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Rôle des lymphocytes T CD4^{+} régulateurs dans la suppression des réponses immunitaires anti-tumorales

Soutenue le 27 septembre 2012 devant le jury composé de :

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Résumé

La génération et/ou le recrutement de cellules immuno-suppressives fait parti des mécanismes majeurs utilisés par les tumeurs afin d'échapper aux réponses anti-tumorales du système immunitaire. Parmi les cellules capables d'inhiber les réponses anti-tumorales, les lymphocytes T $CD4^+$ régulateurs et les macrophages de type II tiennent un rôle de premier ordre dans le contexte tumoral. Au cours de ma thèse, j'ai pu étudier l'impact de ces deux populations dans la suppression des réponses immunitaires anti-tumorales dans le modèle MT/ret de mélanome spontané métastatique.

L'ensemble de nos résultats met en avant plusieurs niveaux d'immuno-suppression dans le modèle MT/ret. D'une part, les lymphocytes T $CD4^+$ régulateurs, de par leur localisation dans les ganglions drainants et dans la peau, semblent impliqués dans la suppression des réponses anti-tumorales aux localisations et aux moments où les tumeurs nécessitent une forte inhibition des effecteurs anti-tumoraux. D'un autre côté, les macrophages de type II présentent, en plus de leurs capacités immuno-suppressives, des fonctions importantes pour la croissance et la dissémination tumorale justifiant leur localisation dans le micro-environnement tumoral.

Dans un second temps, nos données suggèrent pour la première fois un rôle des monocytes Ly-$6C^{fort}$ dans le contrôle tumoral via la lyse de ces dernières ou encore le maintien de la dormance des cellules tumorales disséminées. En conséquence, nous proposons de les ajouter à la liste des acteurs immunitaires directement impliqués lors des phases d'élimination et d'équilibre de la théorie de l'immuno-éditing. De plus, nous mettons en évidence leur inhibition par les lymphocytes T $CD4^+$ régulateurs, ce qui n'avait pas non plus été décrit précédemment. Ceci nous pousse à suggérer de prendre plus en compte l'impact des lymphocytes T $CD4^+$ régulateurs sur d'autres populations immunitaires que les lymphocytes T dans le contexte tumoral

Liste des abréviations

Ac : Anticorps

ADCC : Cytotoxicité Cellulaire Dépendante des Anticorps (Antibody Dependant Cell Cytotoxicity)

Ag : Antigènes

AMPc : Adénosine Mono-Phosphate Cyclique

APC : Cellule Présentatrice d'Antigène (Antigen Presenting Cell)

ATP : Adénosine Tri-Phosphate

CD : Cluster de Différenciation (Cluster of Differentiation)

CMH : Complexe Majeur d'Histocompatibilité

COX-2 : Cycloxygénase-2

CTL : Lymphocytes T Cytotoxique (Cytotoxic T Lymphocytes)

CTLA-4 : Cytotoxic T Lymphocytes Antigen-4

DCs : Cellules Dendritiques (Dendritic Cells)

DMBA/TPA : 7,12-diméthylbenzanthracène/12-O-tetradecanoylphorbol-13-acetate

DR5 : Récepteur de Mort 5 (Death Receptor 5)

EGF : Facteur de Croissance Epidermique (Epidermal Growth Factor)

Foxp3 : Forkhead Box p3

Gal-1 : Galectine-1

GDNF : Facteur de Croissance Neurotrophique Dérivé des Cellules Gliales (Glial cell-Derived Neurothrophic Factor)

GITR : Récepteur au TNF Induit par les Glucocorticoïdes (Glucocorticoïd Induced TNF Receptor)

GM-CSF : Facteur de Croissance Granulocytaire et Monocytaire (Granulocyte Monocyte Colony Stimulating Factor)

HLA : Human Leukocyte Antigen

Hsp : Heat Shock Protein

IBD : Maladie Inflammatoire de l'Intestin (Inflammatory Bowel Disease)

ICOS : Induced Costimulation

IDO : Indoleamine 2,3 Dioxygenase

IFN : Interféron

IL : Interleukine

IPEX : Immune dysregulation, Polyendrocrinopathy, Enteropathy, X-linked syndrome

iTregs : Tregs induits

LAG-3 : Lymphocyte-Activation Gene-3

LB : Lymphocyte B

LPS : Lipopolysaccharide

LT : Lymphocyte T

MCA : méthylcholanthrene

MDSCs : Cellules Myéloïdes Suppressives (Myeloid Derived Suppressor Cells)

NK : Lymphocyte Natural Killer

NKT : Lymphocyte T Natural Killer

NO : Nitric Oxide

Nrp-1 : Neuropiline-1

nTregs : Tregs naturels

PAMP : Pathogen Associated Molecular Pattern

PD-1 : Program Death-1

PD-L1 : Program Death Ligand 1

PGE_2 : Prostaglandine E_2

PRR : Pattern Recognition Receptors

RAG : Recombination Associated Gene

ROS : Espèces Réactives de l'Oxygène (Reactive Oxygen Species)

TAMs : Macrophages Associés aux Tumeurs (Tumor Associated Macrophages)

TcR : Récepteur à l'antigène des lymphocytes T (T Cell Receptor)

TGF : Facteur de Croissance Transformant (Transforming Growth Factor)

Th : Lymphocytes T auxiliaries (T helper)

TILs : Lymphocytes Infiltrant les Tumeurs (Tumor Infiltrating Lymphocytes)

TLR : Toll Like Receptor

TNF : Facteur Nécrosant des Tumeurs (Tumor Necrosis Factor)

TRAIL : Tumor necrosis factor Related Apoptosis Inducing Ligand

Tregs : Lymphocytes T Régulateurs

VEGF : Facteur de Croissance Vasculaire Endothélial (Vascular Endothelial Growth Factor)

AVANT-PROPOS

Avant-propos : Historique de l'immunologie

L'immunologie est définie comme étant l'étude de l'immunité. C'est pourquoi la majorité des écrits relatant son histoire commence par définir l'immunité, rapidement extrapolé au concept « d'immunité contre les maladies ». Ceci explique que l'on retrouve très souvent des descriptions détaillées des travaux de Jenner, Koch et Pasteur. Or, il est à présent bien établi que l'immunologie recouvre un champ plus large que la lutte contre les agents pathogènes, et va au-delà du concept d'immunité. Je me permettrai donc de passer sous silence ces travaux que je considérerais comme « pré-immunologie », sans pour autant minimiser leur importance fondamentale dans l'émergence de cette discipline et commencer cet historique à la fin du 19ème siècle, soulignant ainsi au passage la « jeunesse » de l'immunologie.

- L'opposition entre « Cellularistes » et « Humoralistes »

Elie Metchnikoff, travaillant d'abord à l'institut de bactériologie d'Odessa puis à l'institut Pasteur, fut le premier à mettre en évidence la contribution de la phagocytose dans la mise en place de l'immunité. En étudiant le système digestif de larves d'étoiles de mer, il a observé que certaines cellules, sans relation avec la digestion, étaient capables d'engloutir les échardes qu'il avait introduites dans les larves. Il appela ces cellules phagocytes, d'après le mot grec signifiant « cellule dévoreuse », et devint ainsi le leader des « cellularistes » qui pensaient que les phagocytes plutôt que les anticorps jouaient un rôle prépondérant dans l'immunité (Metchnikoff 1901). Dans le même temps le courant « humoraliste » s'est développé, basé sur les travaux d'Emil Von Behring et Kitasato Shibasaburō (ayant tous deux travaillé avec Robert Koch) qui parvinrent pour la première fois à transférer une immunité contre la diphtérie grâce à une « anti-toxine » contenue dans le sang (Grundmann 2001). Par la suite, Paul Ehrlich mit au point une méthode de standardisation des sérums anti-diphtériques. Mais sa contribution ne s'arrête pas là puisqu'il propose également l'existence de corps immunitaires (anticorps) et de leurs récepteurs (antigènes) dans le sang, et à suggérer que cette fonction immunitaire soit une caractéristique des tissus hématopoïétiques. Enfin, il introduit le concept de discrimination entre le soi et le non-soi

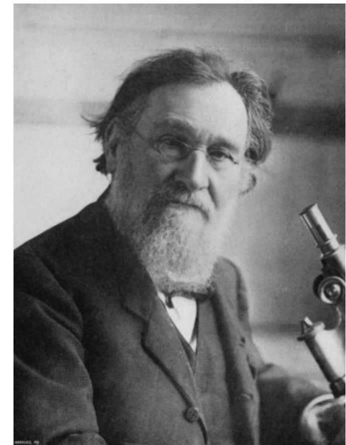

Figure 1 : Elie Metchnikoff

qu'il décrit comme un mécanisme « *prévenant la production dans l'organisme d'amborecepteurs* (anticorps) *dirigés contre ses propres tissues* » (Kaufmann 2008).

- La domination des théories humorales puis la résurgence des théories cellulaires

Entre 1900 et 1942, la théorie humorale a dominé l'immunologie. Ceci repose sur plusieurs observations. Tout d'abord, la majorité des pathologies étudiées était associée à des anticorps spécifiques circulants. De plus, le transfert d'une immunité par des facteurs solubles vint renforcer cette observation. Enfin, l'étude de la réaction anticorps/antigène par Heidelberger et Kabat, dans un contexte où aucune autre spécificité immunologique n'avait été mise en évidence, finit de renforcer l'idée que les anticorps seraient l'unité fondamentale de l'immunologie (Silverstein 2009).
Cependant, plusieurs observations continuaient à aller à l'encontre de la théorie humorale. En effet, l'hypersensibilité retardée et le rejet de greffe apparaissaient être indépendants de la présence d'anticorps sériques. La preuve définitive de l'importance de l'immunité cellulaire vint en 1942 des expériences de Landsteiner et Chase. Le transfert de cellules de cobayes immunisés avec *M. tuberculosis* dans des cobayes naïfs permit à ces derniers de mettre en place une réponse immunitaire anti-bactérienne alors que ceci n'était pas observé avec le transfert de sérum (Silverstein 2009). Les cellules immunes impliquées n'ont été découvertes que bien plus tard par Gowans en 1962 (Gowans 1962).

- La théorie de la sélection clonale

Avant les années 1950, les mécanismes de génération du répertoire des anticorps n'étaient pas connus. Les premières théories estimaient que l'antigène instruisait la spécificité de l'anticorps. A la fin des années 1950, trois scientifiques travaillant indépendamment (Niels Jerne, David Talmage et Frank Macfarlane Burnet) ont développé ce que nous appelons maintenant la théorie de la sélection clonale. En 1955, Jerne décrit « l'hypothèse sélective », où chaque organisme possède une grande variété d'anticorps naturels qui se

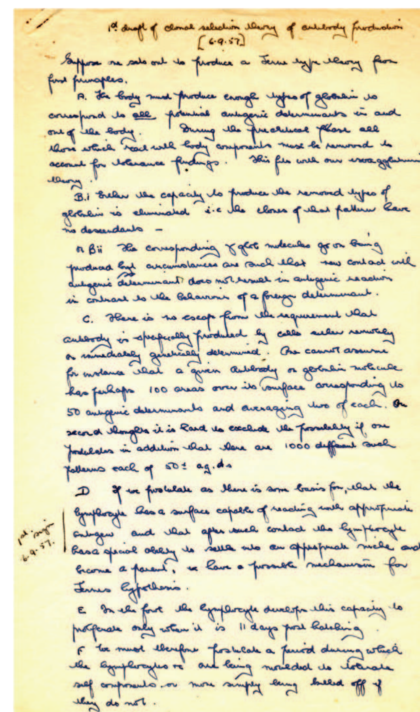
1st draft of clonal selection theory of antibody production
[6.9.57]
Suppose one sets out to produce a Jerne type theory from first principles.
A. The body must produce enough types of globulin to correspond to all potential antigenic determinants in and out of the body. During the pre-critical phase all those which react with body components must be removed to account for tolerance findings. This fits with our [illegible] theory.
B.i Either the capacity to produce the removed types of globulin is eliminated i.e. the clones of that pattern have no descendants –
or B.ii The corresponding γ glob molecules go on being produced but circumstances are such that new contact with antigenic determinant does not result in antigenic reaction in contrast to the behaviour of a foreign determinant.
C. There is no escape from the requirement that antibody is specifically produced by cells either remotely or immediately genetically determined. One cannot assume for instance that a given antibody or globulin molecule has perhaps 100 areas over its surface corresponding to 50 antigenic determinants and averaging two of each. On second thoughts it is hard to exclude the possibility if one postulates in addition that there are 1000 different such patterns each of 50± ag.ds
D If we postulate as there is some basis for, that the lymphocyte has a surface capable of reacting with appropriate antigen and that after such contact the lymphocyte has a special ability to settle into an appropriate niche and become a parent, we have a possible mechanism for Jerne's hypothesis.
1st sugn 6.9.57.
E In the fowl the lymphocyte develops this capacity to proliferate only when it is 11 days post hatching.
F We must therefore postulate a period during which the lymphocytes are being moulded to tolerate self components or more simply being killed off if they do not.

Figure 2 : La théorie de la sélection clonale six semaines avant publication

combinent avec les antigènes correspondants. Selon lui, l'antigène servirait de transport jusqu'à la cellule productrice d'anticorps pour qu'elle synthétise des anticorps identiques à celui présenté (Jerne 1955). Ceci ouvrit la voie aux publications de Talmage et de Burnet en 1957. Selon Talmage « *Il est tentant de considérer que l'une des multiples unités de la réponse anticorps est la cellule elle-même. Selon cette hypothèse, sont sélectionnées les seules cellules ayant une affinité pour l'antigène injecté. Cela aurait le désavantage de nécessiter une cellule pour chaque antigène, mais n'accroitrait pas la quantité d'information requise dans le processus héréditaire* » ; pour appuyer ceci, il se base sur l'existence d'une mémoire immunitaire ainsi que sur le fait que les myélomes sont capables de produire « *un anticorps au hasard parmi la famille des anticorps normaux* » (Talmage 1957).
C'est Burnet qui énonça la théorie de la sélection clonale telle que nous la connaissons. Selon lui : 1. Les animaux possèdent un grand nombre de cellules appelées lymphocytes ; 2. Chaque lymphocyte répond à un antigène particulier grâce à des récepteurs de surface spécifiques ; 3. Lors d'un contact avec son antigène, le lymphocyte est stimulé, prolifère et se différencie ; 4. Les clones issus de cette expansion sont responsables d'une réponse secondaire alors que les cellules différenciées sécrètent les anticorps (Burnet 1957).

- La découverte du complexe majeur d'histocompatibilité (CMH) et de la restriction au CMH

La théorie de la sélection clonale représente une avancée majeure dans l'histoire de l'immunologie mais n'explique pas les mécanismes de reconnaissance des antigènes par les lymphocytes. La compréhension de cette reconnaissance spécifique provient majoritairement des travaux de George Snell dans les années 1930 et de Jean Dausset dans les années 1950 (Silverstein 2009). Snell observe que la transplantation est acceptée chez des souches de souris identiques mais pas dans des souches différentes. Snell nomma les gènes responsables de ce phénomène gènes d'histocompatibilité. Il a ensuite établi que le locus majeur impliqué est le locus codant pour l'antigène II et l'a donc renommé en histocompatibilité 2 ou H-2. Dausset a, quand à lui, observé que les patients recevant beaucoup de transfusions sanguines produisent des anticorps capables d'agglutiner les leucocytes du donneur mais pas ceux du patient. Les études génétiques ont par la suite permis d'identifier la région chromosomique responsable qui fut appelée Human Leucocyte Antigen (HLA) et s'est avérée l'analogue du H-2 chez la souris. En 1943, le conseil médical britannique nomma Peter Medawar pour étudier les problèmes de rejet de greffe. Medawar observe que les autogreffes de peau chez les grands brulés ne sont pas rejetées, contrairement aux allogreffes. Il émit l'hypothèse que

ce rejet était un mécanisme immunologique, ce qu'il confirme par des expériences sur des animaux de laboratoire (Medawar 1944).
En 1974, Peter Doherty et Rolf Zinkernagel observent que des cellules de cerveau infectées par le virus de la méningite virale ne sont détruites par les lymphocytes T que si elles proviennent de la même souche de souris. En revanche, les cellules infectées provenant d'un fond génétique différent sont ignorées. Ces expériences mettent pour la première fois en avant la restriction au CMH : Les lymphocytes T reconnaissent leur antigène uniquement dans le contexte des molécules du CMH. (Zinkernagel 1974).

- Les rôles complémentaires de l'immunologie cellulaire et moléculaire

Depuis 1974, de grandes avancées ont été faites dans la compréhension du fonctionnement du système immunitaire. Ceci a été permis par le développement conjoint des approches moléculaires, comme le clonage du récepteur des lymphocytes T (TcR, 1984) ou la résolution de la structure cristallographique du complexe CMH-peptide (1987), et cellulaires. En 1978, Ralph Steinman identifie les cellules dendritiques comme principales cellules présentatrices d'antigènes du système immunitaire (Steinman 1978) et en 1986 Tim Mosmann et Bob Coffman découvrent la dichotomie Th1/Th2 (Mosmann 1986).

- L'immunité innée

Le système immunitaire adaptatif, tel que nous le connaissons classiquement, est l'apanage des vertébrés, pour autant les invertébrés et les végétaux ne sont pas moins pourvus d'immunité. Charles Janeway en 1989 propose de considérer que les fondements de la résistance aux pathogènes se sont mis en place chez les invertébrés avant de se retrouver chez les vertébrés et prédit l'existence de récepteurs qu'il nomme pattern recognition receptors (PRR) capables de reconnaitre des motifs bien conservés dans l'évolution qu'il appelle les pathogen-associated molecular patterns (PAMP) (Janeway 1989). En 1996, Jules Hoffmann met en évidence chez la drosophile le rôle des Toll dans l'immunité anti-fongique et anti-bactérienne. Janeway identifie alors des homologues chez l'humain et les nomment Toll-like receptors (TLR), et démontre leur spécificité de reconnaissance de motifs bactériens tel que le LPS (Lemaitre 1996).

Mais l'immunité innée ne se limite pas aux TLR. En 1973, les lymphocytes NK (Greenberg 1994) et en 1986, les lymphocytes T γδ sont identifiés (Heilig 1986). Ces cellules jouent, comme les TLR, un rôle de première ligne de défense.

- Le critère d'immunogénicité

Pour terminer cet avant-propos, j'aimerais quitter quelque peu l'ordre chronologique des évènements. L'une des grandes questions de l'immunologie reste encore celle du critère d'immunogénicité, à savoir à quelles conditions se met en place une réponse immunitaire ?

La très grande majorité des immunologistes utilise les termes de soi et non-soi, se référant ainsi à la théorie de Burnet (Burnet 1969) selon laquelle l'organisme déclenche une réponse immunitaire contre tout organisme étranger (non-soi) mais pas contre toute entité qui lui est propre (soi). Or, cette théorie ne peut rendre compte de tous les phénomènes immunitaires et Burnet lui-même émettait des doutes sur son bien-fondé : « *Pour moi, il a été en apparence gratifiant de voir la manière dont la pensée immunologique a évolué comme si elle suivait le chemin que j'avais tracé, mais je n'y ai jamais complètement cru.*» En effet, l'auto-immunité ainsi que la tolérance immunitaire, notamment aux bactéries commensales et fœto-maternelle vont à l'encontre de cette dichotomie, de même que le rejet des greffes. Sur cette base, plusieurs scientifiques ont essayé d'élaborer des théories plus appropriées.

Figure 3 : Couverture du livre de Burnet en 1969

En 1974, Niels Jerne publie la théorie du réseau idiotypique (Jerne 1974). Celle-ci postule qu'un anticorps peut lui-même être un antigène via son idiotype. Des anticorps seraient reconnus par des auto-anticorps et eux-mêmes par des auto-auto-anticorps, et ainsi de suite à l'infini. Selon Jerne, le système immunitaire

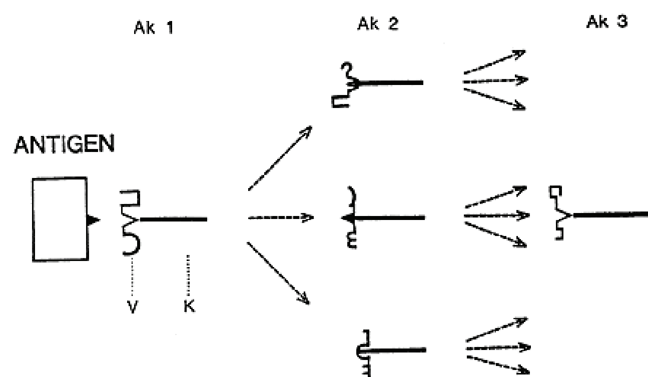

Figure 4 : La théorie du réseau telle que décrite par Jerne

réagit non pas aux antigènes environnementaux, mais à « l'image interne » de ces antigènes et ce parce que les anticorps du système immunitaire expriment déjà tous les antigènes possibles. La théorie de Jerne a le mérite d'introduire la notion d'auto-réactivité normale et de prendre en compte l'auto-immunité. Malgré tout, elle se base sur le fait que le système immunitaire ne serait composé que de lymphocytes et ignore les composants de l'immunité innée ainsi que la non-réponse contre les tumeurs qui devrait constituer une rupture de l'équilibre (à noter que le système immunitaire répond effectivement aux tumeurs, mais ceci n'était pas connu à cette époque).

En 1989, Charles Janeway propose la théorie du non-soi infectieux (Janeway 1989) comme prolongement de la théorie du soi et du non-soi. Selon Janeway, les cellules présentatrices d'antigènes (APC) peuvent reconnaître des pathogènes distants sur le plan de l'évolution. Ces cellules auraient évolué pour interagir avec des motifs microbiens présents de façon répétée dans la nature. Elles ne reconnaîtraient pas tout non-soi, mais seulement ces motifs étrangers fidèlement conservés au cours de l'évolution. La théorie de Janeway renforce le rôle de l'immunité innée comme initiatrice de l'immunité adaptative mais ne peut expliquer la tolérance à la flore commensale ainsi que le rejet des greffes.

