by myeloid antigen-presenting cells (APCs), both in blood and in inflamed tissues of adult and pediatric SLE patients (16). The frequency of circulating OX40L-expressing myeloid APCs correlated with disease activity as well as with the frequency of activated blood follicular helper T (Tfh) cells. OX40L stimulation induced human $CD4^+$ T cells to express Tfh cell–associated molecules and was sufficient to induce memory and naive $CD4^+$ T cells to become functional B cell helpers. Interestingly, besides this newly described role in promoting Tfh cell differentiation (17), OX40L has been shown to block Treg functions both in mice and in humans (18). Its role in the recently described Tfr cell function remains unknown (13). Thus, in the present study, we investigated the role of the OX40L/OX40 axis on the functions of both Tregs and Tfr cells in human SLE.

Results

Soluble and membrane-bound OX40L impair the suppressive functions of Tregs. We recently observed that soluble OX40L (sOX40L) strongly downregulated Treg-related genes, including Foxp3 and the IKZF2-encoding Helios gene, in naive and memory T cells stimulated with anti-CD3 and anti-CD28 after 48 hours of culture (16). This observation suggests that the OX40L/OX40 axis not only promotes Tfh cell differentiation of Th cells, but can also affect the generation and/or function of the Treg compartment. In order to analyze the effect of sOX40L on the Treg function, purified Tregs ($CD4^+CD25^{hi}CD127^-$) were stimulated with recombinant sOX40L and cultured with CFSE-labeled healthy donor (HD) effector T4 (Eff.T4) cells ($CD4^+CD25^-CD127^+$) in the presence of anti-CD3 and anti-CD28. We observed that sOX40L decreased Treg-mediated suppression of Eff.T4 cell proliferation (Figure 1, A and B, $P = 0.028$), without inducing Treg death. To evaluate whether membrane-bound OX40L was also capable of altering Treg function, we took advantage of the ability of anti-RNP^+ SLE sera to upregulate OX40L expression on HD monocytes (SLE DCs) (Supplemental Figure 1, C and D) (3, 16). Indeed, within circulating APCs, SLE $CD11c^+DR^+$ DCs and monocytes (but not B cells) showed increased OX40L expression compared with that in HD DCs and monocytes (Supplemental Figure 1, E and F). Eff.T4 cells and Tregs were purified from blood of HDs and cultured along with DCs differentiated with GM-CSF and IL-4 ($GM\text{-}CSF^+$IL-4 DCs) or SLE DCs. As compared with $GM\text{-}CSF^+$IL-4 DCs, coculture with SLE DCs was associated with a substantial decrease of the ability Tregs to suppress Eff.T4 cell proliferation in a dose-dependent manner (Figure 1C). As a control, the SLE DC–dependent decrease of Treg function was maintained independently of the Eff. T4/Treg ratio (Figure 1D). This process was OX40L dependent, as Treg-suppressive function was restored when SLE DCs were preincubated with a blocking anti-OX40L mAb (Figure 1, E and F). Furthermore, OX40 costimulation did not alter the proliferation capacities of Eff.T4 (Supplemental Figure 2), supporting the hypothesis that OX40L acts on Treg functions. Altogether, these results demonstrate that both sOX40L and membrane-bound OX40L block the suppressive function of purified allogeneic $FoxP3^+$ Tregs in vitro.

OX40L-expresssing APCs from patients with active SLE mediate Treg dysfunction. In order to confirm that an OX40L-dependent Treg dysfunction could operate in SLE patients, we monitored OX40L and OX40 expression in SLE patients. We observed that circulating monocytes from patients with active SLE expressed OX40L (Supplemental Figure 1, E and F) (16) and that SLE patients ($n = 25$) had a higher serum concentration of sOX40L than that in HDs ($n = 15$) (Supplemental Figure 3A). A positive correlation between sOX40L blood concentration and SLE Disease Activity Index (SLEDAI) was observed in SLE patients (Supplemental Figure 3B). Circulating Tregs from SLE patients had a higher expression of OX40 than those from HDs (Supplemental Figure 3, C and D, $P = 0.0055$). To analyze the functional consequences of upregulated OX40L expression by monocytes on Tregs, we purified $CD14^+CD11c^+$HLA-DR^+ APCs from the blood of HDs and SLE patients and cultured them with purified allogeneic HD Eff.T4 cells in the presence or absence of Tregs (Supplemental Table 3). Figure 2A depicts a representative experiment showing the impairment of the Treg-suppressive function in the presence of APCs purified from SLE patient with an active disease. Blocking OX40L signaling with a blocking anti-OX40L mAb restored the Treg-suppressive function (Figure 2A). This observation was further confirmed with APCs purified from 5 patients with active SLE, as compared with APCs purified from 7 HDs (mean percentage of inhibition, 25.5% ± 22% vs. 72.9% ± 9.6%, respectively, $P = 0.01$) (Figure 2B). APCs from 5 SLE patients with inactive disease only had a minor effect on Treg-suppressive function, and anti-OX40L mAb had no significant effect (Figure 2B). Conversely, we observed an inverse correlation between OX40L expression on SLE APCs and their ability

to hamper Treg-suppressive function (Figure 2C, r = –0.85, $P = 0.0001$). In order to see whether the interaction between OX40L$^+$ cells and Tregs was plausible in vivo, we stained skin biopsies from SLE patients with OX40L and Foxp3. We observed a close proximity between infiltrating Foxp3$^+$ cells and OX40L$^+$ cells in affected skin biopsies from SLE patients, suggesting that OX40L$^+$ cell–Treg contact actually operates in vivo within inflammatory tissues (Figure 2D). Altogether, these data suggest that APCs from patients with active SLE can suppress the function of Foxp3$^+$ Tregs in an OX40L-dependent manner.

OX40L-expressing APCs do not confer Treg resistance to effector T cells. The OX40L-dependent decrease of Eff.T4 cell proliferation in the coculture experiments may be the consequence of either an impaired suppressive function of Tregs or an Eff.T4 cell resistance to Treg-mediated suppression (19). In order to evaluate whether SLE DCs could promote Eff.T4 cells to become resistant to Treg suppression, we set up a 3-day coculture of GM-CSF$^+$IL-4 DCs or SLE DCs with purified allogeneic Eff.T4 cells. Eff.T4 cells were then purified and further activated and cultured with HD-purified Tregs and GM-CSF$^+$IL-4 DCs (schematic of the experiment shown in Supplemental Figure 4). As shown in Figure 3A, suppression of Eff.T4 cell proliferation was equivalent, regardless of the origin of Eff.T4 cells (GM-CSF$^+$IL-4- or SLE DC culture), demonstrating that OX40L signals provided by SLE DCs to Eff.T4 cells did not render these cells (the Eff.T4 cell) resistant to Tree-mediated suppression. Moreover, CD4$^+$ T cell expression of TNF receptor II (TNFR2), a marker that has been previously shown to be associated with Eff.T4 cell resistance to Treg suppression (20), as well as the concentration of TNF-α in coculture supernatant (data not shown) were equivalent in both GM-CSF$^+$IL-4 DC and SLE DC culture conditions (Figure 3, B and C). Therefore, these data support that impaired Treg suppression is the consequence of direct Tregs OX40L-mediated signaling.

OX40L-expressing SLE DCs modulate Treg function through downregulation of Foxp3 expression. In order to assess the underlying mechanisms involved in OX40L-mediated Treg dysfunction, we next analyzed the expression of Treg-associated surface markers and intranuclear transcription factors, including CD25, cytotoxic T lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor (GITR), and Foxp3, in Tregs cocultured with GM-CSF$^+$IL-4 DCs or SLE DCs and Eff.T4 cells. As shown in Figure 4A, the expression of CD25, CTLA-4, and GITR was similar in Tregs cultured under different conditions, whereas intranuclear Foxp3 expression was significantly lower in SLE DC compared with GM-CSF$^+$IL-4 DC conditions ($P = 0.02$) (Figure 4B). Moreover, Foxp3 downregulation was inhibited by the addition of a blocking anti-OX40L mAb ($P = 0.03$) (Figure 4B). OX40L-mediated Foxp3 downregulation was further confirmed in Tregs cultured in presence of sOX40L, murine fibroblasts transfected with human OX40L (Figure 4, C and D, respectively), and in the different subsets of Tregs (resting and active Tregs) (Supplemental Figure 3E). Foxp3 downregulation was associated in vitro with an OX40L-dependent Helios downregulation as well (Supplemental Figure 5). We measured cytokine levels in the supernatant of cocultures (IL-6, IL-17A, IL-17F, IFN-γ, IL-10, TGF-β, and IL-2) but did not observe any significant difference among the different conditions (data not shown). In vivo, we did not observe any difference in either CD4$^+$CD25^{hi}Foxp3$^+$ Treg proportion among CD4$^+$ T cells or in Treg absolute counts between HDs and SLE patients ($n = 10$ and $n = 49$, respectively), regardless of disease activity (Supplemental Figure 3, F and G), thus ruling out an OX40L-dependent defect in Treg generation in human SLE. Foxp3 expression levels in blood Tregs were lower in SLE patients as compared with those in HDs (Figure 4E) and were negatively correlated with the proportion of OX40L-expressing CD11c$^+$DR$^+$ DCs, thus strongly suggesting that OX40L-dependent Foxp3 downregulation also operates in vivo (Figure 4F). To gain further insight into the mechanisms leading to Foxp3 downregulation, we next measured IL-2 and IL-6 concentrations in the supernatants of GM-CSF$^+$IL-4/SLE DCs and Treg cocultures, as these cytokines have been shown to modulate Foxp3 expression (21–23). No difference in IL-2 production was detectable between culture conditions, whereas IL-6 concentration was higher in the presence of SLE DCs as compared with GM-CSF$^+$IL-4 DCs (data not shown). Taken together, these results show the critical role of aberrant OX40L-dependent signaling in Foxp3 downregulation associated with Treg dysfunction in patients with active SLE.

The OX40L/OX40 axis modulates follicular Treg functions. Tfr cells are a follicular subset critical for the regulation of the GC response through suppression of Tfh cell and B cell responses (24). Given that OX40L signaling affects Treg function, we hypothesized that OX40L signals may also effect the suppressive function of Tfr cells. In a first step, we purified human tonsil cells from HDs and studied extensive phenotyping of Tregs (Supplemental Figure 6A). Based on the expression of the chemokine receptor CXCR5, we were able to identify two populations of tonsil Foxp3$^+$ Tregs. The first subset, CD4$^+$CXCR5$^+$FoxP3$^+$ cells, also expressed CD25, GITR, PD-1, ICOS, and Bcl-6, which was consistent with the phenotype of Tfr cells

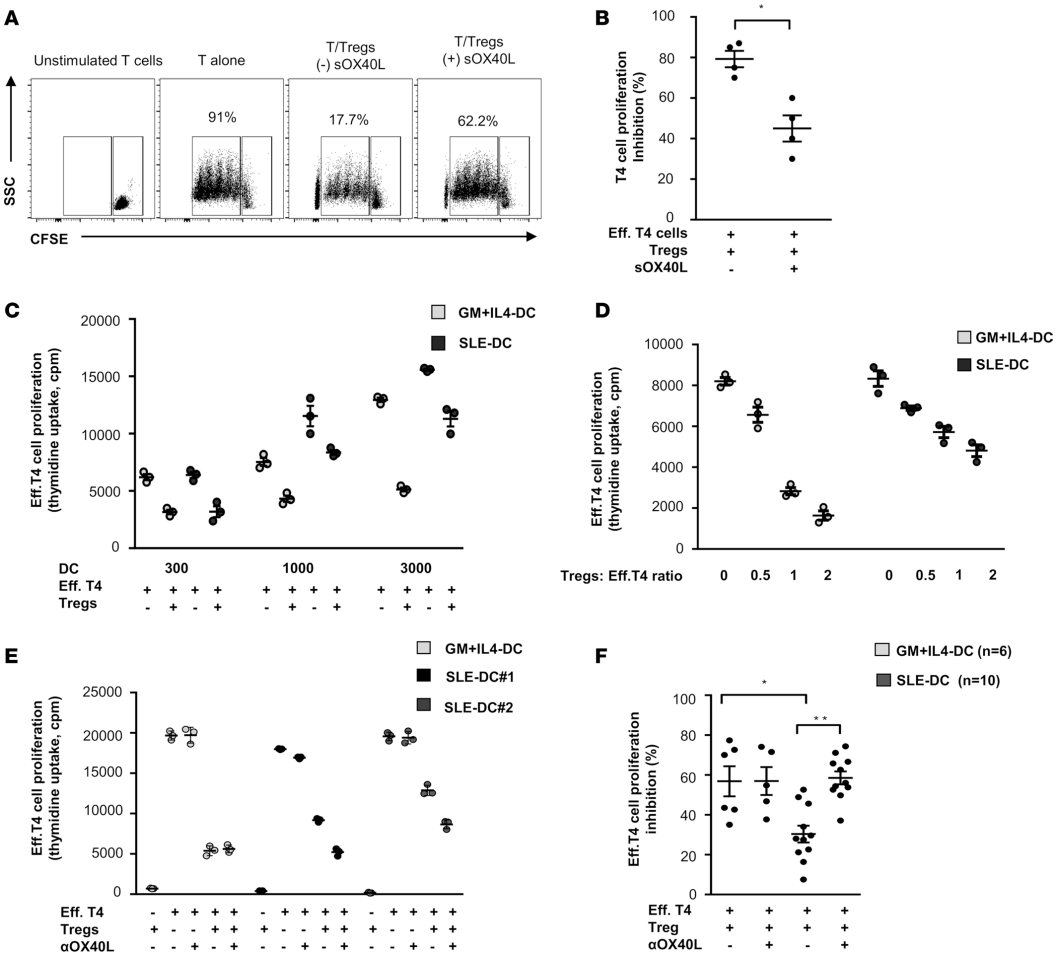

Figure 1. OX40L impairs the suppressive function of Tregs. (**A** and **B**) Sorted effector T4 (Eff.T4) cells (10^4 cells) were labeled with CFSE (5 μM), activated (anti-CD3, 1 μg/ml and anti-CD28, 3 μg/ml) or not for unstimulated condition, and cultured for 3 days alone or with sorted Tregs (10^4 cells) in the presence or absence of soluble OX40L (sOX40L) (100 ng/ml). Eff.T4 cell proliferation was assessed after 3 days of culture. (**A**) Representative dot plot showing proliferation ($CFSE^{dim}$) of Eff.T4 cells after 3 days of culture. (**B**) Percentage of inhibition of Eff.T4 cell proliferation. The percentage of inhibition was calculated in reference to proliferation observed with stimulated Eff.T4 cells cultured alone. Error bars indicate the mean ± SEM, $n = 4$ independent experiments. Statistical analysis was undertaken using the Mann-Whitney U test. $^*P < 0.05$. (**C–F**) $GM\text{-}CSF^+IL\text{-}4$ DCs or SLE DCs were cultured with purified Eff.T4 cells and Tregs for 3 days. Analysis of Eff.T4 cell proliferation was performed by (3H) thymidine incorporation measurement. (**C**) Analysis of Treg-suppressive function toward Eff.T4 cell proliferation at 3 different ratios of $GM^+IL\text{-}4$ DCs or SLC DCs with Eff.T4 cells or Tregs (0.03:1:1, 0.1:1:1 and 0.3:1:1) of 3 independent experiments. (**D**) Analysis of Treg-suppressive function toward Eff.T4 cell proliferation at 4 different Treg/Eff.T4 cell ratios (0:1, 0.5:1, 1:1, and 2:1) of 3 independent experiments. (**E**) Representative experiment performed in triplicate showing that DCs, Tregs, and Eff.T4 cells were cocultured at a 0.1:1:1 ratio, respectively. Anti-OX40L blocking mAb restores Treg-suppressive function. (**F**) Cumulative data obtained with 6 $GM\text{-}CSF^+IL\text{-}4$ DCs and 10 SLE DCs. $GM\text{-}CSF^+IL\text{-}4$ DCs or SLE DCs, Eff.T4 cells, and Tregs were cultured at 0.1:1:1 ratio, respectively. Treg-suppressive function was defined as the percentage of Eff.T4 cell proliferation inhibition and calculated as follows: $(\text{Eff.T4} + \text{Treg})_{\text{condition cpm}}/(\text{Eff.T4})_{\text{condition cpm}}) \times 100$. Statistical analysis was done using the Kruskal-Wallis test followed by Dunn's multiple comparison correction. $^*P < 0.05$, $^{**}P < 0.002$.

(24). In contrast, the $CD4^+CXCR5^-FoxP3^+$ subset did not express Bcl-6 and PD-1 and was defined as Tregs (Supplemental Figure 6A, right). In line with previous reports, Bcl-6 was downregulated in blood follicular counterparts (13, 14) (Supplemental Figure 6B). We also analyzed the frequency and the phenotype of the blood circulating counterparts of T follicular subsets in SLE patients (cTfh cells and cTfr cells). As compared

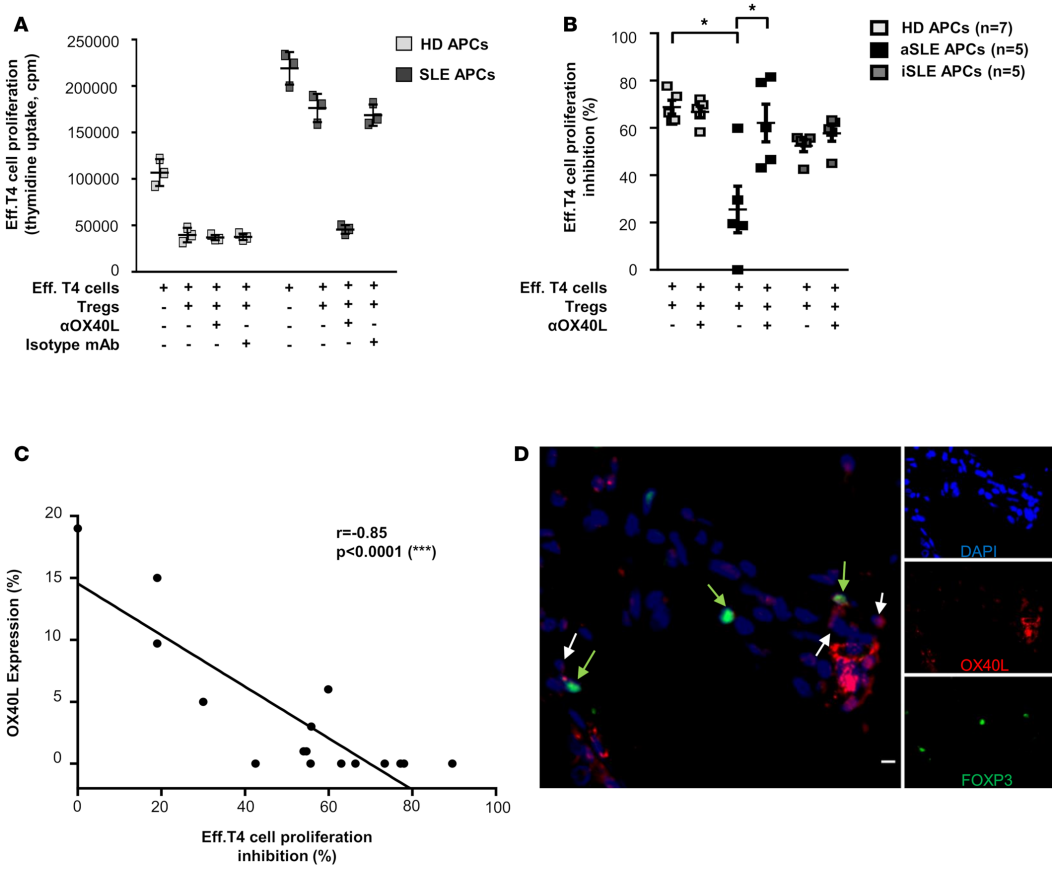

Figure 2. Circulating DCs from SLE patients suppress Treg function in an OX40L-dependent manner. (**A–C**) Blood-purified antigen-presenting cells (APCs) from active SLE (aSLE) patients, inactive SLE (iSLE) patients, or healthy donors (HD) were cultured in the presence of allogeneic Eff.T4 cells and Tregs at a 5:1:1 ratio, respectively, with or without blocking anti-OX40L mAb. An isotype control mouse IgG1 was used when indicated. Treg-suppressive function was calculated as described above. (**A**) This SLE patients' cells, used in this representative experiment, were clinically active, and his disease activity score (SLEDAI) was 16. (**B**) Cumulative data obtained from 5 aSLE (SLEDAI: 29, 4, 4 (clinical activity), 16, and 7, respectively) and 5 iSLE patients (SLEDAI: 0, 0, 0, 4 (biological activity only), 1, respectively) and 7 HD APCs. Statistical analyses were undertaken using the Kruskal-Wallis test followed by Dunn's multiple comparison correction. Error bars indicate the mean ± SEM. (**C**) Correlation between Treg-suppressive function and circulating OX40L-expressing DCs. Statistical analysis was performed using Spearman's rank correlation test. (**D**) Skin biopsies from SLE patients were analyzed for OX40L (in red) and Foxp3 (in green) expression by immunofluorescence. In a cell infiltrate, yellow arrowheads show Foxp3-expressing Tregs in close contact with OX40L-expressing cells denoted by white arrowheads. Scale bar: 10 μm. Data are representative of 3 patients and controls. $^{*}P < 0.05$, $^{***}P < 0.0001$.

with HDs and, in line with previous studies (17), we confirmed that SLE patients display a higher frequency of activated $ICOS^+PD1^+$ cTfh cells, which was positively correlated with $CD38^+$ plasmablasts and SLEDAI. Regarding the Tfr counterpart, we did not observe any difference between HDs and SLE patients (Figure 5), and the disease activity did not affect the proportion of circulating Tfr cells as well.

Next, we analyzed the function of purified tonsil Tfr cells ($CD4^+CD25^+CXCR5^+ICOS^+$) and Tregs ($CD4^+CD25^+CXCR5^-ICOS^+$). Sorted cells of both subsets expressed Foxp3 (data not shown). When cultured with tonsil CFSE-labeled Tfh cells ($CD4^+CXCR5^+$), both Tfr cells and Tregs were able to suppress the proliferation of Tfh cells. Preincubation of Tregs or Tfr cells with sOX40L resulted in a significant increase of Tfh cell proliferation (Figure 6, A and B), demonstrating that OX40L signal also affects the suppressive

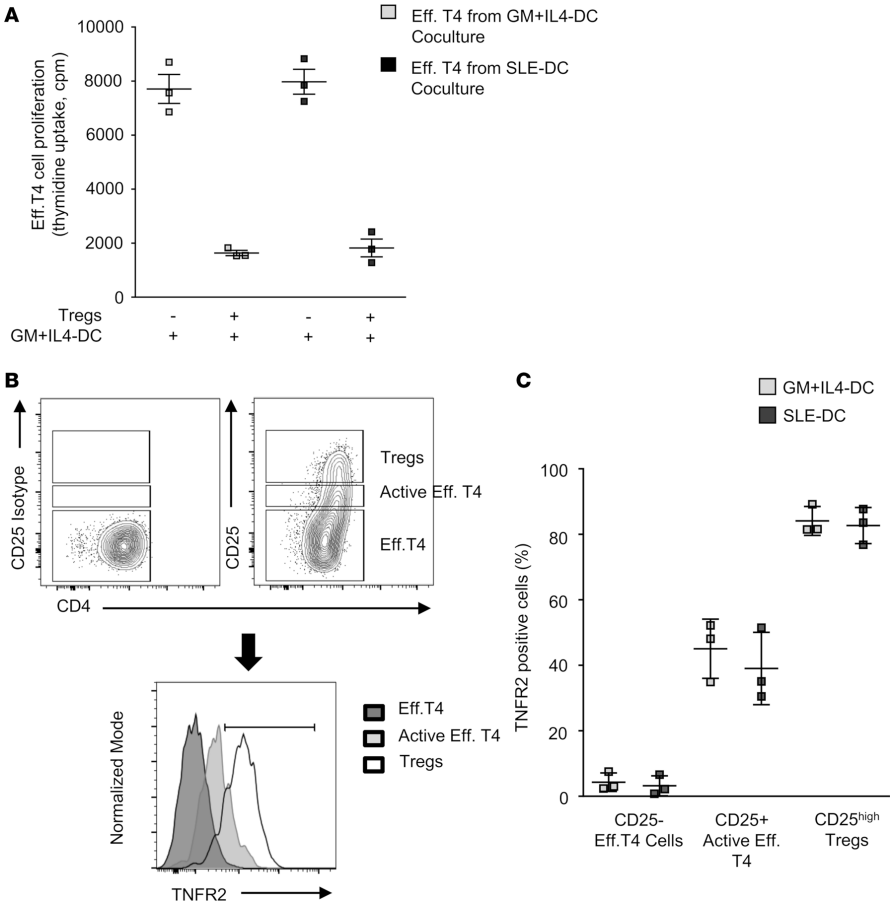

Figure 3. SLE DCs do not confer Tregs resistance to effector T4 cells. (**A–C**) Allogenic HD Eff.T4 cells (5×10^4) were cocultured either with GM-CSF$^+$IL-4 DCs or SLE DCs (5×10^3) for 3 days. At 3 days, Eff.T4 cells were purified from culture and were further cocultured with GM-CSF$^+$IL-4 DCs in the presence or absence of Tregs at a Eff.T4/Tregs/DC ratio of 1:1:0.1 respectively. (**A**) Analysis of Eff.T4 cell proliferation, performed by (3H) thymidine incorporation measurement. (**B**) Analysis of TNFR2 surface expression on CD25$^-$ Eff.T4 cells, CD25$^+$ active Eff.T4 cells, and CD25^{hi} Tregs from GM-CSF$^+$IL-4 DC or SLE DC cocultures. Representative dot plot (top) and histogram (bottom) showing TNFR2 expression on CD25$^-$ Eff.T4 cells, CD25$^+$ active Eff.T4 cells, and CD25^{hi} Tregs. (**C**) Cumulative data obtained from 3 cultures with purified T cells from GM-CSF$^+$IL-4 or SLE DC cocultures represent the percentage of cells expressing surface TNFR2. Error bars indicate the mean ± SEM, $n = 3$.

function of the follicular subset. We next evaluated whether the efficiency of Tfr cells in regulating B cell responses was also altered by OX40L signaling. Tfr cells previously cultured or not with sOX40L were cultured with purified Tfh cells and memory B cells in the presence of Staphylococcal Enterotoxin B (SEB). We observed a higher immunoglobulin production and an increased differentiation of B cells into CD38$^+$ plasmablasts in cocultures with Tfr cells exposed to sOX40L (Figure 6, C and D). Cytokine profiles of coculture supernatants revealed a marked decrease of TGF-β when adding sOX40L (data not shown). Altogether, these data show that OX40L not only impairs the suppressive functions of Tregs, but also those of Tfr cells.

Discussion

Tregs represent a phenotypically and functionally heterogeneous group of lymphocytes that exert immunosuppressive activities on effector immune responses. Tregs play a key role in maintaining immune tolerance and

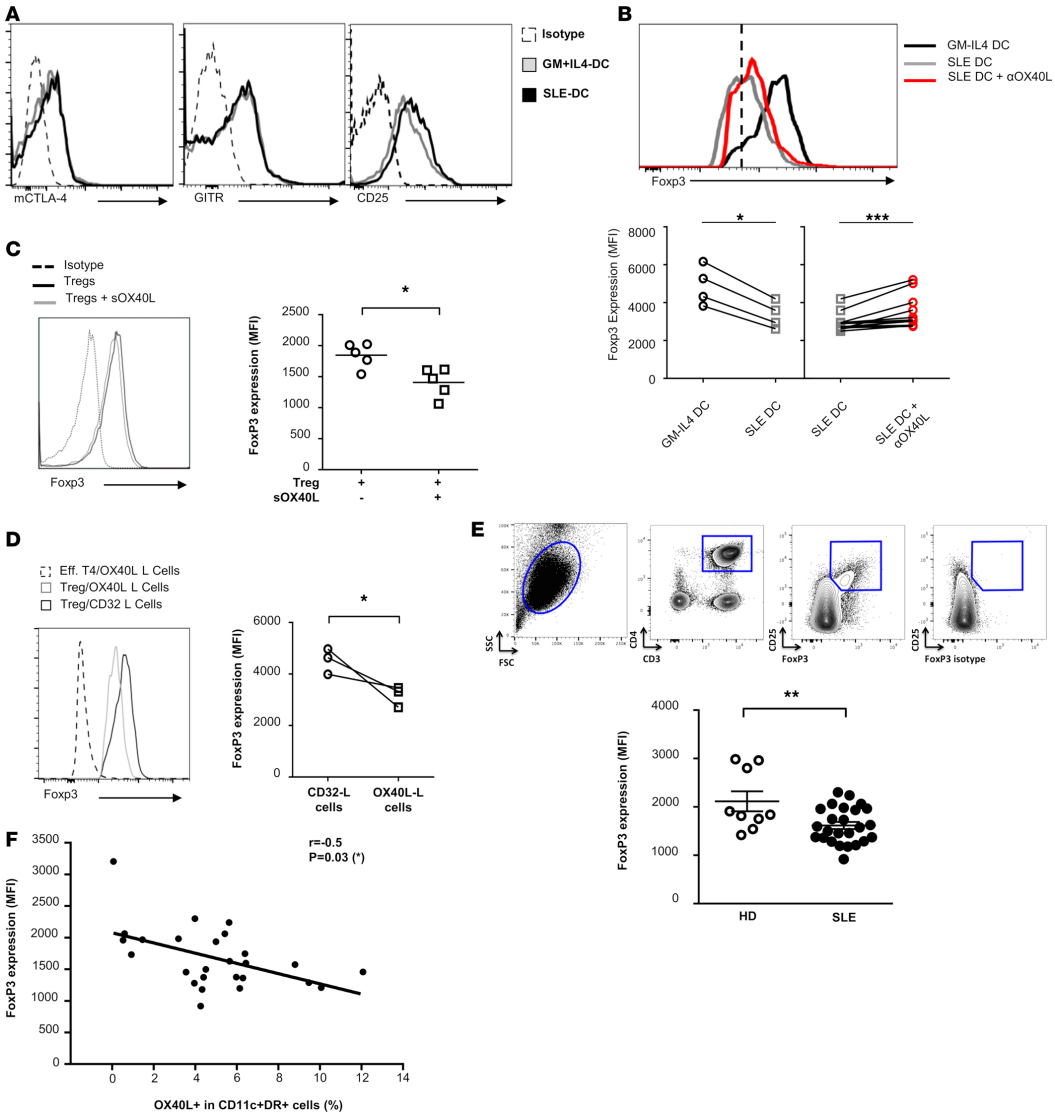

Figure 4. OX40L-dependent downregulation of Foxp3 expression in Tregs. (**A** and **B**) Tregs (10^4 cells) were cocultured in the presence or absence of anti-OX40L mAb (10 μg/ml) for 3 days with Eff.T4 cells (10^4 cells) and either GM-CSF$^+$IL-4 DCs or SLE DCs. (**A**) Representative histogram showing surface expression of CTLA-4, GITR, and CD25 on Foxp3$^+$ Tregs. (**B**) Expression of Foxp3 in CD4$^+$CD25^{hi}CD127$^-$ Tregs from cocultures with Eff.T4 cells and GM-CSF$^+$IL-4 DCs or SLE DCs with or without anti-OX40L mAb. Representative histogram showing Foxp3 expression in Tregs cultured with GM-CSF$^+$IL-4 DCs (black line), SLE DCs (gray line), and SLE DCs $^+$ anti-OX40L mAb (red line) (top). Cumulative data obtained with 4 different GM-CSF$^+$IL-4 DCs and 13 different SLE DCs (bottom). Four independent experiments were undertaken and compared using nonparametric 2-tailed paired Wilcoxon test. *$P < 0.05$; ***$P < 0.001$. (**C**) Expression of Foxp3 in Tregs cultured with or without sOX40L (100 ng/ml). Representative histogram showing Foxp3 expression in Tregs cultured with (gray line) or without (black line) sOX40L (left). Dotted line represents Foxp3 isotype. Cumulative data from 5 independent experiments are shown and compared using nonparametric 2-tailed Mann-Whitney test (right). *$P < 0.05$. Results are expressed as Foxp3 MFI in Tregs. (**D**) Expression of Foxp3 in Eff.T4 cells and Tregs cultured for 3 days with OX40L or CD32-transfected L cells. Representative histogram showing Foxp3 expression in Eff.T4 cells (dashed line) and Tregs cultured with OX40L-transfected L cells (gray line) or CD32-tranfected L cells (black line) (left). Cumulative data from 3 independent experiments, compared using nonparametric 2-tailed paired Wilcoxon test (right). *$P < 0.05$. Results are expressed as Foxp3 MFI in Tregs. (**E**) Foxp3 expression in CD3$^+$CD4$^+$CD25^{hi} blood cells of HDs and SLE patients.