Polly Matzinger élabore en 1994 la théorie du danger (Matzinger 1994). Elle y soutient que toute réponse immunitaire est due à l'émission de signaux de danger. Le système immunitaire réagirait à un ensemble de signaux de danger émis par les cellules ou les tissus endommagés. Par exemple, la tolérance fœto-maternelle s'expliquerait par le fait que le fœtus n'est pas « dangereux » pour la mère, et il en irait de même pour la tolérance aux bactéries commensales ou de l'auto-immunité normale. De plus, la théorie du danger prend bien en compte l'immunité innée. La théorie de Matzinger a suscité beaucoup d'enthousiasme mais aussi beaucoup de critiques. En effet, la définition des signaux de danger reste très imprécise. De plus, Polly Matzinger prétend pouvoir systématiquement remplacer « non-soi » par « danger » ce qui ne peut être exact. Par exemple, Matzinger explique le rejet de greffe par le fait que l'acte chirurgical provoque un danger, or pourquoi les auto-greffes ne sont-elles pas rejetées ? Enfin, la plus grosse erreur de Matzinger est probablement d'avoir clamé que seule la théorie du danger peut expliquer la non-réponse contre les tumeurs, se basant sur des travaux anciens montrant que les souris immuno-déficientes ne développent pas plus de tumeurs, ce qui est à présent vu comme inexact. Il est aujourd'hui établi que le système immunitaire répond au

développement des tumeurs. La théorie du danger ne prend donc pas suffisamment en compte les réponses immunes tolérogènes ou chroniques.

D'autres théories ont tenté d'améliorer ces différents concepts. On peut citer par exemple la théorie de l'autopoïèse de Maturana et Varela (Maturana 1994) qui prolonge le réseau idiotypique, ou encore la théorie de l'auto-organisation d'Irun Cohen (Cohen 1992). Ces dernières n'ayant pas eu le même retentissement et n'ayant pas apporté le même progrès que le réseau, le non-soi infectieux ou le danger, je ne les développerai pas ici. De plus, mon propos n'est pas de faire un inventaire exhaustif de toutes les théories ni d'en proposer une nouvelle, mais de mettre en avant le fait que l'une des questions, selon moi, fondamentale à la compréhension de l'immunologie reste en suspend.

De la même manière, cet historique ne peut être considéré comme complet. En effet, beaucoup d'autres avancées majeures pourraient être listées, comme le développement des connaissances sur le répertoire des lymphocytes T et B, le modèle des deux signaux nécessaires à l'activation des lymphocytes T, le fonctionnement des cellules NK ou bien la découverte des lymphocytes T régulateurs (Tregs, sur lesquels je reviendrai de manière plus exhaustive dans le corps de ce manuscrit). Je me suis contenté de revenir sur certains des travaux ayant fait très significativement avancer cette discipline.

On ne sait bien où l'on va que lorsqu'on connait d'où l'on vient, et la thèse a beau être un accomplissement personnel important, elle n'en reste pas moins que le début.

INTRODUCTION

A. La réponse immunitaire dans le cadre du cancer

1) Introduction

L'idée que le système immunitaire peut contrôler le développement tumoral est longtemps restée sujette à débat. Au début des années 1900, Paul Ehrlich fut probablement le premier à suggérer que des cellules tumorales se développeraient régulièrement, mais seraient contrôlées par le système immunitaire (Ehrlich 1909). Les connaissances concernant le système immunitaire étaient si limitées à cette époque qu'il était parfaitement impossible de valider cette théorie. Il aura fallu plus de 50 ans pour que cette idée resurgisse grâce à une meilleure compréhension des mécanismes immunitaires et à la découverte des antigènes tumoraux (Old 1964). C'est sur ces bases que Burnet et Thomas ont fondé l'hypothèse de l'immuno-surveillance (Burnet 1957, Thomas 1959). Cependant, des expériences montrant que la susceptibilité au développement de tumeurs chez les souris immuno-compétentes et athymiques (nudes) est similaire ont provoqué la chute de l'hypothèse de l'immuno-surveillance (Stutman 1974). Plusieurs arguments expliquant pourquoi il n'y aurait pas d'immuno-surveillance ont été avancés. Certains envisagent que les cellules tumorales ne possèdent pas les signaux de danger appropriés (Matzinger 1994), d'autres que les cellules tumorales sont trop similaires aux cellules normales (Pardoll 2003) ou encore que l'inflammation due à l'activation persistante du système immunitaire innée facilite l'initiation tumorale (Balkwill 2001). L'émergence de modèles murins immuno-déficients sous fond génétique pur a permis de remettre au goût du jour le concept de l'immuno-surveillance, notamment en mettant en évidence la plus grande susceptibilité au développement tumoral des souris déficientes en interféron γ (IFNγ) (Dighe 1994) ou en RAG2 (Recombination actived gene 2) (Shankaran 2001). D'autres travaux, sur lesquels je reviendrai plus en détail, ont par la suite permis de confirmer le rôle du système immunitaire dans le contrôle des tumeurs (Vesely 2011).

Il est maintenant admis que le système immunitaire joue au moins trois rôles dans la prévention tumorale. Il protège l'hôte contre les infections virales et donc empêche le développement des tumeurs viro-induites. De plus, il prévient l'établissement de conditions inflammatoires propices au développement tumoral. Enfin, il présente une activité anti-tumorale. Mais les interactions entre tumeurs et système immunitaire sont plus complexes et ne se limitent pas à l'élimination de l'un par l'autre. En 2002, Dunn démontre que le système

immunitaire peut à la fois contrôler la progression tumorale, mais aussi limiter l'immunogénicité des cellules tumorales. Cela a conduit à la révision de l'hypothèse de l'immuno-surveillance (Dunn et al 2002). Cette étude suggère que les tumeurs se développant chez des souris immuno-déficientes sont plus immunogènes que celles développées par des souris immuno-compétentes. Ces observations ainsi que la prise en compte du fait que le système immunitaire peut avoir des effets pro-tumoraux conduisent à la théorie de l'immuno-éditing. Celle-ci s'articule en trois phases : Elimination, équilibre et échappement sur lesquelles nous allons maintenant revenir.

2) La théorie de l'immuno-éditing

2.1 Elimination : Rejet immun des tumeurs

La première phase de l'immuno-éditing est l'élimination. Elle correspond à une vision moderne de l'immuno-surveillance de Burnet et Thomas. Durant cette étape, les cellules du système immunitaire localisent, reconnaissent et détruisent les cellules tumorales.

2.1.1 Mise en évidence du rôle du système immunitaire dans l'immuno-surveillance

Plusieurs modèles tumoraux ont permis la mise en évidence de la phase d'élimination : tout d'abord, les modèles de tumeurs induites par des carcinogènes puis le développement tumoral spontané survenant avec l'âge, et enfin les modèles génétiques de souris prédisposées au cancer. Je mets de coté, pour cette étape, les modèles de transplantation tumorale. En effet, les cellules tumorales transplantées provenant à l'origine de tumeurs ayant déjà échappé au système immunitaire et ayant donc déjà été immuno-éditées, il ne semble pas pertinent d'utiliser ces modèles pour mettre en évidence l'immuno-surveillance.
Ce processus a été démontré dans différents modèles murins déficients pour des populations cellulaires ou des voies de signalisations du système immunitaire. Le rôle important des lymphocytes a pu être démontré grâce à l'utilisation de souris déficientes pour la molécule RAG2. Ces souris sont incapables d'opérer les recombinaisons nécessaires aux réarrangements des chaînes $\alpha\beta$ et $\gamma\delta$ du TcR ainsi que des chaînes lourdes et légères des anticorps et ne possèdent donc ni lymphocytes T ni lymphocytes B. Ces souris sont plus susceptibles aux tumeurs induites par le méthylcholanthrene (MCA) et développent également plus de tumeurs spontanées (Figure 5) (Shankaran 2001). De la même façon, le rôle crucial de l'interféron γ (IFNγ) a été démontré grâce à la plus grande susceptibilité des souris IFNγ^{KO} et

IFNγR^{KO} au développement de tumeurs induites par le MCA (Kaplan 1998, Wakita 2009) et de tumeurs spontanées (Street 2002) ou génétiquement induites (Kaplan 1998, Mitra-Kaushik 2004). De façon plus fine, les rôles distincts (Girardi 2001) et complémentaires (Girardi 2003) des lymphocytes Tαβ et Tγδ ont été établis dans le modèle de tumeurs induites par le MCA, mais également par le 7,12-diméthylbenzanthracène/12-O-tetradecanoylphorbol-13-acetate (DMBA/TPA). De plus, l'importance des lymphocytes natural killer (NK) a été suggérée par l'incidence accrue de tumeurs induites par le MCA dans des souris déplétées en NK (Smyth 2001).

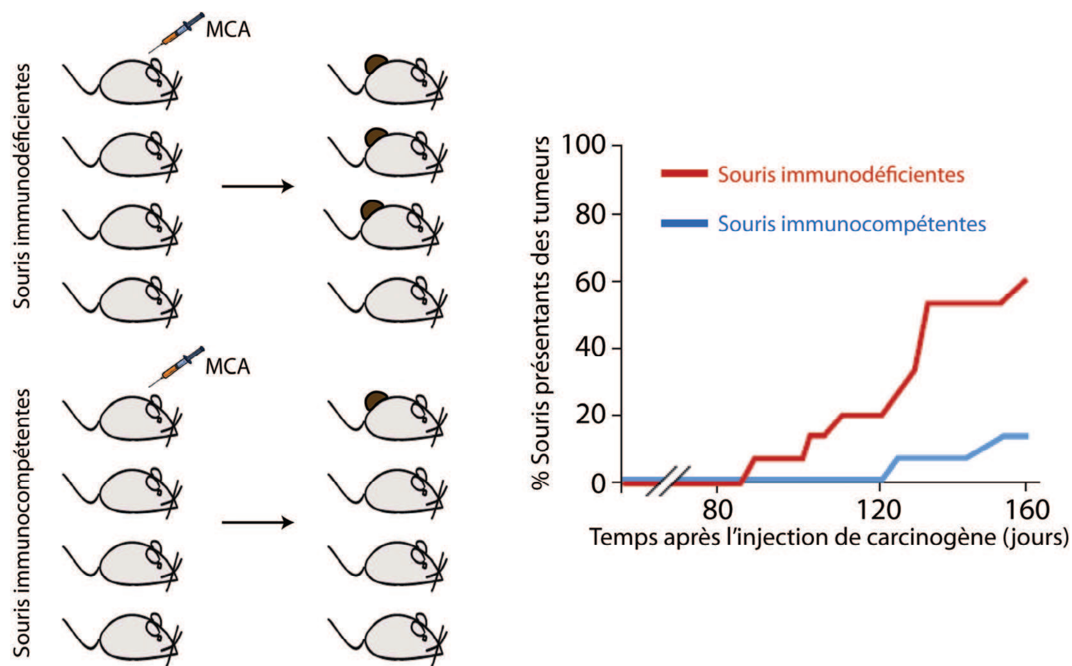

Figure 5 : Démonstration de la plus grande susceptibilité des souris immuno-déficientes à l'induction tumorales. D'après Shankaran *et al* Nature 2001.

Sans être exhaustif, cet inventaire de différentes expériences démontre l'implication de l'immunité innée, aussi bien qu'adaptative, dans la surveillance des tumeurs.

2.1.2 Les effecteurs du système immunitaire dans la réponse anti-tumorale

Je vais maintenant m'attacher à détailler le rôle des différents effecteurs du système immunitaire. La plupart des populations décrites ci-dessous ont des rôles ambivalents dans l'immunité anti-tumorale. Je ne décrirai dans ce paragraphe que les rôles anti-tumoraux attribués à ces populations et reviendrais sur les rôles pro-tumoraux dans la partie de ce manuscrit consacrée aux mécanismes d'échappement tumoral.

- Macrophages

Les macrophages de type M1 sont les macrophages « classiques » induits par des cytokines de type Th1 telles que l'IFNγ et l'interleukine (IL)-1β ou par le lipopolysaccharide (LPS) (Allavena 2008). Leur action anti-tumorale vient du fait qu'ils sont capables de présenter les antigènes (Ag) aux lymphocytes T (LT) ainsi que de sécréter de l'IL-12. Les M1 peuvent également avoir une activité cytotoxique sur les cellules tumorales grâce à leur production de réactifs oxygénés ou bien de molécules de la famille du Tumor Necrosis Factor (TNF). De plus, les M1 peuvent être les effecteurs de la cytotoxicité cellulaire dépendante des anticorps (ADCC).

- Polynucléaires neutrophiles

La majorité des tumeurs sécrètent des cytokines et chimiokines capables de recruter les neutrophiles dans le micro-environnement tumoral (Di Carlo 2001). Les neutrophiles ainsi recrutés peuvent exercer des fonctions anti-tumorales grâce à des médiateurs cytotoxiques tels que des réactifs oxygénés, des protéases ou encore des médiateurs solubles comme le TNFα, l'IL-1β ou les IFNs.

- Polynucléaires éosinophiles

Les éosinophiles attirent de plus en plus l'attention des chercheurs dans le cadre de l'immunité anti-tumorale et ce pour plusieurs raisons (Costello 2005). En effet, les éosinophiles sont des cellules possédant des capacités cytotoxiques importantes et ils exercent une activité anti-tumorale potentielle *in vitro*. De plus, l'infiltration d'éosinophiles dans le micro-environnement tumoral est bon pronostic dans les cancers non-Hodgkiniens.

- Cellules dendritiques (DCs)

Les DCs sont une population cellulaire clé de la réponse immunitaire. Ce sont les cellules présentatrice de l'Ag (APC) professionnelles qui sont les plus aptes à induire une activation des LT et donc à initier une réponse anti-tumorale (Apetoh 2011). En outre, les DCs plasmacytoïdes (pDCs) sont capables de sécréter de très grandes quantités d'IFN de type 1 (α et β) très rapidement après activation. Ceci fait des DCs un acteur anti-tumoral majeur.

- Lymphocytes Natural Killer (NK)

Les NK sont des lymphocytes spécialisés de l'immunité innée capables de discriminer les cellules normales des cellules infectées ou tumorales (Hayakawa 2006). Ils ont d'ailleurs été identifiés grâce à leur cytotoxicité naturelle contre les cellules tumorales. Les NK ont la capacité de lyser les cellules ayant des molécules de CMH de classe I altérées ou manquantes. Ils utilisent majoritairement la voie des granules cytotoxiques (perforine/granzyme) et sont également capables de produire de grandes quantités d'IFNγ et d'utiliser la voie des récepteurs de mort. Ces capacités spontanées en font des acteurs majeurs de l'immuno-surveillance.

- Lymphocytes T non conventionnels

Les LTγδ ont un récepteur à l'antigène des LT (TcR) composé des chaînes γ et δ (Kabelitz 2007). Ils reconnaissent les phospho-Ag exprimés à la surface des cellules tumorales et participent ainsi au contrôle immunitaire de ces dernières. Ils possèdent une activité cytotoxique importante utilisant la voie des granules cytotoxiques et des récepteurs de mort, mais aussi via leur production importante de TNFα et IFNγ.

Les LT NK (NKT) sont des LTαβ possédant également des caractéristiques de NK (Berzofsky 2009). Ils reconnaissent des motifs moléculaires lipidiques ou glyco-lipidiques présentés dans le contexte CD1d. La protection conférée par les NKT repose sur leur sécrétion importante de cytokines telles que l'IFNγ qui active les NK et les LT $CD8^{+}$.

- Lymphocytes B (LB)

De par leur sécrétion d'Ac, les LB jouent un rôle déterminant dans la réponse anti-tumorale. Les Ac reconnaissant les cellules tumorales peuvent adresser les cellules du système immunitaire possédant les récepteurs Fc adéquats, tel que les NK, les neutrophiles, les macrophages ou les DCs, et ainsi provoquer l'ADCC. De plus, les Ac fixés aux cellules tumorales peuvent activer le système du complément conduisant à la lyse des cellules (Weiner 2010).

• Lymphocytes T conventionnels

Les LT conventionnels peuvent être divisés en plusieurs sous-populations (Muranski 2009).

- LT CD4^{+} ou LT auxiliaires (Thelper Th)

Lors d'une stimulation antigénique, les LT CD4^{+} naïfs, ou Th0, ont la capacité de s'engager dans diverses voies de différenciation (Figure 6). Les cellules Th1 produisent de l'IFNγ et favorisent les réponses immunitaires à médiation cellulaire. A l'inverse, les cellules Th2 se révèlent incapables de produire de l'IFNγ, mais sont caractérisées par leur production d'IL-4, d'IL-5 et d'IL13 et favorisent la réponse humorale en aidant à la production d'anticorps par les LB. Les Th17 sont caractérisés par leur production d'IL17A, IL17F et IL22. Enfin, deux populations régulatrices majeures dérivant des LT CD4^{+} : les Tregs d'origine thymique (appelées naturelles, nTregs) et les Tregs induits à partir de LT CD4^{+} naïfs (iTregs). Chacun de ces lignages de LT effecteurs est associé à des facteurs de transcriptions spécifiques. Ainsi, T-bet, GATA-3, Foxp3 et RORγt ont, respectivement, un rôle majeur dans la différenciation et l'homéostasie des cellules Th1, Th2, Tregs et Th17 (Figure 6).

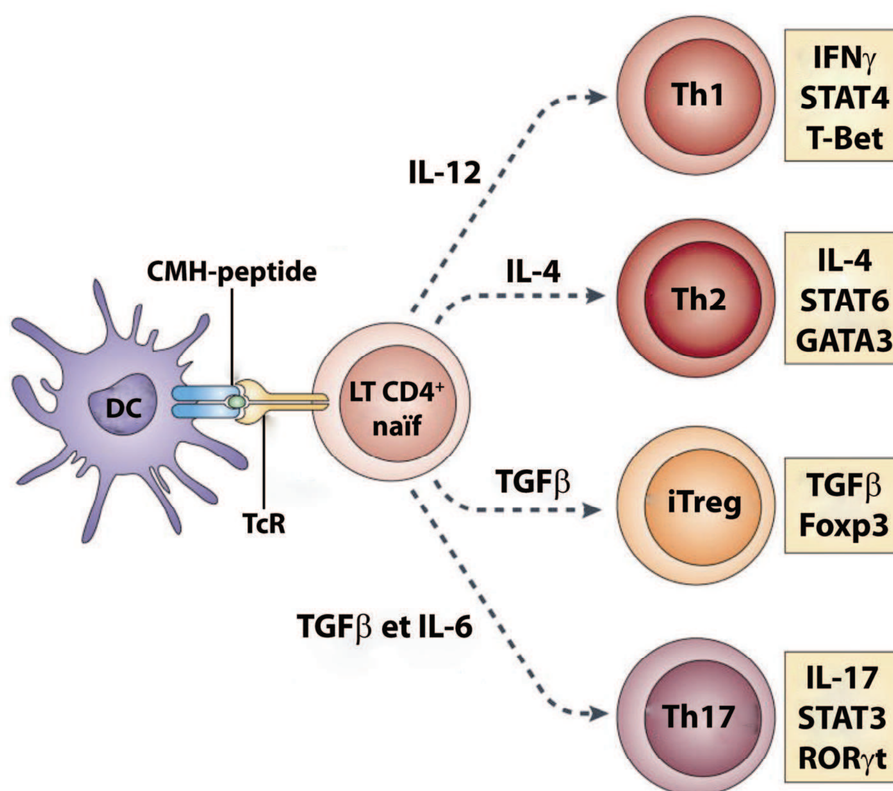

Figure 6 : Voies de différenciation des LT CD4^{+} naïfs. Zou *et al* Nature Reviews Immunology 2010.

Alors qu'*in vivo*, des processus complexes semblent dicter l'orientation des LT naïfs en LT effecteurs / mémoires, *in vitro* ce choix dépend du stimulus cytokinique présent au moment de l'activation. Il a ainsi pu être démontré pour les LT CD4$^+$ naïfs, que l'IL-12 favorise l'induction de cellules Th1 et que la polarisation en Th2 dépend de la présence d'IL-4 et d'IL-2. De façon intéressante, l'engagement des Th0 dans la voie de différenciation des Th17 et iTregs est dépendent d'une même cytokine, le facteur de croissance transformant-β (TGFβ). Le choix entre ces deux voies de différenciation se fait en fonction de la présence d'IL-6 qui favorise la polarisation en Th17.
Dans le cadre de la réponse anti-tumorale, les Th1 de par leur activation des LT CD8$^+$ et leur sécrétion d'IFNγ jouent un rôle majeur. Même s'il a été montré que ce type de réponse est moins efficace, les Th2 de par leur activation des LB peuvent également jouer un rôle. Le rôle des Th17 reste encore controversé. En effet, d'un côté l'induction de processus de type auto-immun contre les tumeurs peut être une stratégie intéressante. Il a en outre été démontré dans un modèle expérimental de mélanome transplanté que les Th17 seraient plus efficaces que les Th1 dans l'immunité anti-tumorale (Muranski 2008). Cependant, le caractère inflammatoire des Th17 peut favoriser l'initiation et la progression tumorale.

- LT CD8$^+$ ou LT cytotoxiques (CTL)
Les LT CD8$^+$ sont les effecteurs terminaux de l'immunité adaptative. Ils possèdent des capacités anti-tumorales très importantes. Ils sont capables de lyser les cellules tumorales par des mécanismes dépendant de la perforine et du granzyme. De plus, ils ont une capacité de sécrétion d'IFNγ et de TNFα très importante qui permet de réguler positivement l'expression des molécules du CMHI et CMHII et d'augmenter l'immunogénicité des cellules tumorales (Restifo 2012).

2.2 Equilibre : Dormance tumorale induite par l'immunité

Historiquement, la dormance des tumeurs est un terme utilisé pour décrire la période de latence des tumeurs qui dure parfois pendant des décennies chez les patients. Les tumeurs en phase d'équilibre sont des tumeurs dormantes contrôlées par le système immunitaire. En effet de rares cellules tumorales peuvent survivre à la phase d'élimination. Durant la phase d'équilibre, le système immunitaire inflige une pression de sélection qui prévient la croissance tumorale mais influence également l'immunogénicité des cellules cancéreuses, favorisant par

la suite l'apparition de mécanismes d'échappement. La première suggestion que le système immunitaire maintient les cellules tumorales à l'état d'équilibre vient d'une expérience menée chez des souris transplantées et réinjectées avec la même tumeur pour induire une dormance (Farrar 1999). Dans ces expériences, la déplétion des LT CD8$^+$ ou l'inhibition de l'activité de l'IFNγ conduit à un temps de dormance plus court. La véritable démonstration de l'existence de la phase d'équilibre vient d'expériences d'induction tumorale par de faibles doses de MCA (Koebel 2007) (Figure 7). Ces expériences montrent la présence de cellules tumorales latentes pendant de longue période chez les souris ne présentant pas de lésions visibles. Ces souris traitées pour dépléter les LT ou neutraliser l'IFNγ ou l'IL-12 développent alors rapidement des tumeurs au niveau du site d'injection du MCA. La déplétion des NK ou la neutralisation de certains de leurs récepteurs ne provoque pas de croissance tumorale. L'ensemble de ces résultats démontre le rôle prépondérant de l'immunité adaptative lors de la phase d'équilibre.