Representative dot plots showing blood $CD3^+CD4^+CD25^{hi}Foxp3^+$ cells (top). Cumulative data analysis from 9 HDs and 25 SLE patients (bottom). Results are expressed as mean Foxp3 MFI in $CD3^+CD4^+CD25^{hi}$ cells. Error bars indicate the mean ± SEM. (**F**) Correlation between proportion of blood OX40L-expressing $CD11c^+DR^+$ cells and Foxp3 MFI expression in $CD3^+CD4^+CD25^{hi}$ blood cells of SLE patients (n = 26). Statistical analysis was performed using Spearman's rank correlation test.

homeostasis through diverse mechanisms, which involve interactions with components of both the innate and adaptive immune systems (25). In SLE, as well as in many autoimmune diseases, Tregs have been proposed to play a relevant role in their pathogenesis. Initial studies regarding frequencies of Tregs in the peripheral blood of SLE patients have generated controversial results. Several groups observed a decreased proportion of $CD4^+CD25^{hi}$ Tregs in SLE patients as compared with healthy controls and observed an inverse correlation of Treg numbers with disease activity (8, 9). However, other studies reported unaltered proportions or even increased proportions of Tregs in SLE patients and a positive correlation with disease activity (11). Our results did not reveal any quantitative defect in either the proportion or in absolute counts of circulating Tregs in SLE patients. Rather, our results identified an OX40L-dependent mechanism responsible for Treg dysfunction in inflammatory tissues. Indeed, we observed that myeloid cells expressing OX40L that migrated to inflammatory tissues in SLE patients colocalized with Tregs and OX40L impairs in vitro immunosuppressive properties of Tregs. Although we cannot rule out a local upregulation of OX40L by myeloid APCs within the tissues, these results established the OX40L/OX40 axis as critical mechanism in explaining dysregulated suppressive functions in the SLE inflammatory microenvironment.

Tregs have been shown to downregulate FoxP3 expression and to potentially revert to or act as effector cells. These cells promote inflammation by the production of inflammatory cytokines, such as IFN-γ or IL-17 (26, 27). It was first suggested that these ex Tregs develop prior to Treg commitment, but a recent study has demonstrated that a fraction of bona fide Tregs with a demethylated TSDR in the Foxp3 locus also downregulates Foxp3 during an inflammatory autoimmune response and acquires effector T cell functions (28). In SLE patients, Foxp3 expression is consistently decreased, possibly due to IL-2 deficiency, in addition to other possible mechanisms (29). In this regard, our results demonstrate that OX40L-dependent signaling on Tregs could be another mechanism explaining Foxp3 downregulation in SLE patients. Interestingly, several authors have demonstrated in mouse models the ability of OX40 agonists to drive Treg expansion (30, 31). This discrepancy may rely on the cytokine microenvironment and the timing of the signal. As an example, OX40 agonist administration influenced experimental autoimmune encephalomyelitis disease severity in opposite directions, depending on the timing of administration (30). The mechanisms and the precise intracytoplasmic pathway by which OX40L hampers Treg functions and Foxp3 expression remain to be discovered.

Regulation of (auto)antibody production primarily takes place in the GC, and an aberrant GC response can contribute to autoimmunity (32). In this GC process, Tfh cells select B cells based on their capacity to bind and present the specific antigen (33). The recently identified Tfr cells are ideal candidates for regulating the normal GC response and preventing emergence of autoreactive B cells. Tfr cells constitute 5%–25% of the GC T cells in mice and originate from $Foxp3^+$ thymic-derived Treg precursors. In the absence of Tfr cells, overwhelming outgrowth of nonspecific B cells leads to lower amounts of antigen-specific B cells (13, 14). Although of interest, no report has addressed the possible role of Tfr cells in the pathogenesis of SLE. Interestingly, based on the evaluation of the circulating $CD4^+CXCR5^+CD25^{hi}CD127^-Foxp3^+$ cells, we could not detect any quantitative defect related to diseases activity in SLE patients. Rather, our results implicate Tfr cells in pathogenesis through their inability to block GC under inflammatory conditions. Our previous work showed that upon TLR7-dependent triggering signals, OX40L is upregulated on myeloid APCs (16) within the inflammatory tissues. This set of data provides evidence that OX40L-deregulated signals block the suppressive functions of Tfr cells, without altering their ability to migrate into the B cell area.

Altogether, our observations identify the OX40L/OX40 axis as an important enhancing inflammatory loop in SLE patients, as it can promote the differentiation of naive and memory T cells into follicular T lymphocytes while blocking the suppressive function of Tregs and Tfr cells. Therefore, blocking of the OX40L/OX40 axis should be considered as a new target option for future clinical trials in lupus.

Methods

Patients. Sixty-one consecutive SLE patients who met the revised criteria of the American College of Rheumatology for SLE (18) were enrolled. All clinically and biologically relevant information concerning the patients is provided in Supplemental Table 1. The active disease patient group was defined as having

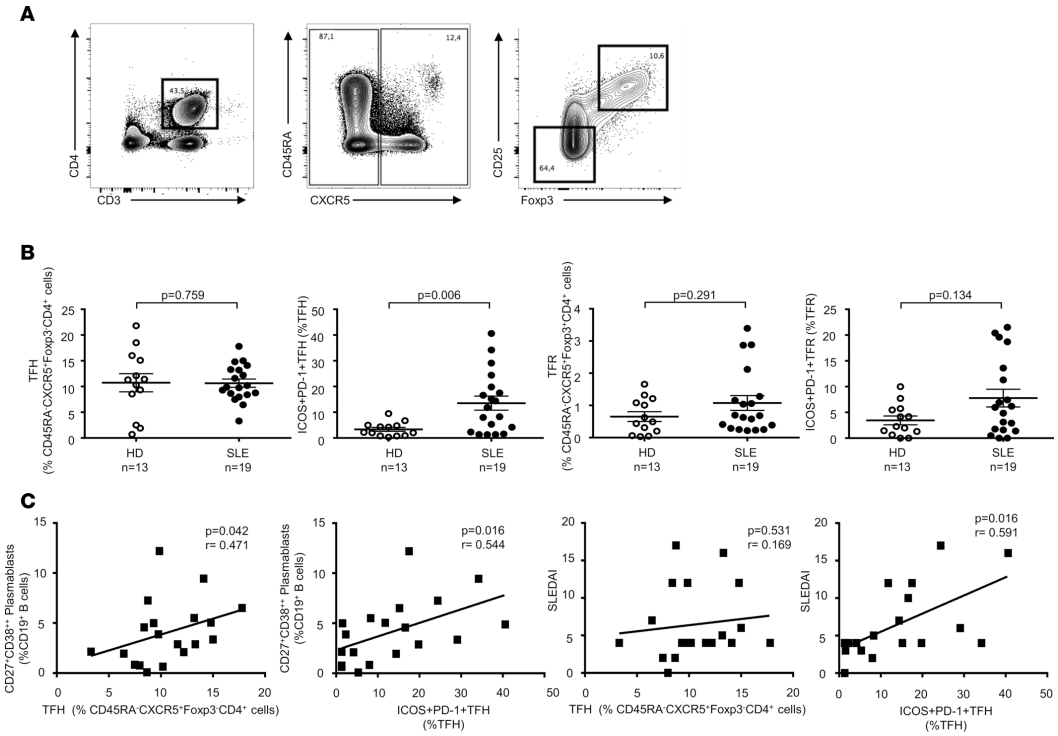

Figure 5. The association of blood Tfh and Tfr cells in HDs and SLE patients with disease activity and plasmablast frequency. (**A**) Representative dot plots showing the gating strategy. (**B**) Frequency of Tfh cells (CD4$^+$CD45RA$^-$CXCR5$^+$FoxP3$^-$), Tfr cells (CD4$^+$CD45RA$^-$CXCR5$^+$FoxP3$^+$), activated Tfh cells (aTfh cell, PD1$^+$ICOS$^+$), and activated Tfr cells (aTfr, PD1$^+$ICOS$^+$) in SLE patients (n = 19) and in HDs (n = 13). Statistical analysis was conducted using the 2-tailed Mann-Whitney U test. Error bars indicate the mean ± SEM. (**C**) Correlation between Tfh cell subsets, plasmablasts (CD27$^+$CD38$^+$CD19$^+$), and SLEDAI. Statistical analysis was conducted using the Spearman's rank correlation test.

a SLEDAI score of ≥6. HDs from our staff (13 females and 3 males, all provided informed consent, approved by the ethics committee of Bordeaux University Hospital) were studied as a control group.

Phenotypic analysis of blood samples. Whole blood analysis of OX40L expression was conducted using anti-human CD14, CD16, CD11c, HLA-DR, and OX40L mAbs incubated in whole blood following red blood cells lysis (Versalyse solution from Beckman Coulter). For Treg analysis, cells were stained with anti-CD3, CD4, and CD25, before intranuclear staining for Foxp3 according to the manufacturer's instructions (eBioscience Foxp3/Transcription Factor Staining Buffer Set). Only the CD25^{hi} Foxp3$^+$ cells were analyzed. For Tfr cell analysis, the following antibody panel was used: anti-CD3, CD4, CD25, ICOS, CD45RA, PD1, and CXCR5; this was followed by intranuclear staining for Foxp3. Data were collected using a BD FACSCanto II or a BD Fortessa and analyzed with DIVA and Flowjo softwares (BD Biosciences and Tree Star, respectively). All antibodies used in this study are listed in Supplemental Table 2.

Phenotypic analysis of tonsil samples. Tonsil samples were obtained from HDs undergoing tonsillectomies, and single cells were collected by mechanical disruption. For surface staining, cells were incubated with fluorochrome-conjugated antibodies: anti-CD4, -CD3, and -CD25 from Beckman Coulter; PD-1, ICOS, and GITR from Biolegend for 15 minutes; followed by intranuclear staining with Foxp3 and Bcl-6. Samples were analyzed using a BD Fortessa and analyzed with DIVA and Flowjo softwares (BD Biosciences and Tree Star, respectively).

Immunofluorescence experiments. Punch biopsy specimens (4 mm) of affected skin were obtained from 3 SLE patients. Immunofluorescence was performed as previously described by Truchetet ME, et al. (34).

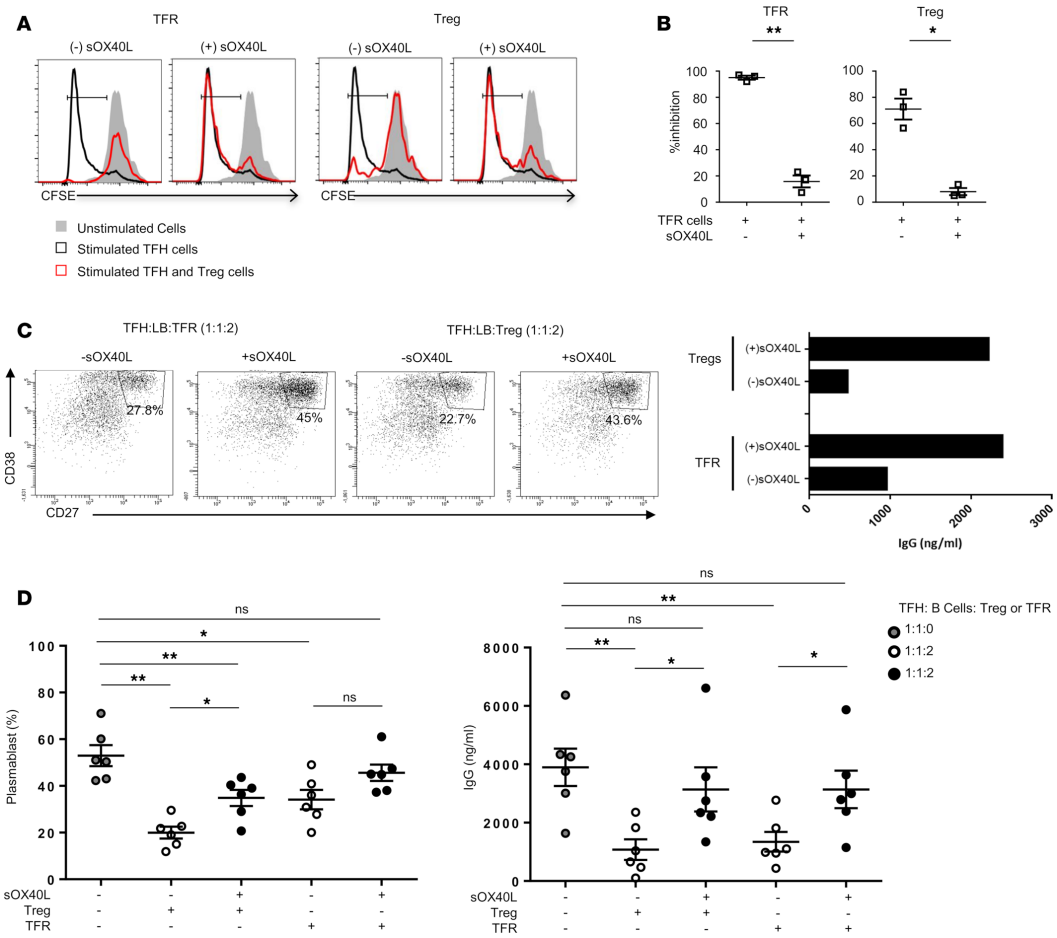

Figure 6. OX40-OX40L axis modulates Tfr cells regulatory functions. (**A** and **B**) Sorted tonsil $CD4^+CD25^-ICOS^+CXCR5^+$ Tfh cells (10^4 cells) were either stimulated (CD3 and CD28 mAb) or not and were cultured for 3 days alone or with sorted tonsil $CD4^+CD25^+ICOS^+CXCR5^+$ Tfr cells (10^4 cells) or $CD4^+CD25^+ICOS^+CXCR5^-$ Tregs (10^4 cells) in the presence or absence of sOX40L (100 ng/ml). Proliferation of Tfh cells was analyzed at 3 days of coculture. (**A**) Histograms of a representative experiment showing proliferation ($CFSE^{dim}$) of unstimulated Tfh cells (gray filled), stimulated Tfh cells (black line), and Tfh cells cultured with either Tfr cells or Tregs (red line) at 3 days of culture. (**B**) Percentage inhibition of Tfh cell proliferation. The percentage of inhibition was calculated in reference to proliferation observed with stimulated Tfh cells cultured alone. Error bars indicate the mean ± SEM, $n = 3$. Statistical analysis was conducted using the 1-way ANOVA test. (**C** and **D**) Soluble OX40L impairs the ability of Tfr cells and Tregs to suppress Tfh cell function. After incubation with sOX40L (100 ng/ml), Tregs or Tfr cells (4×10^4 cells) were cultured for 7 days along with Tfh cells (2×10^4) and memory B cells (2×10^4) in the presence of SEB (0.25 ng/ml). $CD27^+CD38^+$ plasmablast percentage in cell culture (**C**) and concentration of IgG in cell culture supernatant (**D**) were determined by flow cytometry and ELISA, respectively, after 7 days of coculture. Individual values are shown with mean and SEM and were compared using nonparametric Kruskal-Wallis test with Dunn's comparison for multiple comparisons. $^*P < 0.05$; $^{**}P < 0.01$.

Monoclonal mouse anti-human OX40L (clone 159403, mouse IgG1) was from R&D Systems, rat anti-human FoxP3 (clone PCH101, rat IgG2κ) was from eBioscience, while Alexa Fluor 488–conjugated donkey anti-rat and Alexa Fluor 547–conjugated donkey anti-mouse were from Invitrogen. After paraffin removal, epitope retrieval, and blocking in PBS–4% BSA, tissue sections were incubated with anti-FoxP3 and anti-OX40L. Binding was revealed with Alexa Fluor 488– or Alexa Fluor 547–conjugated anti-rat or anti-mouse sera. Nuclei were stained with DAPI. Laser-scanning confocal images were acquired using a

Zeiss LSM 510 META confocal laser-scanning microscope (Carl Zeiss). Negative controls, stained only with secondary antibodies, did not result in significant fluorescence and were omitted from the figures.

Monocyte-derived DC generation and purification of DCs from patients and HDs. Monocyte-derived DC preparations were generated as previously described (3). Briefly, $CD14^+$ monocyte cells were purified from PBMCs using anti-CD14 MicroBeads (Miltenyi Biotec). 25 ng/ml recombinant human GM-CSF and 10 ng/ml recombinant human IL-4 purchased from PeproTech were add to $CD14^+$ purified monocytes in complete RPMI 1640 medium (supplemented with 8% FCS). At day 4, DCs were collected and analyzed by flow cytometry to determine the expression levels of CD14, HLA-DR, CD11c, and OX40L. For ex vivo experiments, the non–T cell fractions were negatively isolated from HDs or the peripheral blood mononuclear cells (PBMCs) of SLE patients using anti-CD3 MicroBeads (Miltenyi Biotec), and the cells were irradiated (35 Gy).

Effector T cell and Treg purification. Eff.T4 cells and Tregs were isolated from HD PBMCs by magnetic cell separation using CD4 MicroBeads (Miltenyi Biotec). After 18 hours at 4°C, the effluent preenriched $CD4^+$ T cells were labeled with anti-CD127 antibody and coupled with anti-Mouse IgG MicroBeads (Miltenyi Biotec). The negative fraction was labeled with CD25 MicroBead II (Miltenyi Biotec) to obtain $CD4^+CD127^{-/lo}CD25^+$ Tregs. Naive T cells were purified from preenriched $CD4^+$ T cells by sorting with FACSAria (BD Biosciences). Purity was controlled by flow cytometry analysis using LIVE/DEAD FIXABLE Aqua (Invitrogen) and was always above 95%.