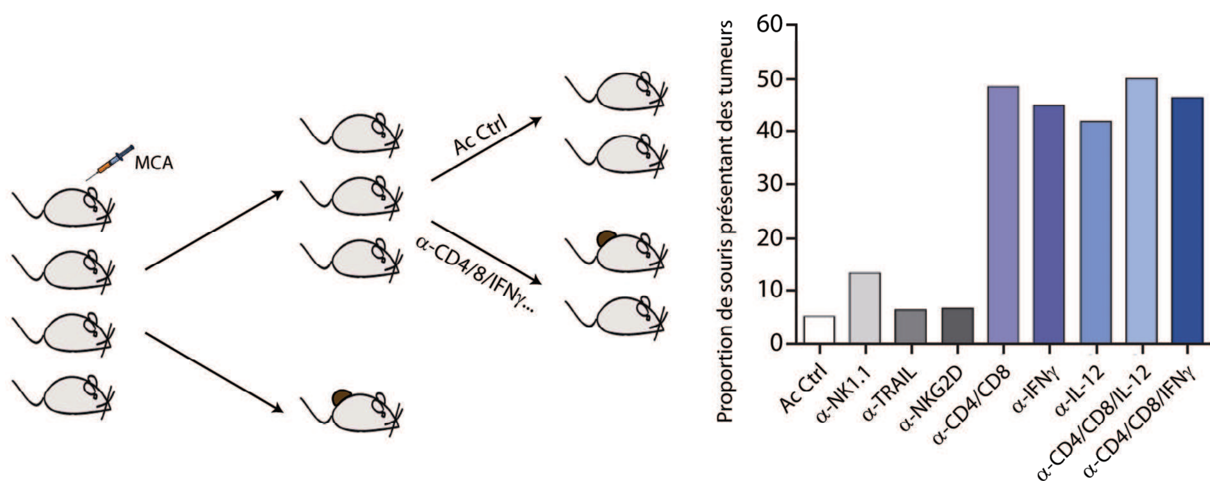

Figure 7 : Démonstration que le système immunitaire peut maintenir les cellules tumorales en dormance. D'après Koebel *et al* Nature 2007.

Le rôle des LT CD8$^+$ dans le phénomène de dormance a également pu être démontré dans le modèle de mélanome spontané Ret. Dans ce modèle, les cellules de la tumeur primaire disséminent rapidement dans de nombreux organes et restent dormantes. La déplétion des LT CD8$^+$ dans ce modèle accélère significativement l'apparition de métastases à distance (Eyles 2010).

2.3 Echappement au système immunitaire

La phase d'échappement représente l'échec du système immunitaire à contrôler les cellules transformées, laissant les variants tumoraux persistants croître dans un environnement immunologique non contrôlé. Les mécanismes d'échappement sont très variés et peuvent être classés en deux catégories : les modifications propres de la cellule tumorale visant à échapper au système immunitaire et l'induction de cellules immunitaires suppressives.

2.3.1 Modification des cellules tumorales permettant l'échappement à la reconnaissance et à la destruction immunitaire

Les cellules tumorales mettent en place plusieurs mécanismes afin d'échapper à la lyse par les CTL. Elles acquièrent un défaut de présentation antigénique tout particulièrement par la perte d'expression des molécules de classe I du CMH (Jäger 1996, Khong 2004) ou de leur machinerie d'expression comme TAP1 ou la β2 microglobuline (β2m) (Restifo 1996). Certains variants tumoraux développent une insensibilité à l'IFNγ et aux IFN de type 1 (Dunn 2006). Les cellules tumorales peuvent également échapper à la lyse par les NK en perdant l'expression des ligands pour les récepteurs activateurs tels que MICA et MICB, ligands de NKG2D (Stern-Ginossar 2008).

Même lorsque les antigènes tumoraux continuent à être exprimés, les cellules tumorales peuvent développer des mécanismes leur permettant d'échapper à la lyse par les cellules du système immunitaire. Ainsi, la surexpression de molécules anti-apoptotiques telles que BCL-XL (Hinz 2000) ou FLIP (Kataoka 1998) rend les cellules tumorales insensibles à l'apoptose induite via les récepteurs de mort. Cette résistance peut être acquise par mutation inactivatrice des récepteurs de mort comme TRAIL (Shin 2001) ou Fas (Takahashi 2006).

Ces stratégies d'échappement sont passives, mettant en jeu des défauts de reconnaissance ou de sensibilité. Mais les cellules tumorales peuvent avoir une action plus directe en exprimant des ligands inhibiteurs du système immunitaire. Ainsi l'expression par les cellules tumorales de PD-L1 (Programmed Death Ligand 1) (Dong 2002), HLA-G (Tripathi 2006) ou HLA-E (Derré 2006) permet de diminuer l'activité cytotoxique ou d'induire l'apoptose des LT.

Les cellules tumorales peuvent également perdre leur capacité de sécrétion de cytokines pro-inflammatoires activatrices du système immunitaire. Il a été tout particulièrement montré que l'activation constitutive de Stat3 dans les cellules tumorales aboutit à un défaut de production

d'IL-6, de TNFα et de CCL5 (Wang 2004). Le blocage de Stat3 permet de restaurer cette sécrétion et d'activer les DCs du microenvironnement tumoral activant elles-mêmes les CTL.

2.3.2 Induction d'un microenvironnement immuno-suppresseur

Un microenvironnement tumoral immuno-suppresseur peut s'établir de deux façons. Les cellules tumorales peuvent secréter des facteurs suppresseurs ou bien favoriser le recrutement de cellules immuno-suppressives.

2.3.2.1 Facteurs sécrétés par les cellules tumorales

Le tableau 1 résume les principaux mécanismes moléculaires mis en place par les cellules tumorales pour induire un environnement immuno-suppresseur.

Molécules	Cibles	Effets	Références
TGFβ	DC, LT, NK	Blocage de l'activation et de la prolifération	Wrzesinki 2007
Stérols	DC	Baisse d'expression de CCR7, Problèmes de migration vers les ganglions	Villablanca 2010
VEGF	DC	Baisse des capacités de présentation antigénique	Gabrilovich 1999
MIC solubles	NK, CTL	Baisse d'expression de NKG2D	Groh 2002
Kynurenines	LT, NK	Produit de l'activité de IDO, Toxicité cellulaire	Löb 2009
Gal-1	LT	Apoptose	Rubinstein 2004
IDO	LT	Privation de tryptophane, Apoptose	Uyttenhove 2003
Arg1	LT	Privation d'arginine, Apoptose	Bronte 2005
N.D.*	DC	Accumulation de lipides intracellulaires, baisse des capacités de présentation antigénique	Herber 2010

* N.D. : Non déterminé

Tableau 1 : Principaux facteurs immuno-suppresseurs sécrétés par les cellules tumorales

2.3.2.2 Cellules immuno-suppressives

Plusieurs cellules immunitaires suppressives peuvent être recrutées ou induites par les tumeurs. Parmi celles-ci, les Tregs jouent un rôle prépondérant. Je reviendrai en détail sur ces dernières plus tard dans ce manuscrit. Le second type de cellules immunitaires régulatrices importantes sont les cellules myéloïdes : DCs tolérogènes, macrophages associés aux tumeurs (TAMs) et cellules myéloïdes suppressives (MDSCs) (Figure 8). Les principaux mécanismes d'immunosuppression mis en place par ces différentes cellules sont récapitulés dans le tableau 2 à la fin de ce chapitre.

- Cellules dendritiques tolérogènes

Il est aujourd'hui bien établi que les DCs d'un hôte porteur de tumeurs ne stimulent pas la réponse immunitaire efficacement et peuvent contribuer à l'échappement tumoral. De nombreuses études démontrent qu'une myélopoïèse anormale serait le mécanisme majeur responsable du mauvais fonctionnement des DCs dans le contexte tumoral. Cette différenciation anormale produit au moins deux effets : une baisse de la production de DCs fonctionnelles et une accumulation de DCs immatures dans le microenvironnement tumoral (Gabrilovich 2004).

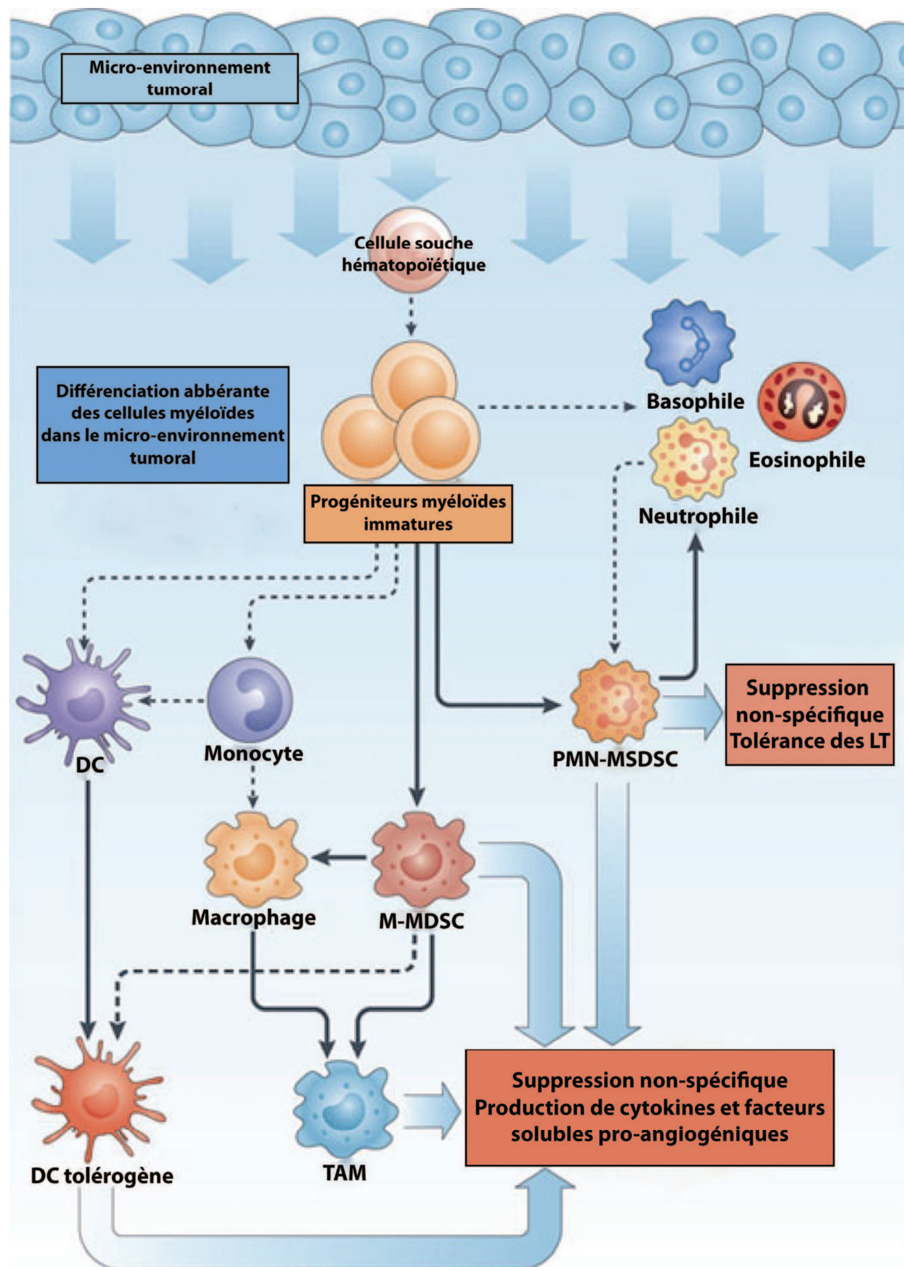

Figure 8 : Changements se produisant chez les cellules myéloïdes lors d'un cancer. D'après Gabrilovich *et al* Nature Reviews Immunology 2012.

- Macrophages associés aux tumeurs

La terminologie de macrophage M1 et M2 a été proposée pour définir les différents états fonctionnels des macrophages et fut originellement basée sur des travaux effectués chez la souris (Mantovani 2002). Les M2 ou « macrophages alternativement activés » sont activés par l'IL-4, l'IL-10, l'IL-13 et les glucocorticoïdes, et les TAMs peuvent être assimilés à des M2. La littérature démontrant, chez la souris aussi bien que chez l'homme, un rôle pro-tumoral des TAMs et leur association à un diagnostic défavorable est très abondante (Qian 2010). Récemment, le rôle central des TAMs dans le cancer a été mis en avant par une étude démontrant que certains TAMs possèdent une signature transcriptionnelle particulière et sont associés à l'échec des traitements de première intention chez les patients atteints de lymphomes Hodgkiniens (Steidl 2010).

Les différentes zones des tumeurs solides présentent des microenvironnements différents et les TAMs retrouvés au sein d'une tumeur varient beaucoup en fonction de cet environnement.

Sept sous-populations de TAMs ont été identifiées dans le carcinome mammaire de la souris et dans l'adénocarcinome du poumon en fonction de leur expression de la molécule Ly-6C, du CMHII, des récepteurs CX3CR1 et CCR2, et de CD62L (Movahedi 2010). Ces sous-populations ont des demi-vies différentes et leur fréquence peut changer à mesure que le microenvironnement tumoral évolue.

- Cellules myéloïdes suppressives

Des cellules myéloïdes immatures sont continuellement générées dans la moelle osseuse d'individus sains et se différencient en cellules myéloïdes matures. Toutefois, dans le contexte tumoral, la différenciation des cellules myéloïdes est détournée de sa voie normale, ce qui favorise la différenciation des cellules myéloïdes pathologiques. Ces cellules ont été nommées MDSCs pour souligner leur origine myéloïde commune ainsi que leurs propriétés immuno-modulatrice (Gabrilovich 2007).

Les MDSCs ont été identifiées à l'origine dans la rate de souris porteuses de tumeurs comme des cellules exprimant CD11b et GR1, mais leur phénotype dans le contexte tumoral est plutôt divers (Peranzoni 2010). Actuellement, deux principales populations de MDSCs ont été caractérisées: Les MDSCs monocytaires (M-MDSCs) et polynucléaires (PMN-MDSCs). Chez les souris porteuses de tumeurs, les PMN-MDSCs sont plus fréquentes mais moins immunosuppressives que les M-MDSCs (Youn 2008). Dans les études effectuées chez l'homme, le nombre de M-MDSCs est en corrélation directe avec la suppression de l'activation *in vitro* des LT (Mandruzzato 2009).

Les M-MDSCs sont définies comme étant CD11b^{+} Ly-6C^{fort}Ly-6G^{-}, et les PMN-MDSCs comme étant CD11b^{+} Ly-6C^{faible}Ly-6G^{+}. En plus de ces marqueurs, les M-MDSCs peuvent exprimer différents niveaux de marqueurs classiques des monocytes comme F4/80, CD115, 7/4 (ou Ly-6B) et CCR2 (Youn 2008).

- Mécanismes immuno-suppresseurs des cellules myéloïdes

Certains des mécanismes mis en place par les cellules myéloïdes sont communs aux trois populations précédemment décrites. Ils peuvent être classés en quatre grands types : Métabolisme des acides aminés, facteurs immuno-suppresseurs et inflammatoires, régulation du trafic cellulaire et polarisation, et induction et expansion de Tregs.

Type de mécanismes	Molécules	Cellules	Effets
Métabolisme des acides aminés	Arg1	DC, TAM, MDSC	Privation de L-Arginine, Baisse d'expression de CD3ζ, Inhibition des LT (Liu 2009, Rodriguez 2004, Mandruzzato 2009)
	IDO	DC	Privation de Tryptophane, Apoptose des LT (Novitskiy 2008)
	iNOS	MDSC	Production NO à partir de L-Arginine, Suppression de la prolifération et migration des LT (Mandruzzato 2009)
	Transporteur XC-	MDSC	Privation de cystéine, Baisse de l'activation et des fonctions des LT (Srivastava 2010)
Facteurs immuno-suppresseurs et inflammatoires	VEGF	DC	Angiogenèse et métastase facilité (Novitskiy 2008)
	TGFβ	DC, TAM, MDSC	Blocage de l'activation et de la prolifération des LT et NK (Novitskiy 2008, Torroella-Kouri 2009)
	IL-10	DC, TAM, MDSC	Inhibition de la sécrétion de cytokines immuno-stimulatrices et de la présentation antigénique (Novitskiy 2008, Murai 2009, Sinha 2007)
	IL-6 et IL-8	DC	Facteurs pro-inflammatoires (Novitskiy 2008)
	Péroxynitrite et ROS	MDSC	Inhibition de la production de cytokines (Schmielau 2001) et de la prolifération des LT (Mazzoni 2002), Nitration du TcR (Nagaraj 2007)
	Galectine-9	MDSC	Induction de l'apoptose des LT (Sakuishi 2011)
	PDL1	TAM	Réduction de la prolifération et apoptose des LT (Kuang 2009)
	TGFβ membranaire	MDSC	Inhibition des fonctions des NK (Li 2009)
Régulation du trafic cellulaire et de la polarisation	COX2	DC, TAM	Production de PGE_2, Attraction des MDSC et polarisation des macrophages en M2 (Novitskiy 2008, Torroella-Kouri 2009)
	A2B	DC	Favorise la polarisation Th2 (Yang 2010)
	ADAM17	MDSC	Diminution de l'expression de CD62L sur les LT, limitation du trafic cellulaire (Hanson 2009)
	Péroxynitrite et ROS	MDSC	Nitration de CCL2, inhibition du recrutement des LT (Molon 2011)
Induction et expansion de Tregs	Multiples	DC, TAM, MDSC	cf Discussion

Tableau 2 : Principaux mécanismes immuno-suppresseurs des cellules myéloïdes

2.4 Bilan

La théorie de l'immuno-éditing ou « théorie des 3E » récapitule les connaissances actuelles concernant l'immunité anti-tumorale. Le schéma ci-dessous (Figure 9) résume cette théorie.

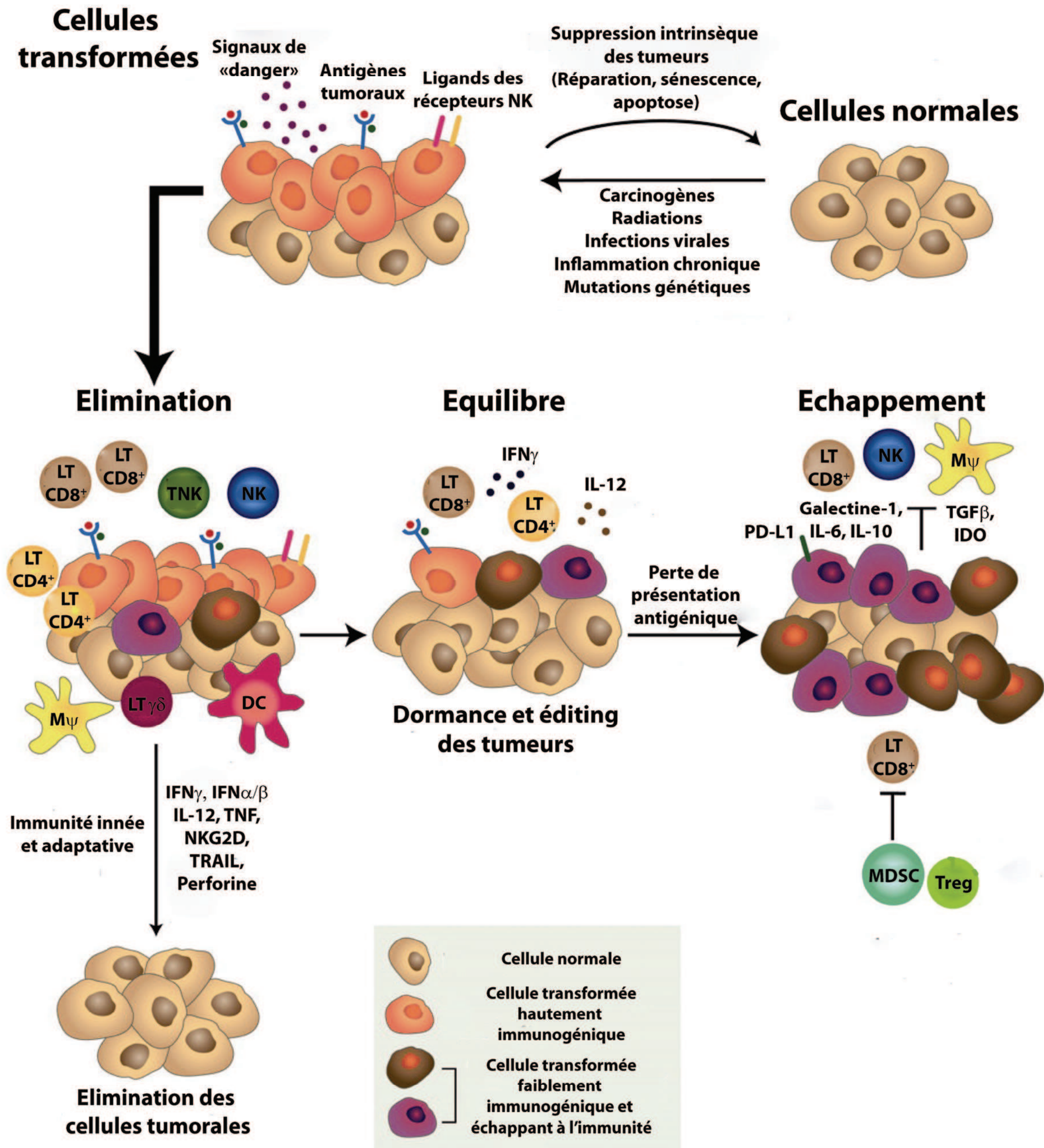

Figure 9 : Schéma récapitulatif de la théorie de l'immuno-éditing. D'après Schreiber *et al*, Science 2011

B. Les lymphocytes T régulateurs $CD4^+$ $Foxp3^+$

1) Mise en évidence et phénotype des Tregs

Malgré le scepticisme qui fut d'abord de mise parmi les immunologistes, il est maintenant clair que le système immunitaire dans les conditions physiologiques possède une population de lymphocytes T spécialisés dans la suppression des réponses immunitaires, les Tregs.

1.1 Les Tregs $CD4^+$: De CD5 à Foxp3

La mise en évidence de l'existence de LT capables de réguler les réponses immunitaires n'est pas nouvelle. En effet, en 1970 et 1971 Gershon et Kondo font la découverte que des LT différents des Th peuvent inhiber les réponses immunitaires (Gershon 1970, Gershon 1971). Cette population appelée LT suppresseurs fut très étudiée dans les années qui suivirent. Cependant, l'engouement pour ces cellules s'arrêta dans les années 1980 pour plusieurs raisons : le manque de marqueurs spécifiques, l'ambiguïté concernant les bases moléculaires de la suppression, la difficulté à obtenir des LT suppresseurs spécifiques et l'absence de preuve qu'un défaut des LT suppresseurs conduisait à des manifestations cliniques (Möller 1988).
Le regain d'intérêt porté aux LT suppresseurs provient de plusieurs études. En 1982, Shimon Sakaguchi démontre dans un modèle de multiples atteintes auto-immunes après thymectomie néo-natale, que les LT sont nécessaires au développement de la pathologie et que celle-ci peut être transférée d'un individu à l'autre (Sakaguchi 1982a). Il démontre également que ces atteintes peuvent être prévenues par le transfert de splénocytes ou de thymocytes syngéniques normaux (Sakaguchi 1982b). De plus, il identifie aussi bien les splénocytes que les thymocytes responsables de la régulation comme étant $CD5^+CD8^-$ et conclut donc qu'il s'agit de LT $CD4^+$. Par la suite, plusieurs équipes ont tenté d'identifier des marqueurs capables de discriminer les LT suppresseurs des autres LT $CD4^+$. En 1985, Sakaguchi met au point un second modèle d'étude de la tolérance immunitaire. Dans ce modèle, le transfert de LT $CD4^+CD5^{faible}$ à des souris athymiques conduit à une auto-immunité multiple alors que le co-transfert avec des LT $CD4^+CD5^{fort}$ prévient l'apparition de la maladie (Sakaguchi 1985). Ce résultat suggère également qu'un déficit en LT suppresseurs pourrait être la cause des maladies auto-immunes. Sur cette lancée, Fiona Powrie démontre, que le transfert de LT

$CD4^+CD45RB^{fort}$ à des rats athymiques induit une colite auto-immune qui peut être prévenue par le co-transfert de LT $CD4^+CD45RB^{faible}$ (Powrie 1990). Cependant, ces marqueurs étant également exprimés par les LT conventionnels (Tconvs), ne suffisent pas à caractériser les LT suppresseurs. En 1995, Sakaguchi identifie CD25 (chaîne α du récepteur à l'IL-2) comme potentiel marqueur des Tregs. En effet, les LT $CD4^+CD25^+$ constituent de 5 à 10% des LT $CD4^+$ périphériques et sont majoritairement compris parmi les fractions $CD5^{fort}$ et $CD45^{faible}$ des LT $CD4^+$. En outre, le transfert de LT $CD4^+CD25^-$ à des souris athymiques entraîne une auto-immunité multiple qui peut être prévenue par le co-transfert de LT $CD4^+CD25^+$ (Sakaguchi 1995). CD25 s'est révélé être un bon marqueur des Tregs. En effet, les Tconvs activés expriment CD25, mais à des niveaux inférieurs aux Tregs (Kuniyasu 2000) et cette expression est transitoire, alors qu'elle est constitutive et stable chez les Tregs (Fisson 2003). La découverte de CD25 comme marqueur des Tregs a permis une étude extensive de ces derniers et d'identifier de nombreux marqueurs souvent en lien avec les propriétés régulatrices des Tregs (Tableau 3).

Marqueurs	Niveau d'expression	Commentaires	Références
CD25	+++	cf texte	Sakaguchi 1995
Foxp3	+++	cf texte	Hori 2003
CTLA-4	+++	Fortement exprimé, important dans la fonction	Read 2000
CD103	+	10-30% des Tregs périphériques, très majoritaire dans la peau et les muqueuses	Lehmann 2002
GITR	+++	Constitutivement exprimé	Shevach 2006a
Nrp-1	+++	Exprimé par la majorité des Tregs	Sarris 2008
ICOS	+++	Fortement exprimé, le niveau d'expression peut délimiter des sous-populations	Burmeister 2008
LAG-3	+++	Exprimé après activation	Huang 2004
CD39	++	Environ 60% des Tregs périphériques	Borsellino 2007
TNFR2	++	30-40% des Tregs périphériques, peut délimiter des sous-populations	Chen 2008

Tableau 3 : Phénotype des Tregs

La découverte en 2001 du gène *Foxp3* et des fonctions de ce facteur de transcription marque une avancée majeure dans l'étude des Tregs. En effet, *Foxp3* est le gène responsable de la maladie des souris scurfy qui développent rapidement après la naissance une auto-immunité létale (Brunkow 2001). Chez l'Homme, la mutation du gène *FOXP3* est responsable du syndrome IPEX (Immune dysregulation, Polyendrocrinopathy, Enteropathy, X-linked syndrome) qui se caractérise par une atteinte auto-immune de multiples organes (Bennet 2001). Par la suite, il a été démontré que l'expression de Foxp3 est limitée aux LT $CD4^+CD25^+$ et que la transfection de LT $CD4^+CD25^-$ avec le gène *Foxp3* leurs confère des propriétés régulatrices (Hori 2003). En outre Foxp3 est d'une importance capitale pour le développement et les fonctions des Tregs (Fontenot 2005a). La délétion de *Foxp3* dans les cellules de l'épithélium thymique et les DCs (Liston 2007) ou dans les macrophages

(Josefowicz 2012) ne conduit à aucune dérégulation immunitaire. Dans les LT naïfs, l'ablation de *Foxp3* ne change pas le seuil d'activation (Hsieh 2006) et les LT effecteurs ne présentent pas de différence dans l'expansion clonale ou la sécrétion de cytokines (Fontenot 2005a). L'ensemble de ces donnés suggèrent que Foxp3 n'est pas un facteur de transcription crucial au développement et aux fonctions des autres populations du système immunitaire. Néanmoins Foxp3 n'est pas spécifique aux Tregs. Chez l'Homme (Miyara 2009) aussi bien que chez la souris (Miyao 2012), il a été démontré une expression transitoire de Foxp3 par les Tconvs au moment de l'activation.

1.2 Les autres LT régulateurs

Les Tregs $CD4^+CD25^+Foxp3^+$ sont les acteurs majeurs de la tolérance immunitaire. Cependant, d'autres types de LT régulateurs existent. Le tableau 4 résume leurs phénotypes et leurs fonctions.

Cellules	Phénotypes	Fonctions	Références
Tr1	$CD4^+CTLA\text{-}4^+Foxp3^-$	Régulent le diabète, l'IBD et l'EAE via l'IL-10 et le TGFβ	Roncarolo 2006
NKT $CD4^+$ type II	$CD4^+Foxp3^-ICOS^+PD\text{-}L1^+$	Suppriment le diabète via ICOS et PD-L1	Kadri 2012
$CD8^+$ Qa-1	$CD44^+CD122^+Ly\text{-}49^+NKG2D^+Foxp3^-$	Inhibent la production d'Ac par les LB	Kim 2011
$CD8^+Foxp3^+$	$CD25^+CD122^+Foxp3^+GITR^+$	Suppriment les fonctions des LT par des mécanismes similaires aux Tregs	Bienvenu 2005
$CD8^+CD28^-$	$CD18+CD27^+CD95^+DNAM\text{-}1^+Foxp3^-$	Suppriment la prolifération des Th in vitro par un mécanisme contact dépendant	Cortesini 2001
$CD8^+CD122^{fort}$	$CD122^{fort}Foxp3^-$	Inhibent les fonctions effectrices des LT par l'IL-10	Suzuki 2008

Tableau 4 : Les autres LT régulateurs

2) Les Tregs dans la tolérance et l'homéostasie

L'identification de Foxp3 comme facteur de transcription spécifique des Tregs a permis l'étude plus approfondie de cette population, notamment grâce à l'utilisation de souris délétées pour le gène *Foxp3* ($Foxp3^{null}$) ou exprimant le récepteur à la toxine diphtérique (DTR) humaine sous le contrôle du promoteur du gène *Foxp3* (DEREG et $Foxp3^{DTR}$). Les souris $Foxp3^{null}$ développent un syndrome auto-immun similaire à celui de la souris scurfy à partir de 12 jours après leur naissance et meurent au bout de 4 semaines. De plus, l'injection de LT $CD4^+CD25^+$ aux nouveau-nés à jour 2 prévient l'apparition de cette pathologie (Fontenot 2003). Les souris DEREG et $Foxp3^{DTR}$ développent également le même syndrome

en deux à trois semaines après ablation des Tregs (Lahl 2007), que ce soit chez les animaux nouveau –nés ou chez les adultes (Kim 2007). L'ensemble de ces résultats démontrent que les Tregs sont les acteurs majeurs de la tolérance périphérique et maintiennent l'homéostasie immunitaire. En outre, ils viennent également confirmer la véracité de la plupart des travaux antérieurs utilisant le CD25 comme marqueur des Tregs.

3) La différenciation des Tregs

Deux types de Tregs existent : ceux qualifiés de naturels (nTregs) se différencient dans le thymus et ceux dits induits (iTregs) se différencient à la périphérie à partir de LT $CD4^+$ naïfs.

3.1 Différenciation des nTregs

Les premières indications de l'origine thymique des Tregs proviennent d'expériences de thymectomie néo-natale. Les souris thymectomisées à J3 développent un syndrome auto-immun du à l'absence de Tregs qui apparaissent à la périphérie dès J4 (Asano 1996). Ceci à été confirmé grâce à l'utilisation de souris où Foxp3 est couplé à la GFP (Green Fluorescent Protein) (Fontenot 2005b).
La mise en évidence de l'importance du signal donné par le TcR dans la différenciation en Tregs s'est faite grâce à l'utilisation de souris possédant un TcR transgénique spécifique de la myéline et déficientes en RAG. Ces animaux ne possèdent pas de Tregs et développent des encéphalites auto-immunes contrairement à ceux possédant RAG (Olivares-Villagómez 1998). Ceci indique que l'avidité du TcR joue un rôle important dans la sélection thymique des Tregs, les RAG^{KO} étant incapables d'effectuer les réarrangements nécessaires à la génération d'un TcR de haute affinité. Par la suite, de nombreuses études ont pu mettre en évidence que l'intensité du signal apporté par le TcR est déterminante dans la différenciation des nTregs (Josefowicz 2012) (Figure 10).

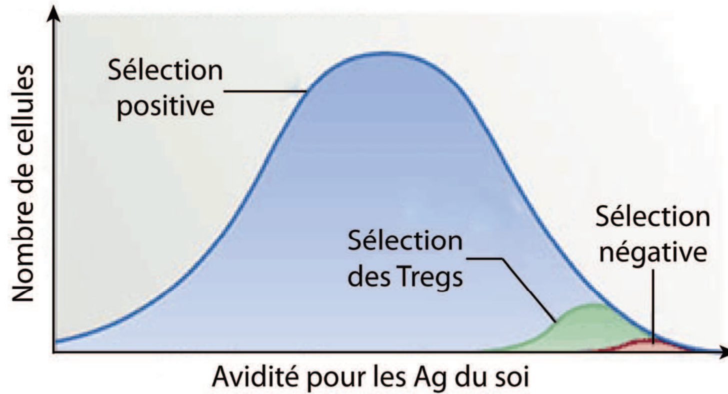

Figure 10 : Importance du signal TcR dans la sélection thymique des nTregs. D'après Hsieh *et al* Nature Review Immunology 2012

L'importance des signaux de co-stimulation a été démontrée chez les souris NOD-CD28^{KO}. Les souris CD28^{KO} ont une incidence de diabète très augmentée due à un nombre de Tregs diminué (Salomon 2000). Les signaux TcR et CD28 jouent un rôle important dans l'augmentation de la sensibilité des précurseurs de Tregs à l'IL-2, qui est le troisième signal important. En effet, c'est cette plus grande sensibilité à l'IL-2 qui va conduire à l'expression de Foxp3 (Lio 2008). La différenciation des nTregs semble reposer sur de nombreux autres facteurs qui ne sont pas complètement élucidés à l'heure actuelle.

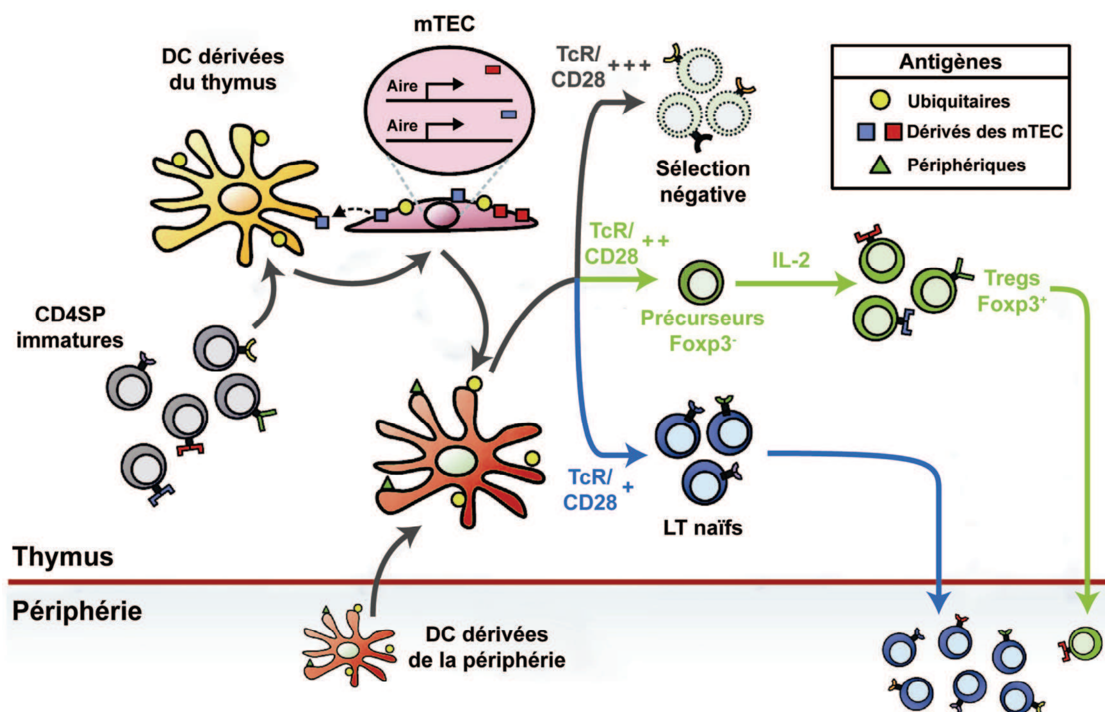

Figure 11 : Résumé de la différenciation thymique des Tregs. D'après Lio *et al* Current opinion in immunology 2011

3.2 Différenciation des iTregs (Bilate 2012)

La différenciation des LT $CD4^+$ naïfs en iTregs est favorisée dans deux conditions. L'activation des LT en absence d'inflammation, appelée condition sub-immunogène, ne permet pas la différenciation en LT effecteurs, mais est suffisante pour générer des iTregs. Lorsque les conditions inflammatoires permettent la différenciation en LT effecteurs, la génération d'iTregs est moins fréquente. De façon intéressante, il semble que les iTregs générés dans ces deux conditions aient des fonctionnalités différentes. Contrairement aux nTregs, l'intensité du signal TcR nécessaire à la différenciation en iTregs est beaucoup plus faible. L'IL-2 est requise comme pour les nTregs mais également le TGFβ.

4) Mécanismes d'action des Tregs

Les mécanismes de suppression mis en place par les Tregs peuvent être classés en quatre catégories : Sécrétion de cytokines suppressives, cytolyse, perturbations métaboliques et action sur les DCs.

4.1 Cytokines immuno-suppressives

4.1.1 L'IL-10

L'importance de l'IL-10 comme mécanisme de suppression des Tregs est longtemps restée controversée à cause de sa non-implication dans la suppression *in vitro* de la prolifération des Tconvs (Shevach 2006b). Cependant, de nombreuses données *in vivo* démontrent que l'IL-10 est un des mécanismes importants pour cette suppression. De nombreux modèles animaux ont mis en évidence le rôle de l'IL-10 dans la prévention de différentes pathologies telles que le rejet de greffe (Molitor-Dart 2007), l'hyper-sensibilité pulmonaire (Kearley 2005) ou les infections mycobactériennes (Kursar 2007). Mais, ces études n'ont pas démontré que l'IL-10 dans ces modèles était produite par les Tregs.

En 1999, l'équipe de Fiona Powrie a démontré que l'IL-10 produite par les Tregs est cruciale pour la prévention de l'IBD (Inflammatory Bowel Disease) induite par le transfert adoptif de LT $CD4^+$ naïfs, les Tregs provenant de souris $IL\text{-}10^{KO}$ étant incapables de prévenir la pathologie (Asseman 1999). C'est en 2008 que l'équipe d'Alexander Rudensky démontre définitivement le rôle de l'IL-10 produite par les Tregs grâce à l'utilisation de souris où l'ablation de l'IL-10 est spécifique des Tregs. Ces animaux ne développent pas de syndrome

auto-immun foudroyant de la même façon que les souris Foxp3^{null} ce qui suggère que l'IL-10 n'est pas indispensable aux fonctions suppressives des Tregs. Malgré tout, ces souris développent une IBD ainsi qu'une hyper-sensibilité pulmonaire et cutanée, indiquant que l'IL-10 produite par les Tregs est cruciale dans la régulation immunitaire aux interfaces environnementales (Rubtsov 2008).

4.1.2 Les autres cytokines immuno-suppressives

Deux autres cytokines immuno-suppressives sont importantes pour les fonctionnalités des Tregs : Le TGFβ et l'IL-35. L'IL-35 a été découverte récemment et est composé de la chaîne α de l'IL-12 et de la chaîne β de l'IL-27.

Cytokines	Effets	Références
TGFβ	Suppression de la prolifération *in vitro* des Tconv	Nakamura 2001
	Contrôle du diabète induit par des LT CD8$^+$ spécifiques des ilots pancréatiques	Green 2003
	La délétion du TGFβ dans les LT induit un syndrome auto-immun	Li 2007
	La délétion du récepteur au TGFβ rend les Tconv résistants à la suppression par les Tregs	Fahlén 2005
	Induction de Tregs par tolérance infectieuse	Waldmann 2006
IL-35	Les Tregs déficients en IL-35 sont moins suppresseurs *in vitro* et *in vivo*	Collison 2007
	Suppression via l'IL-35 potentialisée par le contact	Collison 2009

Tableau 5 : Autres cytokines immuno-suppressives utilisées par les Tregs

4.2 Cytolyse

Les Tregs peuvent utiliser la voie des récepteurs de mort pour induire l'apoptose. L'expression par les Tregs de TRAIL (Tumor necrosis factor related apoptosis inducing ligand) après activation permet l'induction de l'apoptose via sa liaison avec DR5 (Death receptor 5) sur la cellule cible (Ren 2007). En outre, la galectine-1 (Gal-1), qui se lie à CD45, CD43 et CD7, est très fortement exprimée par les Tregs. La liaison de la Gal-1 induit l'apoptose de la cellule cible. De plus, le Tregs provenant de souris déficientes pour la Gal-1, sont moins suppresseurs *in vitro* (Garin 2007).

Les Tregs peuvent également utiliser la voie de cytolyse par les granules cytotoxiques. En effet, les Tregs déficients en granzyme B voient leurs capacités suppressives *in vitro* amoindries (Gondek 2005). Il a, par la suite, été démontré que les Tregs peuvent directement lyser leurs cibles par la voie perforine/granzyme (Cao 2007).

4.3 Perturbations métaboliques

Les Tregs peuvent perturber le métabolisme et ainsi inhiber les fonctions effectrices ou bien induire l'apoptose de leurs cibles (Figure 12) :

- CD39 et CD73, deux ecto-enzymes exprimées à la surface des Tregs, catalysent la transformation de l'ATP en adénosine aux capacités inhibitrices.

- De par leur forte expression de CD25, les Tregs consomment beaucoup d'IL-2, en privant les Tconvs.

- Les Tregs possèdent une concentration importante d'AMPc intracellulaire et peuvent transférer cet inhibiteur de la croissance et de la prolifération des LT aux Tconvs via des jonctions Gap.

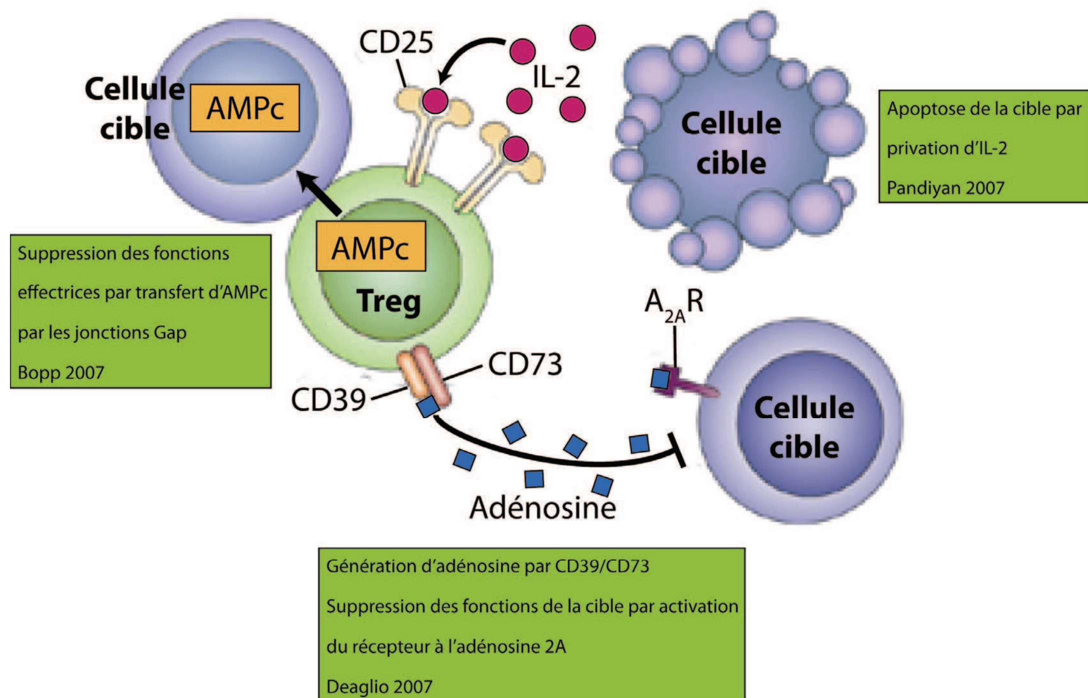

Figure 12 : Mécanismes d'immuno-suppression par perturbation métabolique. D'après Vignali *et al*, Nature Reviews Immunology 2008

4.4 Modulation des cellules dendritiques

Les Tregs peuvent agir sur les DCs pour les empêcher d'activer les LT effecteurs (Figure 13) :

- CTLA-4, une molécule de co-stimulation similaire à CD28, se lie également à CD80 et CD86. Contrairement à CD28, CTLA-4 transmet un signal inhibiteur.