Treg functional assays. For (^{3}H) thymidine–based suppression assays, freshly isolated Tregs (10^4 cells) and Eff.T4 cells (10^4 cells) were cultured in complete RPMI 1640 medium (supplemented with 8% FCS) in 96-well round-bottomed microplates (Becton Dickinson) with 1 μg/ml plate-bound anti-CD3 and SLE or GM-CSF$^+$IL-4 DCs (10^3 cells) with or without 10 μg/ml blocking anti-OX40L mAbs (Ancell) or isotype-matched mAbs. For APC-based suppression assay, Tregs (10^4 cells) and Eff.T4 cells (10^4 cells) were then cultured together with irradiated allogenic (35 Gy) $CD3^+$ cells depleted PBMCs (APCs) (5×10^3 cells). After 3 days of culture, (^{3}H) thymidine (1 μCi) was added for 18 additional hours of culture. Proliferation was determined using a scintillation counter (Microbeta Trilux, Wallac). All clinically and biologically relevant information concerning the patients included in the Treg functional assay are provided in Supplemental Table 3.

Th and B cell coculture. Tfh cells, Tregs, and Tfr cells were purified from preenriched $CD4^+$ T cells and sorted with a FACSAria. Autologous memory LB were purified from preenriched $CD19^+$ cells and sorted with a FACSAria ($CD3^-CD19^+CD27^+IgD^-$). Tfr cells or Tregs were incubated with or without 100 ng/ml sOX40L (2×10^4 or 4×10^4) during one in complete RPMI medium. After 1 hour the cells were washed and cocultured with Tfh cells and memory LB in complete RPMI medium in the presence of endotoxin-reduced SEB (Toxin Technology). The percentage of plasmablast was analyzed by flow cytometry, and IgG produced in supernatants was analyzed by ELISA at day 7.

Analysis of Foxp3 expression. Purified Tregs and/or Eff.T4 cells (5×10^4 cells) were cultured with or without of sOX40L (100 ng/ml) or cocultured with SLE or GM-CSF$^+$IL-4 DCs (5×10^3 cells) in 96-well round-bottom plates precoated with 1 μg/ml anti-CD3. After 48 hours of culture, cells were incubated with fluorochrome-conjugated antibodies CD4, CD3, CD25, CTLA-4, and GITR. After surface staining, cells were fixed, permeabilized, and stained for Foxp3 per the manufacturer's instructions (ebioscience, Foxp3/Transcription Factor Staining Buffer Set).

Irradiated (60 Gy) CD32-expressing L cells or OX40L-expressing L cells were cultured in RPMI complete medium for 3 days with Eff.T4 cells or Tregs (2.5:1 for T cells/L cell ratio) in 96-well round-bottom plates precoated with 1 μg/ml anti-CD3. After 3 days, cells were collected, surface stained with anti-CD4 and -CD25, and intranuclear stained for Foxp3.

sOX40L and cytokines measurements. sOX40L was measured by ELISA (Cusabio) in sera of SLE patients and HDs, in accordance with the manufacturer's recommendations. Cytokines were measured using Cytometric Bead Array from Biolegend (Legendplex).

Statistics. The normality of the variable distribution was assessed using the Kruskal-Wallis test. In case of normality of data distribution, 2-tailed Student's *t* tests were performed, or in case of more than two groups, a 1-way ANOVA test with Holm-Sidak's correction for multiple comparisons was used. When the normality of the distribution was rejected, statistical analyses were performed using the nonparametric paired Wilcoxon test or unpaired Mann-Whitney *U* test as appropriate or with Kruskal-Wallis followed by Dunn's correction for multiple comparisons. Correlation between variables was determined using the Spearman test. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism 6.0 software (SAS Institute).

Study approval. The study protocol was approved by the ethics committee of Bordeaux University Hospital. Each patient enrolled in the study signed a consent form approved by the ethics committee.

Author contributions

CJ, JFA, ID, CCB, and MS analyzed the phenotype and performed statistical analysis of blood samples. CJ, ID, JFA, and CCB performed the in vitro experiments with DCs. MET, EL, and JS performed tissue staining. ID, JFA, CCB, and EF performed the in vitro experiments with effector, Trf, and B cells and ELISA. CCB was involved in the experimental design. CR, EL, PD, MET, LC, TS, JFV, and JLP provided adult SLE samples and clinical information. MS performed Helios staining and multiplex cytokine assays. VP was involved in the design of the experiments and in the providing of SLE patient samples with clinical information. NG was involved in performing in vitro DC experiments and phenotyping analysis of blood samples. Data interpretation was performed by all authors. CJ, JFA, and PB wrote the manuscript. PB conceived the project and oversaw the entire work.

Acknowledgments

We thank J.-C. Caron, K. Eschel, M. Gassie, and C. Cognet for technical help provided for flow cytometry. We thank V. Pitard and V. De Luca for cell sorting. We thank H. Ueno and V. Pascual for discussions. This study was supported by research funding from Centre national pour la Recherche Scientifique, the Société Française de Rhumatologie, Société française Nationale de Médecine Interne, Arthritis Fondation Courtin, Ministère de la Recherche et de l'Enseignement supérieur, the NIH (grants U19-AI057234, U19-AI082715, U19-AI089987), Alliance for Lupus Research, and Baylor Health Care System.

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III) DISCUSSION

Le lupus érythémateux systémique est caractérisé par une physiopathologie complexe et une grande hétérogénéité clinique et biologique. A titre d'exemple, la signature interféron souvent décrite dans la maladie n'est retrouvé que chez deux tiers des patients (beaucoup plus dans la population pédiatrique), tandis que d'autres patients portent une signature B ou granuleuse *(164, 165)*. Plusieurs travaux mettent également en évidence une activation du système plaquettaire, concomitante à l'activité de la maladie dans le LES *(166–168)*. En effet, le sérum de patients ayant un LES, a fortiori un LES actif, induit l'activation plaquettaire *in vitro*, tel qu'évalué par l'expression membranaire de P-selectine ou l'aggrégation plaquettaire *(169)*. Les auto-anticorps (anti-dsDNA) *(160)*, les anti-phospholipides *(161)*, les complexes immuns (via le FcγRII *(159)* ou le TLR7) et les stimulus viraux *(162)* sont autant de facteurs activateurs des plaquettes jouant un rôle dans le LES. Cette activation plaquettaire va induire, entre autre, l'expression membranaire de CD40-ligand (CD40L) et de la selectine plaquettaire (P-selectine) et l'augmentation des taux solubles de P-selectine par clivage de la P-selectine membranaire *(170)*.

Notre équipe a récemment montré que le CD40L exprimé par les plaquettes activées interagissait avec le CD40 exprimé par les pDC, augmentant la production d'IFN de type I par ces dernières en réponse aux complexes immuns circulants *(159)*. Le rôle des plaquettes dans la physiopathogénie du LES a été confirmé dans ce même travail en montrant que l'inhibition de l'activation plaquettaire à l'aide du clopidogrel (un inhibiteur pharmacologique de $P2Y_{12}$) permettait d'améliorer le phénotype de deux modèle de LES (NZB/NZW et MRL/Lpr). Ainsi, l'activation plaquettaire retrouvée dans le LES pourrait participer activement à la physiopathogénie de la maladie.

Dans notre travail (article 2), nous avons montré que les Tregs, exprimant la forme fucosylée et donc fonctionnelle du ligand de la P-selectine (le sialyl lewis X, sLe^x ou CD15s), interagissaient de manière préférentielle avec les plaquettes. Nous avons montré que le pourcentage d'agrégats Treg/plaquettes était augmenté dans le LES, et ce de manière corrélée à l'activité de la maladie. Les Teff n'exprimant

pas la forme fucosylée du PSGL-1, la corrélation entre le pourcentage d'agrégats Teff/plaquettes et l'activité de la maladie n'était pas retrouvée. Témoin de l'activation plaquettaire retrouvée chez les patients atteints de LES actif, nous retrouvions des taux augmentés de P-selectine soluble et microparticulaire chez les patients ayant une maladie active, et ce de manière corrélée à l'activité de la maladie. Ainsi, l'activation plaquettaire retrouvée dans le LES actif induit une interaction spécifique avec les Tregs par le biais du couple P-selectine/PSGL1-CD15s.

Dans l'objectif d'analyser l'impact de cette interaction, nous avons étudié les fonctions immunosuppressives de Tregs humains *in vitro*, avec ou sans exposition aux plaquettes puis à la P-selectine. Nous avons montré que les plaquettes, via l'interaction P-selectine/PSGL-1 inhibaient les fonctions immunosuppressives des Tregs *in vitro* (figure 2). Une inhibition des fonctions suppressives des Tfr sur la réponse humorale était également retrouvée *in vitro* par le biais de co-culture B-Tfh-Tfr.

Nous avons par le suite étudié les voies de signalisation intracellulaires activées par la P-selectine. Celle-ci induit une phosphorylation de Syk, spécifique du Treg (non retrouvée avec les Teff) *in vitro* et *in vivo.* L'incubation des Treg (mais pas des Teff) avec de la P-selectine induisait un flux calcique intracellulaire, qui était bloqué spécifiquement par un inhibiteur de Syk ou un anticorps bloquant PSGL-1. Dans l'objectif de montrer l'importance de cette voie de signalisation, nous avons démontré par la co-culture Teff/Treg que l'ajout d'un inhibiteur de Syk permettait de restaurer les fonctions immunosuppressives des Tregs exposés à la P-selectine.

D'un point de vue mécanistique, nous avons montré que la P-selectine induisait une diminution d'expression du TGF-β et de GARP à l'échelle transcriptomique et protéique. De manière concomitante, la P-selectine induisait une diminution d'expression de FoxP3 et d'Helios, deux facteurs de transcription précédemment décrits dont le niveau d'expression est étroitement corrélé à l'activité des Tregs. De manière intéressante, l'ajout de TGF-β aux Tregs cultivés en présence de P-selectine permettait de maintenir les niveaux d'expression de FoxP3 et d'Hélios et de rétablir les fonctions suppressives des Tregs. Ces résultats suggèrent que l'inhibition de la voie du TGF-β serait le *primum*

movens de l'effet de la P-selectine sur le Treg, induisant une baisse de l'expression de FoxP3 par diminution de l'effet autocrine du TGF-β.

Nous avons ensuite montré que chez le patient atteint de LES, les taux de P- (et de E-) selectine solubles et microparticulaires étaient augmentés, avec une corrélation à l'activité de la maladie. Ces résultats sont cohérents avec ceux de la littératures et des modèles murins de LES *(161, 171)*.

Afin d'explorer l'intérêt thérapeutique du blocage de la P-selectine, nous avons utilisé le modèle murin DNAse1L3-KO de LES reposant sur un terrain génétique Black-6. Il s'agit d'un modèle spontané de LES dans lequel une perte de l'activité DNAse1L3 induit l'accumulation de microparticules riches en acides nucléiques induisant une apparition précoces d'anticorps anti-ADN, puis l'apparition d'une glomérulonéphrite ressemblante à celle du LES *(172)*. Nous avons choisi ce modèle car il s'agit d'un modèle de LES spontanée dont la physiopathologie est proche du LES et qui était disponible dans notre laboratoire de par le recrutement récent du Dr Vanja Sisirak dans notre unité. Nous avons dans un premier temps confirmé dans ce modèle qu'il existait une augmentation de la P-selectine microparticulaire. Les Tregs de souris black 6 sauvages répondaient par un signal calcique à la P-selectine de manière identique aux Tregs humains. Nous avons ensuite traités des souris DNAse1L3-KO par des injections intra-péritonéales d'anticorps anti-P-selectine ou d'isotype contrôle durant 10 semaines. Nous avons montré que l'anti-P-selectine induisait une diminution significative des taux circulants d'anti-ADN ainsi que des lésions rénales (histologiques et en terme de dépôts d'IgG et de complément évalués en immunofluorescence).

Suite à l'évaluation de notre travail par une revue à comité de lecture, nous avons entrepris de confirmer ces résultats *in vivo* dans un deuxième modèle murin de LES : le modèle NZB/NZW. Nos travaux apportent des éléments d'explications à la dysfonction du compartiment Treg retrouvée dans le LES, et relient cette dysfonction à l'activation du compartiment plaquettaire. Le blocage de la voie P-selectine/PSGL-1 pourrait constituer une voie thérapeutique innovante dans le LES.

Actuellement, la prise en charge du LES repose principalement sur la corticothérapie et les traitements immunosuppresseurs. Le seul traitement validé dans les 50 dernières années dans le LES est le belimumab, un anticorps monoclonal ciblant Blys (BAFF), un facteur de croissance des lymphocytes B *(5)*. L'anifrolumab, un anticorps ciblant le récepteur des interférons de type I semble très prometteur même si les deux essais de phase 3 ont rapporté des résultats discordants *(6, 173)*. Cependant, les traitements actuellement à disposition sont responsables d'une augmentation du risque infectieux ainsi que du risque cardiovasculaires qui constituent aujourd'hui les premières causes de décès dans le LES *(174, 175)*. Ainsi, le développement de thérapies permettant de rétablir l'homéostasie immunitaire sans pour autant augmenter les risque infectieux et cardiovasculaires est cruellement nécessaire.

Le blocage de la P-selectine pourrait répondre à ces impératifs. Concernant l'athérosclérose, la P-selectine a été associée au développement de l'athérosclérose chez l'homme *(176, 177)*, avec une relation causale démontrée chez la souris *(178)*. D'autre part, le rétablissement de l'activité immunosuppressive des Tregs est à même de diminuer le risque cardiovasculaire. En effet, le compartiment Treg exerce un rôle protecteur dans l'athérosclérose comme le suggère le traitement par IL-2 dans le modèle murin athérogénique LDL-$R^{-/-}$ *(52)*, et le fait que la plasticité des Tregs en Tfh à l'intérieur des plaques d'athérome induit une progression de l'athéromatose chez la souris *(53)*. Concernant le risque infectieux, le crizanlizumab, un anticorps monoclonal ciblant la P-selectine humaine a montré son efficacité dans la prévention des crises vaso-occlusives drépanocytaires chez l'Homme *(179)*. Il n'y avait pas d'augmentation du risque infectieux chez ces patients pourtant à risque d'infections graves (du fait de l'asplénie fonctionnelle), mais ces données devront être confirmées sur de plus larges échantillons de patients. Si nos données sont confirmées, le crizanlizumab pourrait faire l'objet d'un développement dans le LES.

Récemment, une équipe chinoise a montré l'efficacité du blocage de la P-selectine sur l'atteinte rénale du modèle MRL/Lpr, un modèle murin très utilisé dans l'étude du LES *(180)*. D'autres maladies

inflammatoires pourraient également bénéficier d'un blocage de la P-selectine. En effet, nous avons également récoltés des données préliminaires montrant une augmentation de la P-selectine soluble et microparticulaire dans les vascularites à ANCA et dans la sclérodermie systémique, des résultats similaires étant également retrouvées dans la littérature *(181, 182)*.

Bien que notre travail se soit concentré sur le compartiment Tregs, les effets de la P-selectine n'y sont pas limités. En effet, d'autres cellules portent le CD15s (PSGL-1 fucosylé) permettant de lier efficacement la P-selectine. Parmi ces cellules, les neutrophiles sont particulièrement intéressants car directement impliqués dans la physiopathologie du LES, notamment par une mort cellulaire spécialisée, la NETose. Lors de la NETose, les neutrophiles relarguent dans le compartiment extracellulaire de nombreuses molécules immunogènes tels que de l'ADN, des histones, mais également des mitochondries et de l'ADN mitochondriale oxydé, particulièrement interferonogénique *(183, 184)*. Des données chez la souris suggèrent que la P-selectine peut induire la NETose *in vitro* et *in vivo (185)*. En outre, la NETose induite par les complexes immuns de patients porteurs d'une thrombopénie induite à l'héparine peut être inhibée par le blocage de l'interaction neutrophile/plaquette via un anticorps anti-P-selectine *(186)*. D'autres cellules tels que les monocytes et les cellules dendritiques portent le CD15s (figure S1, papier 2). Un très récent article publié dans la revue Science Advances a montré que les plaquettes activée pouvait interagir avec les monocytes circulant via l'axe P-selectine/PSGL-1 *(187)*. Cette interaction induit un flux calcique intracellulaire et une différentiation du monocyte en cellule présentatrice d'antigène capable d'induire une réponse T robuste par une augmentation d'expression des molécules de HLA de type II et de costimulation *(187)*. Enfin, ils ont montré *in vitro* que la réponse cytotoxiques, la prolifération et la production d'IFN-γ de PBMC murins ou humains envers des lignées cancéreuses étaient fortement majorées en cas de présence de plaquettes.

Il est important de souligner que PSGL-1 semble avoir plusieurs fonctions majeures ne se limitant pas à son rôle de ligand à la P-selectine. En effet, il a été montré dans un modèle murin que la délétion du

PSGL-1 induisait une augmentation de la réponse anti-virale $CD8^+$ par une perte des capacités d'exhaustion *(188)*. Ce même modèle développait secondairement et de manière spontanée un phénotype auto-immun systémique, suggérant un rôle de point de contrôle immunitaire du PSGL-1 *(188)*. Cependant, plusieurs arguments vont à l'encontre du rôle de la P-selectine comme intervenant dans l'effet d'immune-checkpoint médié par le PSGL-1 dans ce modèle. Tout d'abord, les lymphocytes T effecteurs y compris les $CD8^+$ expriment une forme non fucosylée de PSGL-1, qui ne peut donc pas servir de ligand à la P-selectine *(32)*. D'autre part, les auteurs ont montré cet effet via l'utilisation d'un anticorps bloquant (clone 4RA10) mais ne parvenaient pas à le reproduire avec d'autres anticorps, ainsi qu'avec les différentes sélectines recombinantes (P-, E- et L-selectine) *(188)*. Cependant, il restait difficile de rallier ces résultats à nos observations. Johnston *et al.* ont très récemment apporté la réponse à cette question. Ils ont démontré qu'en condition inflammatoire (pH acide), le PSGL-1 faisait office de ligand au V-domain immunoglobulin suppressor of T cell activation (VISTA), une molécule de point contrôle immunitaire *(189)*. Le PSGL-1 revêt donc des fonctions différentes entre les cellules portant sa forme fucosylée (rôle de ligand plaquettaire/endothélial) et celles portant la forme native (rôle de point de contrôle immunitaire). Ainsi, si un blocage pharmacologique de l'axe P-selectine/PSGL-1 était envisagé en clinique, il semblerait plus sage de bloquer directement la P-selectine. En cas de ciblage de PSGL-1, il faudrait s'assurer que l'agent utilisé ne bloque pas l'interaction avec VISTA, sous peine de favoriser l'auto-immunité.

Une autre stratégie pourrait viser à inhiber l'activation plaquettaire. L'inhibition pharmacologique de l'activation plaquettaire par du clopidogrel (un inhibiteur de $P2Y_{12}$ utilisé en clinique) a montré une efficacité *in vivo* dans deux modèles murins de LES (NZB/NZW et MRL/Lpr) *(159)*. Un essai exploratoire a été mené chez des patients atteints de LES au CHU de Bordeaux, et son analyse est en cours (CLOPUS, NCT02320357).

Dans le troisième article, nous avons étudié le rôle de l'OX40 dans l'homéostasie des Tregs. L'OX40 (TNFSR4 ou CD134) est une protéine de la famille des récepteurs au TNF, exprimée de manière constitutionnelle par les Tregs. Nous avons montré que l'interaction entre l'OX40-ligand (OX40L) et son récepteur OX40 exprimé par les Treg était responsable d'une diminution des capacités immunosuppressives des Tregs et des Tfr, via une diminution d'expression de FoxP3. L'OX40L est exprimé par les monocytes circulants en condition inflammatoire, ainsi que chez les patients ayant un LES actif. D'autre part, nous avons montré que dans les biopsies cutanées de patients atteints de LES, une interaction physique existait entre les Tregs et les monocytes exprimant OX40L, renforçant le l'importance physiopathologique de cet axe. Il existait une corrélation négative entre le pourcentage de monocytes circulants $OX40L^+$ et l'intensité d'expression de FoxP3 par les Tregs de patients. Enfin, le blocage *in vitro* d'OX40L par le biais d'un anticorps monoclonal permettait de restaurer l'expression de FoxP3 par les Tregs. Ces résultats, associés à une autre publication de notre équipe montrant que l'OX40L favorisait la différentiation des lymphocytes T en Tfh stimulant la production d'auto-anticorps pathogènes *(190)*, soutiennent le potentiel développement d'un anticorps bloquant OX40L en clinique.

En conclusion, nous avons décrit dans ce travail plusieurs interactions entre des cellules circulantes (monocytes et plaquettes activées) et les lymphocytes T régulateurs responsables d'une inhibition des capacités immunosuppressives de ces derniers. L'identification de l'axe OX40/OX40L et de l'axe

P-selectine/PSGL-1 comme responsable de l'inhibition des capacités immunosuppressives des Treg pourrait ouvrir la voie à l'essai d'anticorps bloquant l'une de ces voies chez les patients atteints de LES. Par exemple, le blocage de l'axe P-selectine/PSGL-1 pourrait restaurer les fonctions des Tregs sans pour autant promouvoir les risques infectieux et thrombotiques qui restent aujourd'hui les causes principales de morbidité et mortalité dans le lupus systémique.

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V) ANNEXES

1) ARTICLE COLLABORATIF : L'ASCORBATE MAINTIEN UNE HYPOXIE PLASMATIQUE

Dans ce travail, nous avons collaboré avec l'équipe du Dr Benoit Marteyn (IGBC, Bordeaux ; ayant déménagé à l'IBMC, Strasbourg). Son équipe travaille sur le polynucléaire neutrophile et plus spécifiquement sur les interaction entre les pathogènes et l'environnement sur cette cellule.

Dans cette étude princeps, l'idée était d'évaluer l'état d'oxygénation des cellules dans le plasma. En effet, la pression partielle en oxygène du plasma n'avait jamais été mesurée au préalable.

À l'aide de tube vidés de leur contenu en oxygène (hypoxytube) ou de tubes classiques, du sang veineux de 12 donneurs sains non-fumeurs a été prélevé. La pression partielle en O2 a ensuite été mesurée dans le sang total.

Nous avons montré que la pression partielle en O2 du sang total était très faible (8,4 mmHg), bien plus basse que celle de l'air ambiant (159,7 mmHg). Ce résultat nous informe que les cellules circulantes, y compris les neutrophiles sont confrontés à un environnement très hypoxique *in vivo*. Nous avons ensuite montré, *in vitro* et *in vivo* que l'ascorbate (vitamine C) était responsable du maintien d'une hypoxie dans le plasma en tamponnant le potentiel oxydatif de l'oxygène.

Ces résultats ont des conséquences majeures concernant l'étude des cellules immunitaires *ex vivo*. En effet, les conditions de purifications habituelles se font en air ambiant, et la culture cellulaire est habituellement réalisée avec une atmosphère composée à 5% de CO2, bien loin des condition *in vivo* que nous décrivons ici. Il est alors probable que ces conditions expérimentales impactent de manière significative le réseau mitochondrial, le métabolisme et par conséquent, la réponse immunitaire de ces cellules étudiées *in vitro*. Ces résultats soutiennent le fait de purifier et cultiver les cellules immunitaires, notamment les neutrophiles dont le métabolisme oxydatif fait partie intégrante de leur physiologie, dans des conditions hypoxiques.

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Ascorbate maintains a low plasma oxygen level

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In human blood, oxygen is mainly transported by red blood cells. Accordingly, the dissolved oxygen level in plasma is expected to be limited, although it has not been quantified yet. Here, by developing dedicated methods and tools, we determined that human plasma pO_2 = 8.4 mmHg (1.1% O_2). Oxygen solubility in plasma was believed to be similar to water. Here we reveal that plasma has an additional ascorbate-dependent oxygen-reduction activity. Plasma experimental oxygenation oxidizes ascorbate (49.5 µM in fresh plasma vs < 2 µM in oxidized plasma) and abolishes this capacity, which is restored by ascorbate supplementation. We confirmed these results in vivo, showing that the plasma pO_2 is significantly higher in ascorbate-deficient guinea pigs ($Ascorbate_{plasma}$ < 2 µM), compared to control ($Ascorbate_{plasma}$ > 15 µM). Plasma low oxygen level preserves the integrity of oxidation-sensitive components such as ubiquinol. Circulating leucocytes are well adapted to these conditions, since the abundance of their mitochondrial network is limited. These results shed a new light on the importance of oxygen exposure on leucocyte biological study, in regards with the reducing conditions they encounter in vivo; but also, on the manipulation of blood products to improve their integrity and potentially improve transfusions' efficacy.

Blood gases are either dissolved in the plasma or transported by hematies. The solubility of O_2 is low compared to CO_2[1]. Only a limited fraction of O_2 is dissolved in plasma, representing less than 2% of the total blood oxygen content. Arterial pO_2 equals 75–100 mmHg (9.9–13.1% O_2) and venous pO_2 equals 30–50 mmHg (3.9–5.6% O_2); in theory the blood plasma pO_2 would be ranged from 0.9 to 3 mmHg (0.1–0.4% O_2), although it has not been experimentally quantified. Until now, it was considered that the solubility coefficient of O_2 in plasma was similar in water[2]. The impact of ascorbate (or Vitamin C), a strong reducing molecule, on plasma oxygen level has not yet been investigated, despite of its abundance (50–70 µM[3,4]) and their respective standard redox potentials ($E'^0_{O2/H2O} = 0.815$ and $E'^0_{DHA/Ascorbate} = 0.08$)[5].

In this report, we confirmed experimentally that plasma is poorly oxygenated and revealed that ascorbate contributes to its low oxygenation level, by reducing O_2. The impact of plasma "physiological hypoxia" on circulating cells' physiology and plasma components' stability has been further investigated.

Results

Blood plasma is poorly oxygenated. In order to quantify the blood plasma oxygen level, we aimed at avoiding non-physiological oxygenation of samples. All commercially available blood collection tubes contain a significant amount of oxygen, since they are sealed under atmospheric conditions (Fig. 1A, 75.7 ± 4.6 mmHg; 9.9 ± 0.6% O_2). To avoid blood experimental oxygenation, we designed and produced tubes containing a limited amount of oxygen (Fig. 1A, 15.9 ± 2.9 mmHg; 2.1 ± 0.4% O_2) hereafter termed Hypoxytube. These non-commercial prototype tubes were produced by the Greiner BioOne company. As opposed to commercial tubes, hypoxic tubes were sealed under a nitrogen atmosphere, hence limiting their oxygen-content. Immediately after blood collection, oxygen level in plasma was quantified with a needle sensor in commercial tubes or Hypoxytubes (Fig. 1B). Plasma pO_2 was 9.8 ± 4.8 mmHg (1.3 ± 0.6% O_2) in commercial tubes versus 8.4 ± 1.0 mmHg

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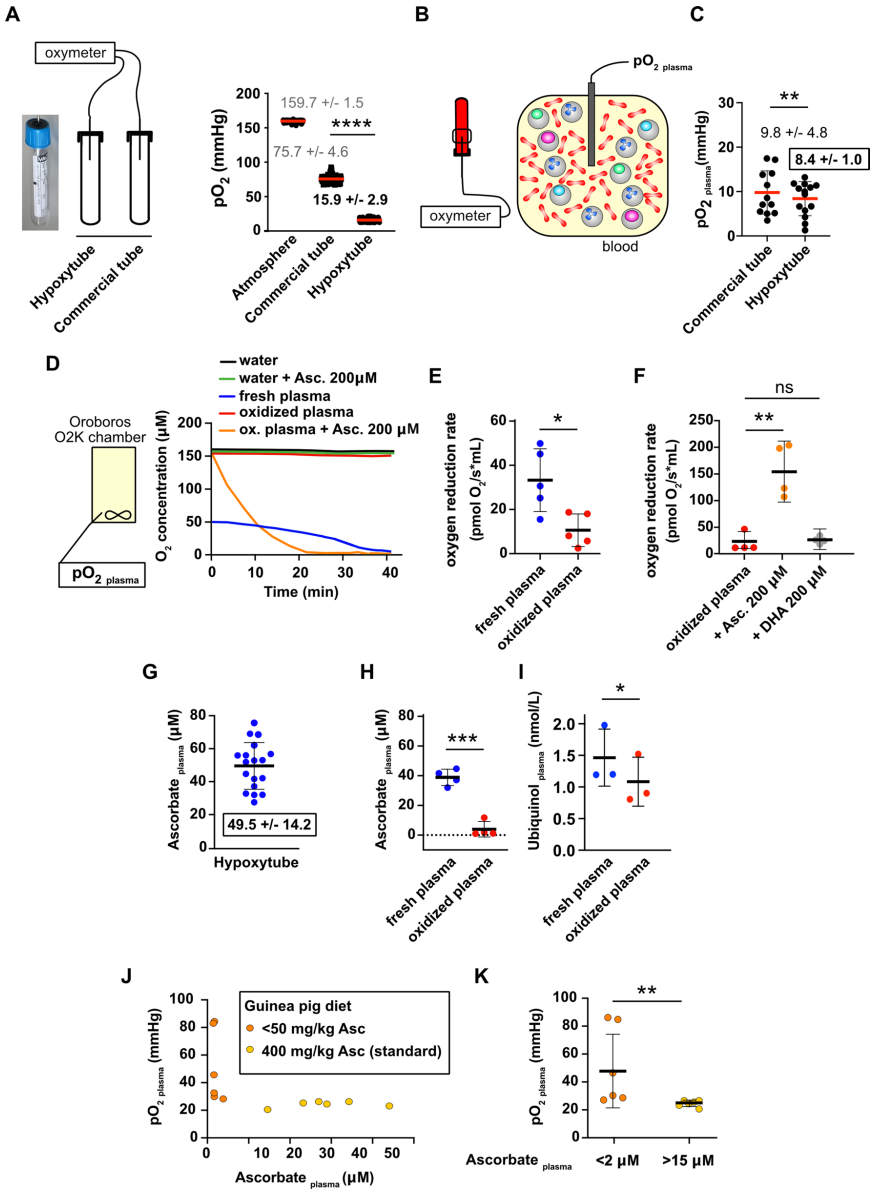

Figure 1. The plasma oxygen level is low, mainly sustained by the ascorbate oxygen. (**A**) Blood collection tubes containing a limited amount of oxygen (Hypoxytubes; picture) have been designed and validated using an oximeter with a microsensor equipped with a steel needle. Commercial tube used as a control was BD Vacutainer K2E (EDTA). Results are expressed as Mean ± S.D.; **** indicates $p < 0.0001$, $n = 20$ (tubes). (**B–C**) Plasma oxygen level was directly quantified in whole venous blood collected in commercial tube or Hypoxytube. Plasma pO_2 quantifications are expressed as Mean ± S.D.; ** indicates $p < 0.01$, $n = 12$ individual donors. (**D**) Plasma samples were loaded in closed cuve to record the time-dependent oxygen availability in fresh plasma, oxidized plasma and water, supplemented or not with 200 µM ascorbate (representative experiment). (**E**) Plasma oxygen reduction rates were quantified in fresh or oxidized plasma samples, as described in (**D**).

Results are expressed as Mean ± S.D.; * indicates $p < 0.05$, n = 5 individual samples. (**F**) The impact of oxidized plasma supplementation with ascorbate or dehydroascrobate (DHA) (200 µM) was quantified as described in (**E**). Results are expressed as Mean ± S.D.; ** indicates $p < 0.01$, n = 4. (**G–H**) Plasma ascorbate concentration in fresh samples was quantified as described in Methods, in blood samples collected in Hypoxytubes (**G**, n = 18 individual samples). The impact of plasma oxygenation on ascorbate concentration is shown in (**H**, n = 4). Results are expressed as Mean ± S.D.; *** indicates $p < 0.001$. (**I**) Ubiquinol concentration in fresh and oxidized plasma samples was quantified, together with other plasma components (see Figure S1). Results are expressed as Mean ± S.D.; * indicates $p < 0.05$, n = 3 individual samples. (**J–K**) The impact of plasma ascorbate deficiency on the control of the oxygen level has been investigated in vivo in guinea pigs (**J–K**). Plasma ascorbate concentration and pO_2 was recorded in animals fed standard (400 mg ascorbate/kg) or ascorbate-deficient diet (< 50 mg ascorbate/kg) (**J**). Plasma pO_2 was average in each group (plasma ascorbate < 2 µM (deficient) and > 15 µM (control)). Results are expressed as Mean ± S.D.; ** indicates $p < 0.01$, n = 6 animals.

(1.1 ± 0.1% O_2) in Hypoxytube ($p < 0.01$): the latest value being the most accurate quantification of the plasma oxygen level (Fig. 1C).

Ascorbate sustains a plasma oxygen-reduction activity. When fresh plasma pO_2 was recorded in a closed chamber (Oroboros), a continuous decrease was observed until anoxia was reached (Fig. 1D). This reaction was significantly lower in oxidized plasma or in water (Fig. 1D,E and S1A). These results strongly suggested that an oxidation-sensitive plasma component was supporting its oxygen-reduction capacity. We hypothesized that plasma ascorbate may play a central role in this reaction. Indeed, the supplementation of oxidized plasma with 200 µM ascorbate restored its oxygen reduction activity, not with dehydroascrobate (DHA) (Fig. 1D,F), supporting this hypothesis. However, this reaction does not occur in water (Figure S1A), indicating that the ascorbate-dependent oxygen reduction involved other plasma redox components. Additionally, we demonstrated that plasma ascorbate concentration (49.5 ± 14.2 µM, Fig. 1G) was drastically reduced in oxidized plasma ($p < 0.001$, Fig. 1H). The concentration of ubiquinol, another oxidation-sensitive plasma component was significantly lower in oxidized plasma ($p < 0.05$, Fig. 1I); in association with an increase of its oxidized form (ubiquinone) (Fig. 1I). The concentration of other tested plasma components was unchanged upon plasma oxygenation (salts, proteins or additional oxidation-sensitive components (α-tocopherol, γ-tocopherol, Figure S1B–D).