- LAG-3 (Lymphocyte-Activation Gene-3) est un homologue du co-récepteur CD4 se fixant aux molécules du CMH de classe II avec une affinité très importante. Sa liaison sur les DCs immatures transmet un signal inhibiteur.

- Nrp-1 se lie au VEGF (Vascular Endothelial Growth Factor) et aux sémaphorines de classe 3 ce qui permet de prolonger les interactions avec la DCs.

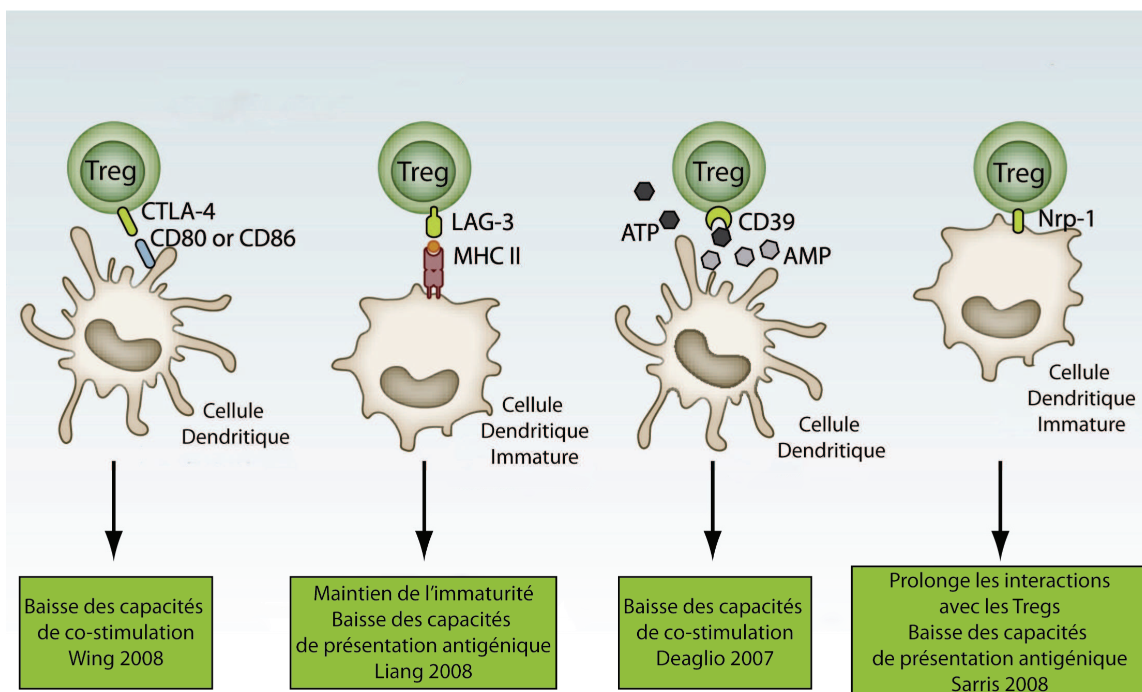

Figure 13 : Mécanismes d'immuno-suppression par modulation des DCs. D'après Shevach *et al*, Immunity 2009

5) Les Tregs : Cellules régulatrices professionnelles

5.1 Cibles des Tregs

Les Tregs peuvent inhiber différentes populations du système immunitaire (Tableau 6).

Cibles	Mécanismes	Références
Tconv	Tous mécanismes	cf paragraphe 4
LB	Perforine/Granzyme	Zhao 2006
NK	TGFβ	Ghiringhelli 2005
NKT	Contact cellulaire	Azuma 2003
Macrophages	TGFβ	Tonkin 2009
DC	cf paragraphe 4.4	cf paragraphe 4.4

Tableau 6 : Cibles des Tregs

5.2 Suppression de différentes classes de réponse immunitaire

Il est important de souligner qu'aucun des mécanismes précédemment cités ne peut à lui seul expliquer le contrôle du système immunitaire par les Tregs. Ces dernières années, plusieurs études ont suggéré que des ensembles de mécanismes sous contrôle de facteurs de transcription différents sont mis en place par les Tregs. Plus précisément, qu'ils soient générés dans le thymus ou à la périphérie, les Tregs peuvent exprimer Tbet, IRF4, GATA3, STAT3 ou Bcl-6 selon les conditions environnementales. Ces facteurs de transcription collaborent ensuite avec Foxp3 pour spécialiser les Tregs dans la suppression des réponses Th1, Th2, Th17 ou Tfh (Figure 14).

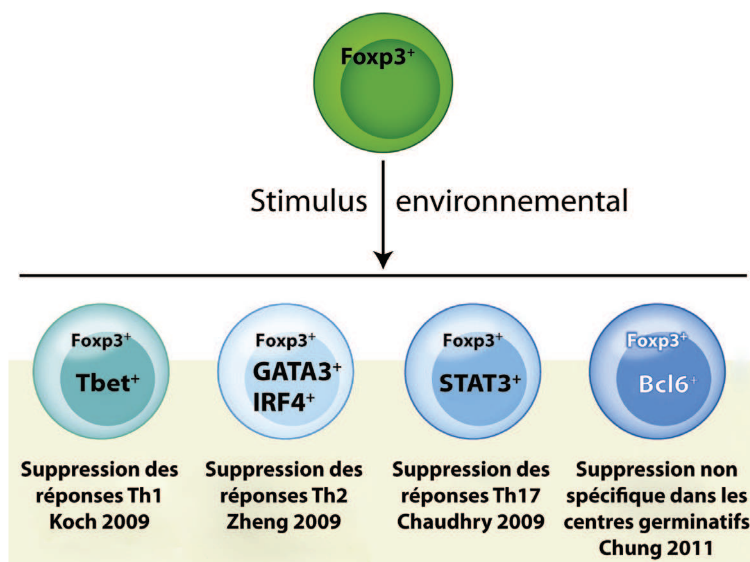

Figure 14 : Contrôle environnemental de l'activité des Tregs. D'après Josefowicz et al, Annual review of Immunology 2012.

C. Les Tregs dans le cancer

1) Mise en évidence de l'importance des Tregs dans le cancer

1.1 Dans les modèles murins

Le regain d'intérêt pour les Tregs dans les années 1990 a conduit à de nombreuses études des Tregs dans le cadre du cancer rapportant leur rôle de premier plan dans l'échappement tumoral. En particulier, le volume tumoral corrèle avec le nombre de Tregs (Shimizu 1999). En outre la déplétion des Tregs *in vivo* grâce à l'anticorps anti-CD25 permet de retarder la croissance tumorale (Onizuka 1999). De façon intéressante, la déplétion des LT $CD4^+$ totaux conduit au même résultat (Yu 2005). Ces données ont été confirmées par de nombreuses équipes aussi bien via l'utilisation de l'anticorps anti-CD25 seul ou en conjonction avec un anticorps anti-CTLA4 (Sutmuller 2001), de l'IL-12 (Nagai 2004) ou un transfert adoptif de DCs (Prasad 2005). Inversement, le transfert adoptif de Tregs s'accompagne d'une diminution de la réponse anti-tumorale (Turk 2004).

1.2 Chez l'Homme

En 2001, le groupe de Carl June met en évidence un nombre accru de Tregs dans le sang des patients atteints de cancer du poumon ou des ovaires. Depuis, de nombreuses études ont mis en avant une modulation des Tregs, aussi bien en fréquence qu'en fonctionnalité, dans le sang, les ganglions drainants ou encore les masses tumorales des patients (Tableau 7).

Types de tumeur	Cancers	Observations	Références
Tumeurs solides	Poumon	↗ sang, Grande proportion parmi les TILs	Woo 2001
	Ovaire	↗ sang, dLNs, Grande proportion parmi les TILs	Wolf 2005
	Mélanome	↗ sang, dLNs	Viguier 2004
	Gastro-oesophagien	↗ sang, dLNs, Grande proportion parmi les TILs	Sasada 2003
	Hepato-cellulaire	↗ sang, Grande proportion parmi les TILs	Ormandy 2005
	Tête et cou	↗ sang	Schaefer 2005
	Prostate	↗ sang, Grande proportion parmi les TILs	Miller 2006
	Sein	↗ sang, dLNs, Grande proportion parmi les TILs	Liyanage 2002
	Colorectal	↗ sang, Grande proportion parmi les TILs	Ling 2007
	Pancréas	↗ sang, dLNs, Grande proportion parmi les TILs	Liyanage 2002
Tumeurs hématologiques	Lymphôme Hodgkinien	↗ sang, Grande proportion parmi les TILs	Marshall 2004
	Lymphôme non-Hodgkinien	↗ sang, Grande proportion parmi les TILs	Yang 2006
	Leucémie lymphoïde chronique	↗ sang	Beyer 2005
	Leucémie myéloïde aigüe	↗ sang	Wang 2005
	Lymphôme cutané T	↘ capacités suppressives	Tiemessen 2006
	Myélôme multiple	↘ sang	Prabhala 2006

↗ : Augmentation ↘ : Baisse dLNs : Ganglions drainant les tumeurs TILs : Lymphocytes infiltrant les tumeurs

Tableau 7 : Proportions des Tregs dans différentes tumeurs humaines.

Dans toutes les tumeurs solides, la proportion de Tregs est augmentée, notamment dans le sang ou parmi les TILs (Lymphocytes infiltrant les tumeurs). Néanmoins, dans les tumeurs hématologiques, une baisse de la proportion de Tregs ou de leurs capacités suppressives est retrouvée.

L'infiltration de Tregs dans les tumeurs humaines donne également une information pronostique dans plusieurs types de cancers (Tableau 8). Dans la majorité des cas, une fréquence de Tregs importante est de mauvais pronostic. Néanmoins, cela dépend du type de tumeur et de sa localisation. Par exemple, dans les cancers colorectaux, une forte proportion de Tregs dans les tissus sains est de mauvais pronostic, alors qu'elle est de bon pronostic dans la tumeur. De même, une faible proportion de Tregs dans les lymphômes est corrélée à un mauvais pronostic ce qui concorde à la nature hématologique de cette tumeur, les Tregs étant capables d'inhiber les lymphocytes tumoraux.

Cancers	Observations concernant les Tregs	Pronostic	Références
Mélanome	Infiltration importante	↗ Progression	Miracco 2007
Ovaire	Infiltration importante	↗ Mortalité	Curiel 2004
	Faible ratio CTL/Tregs	↗ Mortalité	Curiel 2004
Gastro-oesophagien	Augmentation avec le stade	↗ Mortalité	Kono 2006
	Infiltration importante	↗ Mortalité	Kono 2006
	Tregs diffus dans le corps tumoral	↗ Progression	Mizukami 2008
	Proportion importante dans le stroma	↘ Mortalité	Haas 2009
Pancréas	Infiltration importante	↗ Progression ↗ Mortalité	Hiraoka 2006
Colorectal	Proportion forte dans les tissus sains	↗ Mortalité	Salama 2009
	Proportion forte dans les tumeurs	↘ Mortalité	Salama 2009
	Infiltration importante (cancers héréditaires)	↘ Mortalité	Frey 2010
Tête et cou	Infiltration importante	↘ Mortalité	Badoual 2006
Sein	Infiltration importante	↗ Progression	Bates 2006
Lymphôme	Faible proportion	↗ Mortalité	Alvaro 2005

↗ : Augmentation ↘ : Diminution

Tableau 8 : Valeur pronostique des Tregs dans différents cancers.

2) Accumulation des Tregs

L'accumulation de Tregs chez des hôtes porteurs de tumeurs peut se faire via quatre mécanismes distincts : Redistribution compartimentale, expansion, génération à partir de LT naïfs et survie préférentielle.

2.1 Redistribution compartimentale

Les Tregs peuvent exprimer beaucoup de récepteurs aux chimiokines en fonction de leur degré d'activation et de leur localisation tissulaire. Ainsi, les Tregs expriment CCR2, CCR4, CCR5, CCR7, CCR8, CCR10, CXCR4 et CXCR5 ce qui leur permet de répondre à une grande variété de chimiokines (Wei 2006). Les tumeurs peuvent sécréter des chimiokines capables d'attirer les Tregs dans le micro-environnement tumoral. Ainsi, les cellules tumorales sécrètent CCL22 (Curiel 2004), CCL17 (Ishida 2006), CCL5 (Tan 2009) et CXCL12 (Kryczek 2005) qui attirent les Tregs en se liant à CCR4, CCR5 et CXCR4 respectivement. Récemment, il a été montré que l'hypoxie, caractéristique du micro-environnement tumoral, induit la sécrétion de CCL28 qui attire les Tregs via sa liaison à CCR10 (Facciabene 2011). Les cellules tumorales ne sont pas les seules à participer au recrutement des Tregs dans le micro-environnement tumoral. En effet, les TAMs produisent également CCL22 qui attire les Tregs (Curiel 2004).
L'IL-2 utilisée comme adjuvant dans les thérapies anti-tumorales peut modifier les propriétés migratoires des Tregs. En effet, l'IL-2 provoque une augmentation de l'expression de CCR4 et CXCR4 à la surface des Tregs (Wei 2007). L'IL-2 étant également cruciale à la génération, à l'expansion et à la survie des Tregs (Malek 2004), la remise en question de ce type de thérapie semble s'imposer.

2.2 Expansion

L'accumulation des Tregs peut également venir de la prolifération de nTregs. Une proportion accrue de Tregs Ki67^{+} (marqueur de cellules proliférantes) est retrouvée dans de nombreux types de tumeurs (Wang 2005). Il a également été montré une activité plus importante de la télomérase dans les Tregs des patients atteints de cancer (Wolf 2005). Ceci indique que la prolifération des Tregs ne conduit pas à l'excision des télomères, augmentant ainsi leur survie et leur potentiel de prolifération. En mourant, les cellules tumorales relarguent des membres

de la famille des Hsp (Heat Shock Protein), tout particulièrement Hsp60 qui se lie à TLR2 et induit la prolifération des Tregs ainsi que l'augmentation de leur production d'IL-10 et de TGFβ (Liu 2006).

Le TGFβ permet la prolifération des Tregs *in vivo* (Huber 2004). De même l'activité d'IDO favoriserait la prolifération des Tregs *in vitro* par un mécanisme non encore élucidé (Chung 2009). Les cellules tumorales ainsi que les MDSCs et les DCs tolérogènes produisent de grandes quantités de TGFβ et expriment IDO (Indoleamine 2,3 Dioxygenase) ce qui contribuerait à la prolifération des Tregs. En outre, les MDSCs peuvent provoquer la prolifération des Tregs *in vitro* par leur expression de CD40 et l'interaction avec son ligand CD40L sur les Tregs (Pan 2010). L'inhibition de l'activité d'Arg1 dans les MDSCs réduit la prolifération des Tregs (Serafini 2008) indiquant un rôle de cette enzyme, mais le mécanisme exact de son action n'est pas encore élucidé.

2.3 Induction à partir de LT $CD4^+$ naïfs

L'induction de Tregs à partir de LT $CD4^+$ naïfs peut se faire par la sécrétion de différents médiateurs solubles par les cellules tumorales (Figure 15).

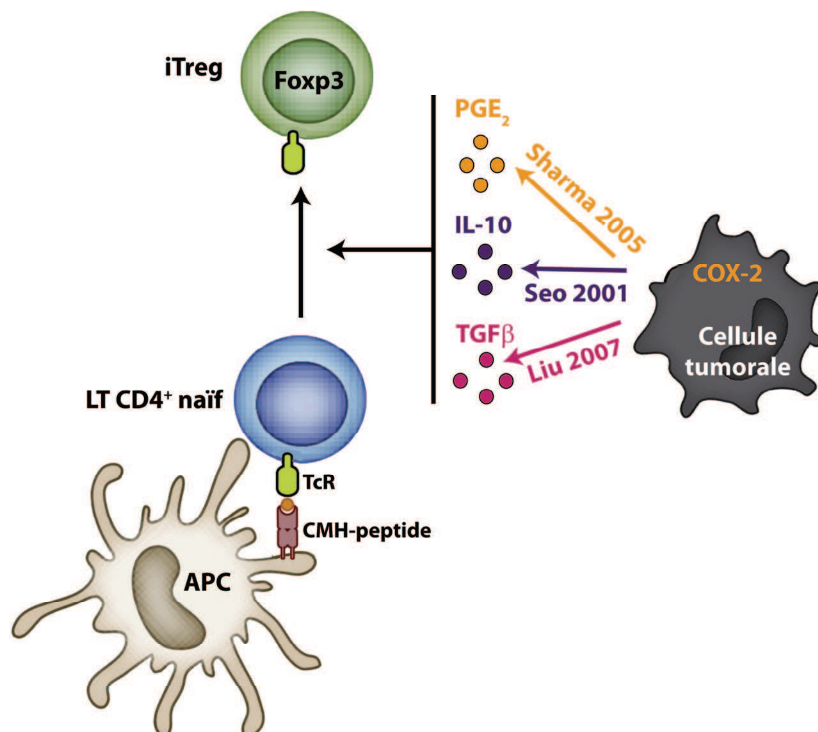

Figure 15 : Mécanismes d'induction de Tregs par les cellules tumorales.

La cycloxygénase-2 (COX-2), très exprimée par les cellules tumorales, produit la prostaglandine E_2 (PGE_2) qui permet l'induction de Foxp3 dans les LT naïfs, et provoque l'augmentation de l'activité des Tregs (Sharma 2005). L'IL-10 et le TGFβ produits en grande quantité par les cellules tumorales permettent également la génération d'iTregs. En outre, l'expression d'IDO par les cellules tumorales pourrait contribuer à l'induction de Tregs dans le micro-environnement bien que ce mécanisme n'ait pas encore été étudié.

Les cellules tumorales ne sont pas les seules à promouvoir l'induction de Tregs dans le micro-environnement tumoral. Les cellules myéloïdes participent à la génération d'iTregs. De par leur production de TGFβ, les DCs tolérogènes (Dumitriu 2009) et les MDSCs (Huang 2006) peuvent induire des Tregs. Ces deux populations peuvent également favoriser la voie de différenciation des iTregs plutôt que des Th17 par un mécanisme dépendant d'IDO pour les DCs (Baban 2009) ou de la production d'acide rétinoïque pour les MDSCs (Hoechst 2011).

2.4 Survie préférentielle

Les Tregs survivent mieux que les Tconvs aux conditions du micro-environnement tumoral. Ils sont plus résistants à la mort induite par le stress oxydatif (Mougiakakos 2009) notamment grâce à l'expression de HO-1 (Brusko 2005). Il a également été démontré que les Tregs ont une susceptibilité moins grande à l'apoptose (Stranzer 2008) grâce à l'expression de niveaux plus importants de Bcl2 et IAP1 que les Tconvs (Jak 2009). De plus, l'analyse génétique des Tregs du micro-environnement tumoral a montré une surexpression des gènes LGALS1 et 3 impliqués dans le contrôle de l'apoptose ainsi qu'une expression diminuée des gènes pro-apoptotiques BAX et TNFRSF25 (Jeron 2009).

3) Spécificité antigénique des Tregs

De la même façon que les Tconvs, les Tregs sont capables de reconnaitre les antigènes associés aux tumeurs. Les premiers Tregs spécifiques d'antigènes tumoraux ont été isolés à partir de lymphocytes infiltrant les tumeurs de patients atteints de mélanome et étaient spécifiques de l'Ag LAGE1 (Wang 2004b). Par la suite, des Tregs spécifiques de gp100, TRP1 et NY-ESO-1 ont été identifiés chez des patients atteints de mélanome (Vence 2007). De plus, cette étude suggère que ces Tregs exercent leurs capacités suppressives de façon dépendante d'un contact cellulaire. D'un autre côté, chez les patients atteints de leucémie

myéloïde aigüe, des Tregs spécifiques de WT1 n'ont pas besoin de contact pour leur suppression *in vitro* (Lehe 2008). Dans le cancer colorectal, des Tregs spécifiques de la télomérase, CEA, EGFR, la mucine-1 et HER2 ont été détectés (Bonertz 2009). L'ensemble de ces résultats suggère que les Tregs peuvent contrôler la réponse dirigée contre les antigènes tumoraux de façon spécifique.

4) Implication des Tregs dans la suppression des réponses anti-tumorales

Les Tregs peuvent inhiber la réponse anti-tumorale en agissant sur de multiples cibles du système immunitaire. Cependant, la majorité des études se sont concentrées sur la suppression des réponses impliquant les Tconvs.
La plupart des études démontrent que les Tregs ont un rôle dans l'inhibition des réponses anti-tumorales. Malgré tout, il a été mis en évidence que les Tregs du micro-environnement tumoral prolifèrent moins et mobilisent moins le calcium en réponse à un anticorps anti-CD3 que ceux se trouvant à la périphérie (Lutsiak 2008). De plus, les Tregs peuvent présenter un rôle anti-tumoral dans les étapes tardives de la progression cancéreuse en inhibant les MDSCs (Zhang 2010).

4.1 Inhibition des réponses immunitaires lymphocytaires T

Les Tregs peuvent jouer sur la réponse anti-tumorale médiée par les LT $CD4^+$ et par les LT $CD8^+$.

4.1.1 Suppression des LT $CD4^+$
L'influence des Tregs sur les LT $CD4^+$ a été mise en évidence aussi bien chez l'Homme (Clarke 2006) que dans des modèles murins (Liu 2009) en démontrant la plus grande fonctionnalité des LT $CD4^+$ effecteurs suite à la déplétion des Tregs. En outre, cette inhibition peut être directe par des mécanismes mettant en jeu l'IL-10 et le TGFβ (Strauss 2007, Loser 2007) ou bien indirecte par l'induction de Tr1 (Strauss 2008). En plus de l'inhibition des fonctions effectrices anti-tumorales, les Tregs empêchent les LT $CD4^+$ de sécréter des facteurs anti-angiogéniques tel que l'IFNγ (Casares 2003), mais aussi d'apporter l'aide nécessaire à l'activité maximale des LT $CD8^+$ (Chaput 2007).