We confirmed the ascorbate-dependent plasma oxygen reduction capacity in vivo, in guinea pigs, which, like humans, do not synthesize ascorbate. When animals were fed a standard diet, the plasma ascorbate concentration was higher than 15 µM and the plasma pO_2 controlled at a low level (24.11 ± 2.23 mmHg; 3.17 ± 0.29% O_2; Fig. 1J–K). These values are higher compared to human plasma, probably due to technical reasons (time between blood collection and pO_2 measurement). When animals were fed an ascorbate-deficient diet, the plasma ascorbate concentration was lower than 2 µM and the plasma pO_2 no longer maintained at a low level (50.40 ± 26.32 mmHg; 6.63 ± 3.46% O_2) (Fig. 1J–K). Altogether these results confirm the in vivo contribution of ascorbate to the maintenance of a low plasma oxygen level.

Circulating leucocytes sense plasma low-oxygenation—mitochondrial network. The adaptation of circulating leucocytes to plasma low oxygen level has not been investigated previously. In other cell-types, it has been reported that under hypoxic conditions, mitochondrial abundance and oxygen consumption is reduced[6–8]. We confirmed by immunofluorescence (Fig. 2A) and flow cytometry (Fig. 2B–D) that, compared to two different cell lines (HEK293T and HEp-G2) cultured under atmospheric conditions (21% O_2), the mitochondrial abundance of leucocytes (granulocytes, monocytes and lymphocytes) was significantly reduced (ANOVA, Fig. 2C–D). These results strongly suggest that leucocytes evolve under low oxygen conditions in the blood plasma fraction.

Further experiments conducted in vitro at 1% O_2, better reflecting the plasma physiological conditions revealed in this study, will have to be performed to support our conclusions.

Discussion

We confirm here experimentally that human plasma has a low oxygen level (< 8.4 mmHg, 1.1% O_2, Fig. 1C) and that ascorbate plays a key role in its maintenance (Fig. 1D). Ascorbate is well described and a strong antioxidant in human plasma, which may either scavenge reactive oxygen species (ROS) or regenerate other plasma antioxidants[9]. Here, we described a physiological and ultimate consequence of the ascorbate reactivity: the dissolved plasma oxygen reduction. Plasma ascorbate is highly susceptible to plasma oxygenation and subsequent oxidation (Fig. 1E). However, our data indicates that ascorbate does not directly react with oxygen (Figure S1A), suggesting that other plasma antioxidants may be involved in its oxygen-depletion capacity. It may be hypothesized that plasma ascorbate acts as a cofactor and increase the oxygen-reduction ability of other plasma components. As an example, ascorbate can bind to human serum albumin, another major antioxidant in the circulation[10]. Further investigations will be required to decipher the overall partners and reactions.

Interestingly, plasma ascorbate concentration is relatively low in plasma (micromolar range, here 50 ± 14 µM, Fig. 1F) compared to human body cells and tissues (millimolar concentrations). Nevertheless, plasma ascorbate concentration is tightly controlled, severe ascorbate deficiency (< 5 µM) is associated with scurvy. We observed in guinea pigs that in these conditions, the plasma low oxygen level was no longer maintained (Fig. 1K). The overall physiological consequences of this regulation defect will have to be further investigated (e.g. red blood cell hemoglobin saturation rate, tissue oxygenation efficiency, integrity of other plasma components, leucocyte

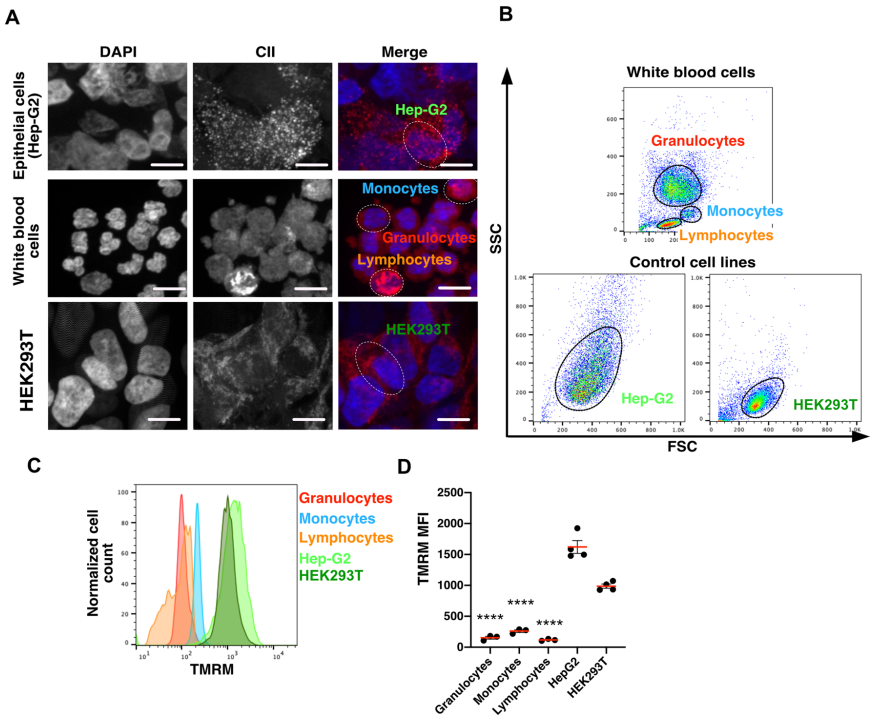

Figure 2. The mitochondria abundance is reduced in circulating leucocytes in low-oxygenated plasma. (**A**) Immunofluorescence staining of white blood cells (WBCs: monocytes, lymphocytes and granulocytes) Hep-G2 cells, and HEK293T cells using anti-CII (mitochondria, red) and DAPI (nuclei, blue). Bars are 10 µm. (**B**) Flow cytometry analysis of WBCs (granulocytes, monocytes and lymphocytes), Hep-G2 and HEK293T. Representation of SSC and FSC profiles. **(C)** TMRM (mitochondria) intensity profiles in granulocytes, monocytes, lymphocytes, Hep-G2 and HEK293T cells (representative experiment) (**D**) Quantification of TMRM mean fluorescence intensity (MFI) in cells described in (**C**). Results are expressed as Mean ± S.D.; **** indicates $p < 0.0001$ (one-way ANOVA with Tukey's test, see Tables in Figure S2A), $n = 4$ independent biological samples.

physiology, among others). In particular, in vitro experiments performed in this study (Fig. 2) were conducted at 0% O_2 due to technical limitations.

Further experiments will have to be performed at 1.1% O_2 to even better appreciate the behavior of plasma proteins (stability, oxidation) and leucocyte physiology in the blood circulation.

Plasma ascorbate concentration varies with daily oral intakes but remains controlled at relatively low levels. If 500 mg ascorbic acid/day is sufficient to maintain a physiological plasma level (50 µM), it was shown that 3 g ascorbic acid oral intake every 4 h leads to a maximal plasma ascorbate concentration of only 220 µM[11]. Millimolar plasma ascorbate concentrations may only be reached upon intravenous administration, as currently investigated in the treatment of various cancers, based on the selective cytotoxicity to tumor cells in vitro[12]. Currently, the impact of such high ascorbate concentrations on the plasma pO_2 is unknown and will have to be determined. In addition, increased plasma ascorbate concentrations have been shown to be associated with an increased production of ascorbate free radicals, a byproduct of self-oxygenation[13]. These free radicals have been proposed to react with transient metal (such as copper and iron), leading to deleterious hydroxyl radical production via the Fenton reaction.

Overall, blood plasma low oxygenation level should be better considered for basic research, diagnostics and therapeutic applications. As an illustration, this statement is critical during blood products collection and preservation prior transfusion to avoid detrimental impact on their quality[14,15].

Methods

Blood collection tubes. Blood samples were collected either in commercial collection tubes (BD Vacutainer K2E (EDTA), ref 368,861) or in Hypoxytubes developed in collaboration with the Greiner Bio One (GBO) company, containing a limited amount of O_2. (tubes were sealed under a nitrogen atmosphere). Internal pO_2 was quantified in commercial tubes and in Hypoxytubes using an oximeter with a microsensor equipped with a steel needle (Unisense).

Blood collection. All participants gave written informed consent and all the study procedures were carried out in accordance with the Declaration of Helsinki principles. Human blood was collected from healthy patients at the ICAReB service of the Pasteur Institut (authorization No. 2020_0120). All donors required to rest in a sitting position for a few minutes before the sampling.

Cell culture. HEK293T (ATCC CRL-1573) and Hep-G2 (ATCC HB-8065) were cultured in DMEM + 8% SVF. Cells were seeded onto 24-well plates and incubated 24 h at 37 °C at 0% (anoxic cabinet) or 21% O_2.

White blood cells (WBCs) were purified form whole blood in an anoxic chamber by the addition of a 6% dextran solution (30 min, RT). The WBC-containing supernatant was collected and resuspended in RPMI 1,640 (Thermofisher); remaining red blood cells were eliminated with a lysis buffer.

Cells were fixed in paraformaldehyde (PFA) 3.3% for immunofluorescent labelling or labeled with fluorescent marker for flow cytometry analysis, as previously described[16].

Plasma pO_2 measurement and components' dosage. Immediately after blood collection, the plasma pO_2 was measured directly in the blood collection tube using an oximeter with a standardized microsensor equipped with a steel needle (Unisense), as previously described[17].

Following centrifugation for 5 min at 2,000×*g*, the plasma was acidified with an equal volume of 10% (w/v) metaphosphoric acid (MPA) containing 2 mmol/L of disodium-EDTA. Ascorbate concentration was quantified by high-performance liquid chromatography with coulometric detection, as described previously[18]. Likewise, using high-performance liquid chromatography with coulometric detection, α- and γ-tocopherol were analyzed as described by Sattler et al.[19], and ubiquinone and ubiquinol as described elsewhere[20].

Plasma potassium, calcium, magnesium, albumin, fibrinogen, Factor V and Factor VIII were quantified by a medical laboratory (Cerballiance, Paris, France).

Plasma oxygen reduction rate quantification. Plasma oxygen consumption rate was measured with an oximeter (Oroboros O2k-FluoRespirometer). Immediately after blood collection, samples were centrifuged, and plasma fractions were loaded in closed cuves (2 mL). Oxygen consumption fluxes were assessed when reaching constant values. Experiments were conducted with fresh plasma and after oxidation (exposure to atmospheric air: at least 30 min on a rotator mixer).

Mitochondria study. *Imaging.* Mitochondria were immunolabeled with anti-SDHA antibody (ab14715, Abcam) and a conjugated Alexa Fluor-568 (2,124,366, Invitrogen); nuclei with DAPI. Cell imaging was performed with a confocal microscope (Leica DM5500 TCS SPE).

Flow cytometry. Cells were resuspended in PBS + 2 mM EDTA, labeled with 100 nM TMRM (T5428, Sigma-Aldrich) and analyzed with FACSCcalibur (BD Biosciences). Data were quantified with the FlowJo software (FlowJo, LLC).

Guinea pig plasma analysis. 3-week Dunkin–Hartley guinea pigs (Charles River) were fed for fifteen days with a standard diet (400 mg ascorbate/kg, Safediet ref. 106) or an ascorbate-deficient diet (< 50 mg ascorbate/kg). Blood samples were collected in Hypoxytubes; plasma ascorbate concentration and pO_2 were determined as described above. Procedure approved by the Institut Pasteur ethics committee (auth. n°190127).

Statistics. Data were analyzed with the Prism 8 software (GraphPad). ANOVA or Student T-test were performed to analyze the different datasets.

Received: 5 March 2020; Accepted: 11 June 2020
Published online: 30 June 2020

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Acknowledgements
This work was supported by the French National Research Agency: ANR JCJC 2017–17-CE15-0012 (BSM). We thank Dr. Naomie Taylor (NIH), Pr. Cormac Taylor (University Dublin) and Dr. Nicolas Prat (Institut de Recherche Biomédicale des Armées) for their critical and challenging reviews of the manuscript.

Author contributions
L.I. conducted quantitative analysis of the data. M.S. performed experiments. J.L. quantified plasma ascorbate in plasma samples. L.I., A.D. and S.R. contributed to data interpretation. B.S.M. designed the study, performed the experiments with L.I., interpreted the data, and wrote the manuscript. All authors critically edited the draft manuscript and approved the final version.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-67778-w.

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2) DONNEES SUPPLEMENTAIRES DE L'ARTICLE 2 :

Supplementary table 1 : characteristics of SLE patients recruited in the study.

Patient number	Age	Sex	Clinical feature (history)	Anti-dsDNA	Other antibodies	Low C3/C4	Current SLEDAI	Associated treatments	Prednisone dose (mg/day)
1	26	F	C,M,R,S	+	Sm/RNP,SSA	+	21	HCQ,ASP	50
2	55	F	H, R, S	+	ApL	+	16*	ASP,CYC	60
3	16	F	M, S	+	SSA, SSB,ApL	+	11	BEL,HCQ,MMF	5
4	43	F	M, C	-	SSA, SSB	-	8	HCQ	5
5	34	F	R	-	SSA, Sm/RNP	+	18*	MMF,HCQ	10
6	32	F	M	-	ApL	-	0	HCQ,	5
7	27	F	C, R	+	-	+	4	THALI,HCQ	5
8	45	M	C,M,R	+	-	+	9	HCQ	0
9	39	F	C,M	+	-	+	4	HCQ,MTX	0
10	31	F	C,S	-	ApL	-	0	-	10
11	56	M	M,S	+	-	-	6	-	0
12	77	M	C,H,M	-	SSA, SSB	+	7	HCQ	0
13	66	M	C,M	-	-	-	0	HCQ,ABA	5
14	22	F	S	+	SSA	-	4	HCQ	10
15	19	F	C,H,M,R	-	Sm,SSA,ApL	-	1	ASP,BEL	5
16	20	F	C,M,R	+	Sm,SSA	+	4	HCQ,MMF,BEL	5
17	21	M	M	+	-	-	2	HCQ	7.5
18	22	F	C,M	-	SSA,ApL	-	0	ASP	0
19	23	M	C,S	-	Sm/RNP,SSA	-	0	HCQ,MTX	5
20	25	F	C,R,S	+	-	-	2	ASP,	0
21	27	F	C,R	+	ApL	-	10	HCQ	0
22	28	F	C	-	SSA	+	12	HCQ	5
23	20	F	C,M	+	-	-	2	-	0
24	41	F	C,M,S	+	Sm/RNP,SSA,ApL	+	10	HCQ,CYC	10
25	62	F	C,M,R	-	Sm/RNP	-	0	HCQ, CYC	20
26	19	F	H,M	-	SSA	-	0	HCQ	5
27	48	F	C,M	+	SSA,RNP	+	4	HCQ	10
28	55	M	M,S	+	-	+	6	HCQ	0
29	43	F	C,M,R	-	SSA	-	0	ASP,MMF	0
30	42	F	C,M,R	+	Sm/RNP	+	4	HCQ,BEL	6
31	57	F	C,M	+	-	-	7	-	0
32	31	F	M,R	-	-	-	0	CIC,HCQ	5
33	45	F	C,M,R	-	-	-	0	HCQ	0
34	44	F	C,R,S	+	SSA	-	6	HCQ	5
35	37	F	S,R	-	SSA	-	2	-	0
36	38	M	C,M,R	+	-	-	2	HCQ,MMF	5
37	55	F	M	-	SSA,SSB	-	0	HCQ	7.5
38	42	F	C,H,M	+	RNP	+	4	HCQ	5
39	35	M	C,M,R	-	-	-	0	HCQ	5
40	53	M	M	-	ApL	-	0	HCQ	0
41	48	M	R	+	-	-	2	HCQ,MMF	4
42	61	F	C,M,S	+	SSA	+	4	HCQ,MMF	5
43	43	F	C,M,R	-	-	-	0	HCQ	0
44	36	F	M	+	RNP	-	0	HCQ	5
45	47	F	R	+	SSA	+	4	HCQ	0
46	40	F	C,H,M,R	-	-	+	2	-	0
47	50	M	C,R,S	+	SSA, ApL	-	6	HCQ, MTX	90
48	71	M	C,	-	ApL	-	0	CLOPI,MMF	0
49	51	F	C,M,R	-	SSA, ApL	-	0	MMF	5
50	48	F	C,M,R	-	Sm/RNP, ApL	-	0	HCQ	0
51	36	F	C,M	+	Sm/RNP, SSA	+	4	HCQ	0
52	33	F	M	+	Sm/RNP	+	8	MTX	7
53	46	F	C,M,R	+	-	-	8	-	0
54	54	M	M	-	SSA,SSB	-	7	-	0
55	27	F	R,S	+	-	-	10	-	0
56	21	F	C,H,M	+	SSA,RNP,ApL	+	15	HCQ,MTX	3
57	38	F	C,H	+	SSA	+	6	HCQ	0
58	50	F	C,S	-	Sm/RNP	-	6	-	70
59	35	F	C,M,R	-	SSA,ApL	+	8	HCQ,MTX	6
60	27	F	C,H,M,R	-	Sm/RNP	+	4	HCQ,MMF	5
61	27	F	C,M,S	+	ApL	-	4	HCQ,MMF	5
62	20	F	C,M,R,S	+	Sm/RNP	+	26*	HCQ	0
63	53	M	C,S	-	ApL	-	0	HCQ	30
64	61	F	C,H,M,R,S	+	Sm/RNP,SSA	+	24*	HCQ	5
65	20	F	M,R,S	+	Sm/RNP	+	22*	HCQ	0

66	31	F	M,R,S	+	-	+	20*	HCQ,AZA	9
67	45	F	M	+	Sm/RNP	+	8	-	40

Clinical features : C, cutaneous; H, haematological; M, musculoskeletal; N, neurological; R, renal; S, serosal.
Treatments : ABA, abatacept; ASP, low-dose aspirin; AZA, azathioprine; BEL, belimumab; CIC, ciclosporin; CLOPI, clopidogrel; CYC, cyclophosphamide; HCQ, hydroxychloroquine; MMF, mycophenolate mofetil; MTX, methotrexate; THALI, thalidomide.
Antibodies : ApL, antiphospholipid antibody.
*, patients with active renal disease, as defined by the SLEDAI.

Supplementary table 2 : antibodies used in the study.

Antibody	Clone	Company (reference)	Dilution
Blood phenotyping (human)			
CD3-APCCy7	UCHT1	Beckman coulter© (A94680)	1/40
CD4-FITC	RPA-T4	BD©(555346)	1/40
CD8-PC7	RPA-T8	BD©(557746)	1/40
CD45RA-ECD	2H4LDH11LDB9	Beckman coulter© (IM2711U)	1/40
CD25-PC7	B1.49.9	Beckman coulter© (A52882)	1/40
CD127-PC5	R34.34	Beckman coulter© (A64617)	1/40
CCR6-APC	REA190	Miltenyi Biotec© (130-117-375)	1/40
CXCR3-BV421	G025H7	Biolegend©(353716)	1/40
CXCR5-BV711	RF8B2	BD©(740737)	1/40
Lin2-FITC	N/A	BD©(643397)	1/40
CD11c-APCVio770	MJ4-27G12	Miltenyi Biotec© (130-100-238)	1/40
CD123-APC	AC145	Miltenyi Biotec© (130-090-901)	1/40
HLADR-PC7	B8.12.2	Beckman coulter© (B49180)	1/40
CD14-FITC	RMO52	Beckman coulter© (B36297)	1/40
CD66b-APC	REA306	Miltenyi Biotec© (130-117-692)	1/40
CD19-PC7	J3-119	Beckman coulter© (IM3628)	1/40
PSGL1-PE	KPL-1	BD© (556055)	1/40
GITR-BV605	V27-580	BD©(747664)	1/40
CD15s-BV421	CSLEX1	BD©(563912)	1/40
FoxP3-PE	236A/E7	Invitrogen©(12-4777-42)	1/40
BCL6-AF647	K112-91	BD© (561525)	1/40
Treg phenotyping (human)			
GARP-BV421	7B11	BD©(563956)	1/100
Helios-AF488	22F6	BD©(563950)	1/40
Tbet-BV786	O4-46	BD©(564141)	1/40
GATA3-AF647	L50-823	BD©(560068)	1/40
RORgt-PE	AFKJS-9	Ebiosciences © (12-6988-82)	1/40
IL17A-PE	CZ8-23G1	Miltenyi Biotec© (130-099-420)	1/40
Mouse T cell differentiation (mouse)			
CD4-BV786	RM4-5	BD© (563727)	1/200
FoxP3-PE	FJK-16S	Ebiosciences © (12-F773-82)	
Tbet-PE	EBio4B10	Ebiosciences © (12-5825-82)	

3) DONNEES SUPPLEMENTAIRES DE L'ARTICLE 3 :

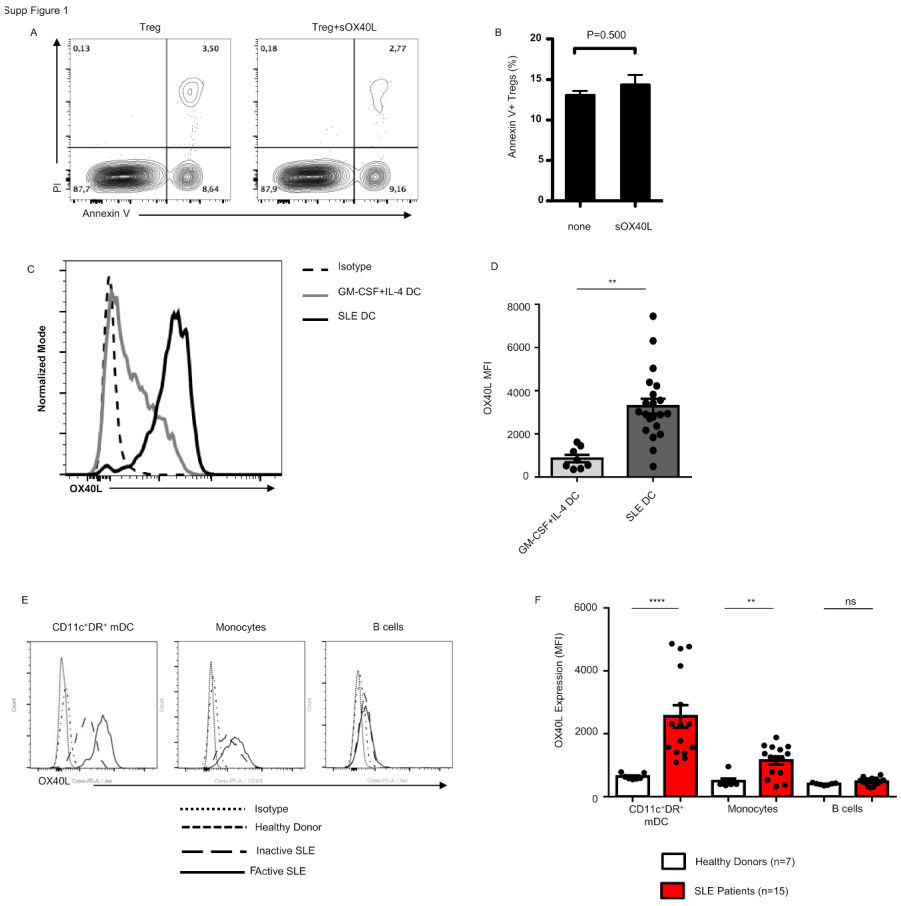


Figure S1: Recombinant sOX40L does not induce Treg cell death and Purified CD14+ monocytes cultured in the presence of SLE sera (SLE-DC) or ex-vivo SLE-mDC expressed OX40L.

(**A-B**), Sorted $CD4^+CD25^+CD127^-$ Tregs cells were cultured with or without sOX40L (100 ng/mL) and Annexin-V/PI staining was performed after 3 days of culture for the assessment of cell death. (**A**), Representative dot plot of Treg Annexin-V/PI staining after 3 days of culture. Annexin-V^+/PI^+ cells were considered as dead cells. (**B**), Cumulative data showing the percentage of dead cells after 3 days of Tregs culture. Statistical analyses were conducted using the two-tailed Mann-Whitney U-test. **(C-D)**, Purified $CD14^+$ monocytes from healthy donors were cultured with GM-CSF+IL-4 or SLE Sera (SLE-DC) for 4 days. OX40L expression was assessed by flow cytometry. (C) Representative staining for OX40L expression. (D). Cumulative results for OX40L-expression (as expressed by MFI) in both conditions. GM-CSF+IL-4 DC (n=8), SLE-DC (n=21). Cumulative data are shown with S.E.M and P value < 0.01 (**). **(E-F)** Ex-vivo OX40L expression level was assessed within circulating APCs such as $CD11c^+DR^+$ mDC, CD14+ monocytes and CD19+ B lymphocytes cells. 7 healthy donors and 15 SLE patients (including 9 active (SLEDAI ≥ 6 and 7 quiescent (SLEDAI < 6) SLE) APCs were analyzed by flow cytometry. The figure shows a representative staining and cumulative data represented as mean with S.E.M. Data are compared using non parametric Mann-Whitney test. ** P<0.01, ****P<0.001.

Supp Figure 2

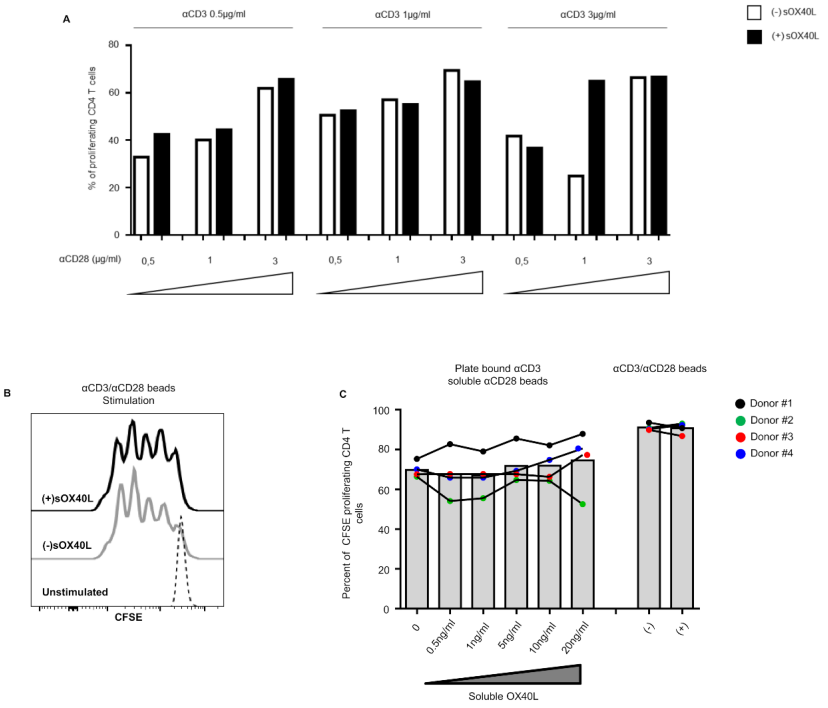


Figure S2: Effect of sOX40L on effector CD4 T cells proliferation alone

(**A**) Effect of sOX40L on the proliferative capacity of Effector CD4+ T cells following activation with anti-CD3 and anti-CD28 at different concentrations. Purified CD4 T cells were stained with CFSE and stimulated with or without agonist soluble OX40L (100ng/ml) for 4 days with different concentration of pre-coated anti-CD3 and soluble anti-CD28. The figure shows one of two independent experiments.
(**B**) Effect of soluble OX40L (100ng/ml) co-stimulation on effector CD4 T cells proliferation stimulated by anti-CD3 and anti-CD28 micro-beads. This figure is representative of 4 independent experiments.
(**C**) Dose effect of soluble OX40L co-stimulation (100ng/ml) on purified effector CD4 T cells stimulated by pre-coated anti-CD3 (1ug/ml) and soluble anti-CD28 (3ug/ml) or anti-CD3+anti-CD28 beads for 4 days. 4 independent experiments were realized using 4 different effector CD4 T cells donor (as show by color plots).

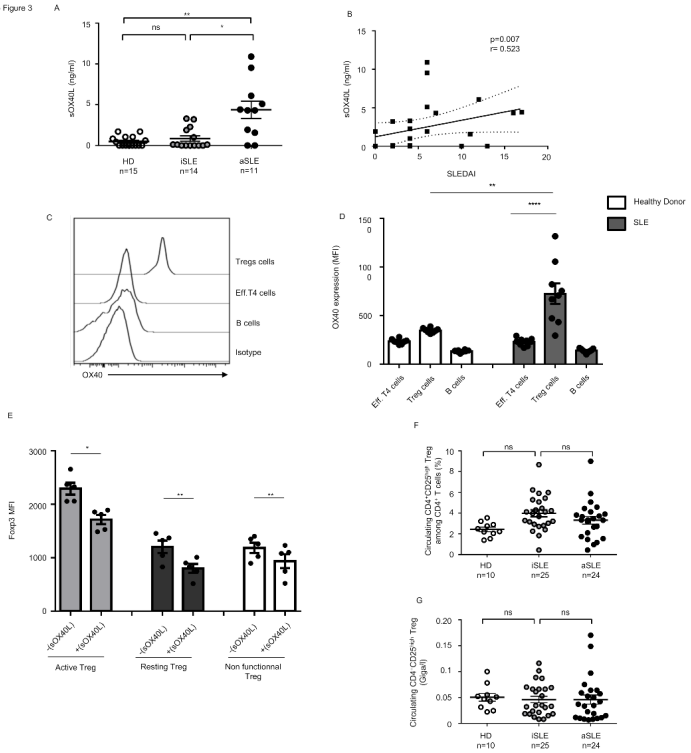
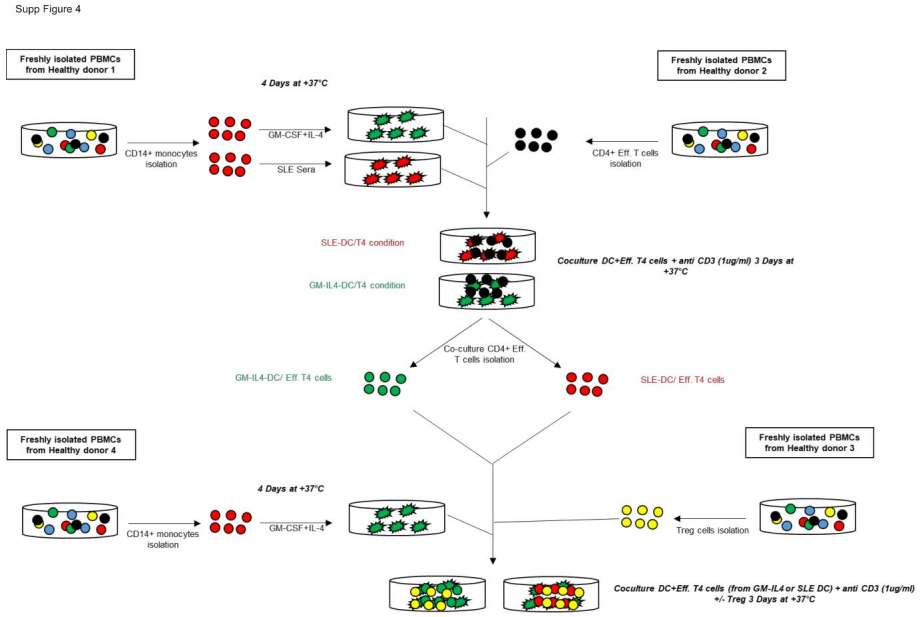


Figure S3: Blood CD4⁺CD25^{high} Tregs and serum concentration of sOX40L in healthy donors and SLE patients.

(**A**) Serum concentration of sOX40L (in ng/ml) from HD (n=15), iSLE (n=14) and aSLE patients (n=11). Individual values are given with mean and S.E.M, and compared using non-parametric Kruskall-Wallis test with Dunn's correction for multiple comparisons. P-value < 0.05 (*), P-value < 0.01 (**).(**B**) Correlation between sOX40L concentration in serum and SLE activity (SLEDAI), using Spearman rank correlation test. (**C-D**)**,** Peripheral Effector CD4 T cells, Tregs, and B cells were investigated for OX40 expression by flow cytometry. PBMCs were isolated and stained for OX40 expression from Healthy donors (n=8) and SLE patients (n=9). **(C)** Representative staining and **(D)** OX40 MFI values with S.E.M are shown and compared using one-way ANOVA with Holm-Sidak's correction for multiple comparisons. P value <0.01 (**), p<0.0001 (****). **(E),** sOX40L impacts Foxp3 expression in different Treg subset. Purified Tregs with or without sOX40L (100ng/ml) pre-treatment were cultured in pre-coated anti CD3 (1ug/ml) supplemented by soluble anti-CD28 (3ug/ml) in 96 wells plate for 3 days. Intra-nuclear Foxp3 expression was assessed by flow cytometry on different Treg sub-population based on CD25 and CD45RA cell surface expression: active Tregs (CD45RA⁻CD25^{high}), resting Tregs (CD45RA⁺CD25⁺) and non-functional Tregs (CD45RA⁻CD25⁺) subset. 5 different donors were studied in 2 independent experiments. Individual data are shown with mean and S.E.M, and compared using non-parametric two-tailed Mann-Whitney test, p<0.05 (*), p<0.01 (**). **(F-G)**, Frequency of blood CD4⁺CD25^{high} Tregs in HD (n=10) and SLE patients with inactive (iSLE, n=25) and active (aSLE, n=24) disease. **(F)** Results are expressed as % of CD4⁺CD25^{high} cells among CD4⁺ T cells, and are compared using non-parametric Kruskall-Wallis test with Dunn's correction for multiple comparisons. **(G)**, Absolute count of CD4⁺CD25^{high} Tregs cell in blood of HD (n=10) and iSLE (n=25) and aSLE patients (n=24). Results are expressed in Giga/L and are compared using non-parametric Kruskall-Wallis test with Dunn's correction for multiple comparisons. iSLE, patients with inactive disease (SLEDAI<6); aSLE, SLE patients with active disease (SLEDAI≥6).