4.1.2 Suppression des LT CD8$^+$

L'inhibition de la réponse anti-tumorale des LT CD8$^+$ est l'aspect le plus étudié du rôle des Tregs dans le contexte tumoral et les mécanismes mis en jeu sont nombreux. Les Tregs inhibent la prolifération (Li 2010, Yu 2005) et la production d'IFNγ (Turk 2004, Yu 2005) des LT CD8$^+$. La déplétion des Tregs *in vivo* induit l'expansion de LT CD8$^+$ spécifiques des Ag tumoraux ainsi que l'augmentation de leur production d'IFNγ (Morse 2008). Le TGFβ produit par les Tregs (Chang 2012) ainsi que l'engagement de PD-1 (Wang 2009) inhibent la prolifération et les fonctions effectrices des LT CD8$^+$. De plus, les Tregs peuvent induire la mort des LT CD8$^+$ de par leur sécrétion de TGFβ (Chang 2012) mais également par cytolyse impliquant le granzyme B (Cao 2007). En outre, il a été démontré que CCL5, produit par les cellules tumorales, est crucial pour l'augmentation des capacités suppressives des Tregs vis-à-vis des LT CD8$^+$ (Chang 2012).

Il a été mis en avant que l'inhibition des LT effecteurs à la fois CD4$^+$ et CD8$^+$, au-delà de l'augmentation du nombre ou des capacités suppressives des Tregs, peut être du au statut d'activation des Tregs par rapport aux LT effecteurs (Darrasse-Jèze 2009). En effet, les Tregs répondent plus rapidement que les LT effecteurs naïfs, créant ainsi un environnement immuno-suppresseur dominant. En revanche, si les LT effecteurs sont mémoires, la dominance va à la réponse effectrice.

4.2 Inhibition des réponses immunitaires non lymphocytaires T

Les Tregs sont capables d'inhiber d'autres acteurs de la réponse anti-tumorale. Les fonctionnalités des NK peuvent être inhibées par le TGFβ (Smyth 2006). Les NK peuvent également être lysés par la voie perforine/granzyme B (Cao 2007). En outre, les Tregs peuvent aussi inhiber l'expression des molécules de co-stimulation CD80 et CD86 à la surface des DCs (Liu 2009) ou bien les tuer dans les ganglions drainants via l'utilisation du granzyme B (Boissonnas 2010).

4.3 Impact des Tregs sur les cellules tumorales

Il a récemment été mis en évidence, aussi bien chez l'Homme que dans un modèle murin, que les Tregs peuvent avoir une influence directe sur les cellules tumorales. En effet, l'interaction entre RANKL, exprimé par les Tregs, et son récepteur RANK exprimé par les cellules tumorales, stimule la prolifération et les de capacités a métastaser ces dernières (Tan 2011). Ainsi, les Tregs agiraient via de nouveaux mécanismes directement sur les cellules tumorales. Néanmoins, cette hypothèse reste a étudier plus avant.

5) Potentielles interventions thérapeutiques

Le tableau 9 regroupe les interventions thérapeutiques ciblant les Tregs. Cependant, même si l'idée de la déplétion des Tregs ou du blocage de leurs fonctions est séduisante, il parait peu probable que cette intervention seule suffise à induire le rejet des tumeurs. En effet, il a été montré que l'utilisation thérapeutique des anticorps anti-CD25, anti-GITR ou anti-récepteur au folate ne peut conduire au rejet tumoral (Teng 2010, Quezada 2008). Même si la déplétion des Tregs est bonne, le manque d'infiltration des effecteurs du système immunitaire dans le corps tumoral ne permet pas une réponse efficace. Cependant, la combinaison de la déplétion de Tregs ou de l'inhibition de leurs fonctions avec des thérapies existantes telles que la radiothérapie ou le transfert adoptif de LT effecteurs pourrait permettre d'augmenter le rejet tumoral (Quezada 2008).

Stratégies	Interventions	Références
Déplétion des Tregs	Anticorps anti-CD25	Zou 2006
	Denileukin diftitox (IL-2 couplé à la toxine diphtérique)	Litzinger 2007
	Cyclophosphamide	Liu 2007b
Blocage des fonctions des Tregs	Anticorps anti-CTLA-4	O'Mahony 2007
	Inhibition de STAT3	Pallandre 2007
	Ciblage de OX-40	Piconese 2008
	Anticorps anti-GITR	Turk 2004
Blocage du trafic des Tregs	Pas de moyen connu à l'heure actuelle	Curiel 2004
Blocage de la différenciation en iTregs	Pas de moyen connu à l'heure actuelle	Mantel 2007
Orienter la différenciation vers Tconv	Pas de moyen connu à l'heure actuelle	Quintana 2008

Tableau 9 : Potentielles interventions thérapeutiques ciblant les Tregs.

D. Objectif du travail et modèle expérimental

Mes travaux de thèse se sont focalisés sur deux aspects importants de la suppression des réponses immunitaires anti-tumorales.

J'ai étudié le rôle des Tregs dans la suppression de la réponse immunitaire anti-tumorale et tout particulièrement les interactions entre les Tregs et l'immunité innée. Comme nous l'avons vu précédemment (cf. paragraphe C.4), il est maintenant établi que les Tregs sont capables d'inhiber la réponse des LT effecteurs spécifiques des tumeurs. Les Tregs peuvent également supprimer la réponse anti-tumorale médiée par les DCs et les NK. Cependant, l'impact des Tregs sur les autres populations du système immunitaire dans le contexte tumoral est beaucoup moins connu. De plus, la majeure partie des observations sont faites dans le micro-environnement tumoral. Je me suis donc attaché à étudier ces cellules à la fois localement et dans les organes lymphoïdes périphériques. En outre, dans le cadre du cancer, les différentes populations de cellules myéloïdes sont majoritairement étudiées pour leur aspect pro-tumoral. En effet, le rôle suppresseur des MDSCs et des TAMs a été mis en avant par de nombreuses études (Gabrilovich 2012). Néanmoins, très peu se sont intéressées au potentiel anti-tumoral des monocytes, des granulocytes et des macrophages.

La seconde partie de mes travaux de thèse a porté sur l'interaction entre LT et TAMs et son impact sur la suppression des réponses immunitaires anti-tumorales. En effet, si les rôles antagonistes, à la fois immuno-suppresseurs et anti-tumoraux, des macrophages dans le cadre du cancer sont bien établis, les mécanismes impliqués dans leur polarisation d'un phénotype M1 à M2 restent encore en grande partie à élucider.

Afin d'adresser ces différentes questions, j'ai disposé d'un modèle expérimental de mélanome métastatique, les souris transgéniques MT/ret (Kato 1998). Le proto-oncogène RET code pour un récepteur à activité tyrosine kinase activant les voies ERK ou PI3K impliquées dans la survie et la prolifération et dont le ligand principal est le facteur de croissance GDNF (Glial cell-Derived Neurothrophic Factor). Une mutation activatrice de RET est impliquée dans le carcinome médullaire de la thyroïde, le neuroblastome ainsi que le mélanome (Narita 2009).
Chez les souris MT/ret, le proto-oncogène RET humain muté et placé sous le contrôle du promoteur ubiquitaire de la métallothionéine est exprimé dans les cellules provenant de la

lignée neuro-ectodermale dont font partie les mélanocytes (Iwamoto 1991). Il en résulte une activation constitutive de ret. Les souris MT/ret présentent, dès quelques jours après leur naissance, une mélanose importante (Kato 1998). Elles développent, dès l'âge de trois semaines, un mélanome primaire uvéal, plus précisément au niveau de la choroïde. Cette tumeur métastase rapidement localement au niveau cutané et à distance (poumons, adénopathie médiastinale et viscérale), mais les cellules tumorales disséminées sont maintenues en dormance (Eyles 2010). A l'âge de 12 semaines, environ 60% des souris MT/ret présentent des métastases cutanées palpables. De façon intéressante, les souris MT/ret développent, dans environ 30% des cas à 12 semaines, un vitiligo associé au mélanome corrélé à une protection contre l'apparition de métastases cutanées (Lengagne 2004). Le vitiligo est un désordre de la pigmentation parfois spontanément observé chez les patients atteints de mélanome et beaucoup plus fréquemment lors d'une immunothérapie. L'apparition d'un vitiligo est corrélée à une réponse clinique positive et à un pronostic favorable.

Les souris MT/ret récapitulent bien l'histoire naturelle du mélanome, depuis la tumeur primaire jusqu'aux métastases distantes, et sont donc un modèle pertinent pour l'étude de la réponse immunitaire anti-tumorale, notamment de la progression tumorale et de la dissémination métastatique.

RESULTATS

Article 1 :

T Cells Contribute to Tumor Progression by Favoring Pro-Tumoral Properties of Intra-Tumoral Myeloid Cells in a Mouse Model for Spontaneous Melanoma

PLoS ONE 6(5): e20235.

Renée Lengagne, Arnaud Pommier, Jonathan Caron, Laetitia Douguet, Marylène Garcette, Masashi Kato, Marie-Françoise Avril, Jean-Pierre Abastado, Nadège Bercovici, Bruno Lucas, Armelle Prévost-Blondel.

Les tumeurs affectent la myélopoïèse et induisent l'expansion de cellules myéloïdes ayant des capacités immunosuppressives. Dans le modèle MT/ret de mélanome spontané métastatique, les cellules myéloïdes sont la population la plus abondante parmi les cellules hématopoïétiques infiltrant les tumeurs. De plus, leur proportion augmente dans les métastases cutanées les plus agressives. Nos données suggèrent que le micro-environnement tumoral favorise la polarisation des cellules myéloïdes en macrophages de type 2 caractérisés par l'expression de F4/80, une faible capacité de sécrétion d'IL-12 et une forte production d'arginase. Les cellules myéloïdes de la tumeur et de la rate des souris MT/ret inhibent la prolifération des lymphocytes T et leur sécrétion d'IFNγ. De façon intéressante, les lymphocytes T jouent un rôle dans la polarisation des cellules myéloïdes vers le type 2. En effet, les cellules myéloïdes intra-tumorales de souris MT/ret dépourvues de lymphocytes T sont moins suppressives que celles des souris MT/ret sauvages et inhibent plus efficacement la prolifération des cellules tumorales. Nos données soutiennent donc l'existence d'un cercle vicieux dans lequel les lymphocytes T pourraient favoriser le développement tumoral en établissant un environnement biaisant les cellules myéloïdes vers un phénotype pro-tumoral et, à leur tour, supprimer la réponse des effecteurs du système immunitaire.

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T Cells Contribute to Tumor Progression by Favoring Pro-Tumoral Properties of Intra-Tumoral Myeloid Cells in a Mouse Model for Spontaneous Melanoma

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Abstract

Tumors affect myelopoeisis and induce the expansion of myeloid cells with immunosuppressive activity. In the MT/ret model of spontaneous metastatic melanoma, myeloid cells are the most abundant tumor infiltrating hematopoietic population and their proportion is highest in the most aggressive cutaneous metastasis. Our data suggest that the tumor microenvironment favors polarization of myeloid cells into type 2 cells characterized by F4/80 expression, a weak capacity to secrete IL-12 and a high production of arginase. Myeloid cells from tumor and spleen of MT/ret mice inhibit T cell proliferation and IFNγ secretion. Interestingly, T cells play a role in type 2 polarization of myeloid cells. Indeed, intra-tumoral myeloid cells from MT/ret mice lacking T cells are not only less suppressive towards T cells than corresponding cells from wild-type MT/ret mice, but they also inhibit more efficiently melanoma cell proliferation. Thus, our data support the existence of a vicious circle, in which T cells may favor cancer development by establishing an environment that is likely to skew myeloid cell immunity toward a tumor promoting response that, in turn, suppresses immune effector cell functions.

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Introduction

Tumor development affects bone marrow myelopoeisis and induces the expansion of myeloid derived suppressive cells (MDSC) [1]. In mouse models, MDSC express the αM integrin CD11b and the myeloid lineage differentiation antigen Ly6C/G recognized by the Gr1 antibody. CD11b$^+$Gr1$^+$ cells represent 2–4% of splenocytes of normal mice, but increase 5- to 20- fold in mice transplanted with tumors [2]. Tumor-induced CD11b$^+$Gr1$^+$ splenocytes are considered to be a heterogeneous population of immature monocytes/macrophages, granulocytes, dendritic cells and their progenitors [3,4,5]. It is established that MDSC suppress conventional T cell proliferation and activation [6]. In addition, MDSC have been suggested to impact the functions of other cells including NK, macrophages and regulatory T cells [7,8,9]. Dissection of their properties is hampered by the lack of specific marker. The α chain of the IL4 receptor (IL-4Rα) [10] and the M-CSF receptor (CD115) [11] have been proposed to help identifying subpopulations of mouse MDSC. Nevertheless, they do not identify immune suppressive populations of MDSC in various tumor models [12].

Macrophages have long been recognized as important components of the innate immunity against tumors [13]. While tumor associated macrophages (TAM) can prevent the establishment and spread of tumor cells, they may also favor tumor growth and dissemination. This paradox is due to the inherent plasticity of macrophages, which can display distinct phenotypes and functions in response to different signals [14,15,16]. M1 macrophages secrete IL-12 and NO, promote antitumor immunity and directly kill tumor cells, whereas alternatively activated M2 macrophages exhibit defective production of IL-12, high IL-10 secretion, produce arginase, suppress the antitumor response and promote angiogenesis and metastasis [17,18,19,20]. Accordingly, TAM favor tumor progression in most mouse models [21,22,23,24], but are vital for tumor eradication in others [25,26], suggesting that TAM display contradictory roles depending on the tumor type which might dictate their polarization [27].

Only few recent data have shown that immune cells within the tumor microenvironment may influence the pro-tumoral properties of tumor infiltrating macrophages. De Visser et al were the first to demonstrate the requirement of B cells in mediating the recruitment of inflammatory cells into premalignant skin associated with neoplastic progression using the model of HPV16 induced epithelial carcinogenesis [28]. More recently, B cells have been shown to play a role in driving M2 like polarization of TAM [29]. Sinha et al have shown that MDSC suppress tumor

immunity by reducing macrophage IL12 production via an IL10 dependent mechanism [9]. In addition, although some subsets of lymphocytes including cytotoxic $CD8^+$ T cells and NK cells exhibit anti-tumor immunity [30], other subsets notably Th2 cells, $CD4^+$ regulatory T cells and NKT cells may exhibit opposite effect on tumor progression by interfering with TAM properties [31]. Thus, various immune cells seem to contribute to myeloid orientation although the relative contribution of the different cell types during spontaneous development of tumors is unclear.

In the model of spontaneous melanoma driven by the RET oncogene (MT/ret mice; [32]), the primary uveal tumor cells disseminate at three weeks of age, but remain dormant for several weeks [33]. 50% of 3 month old mice display cutaneous metastasis and finally develop visceral metastasis [34]. In this MT/ret model, we have shown that $CD8^+$ T cell depletion does not accelerate the onset of cutaneous metastasis, suggesting the presence of tumor induced immunosuppressive factors locally. In the present study, we focused our interest on the characterization of myeloid cells within cutaneous metastasis. Given the critical role of adaptative immunity in regulating innate immune cell functions in some mouse models of cancer development [14], we addressed the possibility that T cells might exert a role in regulating recruitment and/or pro-tumor properties of tumor infiltrating myeloid cells in MT/ret mice. We report that myeloid cells are the most abundant hematopoietic population within the cutaneous metastasis and that they display immune suppressive functions. Our data further established the critical role of T cells in the acquisition of pro-tumoral properties of intra-tumoral myeloid cells in the course of melanoma development.

Materials and Methods

Ethics statement

All animals were handled in strict accordance with good animal practice in compliance with French Ministry of Agriculture regulations for animal experimentation. The animal experiment protocol approval number is 75–510 and was delivered by the veterinary departement of Paris. All experiments were performed in animal facilities which also received an approval number (A75-14-02).

Mice

MT/ret transgenic mice express the human Ret oncogene [32]. 3 to six month old mice at different stages of malignancy were used and age-matched non-transgenic MT/$ret^{-/-}$ littermates were used as control (ctrl). MT/ret mice were crossed with C57BL/6 $CD3\varepsilon^{-/-}$ mice [35] to obtain RetCD3εKO and $RetCD3\varepsilon^{+/-}$ (called $RetCD3\varepsilon^+$ later) mice. MT/ret, RetCD3εKO and $RetCD3\varepsilon^+$ mice were diagnosed for recording the development of exophthalmus related to the uveal primary melanoma, and subsequent cutaneous metastasis. All mice are maintained in our own pathogen free animal facilities. OT-1 mice expressing a TCR specific for Ova257-264 were purchased from Charles River Laboratories.

Cell suspension procedures from lymphoid organs and tumors

Spleens and cutaneous tumor masses were mechanically dissociated and digested with 1mg/mL collagenase A and 0,1mg/mL DNase I (Roche, Mannheim, Germany) for 25 min at 37°C. Single cell suspensions were filtered, washed in PBS 1X, 5% FCS, 0.5 mM EDTA and resuspended in RPMI 1640.

Flow cytometry

After blocking with anti-FcγR Ab, cell suspensions were stained with the following mAbs against CD45.2, CD11b, Gr1, TcRαβ, TcRγδ, CD8α, CD4, CD19 and CD124 from Pharmingen (BD Biosciences, Le Pont de Claix, France), NK1.1 from eBiosciences (San Diego, CA) and F4/80 from Serotec (Düsseldorf, Germany). For IL-12 intracytoplasmic staining, cell suspensions were stimulated overnight with 2 µM Golgi Stop Monensin, 0.1 µg/ml LPS and 101ng/ml IFNγ. The staining was performed following the manufacturer's instructions (kit Cytofix/cytoperm, BD Biosciences) using the combination CD45.2, CD11b, and IL-12. Analyses were performed on a FacsCalibur cytofluorometer (BD Biosciences).

Isolation of myeloid cells and supernatants

$CD11b^+$ cells from spleens and cutaneous nodules were selected via magnetic microbeads conjugated to anti-mouse CD11b mAb (Mac-1; Miltenyi Biotec) using MS columns according to the manufacturer's specifications (Miltenyi Biotec). For supernatant collection, 5.10^5 cells per ml were cultured for 48 h in complete medium at 37°C.

IFNγ-ELISpot

Ctrl mice were immunized with 50 µg of peptide 33–41 from LCMV glycoprotein (GP33:KAVYNFATM) in IFA. Eight days later, inguinal lymph node cells were collected. ELISpot was performed using the mouse IFNγ ELISpot kit (BD Biosciences). GP33-specific T cells (2.10^5 cells/well) were stimulated 24 h with GP33 (10-7M) in the presence of $CD11b^+$ or $CD11b^-$ cells (10^4 cells/well) or corresponding myeloid derived supernatants. In another setting, freshly isolated splenocytes (2.10^5 cells/well) from tumor bearing mice were cultured with the syngeneic TIII melanoma cell line (10^3 cells/well) [34]. Splenocytes were used either directly or after depletion of $CD11b^+$ cells.

CFSE labeling and IFNγ secretion of TCR transgenic T cells

CD8 T cells from lymph nodes of OT-1 mice were prepared using a mouse CD8 negative isolation kit (Dynal Biotec, Oslo,

Table 1. Primer pairs used for real time PCR experiments.

Target cDNA	Upper/Lower	Sequences (5′ to 3′)
GAPDH	U	GCC GGT GCT GAG TAT GTC GT
	L	GGA GAT GAT GAC CCG TTT GG
IL10	U	GGT TGC CAA GCC TTA TCG GA
	L	ACC TGC TCC ACT GCC TTG CT
ARG1	U	ATG GAA GAG ACC TTC AGC TAC
	L	GCT GTC TTC CCA AGA GTT GGG
FIZZ1	U	CCC AGG ATG CCA ACT TTG AA
	L	GGC CCA TCT GTT CAT AGT CT
MGL1	U	ATG ATG TCT GCC AGA GAA CC
	L	ATG ATG TCT GCC AGA GAA CC
EMR1	U	CTC ACC GGT ATA GAC AA
	L	GCA GGC GAG GAA
CCL2	U	TTA AAA ACC TGG ATC GGA ACC AA
	L	GCA TTA GCT TCA GAT TTA CGG GT

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Norway). Purified cells were labeled with CFSE (Molecular Probes). 5.10^4 CFSE$^+$ cells were cultured for 72 h in presence or not of Ova257 (8.10^{-4} µM) with 5.10^5 CD11b$^+$ cells. The supernatants were analyzed for IFNγ by ELISA using IFNγ capture biotinylated mAb, and streptavidin-alkaline phosphatase (BD biosciences). CFSE dilution was determined by flow cytometry.

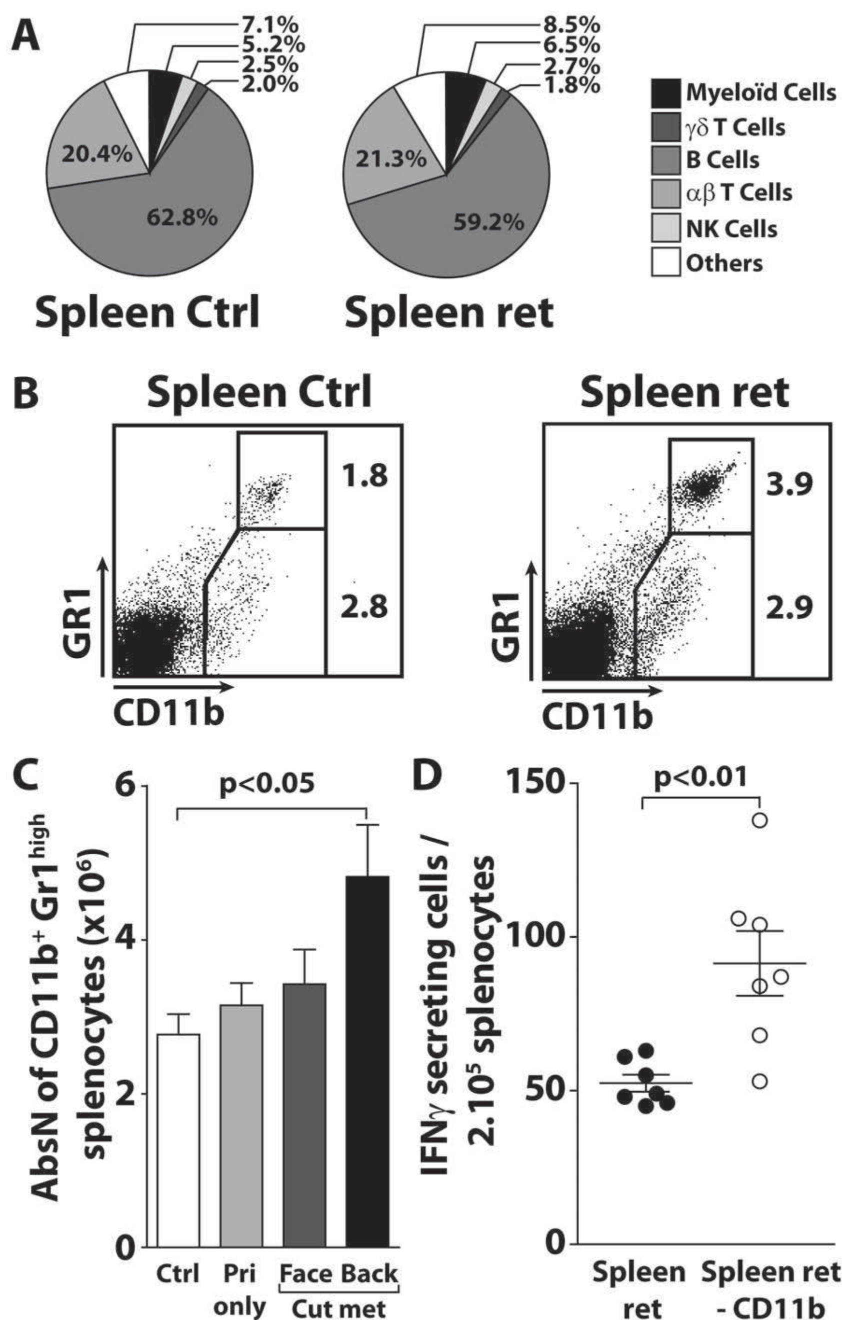

Figure 1. Accumulation of CD11b$^+$Gr1^{high} myeloid cells in spleen of tumor bearing MT/ret mice. (**A**) Comparison of the proportion of hematopoietic cells in spleen of MT/ret and ctrl mice. Myeloid cells are CD11b$^+$ cells. αβT cells are defined as CD4$^+$ αβTCR^{high} cells and CD8$^+$ αβTCR^{high} cells. γδT cells are defined as CD8- γδTCRhigh cells. B cells are defined as CD19$^+$CD8$^-$ cells and NK cells are NK1.1$^+$CD4$^-$CD8$^-$ cells. The pie diagram summarizes data from spleens of MT/ret (n = 29) and Ctrl (n = 13) age matched mice. (**B**) Single cell suspensions derived from spleens of MT/ret or Ctrl mice were stained for CD45, CD11b and Gr1. Representative CD11b/Gr1 dot plots were generated from gated CD45$^+$ cells. (**C**) The histograms correspond to the absolute numbers of both CD11b$^+$Gr1^{high} cell subsets in ctrl spleens (n = 13) and in spleens of MT/ret mice displaying either primary tumors (n = 8), facial (face, n = 13) or dorsal cutaneous metastasis (back, n = 12). (**D**) Myeloid cells from MT/ret spleen inhibit the spontaneous response specific for melanoma. The number of IFNγ-secreting cells was assessed by an ELISPOT assay using splenocytes from MT/ret mice as effectors and Melan-ret cells as targets. Ex vivo splenocytes were used either in total or after depletion of CD11b$^+$ cells. The significance was assessed using unpaired t test.
doi:10.1371/journal.pone.0020235.g001

Quantitative RT-PCR

Total RNA was isolated from purified CD11b$^+$ cells using RNAlater and RNeasy columns (Qiagen, Courtaboeuf, France). RNA was reverse transcribed with SuperScript^{TM} II (Invitrogen) and oligo-dT18 primers. Quantitative PCR was carried out using fast SYBR Green Master Mix (Applied Biosystems) and a real time PCR system (Light Cycler 1.5, Roche Diagnostics, Division Applied Sciences, Meylan, France) according to standard PCR conditions. For quantitative calculations, values were normalized to GAPDH expression. Primer sequences are listed in Table 1.

Tumor cell proliferation assay

The xCELLigence System (Roche Diagnostics) monitors cellular events in real time without the incorporation of labels. The System measures electrical impedance across interdigitated micro-electrodes integrated on the bottom of tissue culture E-Plates. The impedance measurement provides quantitative information about the status of the adherent cells, including cell number, viability, and morphology. Melan-ret melanoma cells (5.10^3) were seeded into the wells of 96X E-Plates in 100 µl of media. Cell adhesion and growth were monitored 48h till their exponential growth phase. Tumor derived CD11b$^+$ purified cells (5.10^4 cells) were added in a volume of 100 µl/well. Co-cultures were assessed by the system with a measure every 5 min for up to 40 h. Results, expressed as Cellular Index, were normalized (nCI) with RTCA Software, and expressed as percentage of specific inhibition = (1-nCI (Melan-ret cells + CD11b$^+$ cells) /nCi (Melan-ret cells))×100.

NO assay

Purified CD11b$^+$ cells (5.10^5) were cultured for 2 days in RPMI, 10% FCS supplemented with LPS and IFNγ. NO was measured using Griess reagents (Promega, Charbonnières-les-Bains). Briefly, 50 µl of culture supernatant was incubated for 10 min at room temperature with 50 µl of Griess reagent A plus 50 µl of Griess reagent B. Absorbance at 540 nm was measured using a microplate reader (Perkin Elmer). Data are mean +/−SD of triplicate wells.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 4.0 software (San Diego, CA).

Results

CD11b$^+$Gr1^{high} myeloid cells accumulate in the spleen in the course of natural melanoma progression

The MT/ret model allowed the monitoring of immune cells within the spleen and the tumor microenvironment during the course of spontaneous tumor progression. Exophthalmus corresponds to the first clinical sign of uveal primary melanoma development. Within 3 months after birth, 50% of mice display cutaneous metastasis that develop first on the face, then in the posterior part of the body [34]. The proportions of hematopoietic populations within spleens derived from age matched MT/ret and ctrl mice were not statistically different (Fig. 1A). However, CD11b$^+$ cells consist of two main populations

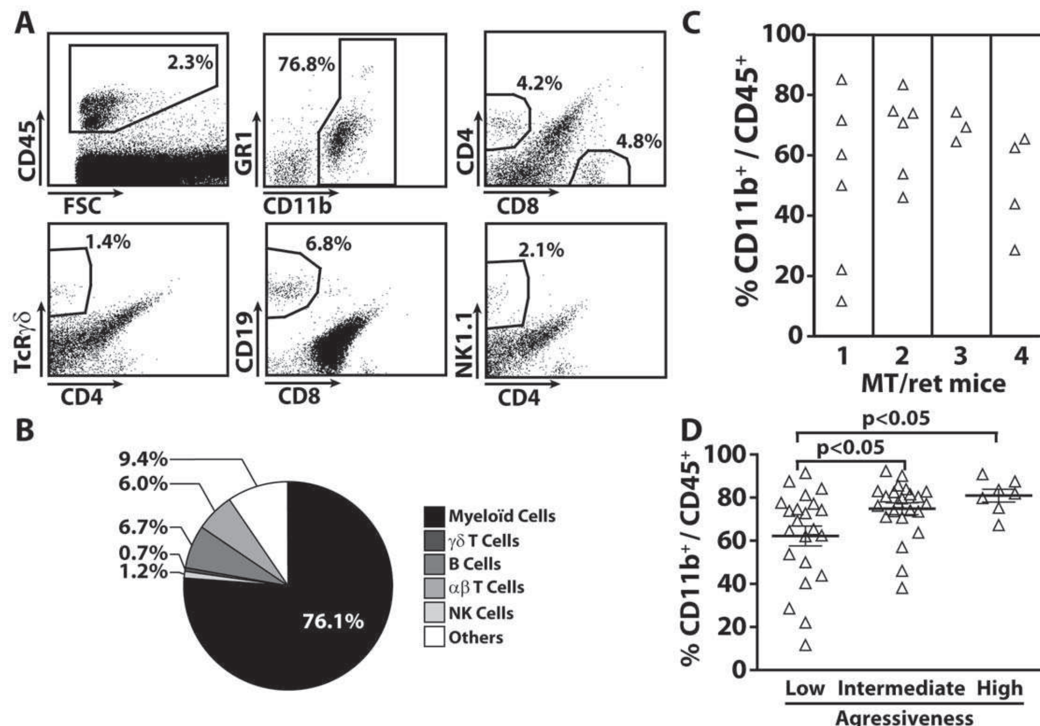

Figure 2. Characterization of hematopoietic cells within the tumor microenvironment of MT/ret mice. (**A**) Dot plots from a cell suspension of one representative cutaneous metastasis. Myeloid and lymphoid stainings were performed as defined in Fig 1A. (**B**) The pie diagram summarizes the proportion of hematopoietic cells from 47 cutaneous metastasis. (**C**) Variability of the proportion of tumor infiltrating CD11b$^+$ cells from cutaneous metastasis. The frequencies of CD11b$^+$ cells gated from CD45$^+$ cells were determined. The graph shows the data for four 3 month old mice. (**D**) Correlation between the tumor aggressiveness and the increase of tumor infiltrating CD11b$^+$ cells. The tumor aggressiveness corresponds to the ratio between the absolute numbers of tumor associated cells and the number of days since appearance of each nodule. Statistical differences were assessed using ANOVA test.
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according to Gr1 expression level, Gr1^{low} (monocytic) and Gr1^{high} (granulocytic), and the proportions of CD11b^{+} subsets in MT/ret mice differ from those in ctrl mice. More precisely, CD11b^{+}Gr1^{high} cells accumulate in spleen of mice displaying dorsal metastasis corresponding to a late melanoma stage (Fig. 1B and C). In addition, we have previously shown that MT/ret mice

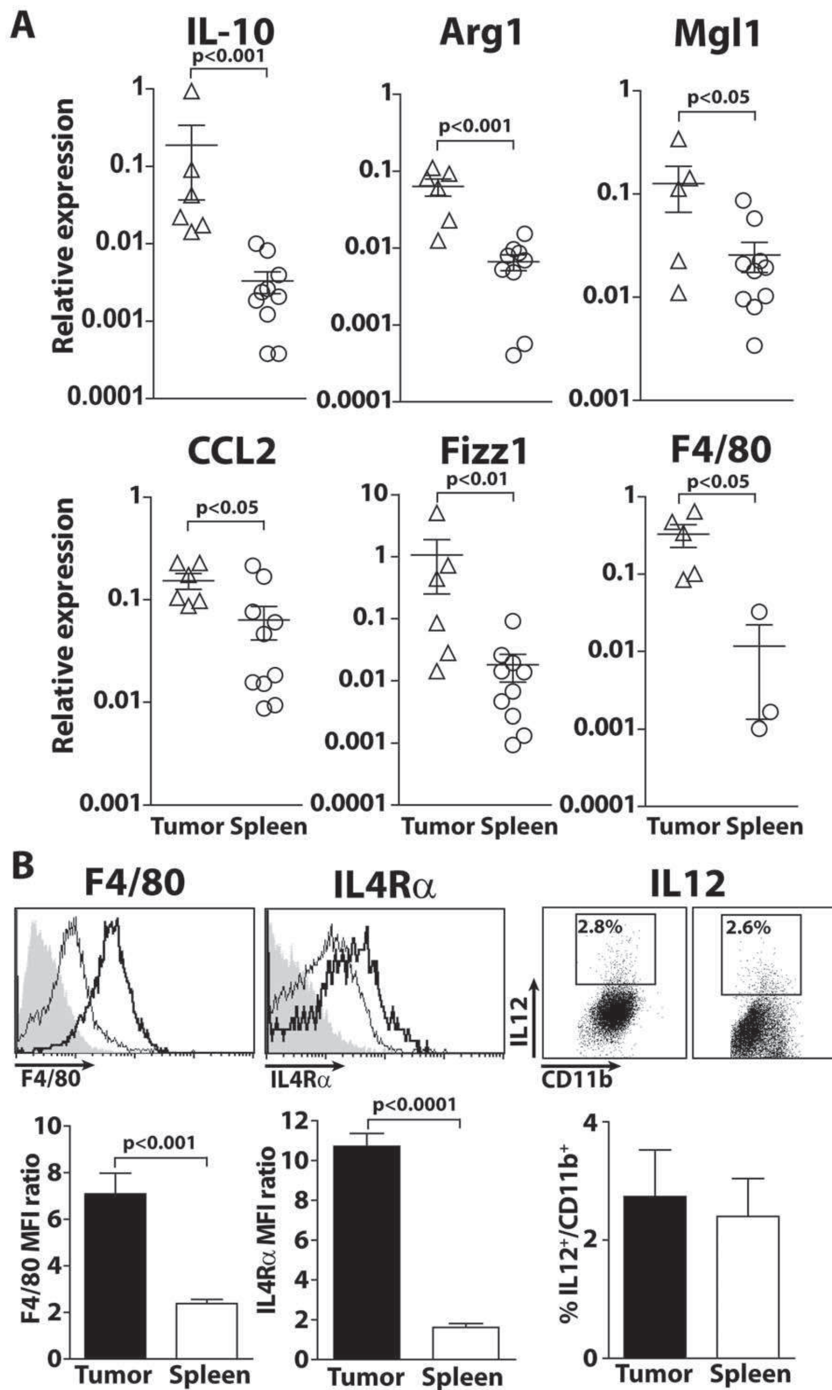

Figure 3. Characterization of myeloid cells from MT/ret mice. (**A**) QPCR. CD11b^{+} cells were isolated from tumors and spleens of tumor bearing MT/ret mice. The transcripts levels of a panel of genes were analyzed by RT-PCR. Mean values+/− SEM of relative expression are shown for indicated genes. (**B**) Phenotype and function of CD11b^{+} cells. Cell suspensions from tumors and spleens of MT/ret mice were stained for CD45, CD11b and IL4-Rα, F4/80 and IL12 and their isotype controls (grey histogram). Representative stainings for spleen (single line) and tumor (bold line) are shown. IL-12/CD11b^{+} dot plots generated from gated CD45^{+} cells are obtained after stimulation with IFNγ and LPS. Representative histograms of more than 3 experiments and performed on more than 10 samples are shown. Results are expressed as the percentage of IL-12^{+} cells from CD45^{+}CD11b^{+}Gr1^{+} cells taking account the two Gr1 subsets within spleens. Statistical differences were assessed using unpaired t test.
doi:10.1371/journal.pone.0020235.g003

develop anti-tumor immune response spontaneously during disease progression [34]. To evaluate if this anti-tumor immune response is negatively influenced by myeloid cells in the spleen, either total splenocytes or $CD11b^+$ cell-depleted splenocytes were stimulated with Melan-ret melanoma cells. The removal of $CD11b^+$ cells raises the number of splenocytes responding to melanoma cells (Fig. 1D). Together our data indicate that, as shown in models of tumor transplantation, myeloid cells accumulate within spleen of MT/ret mice and prevent optimal anti-tumor T cell response.

Accumulation of myeloid cells within cutaneous metastasis correlates with the tumor aggressiveness

To extent these data to the monitoring of the tumor microenvironment, we first compared the proportion of hematopoietic cells that infiltrate cutaneous metastasis derived from 3 to 6 month old MT/ret mice (Fig. 2*A*, 2*B*). $CD45^+$ cells represent 2.3% of total cells. αβT and B cells represent on an 6+/−0.5% and 6.7+/−0.8% of hematopoietic cells respectively. The percentages of γδT and NK cells are pretty low. More interestingly, the percentage of $CD11b^+$ cells ranges from 11.6 to 92.4% with an average 76.1% of $CD45^+$ cells and they are almost exclusively $Gr1^{low}$. In a given mouse, the proportion of $CD11b^+$ cells could be variable from tumor to tumor as shown in Fig. 2*C* for 4 mice. In order to evaluate the association of tumor infiltrating myeloid cells with tumor progression for one given nodule, we defined a "tumor aggressiveness score" corresponding to the ratio between the absolute number of cells in the tumor and the number of days since its appearance. The most aggressive tumors (>2.10^5 cells/day) displayed a high proportion of $CD11b^+$ cells, whereas the less aggressive ones (<2.10^4 cells/day) are significantly less infiltrated by myeloid cells (Fig. 2*D*). Tumors with intermediate aggressiveness already displayed an increased proportion of $CD11b^+$ cells.

Tumor infiltrating myeloid cells express markers of M2 type macrophages

To further compare myeloid cells that accumulate during tumor progression, quantitative PCR were carried out on $CD11b^+$ purified cells from spleen and tumor samples using a set of type 2 myeloid-associated marker genes. QPCR analysis revealed that il10, arginase I, mgl1 fizz1 and the inflammatory chemokine ccl2 mRNA levels were all significantly higher in tumor derived $CD11b^+$ cells (Fig. 3*A*). In addition, these cells were strongly positive for F4/80 mRNA compared to related cells in spleen. Flow cytometric analysis further showed that tumor infiltrating myeloid cells express F4/80 at the protein level, revealing a significant upregulation of this macrophage marker in the tumor microenvironment (Fig. 3*B*). In addition, tumor infiltrating myeloid cells express IL-4Rα (Fig. 3B). Contrasting with transplanted tumor models [12], IL-4Rα expression in spleen of tumor-bearing MT/ret mice is low (Fig. 3B) and similar to the level observed in splenocytes from control mice (data not shown). A relatively low proportion of tumor infiltrating myeloid cells secrete IL-12 upon a short IFNγ/LPS stimulation (2.7+/−0.8; Fig. 3B), *a pro*portion quite similar to that of related splenic myeloid cells. Overall, tumor infiltrating myeloid cells are enriched in $F4/80^+$, $IL\text{-}4R\alpha^+$ cells and only a minority of them have the capacity to produce IL-12.

Tumor and spleen derived myeloid cells impairs T cell functions

To compare the impact of myeloid cells from tumor bearing MT/ret mice on T cell functions, we first stimulated T cells from GP33 immunized mice with GP33 in the presence of $CD11b^+$ cells. $CD11b^+$ cells isolated from tumors or spleens of MT/ret mice inhibit IFNγ secretion (78% and 61% inhibition respectively) (Fig. 4*A*, *upper histogram*). Supernatants from tumor- or spleen-derived $CD11b^+$ cells of MT/ret mice also reduced the proportions of IFNγ secreting T cells (49% and 40% inhibition respectively), while supernatant from control mice had no effect (Fig. 4*A*, *lower histogram*). In addition, we cultured $CD11b^+$ cells with $CD8^+$ T cells specific for Ova257 peptide from OT-1 mice. In the presence of Ova257 and control $CD11b^+$ cells, a majority of OT-1 cells undergoes three to four cycles, whereas $CD11b^+$ cells derived from tumors or spleens of MT/ret mice reduced Ova257 specific T cell division (Fig. 4*B*). Together our data indicate that despite their phenotypic differences described above, both splenic and tumor derived myeloid populations inhibit $CD8^+$ T cell proliferation and IFNγ secretion.

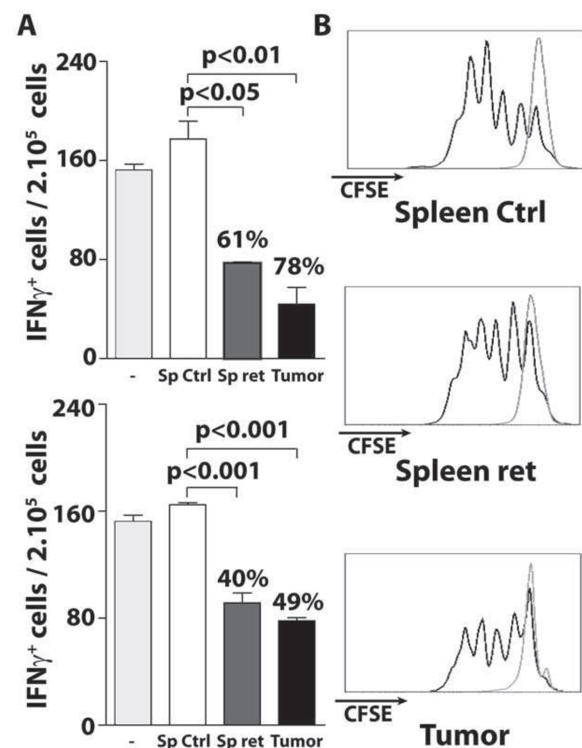

Figure 4. $CD11b^+$ cells from MT/ret mice suppress T cell functions. (**A**) GP33-specific T cells from GP33-immunized mice were stimulated 24 h with GP33 in presence of $CD11b^+$ cells isolated either from tumors or spleens of MT/ret mice or ctrl spleens. The frequency of IFNγ secreting T cells was determined by an ELISPOT assay. The percentage of inhibition indicated on the graph corresponds to the ratio between the number of spots in presence and in absence of $CD11b^+$ (upper histogram). GP33-specific T cells were also stimulated with GP33 together with supernatants of $CD11b^+$ cells isolated from tumors or spleens of MT/ret or non transgenic mice and tested as above (lower histogram). (**B**) Purified OT-1 $CD8^+$ T cells labeled with CFSE were cultured in presence of $CD11b^+$ cells isolated from spleens or tumor nodules from MT/ret mice or from ctrl spleen, and stimulated in presence or not of Ova257. Three days later, proliferation was determined. CFSE fluorescences are shown after culture with (bold lines) or without Ova257 (thin lines).
doi:10.1371/journal.pone.0020235.g004

T cells contribute to the immunosuppressive profile of tumor infiltrating myeloid cells

To investigate the impact of T cells on the composition of hematopoietic cells within the tumor microenvironment and in particular on tumor infiltrating myeloid cells, we crossed MT/ret mice with CD3εKO mice. We found no change in the proportion of hematopoietic cells in mice competent (RetCD3ε$^+$) and deficient (RetCD3εKO) for T cells. Tumor infiltrating myeloid cells represent 79% and 81% of CD45$^+$ cells (Fig. 5*A*) and express a similar level of IL-4Rα (Fig. 5*B*) in the presence or in absence of T cells respectively. Interestingly, myeloid cells derived from RetCD3εKO mouse tumors exhibit a better capacity to secreteIL-12 (Fig. 5*B*) and NO (Fig. 5*C*) than the related cells from age-matched RetCD3ε$^+$ mice. Moreover, they display a poor inhibitory effect on GP33-specific T cells stimulated with GP33 compared to myeloid cells from tumors of mice competent for T cells (Fig. 5*D*). By contrast, the proportion of IL-12 producing CD11b$^+$ cells in spleen and their capacity to impair T cell functions are similar in both groups of animals lpar;not shown).