Figure S4: Schematic showing experimental design for experiment in Figure 3A.



Supp Figure 5

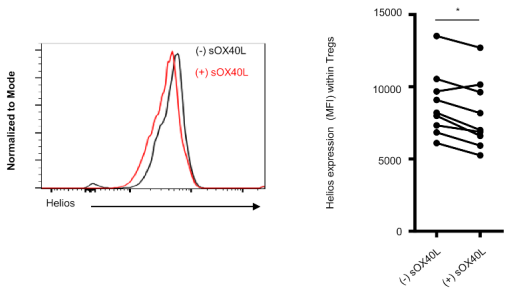


Figure S5: Tregs intranuclear Helios expression following OX40L co-stimulation

Isolated Tregs from healthy donors were cultured using pre-coated anti-CD3 (1ug/ml) 96 well plate with or without soluble OX40L (100ng/ml) for 48 hours. Intranuclear Helios expression level was evaluated by flow cytometry. 9 independents experiments using 9 different Tregs donors were realized. Data are shown as mean MFI and compared using two-tailed paired non-parametric Wilcoxon test. *, $p < 0.05$.

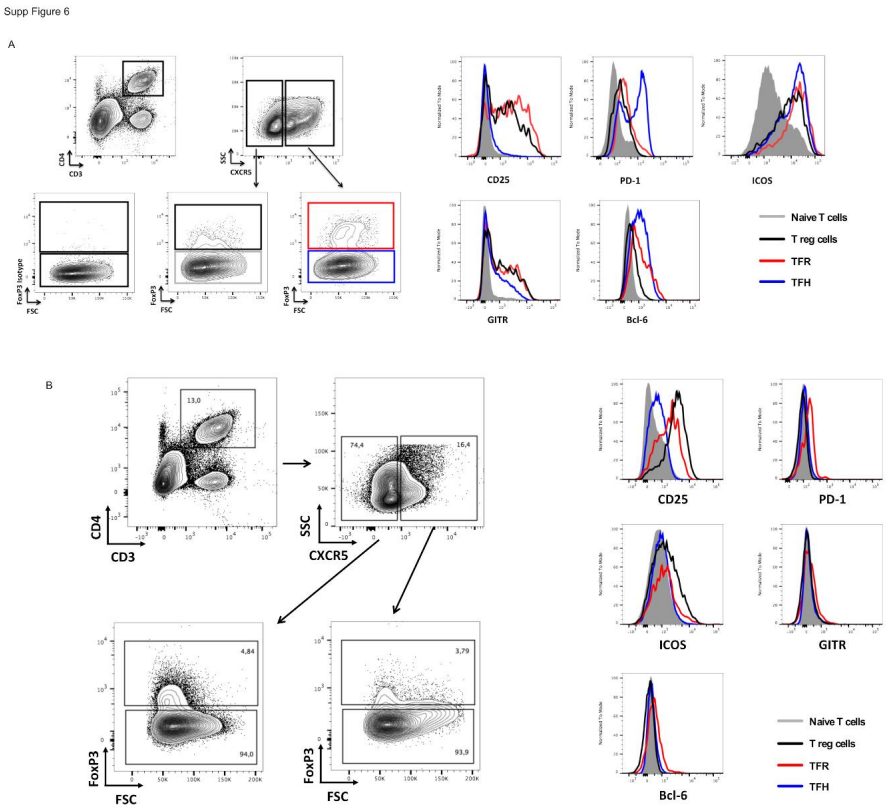


Figure S6: Cell surface expression of CD25, PD-1, ICOS, GITR and Bcl-6 of Tonsil and blood TFH, TFR, Tregs and naïve T cells in HD.

(**A, left and right panel**), cell surface expression of CD25, PD-1, ICOS, GITR and Bcl-6 in Tonsils TFH, TFR, Tregs and naïve T cells. (**A, left panel**), Representative dot plots showing $CD3^+CD4^+FoxP3^-CXCR5^+$ TFH cells (blue line), $CD3^+CD4^+FoxP3^+CXCR5^+$ TFR cells (red line), $CD3^+CD4^+FoxP3^+CXCR5^-$ Tregs cells (black line) and $CD3^+CD4^+FoxP3^-CXCR5^-$ naïve T cells (grey filled). (**A, right panel**), expression of cell surface markers according to cell subsets. Representative of one out of 2 experiments. (**B, left panel**), Representative dot plots showing $CD3^+CD4^+Foxp3^-CXCR5^+$ TFH cells (blue line), $CD3^+CD4^+Foxp3^+CXCR5^+$ TFR cells (red line), $CD3^+CD4^+Foxp3^+CXCR5^-$ Tregs cells (black line) and $CD3^+CD4^+Foxp3^-CXCR5^-$ naïve T cells (grey filled) in blood and (**B, right panel**) expression of cell surface markers according to cell subsets. Representative of one out of 2 experiments.

Supplementary Table 1: clinical and biological informations concerning SLE patients

Patient number	Age	Sex	Clinical failure	Fibre	Corticotherapy (dose, mg/day)	Associated treatment	SLEDAI
1	54	M	A, C, H, R		10	HCQ	2
2	27	F	A, C, H, N, PP, APS	A		MTX, RTX	8
3	35	F	A, H	A	20	HCQ	4
4	33	F	C, H, APS		10	HCQ, MMF	0
4	34	F	C, H, APS		10	HCQ, MMF	2
5	35	F	A, C, R		7	HCQ, AZA	2
6	35	F	A, C, H, R, APS		50	CYC	4
7	41	F	A, C, H, R	N, R	10	MPA	26
8	34	M	A, C, H, R	C, R, V			20
9	40	F	A, C, H			HCQ, MTX	0
10	32	F	A, C, H, R	A, C, R		HCQ	22
10	31	F	A, C, H, R		25	HCQ, CYC	4
10	31	F	A, C, H, R		25	HCQ, CYC	8
10	31	F	A, C, H, R	R	20	HCQ, CYC	8
11	41	F	A, H, APS	R	3	HCQ, MMF	4
12	72	M	C, H, N, PP, R	C, N, PP, R		HCQ	24
13	36	F	A, C, R		10	AZA	4
14	22	F	A, C, R			AZA	2
15	22	F	A, C, H, R	R	2,5	MMF	12
16	23	M	H, R	R		MMF	8
16	22	M	H, R			MMF	4
17	60	M	A, H, N, R	A, N, R	10	HCQ	19
17	58	M	A, H, N, R		10	HCQ	2
17	59	M	A, H, N, R		5	HCQ	2
18	25	F	A, C, H, PP, APS		30	HCQ, RTX	4
18	25	F	A, C, H, PP, APS		25	HCQ, RTX	4
18	25	F	A, C, H, PP, APS	A	40	HCQ, RTX	8
19	18	F	A, C, H, R	A, C, N, R	20	HCQ, MMF	24
19	19	F	A, C, H, R		30	MPA	0
19	19	F	A, C, H, R		7	MPA	0
19	20	F	A, C, H, R	A	6	MPA	8
20	40	F	A, C, H, R		6	HCQ, MPA	2
21	58	F	A, C, R, APS		6	MMF	2
22	42	F	A, C, H		3	HCQ, MTX	4
23	44	M	A, H, R	R, V			33
24	18	F	A, H, PP, R		8	MMF	4
25	27	F	H				3
26	48	F	A, C, R	A	10	HCQ, MMF	8
26	46	F	D, H, M, PP, R	R	40	AZA	16
26	46	F	D, H, M, PP, R		20	MMF	4
26	46	F	D, H, M, PP, R		5	HCQ, MMF	4
27	39	F	A, H, N, R, APS		5	HCQ, AZA	2
28	35	F	A, C, H, N	A	10		8
28	35	F	A, C, H, N	A	15		8
28	28	F	A, C, H, N		7,5	AZA	4
29	58	F	A, H	A	10	AZA	8
29	57	F	A, H	A	15	HCQ, AZA	6
30	35	F	A, C, H, M, N, R	A, R	20	HCQ, MMF	16
31	58	F	A, C		17,5	HCQ	2
32	25	F	A, H	A	17,5	MTX	4
32	25	F	A, H	R	10	MTX	8
33	38	F	A, H, R			MMF	16
34	17	M	A, C, PP, R	R	10	HCQ, MMF	12
35	21	F	A, C, APS			HCQ	4
35	21	F	A, C, APS			HCQ	4
36	19	F	A, C, R	R	5	HCQ, MMF	8
37	39	F	A, C, R		3	MMF, RTX	2
38	51	F	A, H, R	A	10	Abatacept	8
38	51	F	A, H, R	R	8	Abatacept	8
38	52	F	A, H, R	R	9	Abatacept	12
38	52	F	A, H, R	A	10		8
39	57	F	A, H, PP, R	R	5	HCQ	17
39	56	F	A, H, PP, R	R	20	HCQ	4
40	65	F	H, APS				0
41	38	F	A, C, PP	C	5	HCQ	6
42	34	F	A, C, H, N		10	HCQ, MMF	4
43	28	F	R, H, A	R	5	HCQ, MMF	9
44	63	F	C, A, R	H, A	5	HCQ, AZA	22
45	22	F	C, A	PP	30	HCQ, CYC	23
46	37	M	A, R, D		5	MMF	2
47	37	F	A		7	HCQ	2
48	57	F	A		5	HCQ	4
49	53	F	A		5	MTX	3
50	80	F	A, N, PP		9		4
51	20	F	R			HCQ, MTX	2
52	41	F	C, A, R				8
53	43	F	A	A	20		8
54	50	F	H			HCQ	4
55	55	F	A, C			MMF	4
56	27	F	A, R				16
57	25	F	A, R, H, PP		12,5	AZA	23
58	18	F	A, H	A	10	HCQ	8
59	35	F	A		10	HCQ	4
60	37	F	A, C	A	10	HCQ	14
61	41	F	A, C, H	A, R	5	HCQ	25

Legends F: female, M: male, A: articular, C: cutaneous, D: digestive, H: haematologic, M : myocardic, N: neurologic, PP: pleuro-pericardic, R: renal, V: vascular, APS: anti-phospholipid syndrom.
HCQ: hydroxychloroquin, AZA: azathioprin, CYC: cyclophosphamide, MMF: mycophenolate mofetil, MPA: mycophenolic acid, MTX : methotrexate, RTX: rituximab

924 **Supplementary Table 2: Antibodies and clones informations**
925

Marker	Clone	Company
CD3 APC	UCHT1	Beckman Coulter
CD4 PC7	SFCI12T4D11	Beckman Coulter
CD8 FITC	B9.11	Beckman Coulter
CD16 FITC	3G8	BD Biosciences
CD11c APC	B-ly6	BD Biosciences
HLA-DR PC7	Immu-357	Beckman Coulter
CD14 PC5	RM052	Beckman Coulter
CD25 PC5	B1.49.9	Beckman Coulter
CD127 PE	R34.34	Beckman Coulter
TNFR2 PE	hTNFR-M1	BD Biosciences
CTLA-4 APC	BNI3	BD Biosciences
OX40L PE	ANC10G1	Ancell
CCR7 FITC	REA546	Miltenyi
CD56 PE	B159	BD Biosciences
GITR APC	DT5D3	Miltenyi
Foxp3 PE	236A/E7	eBioscience
CD45RA ECD	2H4LDH11LDB9	Beckman Coulter
CXCR5 AF647	RF8B2	BD Biosciences
PD-1 FITC	EH12.2H7	Biolegend
ICOS PC7	C398.4A	Biolegend
Bcl-6 AF647	K112-91	BD Biosciences
CD38 PC7	HIT2	Biolegend
CD27 PC5	O323	eBioscience
IgD FITC	IA6-2	BD Biosciences
CD19 PE	LT19	Miltenyi

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928 **Supplementary Table 3: Clinical information concerning patients used in Tregs**
929 **experiment**
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Patient number	Clinical disease activity	Age	Sex	Flare	Current organ involvement	steroids (dose, mg/day)	Associated treatments	SLEDAI	%CD11cDR OX40L
#1	Active	65	F	yes	N	0	-	8	15
#2	Active	42	F	yes	R, C, H, V, PP	80	HCQ	29	5
#3	Active	41	F	-	A	3	HCQ	4	9,7
#4	Active	35	F	yes	R	60	HCQ - MMF	16	19
#5	Active	25	F	-	A	0	MTX	4	6
#6	Quiescent	60	F	-	-	5	MTX	1	1
#7	Quiescent	58	F	-	-	15	HCQ	4	0
#8	Quiescent	27	M	-	-	0	-	4	0
#9	Quiescent	19	F	-	-	7	MMF	0	3
#10	Quiescent	25	F	-	-	30	RTX	4	1

Legends:
F: female, M: male, A: articular, C: cutaneous, D: digestive, H: haematologic, M : myocardic, N : neurologic, PP: pleuro-pericardic, R: renal, V: vascular
HCQ: hydroxychloroquine, MMF: mycophenolate mofetil, MTX : methotrexate, RTX: rituximab
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Titre de la thèse en anglais : Selectins impair regulatory T cell function and contribute to systemic lupus erythematosus pathogenesis

Résumé de la thèse en anglais :

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by a loss of tolerance toward self-nucleic acids, autoantibody production, an interferon signature, and a defect in the T regulatory cells (Tregs) compartment. In this work, we identified that platelets from active SLE patients preferentially interacted with Tregs *via* the P-selectin/PSGL-1 axis. Selectin interaction with PSGL-1 blocked the regulatory/suppressive properties of Tregs and follicular Tregs by triggering Syk phosphorylation and an increase in intracytosolic calcium. Mechanistically, P-selectin engagement on Tregs induced a downregulation of the TGF-beta axis, altering Tregs phenotype and limiting their immunosuppressive response. In patients, we found a significant upregulation of P- and E-selectin levels both expressed by microparticles and in their soluble forms that correlated with SLE disease activity. Finally, blocking P-selectin in a mouse model of SLE improved cardinal features of the disease. Overall, our results identify a selectin-dependent pathway active in SLE patients and validate it as a potential therapeutic avenue.

Keywords : Systemic lupus erythematosus; T regulatory cells; platelets; selectins; treatment

Titre de la thèse en français : Les sélectines inhibent la fonction des lymphocytes T régulateurs et contribuent à la pathogénie du lupus érythémateux systémique

Résumé de la thèse en français :

Le lupus érythémateux systémique (LES) est une maladie auto-immune systémique caractérisée par une perte de tolérance vis-à-vis des autoantigènes nucléaires, une production d'auto-anticorps, une signature interféron et une dysfonction du compartiment des lymphocytes T régulateur (Tregs). Dans ce travail, nous avons identifié que les plaquettes des patients LES actifs interagissaient de manière préférentielle avec les Tregs via l'axe P-sélectine/PSGL-1. L'interaction de la P-sélectine plaquettaire avec son ligand le PSGL-1 abolissait les fonction immunosuppressives des Tregs et des Tregs folliculaires par le biais d'une phosphorylation de Syk et d'un signal calcique intracellulaire. D'un point de vue mécanistique, l'interaction P-sélectine/PSGL-1 induisait une sous-expression de la voie TGF-béta, altérant le phénotype et les fonctions suppressives des Tregs. Chez les patients, nous avons montré une majoration significative des taux des P- et E-sélectine circulantes, sous forme soluble et microparticulaire, et ce de manière corrélé à l'activité du LES. Enfin, le blocage de la P-selectine dans un modèle murin de LES améliorait des symptômes cardinaux de la pathologies (atteinte rénale notamment). Au total, nos résultats identifient une nouvelle voie physiopathologique impliquant la P-selectine dans le LES, et ouvre la voie pour des essais thérapeutique visant à bloquer la P-sélectine dans le LES.

Mots clés : lupus érythémateux systémique ; lymphocyte T régulateur ; plaquettes ; sélectines ; traitement