T cells favor pro-tumoral properties of tumor infiltrating myeloid cells

The proliferation of Melan-ret cells was dynamically monitored in vitro in presence of tumor derived CD11b$^+$ cells from RetCD3εKO or RetCD3ε$^+$ mice. These latter were added at 48 h when tumor cells reached their exponential growth phase (Fig 6A). According to their M2 phenotype, intratumoral myeloid cells from T cell competent mice promote tumor cell proliferation as shown by the cell index increase, whereas no significant cell index is recorded in wells with CD11b$^+$ cells alone (Fig. 6A). Conversely, myeloid cells from RetCD3εKO mice inhibited Melan-ret cell proliferation within few hours. Indeed, in 7 cases out of 10, CD11b$^+$ cells from RetCD3εKO mice inhibited from 5 to 98% of the proliferation, whereas related CD11b$^+$ cells from RetCD3ε$^+$ mice do it in only 1 case out of 8 (Fig 6B). After 40 hours, the tumor cell proliferation tested in three independent experiments is statistically different between the two groups (Fig 6C). Thus, our data suggest that T cells do not interfere with the recruitment of myeloid cells within the tumor microenvironment, but improve their suppressive and pro-tumoral functions. To assess whether such a different myeloid cell properties correlated with reduced melanoma progression, we diagnosed carefully melanoma symptoms in mice competent and deficient for T cells. Mice from both groups develop primary melanoma with a similar kinetic (Fig. 7*A*). No significant difference was also observed in the onset of cutaneous metastasis (Fig. 7*B*). Interestingly, at 6 month of age, the absolute number of tumor cells within metastasis per mouse is significantly lower in RetCD3εKO mice than in T cell competent

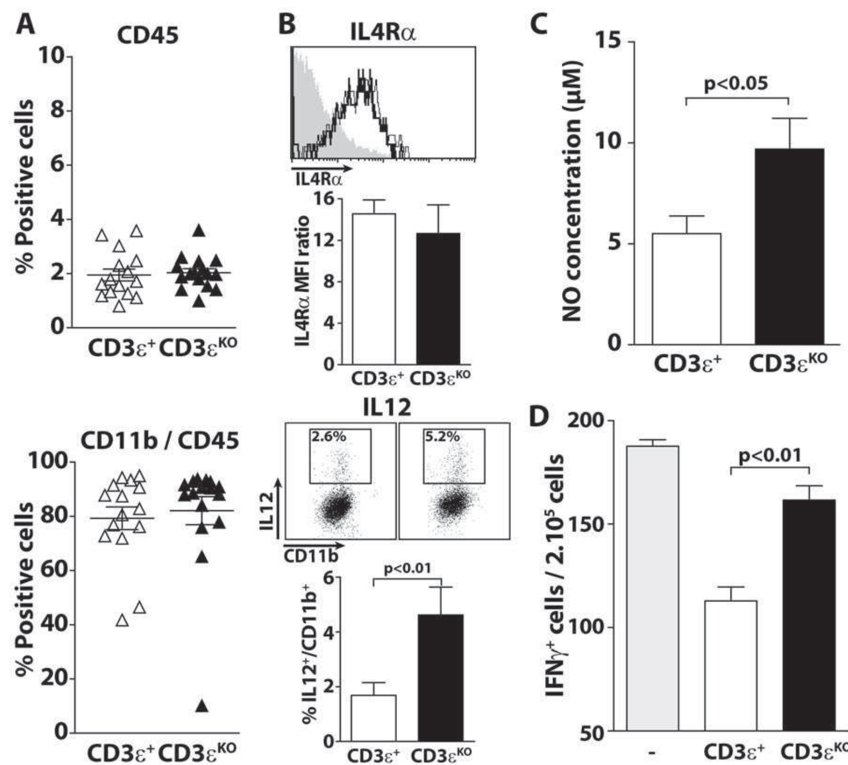

Figure 5. T cells contribute to the immunosuppressive function of tumor infiltrating myeloid cells. (**A**) The graphs indicate the proportion of CD45$^+$ cells from live cells and myeloid cells from CD45$^+$ cells from tumors of RetCD3ε$^+$ (n = 15) and RetCD3εKO (n = 16) age matched mice. (**B**) Cell suspensions from tumors derived from RetCD3ε$^+$ and RetCD3εKO mice were stained for CD45, CD11b and IL4-Rα. Representative histograms are shown for IL4-Rα expression from CD45$^+$CD11b$^+$ cells. The histograms below summarize the MFI ratio of IL4-Rα specific staining on the isotype staining. Cell suspensions were also stained for CD45, CD11b and IL-12 after LPS and IFNγ stimulation. Representative IL-12/CD11b dot plots generated from gated CD45$^+$ cells are shown. The histograms below summarize the proportion of IL-12 secreting cells from tumor infiltrating CD11b$^+$ cells. Purified tumor infiltrating CD11b$^+$ cells were activated with LPS and IFNγ for two days and assessed for NO production (**C**). (**D**) GP33-specific T cells (as in Figure 4.A) were stimulated 24 h with GP33 in presence of CD11b$^+$ cells isolated from tumors of RetCD3ε$^+$ (n = 5) or RetCD3εKO (n = 5) mice. T cell inhibition by myeloid cells is determined by comparing the frequency of IFNγ secreting cells in absence and in presence of CD11b$^+$ cells.
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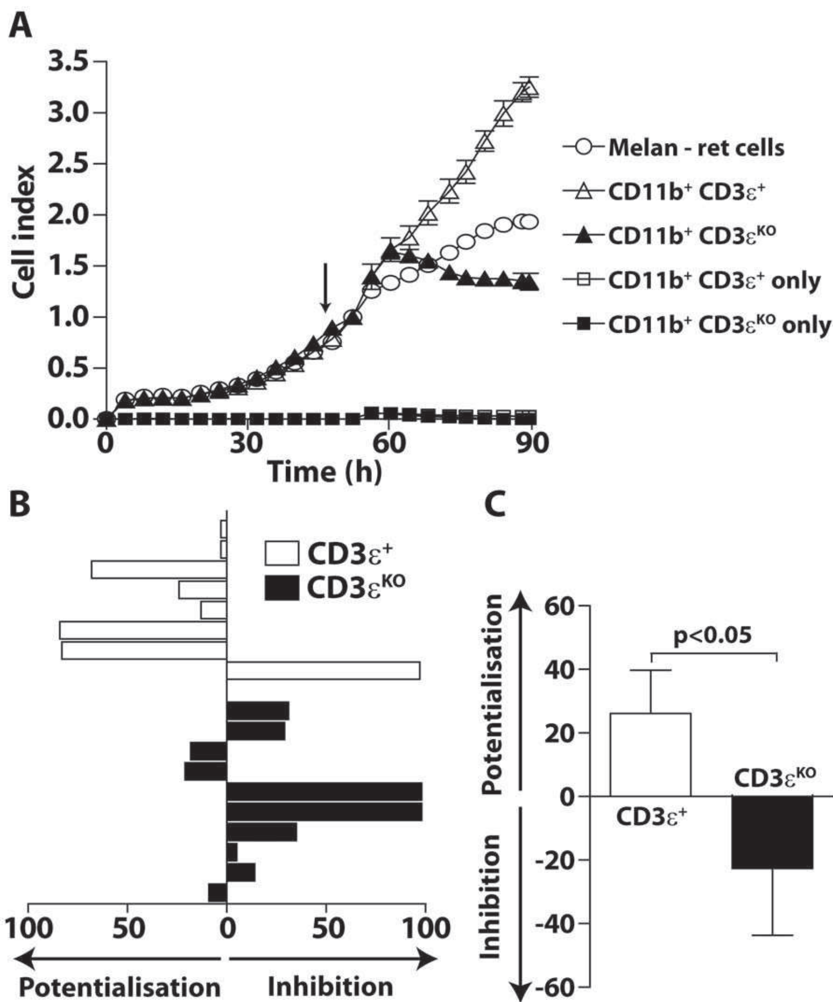

Figure 6. T cells contribute to the pro-tumoral properties of tumor infiltrating myeloid cells. (**A**) Melan-ret cells were seeded in the wells of E-plates. After 48 h, at the time tumor cells were in exponential growth phase (arrow), tumor infiltrating $CD11b^+$ cells isolated either from $RetCD3\varepsilon^+$ and RetCD3εKO mice were added and the tumor cell proliferation was assessed by dynamically monitored every 15 min on cell impedance. The graph shows the nCI values obtained by the RT-CES system for two representative $CD11b^+$ cells isolated from mice deficient or competent for T cells. (**B**) The percent of inhibition of Melan-ret cell proliferation was calculated as described in Materials and Methods, 40 hours after addition of myeloid cells. (**C**) The statistical significance between the effect of $CD11b^+$ cells from $RetCD3\varepsilon^+$ (n = 8) and RetCD3εKO (n = 10) mice tested within three independent experiments was assessed using unpaired t test.
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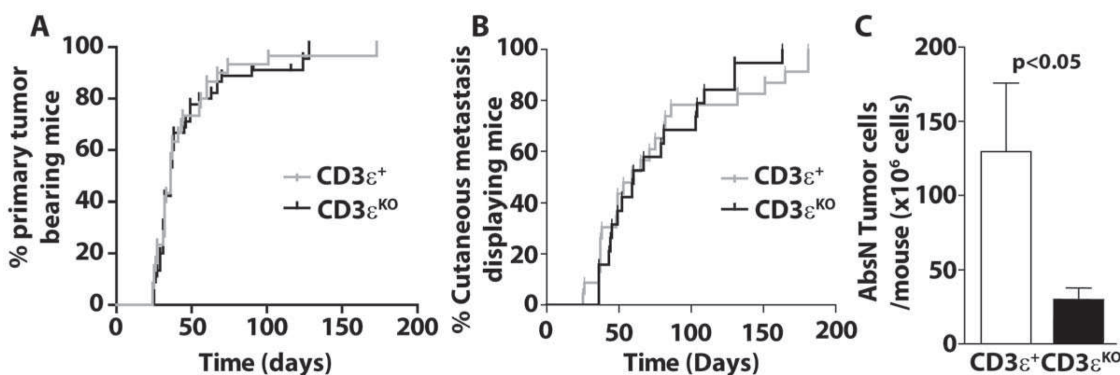

Figure 7. Cutaneous metastasis are smaller in T cell deficient mice than in T cell competent mice. The onset of the primary tumor (**A**) and of cutaneous metastasis (**B**) is shown for $RetCD3\varepsilon^+$ (n = 16) and RetCD3εKO (n = 11) mice. (**C**) The mean absolute number of tumor cells within cutaneous metastasis per mouse is determined after enzyme digestion for more than 5 mice per group.
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mice (Fig. 7*C*), supporting a better control of metastasis growth in the absence of T cells.

Discussion

It has been well established that myeloid cells accumulate in the spleen during tumor progression and inhibit the anti-tumor T cell response [12]. Movahedi et al identified Ly6G$^-$ mononuclear and Ly6G$^+$ polymorphonuclear myeloid cells in the spleen of tumor-bearing mice [3]. In MT/ret mice, splenic CD11b$^+$ cells with a low or high Gr1 expression may correspond to the former and the latter fractions respectively. In the spleen of MT/ret mice, the CD11b$^+$Gr1^{high} myeloid subset tends to accumulate as tumors progress, but the expansion becomes significant only at the latest stage of the disease. By contrast, myeloid cells always dominate the tumor infiltrate and the proportion of tumor infiltrating CD11b$^+$ cells is associated with clinical aggressiveness. Accordingly, Soudja et al have recently shown that CD11b$^+$ cells were more frequent in more aggressive melanomas in the induced melanoma model of TiRP mice [36].

We show that, during spontaneous tumor progression, M2 type marker genes (e.g. arginase1, il10, mgl1, fizz1,) are significantly upregulated in tumor infiltrating CD11b$^+$ cells compared to related splenic cells. The low level of IL-4Rα on splenic myeloid cells from MT/ret mice, similar to that in negative littermates, is consistent with data in transplanted tumor models indicating that the suppressive function of splenic MDSC does not always involve a high expression of this marker [12,37]. Arginase production by myeloid cells requires IL4 and IL13 signaling [38]. In MT/ret mice, IL-4Rα is significantly more expressed on tumor infiltrating CD11b$^+$ cells than on their splenic counterparts. Accordingly, myeloid cells produce more arginase at the tumor site than in the spleen. Finally, all tumor infiltrating myeloid cells express high levels of F4/80 which clearly contrast with splenic myeloid cells that weakly express this marker. Thus, tumor microenvironment contains mononuclear cells that have differentiated into macrophages. It has been proposed that classical M1 macrophages infiltrate the site of chronic inflammation where tumors initially develop, while tumor-promoting M2 like macrophages expressing low levels of inflammatory cytokines such as IL-12 progressively replace them in established tumors [39]. Consistent with this model, the proportion of TAM able to produce IL-12 decreases in MT/ret mice with melanoma progression (not shown). Finally, spleen and tumor derived myeloid cells from MT/ret mice inhibit efficiently antigen specific T cell proliferation and IFNγ secretion. Splenic myeloid cells also impair the anti-tumor reactivity of autologous splenocytes indicating that these cells correspond to tumor-induced MDSC. Thus, tumor growth in the MT/ret model induces the replacement of normal splenic myeloid cells by suppressive cells and the recruitment of immunosuppressive myeloid cells at the tumor site.

While the role of innate immune cells in polarizing the adaptative immune response is well established [40], little is known on the reciprocal involvement of T cells in promoting the expansion and/or suppressive activity of myeloid cells [14,31]. In a murine HPV16-induced epithelial cancer and more recently in a model of transplanted ovarian cancer, CD4$^+$ T cells have been shown to promote the recruitment of myeloid cells into tumors [41,42]. In our model, the proportion of tumor infiltrating myefloid cells is comparable in RetCD3εKO and RetCD3ε^+ mice, indicating that their recruitment does not require T lymphocytes. Tumor infiltrating lymphocytes may rather condition intra-tumoral myeloid cells towards a M2 type macrophage profile, as suggested by data obtained in nude mice inoculated with tumor cells [43]. Our data show that tumor infiltrating myeloid cells from RetCD3εKO mice display a better capacity to secrete IL-12 and produce more NO than related cells in RetCD3ε^+ mice. They only poorly impair the capacity of T cells to secrete IFNγ in response to antigen specific stimulation. Finally, they inhibit Melan-ret cell proliferation within few hours in contrast to intra-tumoral myeloid cells from T cell competent mice, revealing their higher cytotoxic activity towards tumor cells. Together, our data suggest that T cells affect myeloid differentiation within the tumor microenvironment and regulate their immunosuppressive and pro-tumoral properties. These data are in agreement with recent data in a model of breast cancer showing for the first time that IL4 producing CD4$^+$ T cells promote pro-tumoral properties of TAM by reducing their expression level of type 1 cytokines [44].

Many reports, including ours in the MT/ret model [33,45], support the idea that T cells exert a protective role against dissemination of metastatic cells. The present data show in addition that the genetic elimination of T cells does not accelerate the primary melanoma onset. Cutaneous metastasis develop with a similar kinetic in both RetCD3εKO and RetCD3ε^+ mice. More surprisingly, cutaneous metastasis were smaller in T cell deficient mice than in RetCD3ε^+ mice, suggesting a pro-tumor activity of T cells. Accordingly, DeNardo et al have recently revealed a significant role of CD4$^+$ T cells as potentiators of pulmonary metastasis of mammary carcinomas through their influence on pro-tumor properties of TAM [44]. In our model, we do not privilege a pro-tumoral role of CD8 T cells, as they interfere with visceral metastasis spreading at early [33] and late [45] stages of melanoma development. Further experiments will be needed to identify which T cell subset (e.g. conventional CD4 T cells, regulatory T cells or NKT cells) was regulating intra-tumoral myeloid cell functions and what kind of mechanism is involved (e.g. IL4, IL10, IL13 or IL17) in this process in the course of melanoma progression. The unexpected clinical impact of T cell deficiency in MT/ret mice may be in part due to a direct regulation of the cytotoxic activity of myeloid cells towards tumor cells as suggested by our dynamic melanoma cell index monitoring. We cannot exclude that they also impact angiogenesis and extracellular matrix remodeling. Alternatively, the higher proportion of intra-tumoral myeloid cells able to secrete IL12 may also improve tumoricidal NK cell activity [46].

Altogether, our present data suggest that T cells establish an environment that is likely to skew tumor infiltrating myeloid cells toward a tumor promoting response. They support the existence of an immunosuppressive vicious circle in which T cells favor melanoma development by inducing a switch towards a suppressive profile of myeloid cells that, in turn, suppress T cell functions.

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Author Contributions

Conceived and designed the experiments: MFA JPA NB BL APB. Performed the experiments: RL AP JC LD MG APB. Analyzed the data: AP JC LD NB BL APB. Contributed reagents/materials/analysis tools: MK. Wrote the paper: BL APB.

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Article 2 :

Ly6C^{high} monocytes are potent anti-tumor effectors controlled by regulatory CD4^{+} T cells

Cet article est actuellement en préparation. En conséquence, la discussion n'est pas incluse dans ce manuscrit. Néanmoins, les résultats sont amplement discutés dans la partie discussion de ce manuscrit. Les parties manquantes seront inclues dans la version finale.

Arnaud Pommier, Arnaud Delpoux, Alexandra Audemard, Bruno Martin, Laetitia Douguet, Renée Lengagne, Masashi Kato, Marie-Françoise Avril, Cédric Auffray, Bruno Lucas and Armelle Prévost-Blondel.

Les interactions entre les lymphocytes T CD4^{+} régulateurs et les cellules non-T du système immunitaire dans le contexte tumoral reste majoritairement méconnues. Afin d'étudier le rôle des lymphocytes T CD4^{+} régulateurs dans la suppression des réponses anti-tumorales, nous avons utilisé un modèle de mélanome spontané (les souris MT/ret) chez lesquelles la tumeur primaire dissémine très tôt, mais reste à l'état de dormance pendant plusieurs semaines. Les souris MT/ret développent ensuite des métastases cutanées puis, à terme, des métastases distantes. Environ un tiers des souris MT/ret développent un vitiligo associé à un retard dans la progression tumorale. De façon intéressante, la proportion de lymphocytes T CD4^{+} régulateurs est augmentée dans les ganglions drainants des souris MT/ret ne développant pas de vitiligo, et leur déplétion conduit à une augmentation de la fréquence de vitiligo corrélées à une baisse de l'incidence de métastases. En déplétant sélectivement différente populations du système immunitaire, nous démontrons ici que les monocytes Ly-6C^{fort} sont des effecteurs anti-tumoraux efficaces jouant un rôle clé dans le développement du vitiligo et dans le contrôle de la dissémination tumorale. L'ensemble de nos données suggèrent que les lymphocytes T CD4^{+} régulateurs freinent les réponses anti-tumorales, non seulement en supprimant les lymphocytes T, mais également en inhibant les monocytes Ly-6C^{fort} par un mécanisme dépendant de l'IL-10.

Ly6C^{high} monocytes are potent anti-tumor effectors controlled by regulatory CD4^{+} T cells

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Running title:

Key words: Regulatory T cells, Ly6C^{high} monocytes, Tumor spread, Melanoma, Vitiligo

Highlights:

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SUMMARY

Little is known about the interactions between regulatory T cells and non-T cells in the context of cancer. To study the role of regulatory $CD4^+$ T cells in the suppression of anti-tumor responses, we used a model of spontaneous melanoma (MT/ret mice) in which the primary uveal tumor disseminates early, but remains dormant for several weeks. Then, MT/ret mice develop cutaneous metastases and finally distant metastases. 35% of MT/ret mice develop a vitiligo associated with a delay in tumor progression. Interestingly, regulatory $CD4^+$ T cells were more frequent in tumor-draining lymph nodes only in MT/ret mice without vitiligo. Their depletion led to an increased occurrence of vitiligo correlated to a significant decrease in cutaneous metastasis spread. By selectively depleting various immune populations, here, we show that Ly-$6C^{high}$ monocytes are potent anti-tumor effectors playing a key role in the development of vitiligo and in controlling tumor-cell dissemination. Altogether, our data suggest that regulatory $CD4^+$ T cells are involved in dampening anti-tumor responses not only by suppressing conventional T-cell responses, but also by inhibiting Ly-$6C^{high}$ monocytes through an IL-10 dependent mechanism.

INTRODUCTION

The idea that the immune system can control cancer has been a subject of debate for over a century. Paul Ehrlich was among the first to argue that the immune system plays a key role against cancer, a common pathology in long-lived organisms (Ehrlich, 1909). However, in the early 1900s, so little was known about the composition and functions of the immune system that it was simply impossible to assess the validity of this prediction. Advances in the understanding of the immune system permit to resurface the idea that immunity may be protective against cancer. Nevertheless, many recent data demonstrate also the potential role of immune actors in tumor promotion.

The concept of suppressive T cells, which was first described in the early 1970s, fell into oblivion until Sakaguchi and colleagues demonstrated the suppressive functions of a subset of $CD4^+$ T cells now called regulatory T cells (Treg cells; (Sakaguchi et al., 1995)). Treg cells express Foxp3 (Hori et al., 2003) and high surface levels of the α chain of the IL-2 receptor (CD25) and are the main mediator of peripheral tolerance under physiological settings (Sakaguchi et al., 1995). They inhibit various immune cells including conventional T lymphocytes, B cells, natural killer cells (NK cells), dendritic cells (DCs) and macrophages (Shevach, 2009). Their mechanisms of action are diverse including notably the production of anti-inflammatory cytokines such as IL-10 (Annacker et al., 2001) and TGF-β, the expression of large amounts of immunosuppressive molecules including CTLA-4 (Read et al., 2000), CD39, CD73 and PDL-1 (Vignali et al., 2008), and the consumption of IL-2 (Pandiyan et al., 2007).

The role of Treg cells in the suppression of anti-tumor immunity was originally described in the 1980s (North and Bursuker, 1984), but remained at first largely underestimated. The demonstration that systemic depletion of Treg cells favors immune-mediated tumor rejection in mouse models (Shimizu et al., 1999) highlighted their contribution in tumor progression. Furthermore, intra-tumor depletion of Treg cells induces regression of established tumors (Yu et al., 2005). Transfer of $CD4^+$ T cells depleted of Treg cells improves anti-tumor immune responses when compared to total $CD4^+$ T cells (Antony et al., 2005). Finally, the prevalence of Treg cells is increased in peripheral blood and/or within tumor microenvironment in patients with various types of cancers including melanoma (Ahmadzadeh et al., 2008; Miracco et al., 2007; Viguier et al., 2004), head and neck (Schaefer et al., 2005), lung (Wolf et al., 2003), hepatocellular carcinoma (Ormandy et al., 2005), gastric and esophageal (Ichihara et al., 2003), pancreas and breast (Liyanage et al., 2002).

Treg cells have been further associated to a reduced survival in ovarian cancer (Curiel et al., 2004).

It is now admitted that Treg cells can inhibit tumor specific $CD8^+$ T cells (Quezada et al., 2011). However, little is known about their impact on other immune populations in the context of cancer. To decipher the role of Treg cells in the suppression of anti-tumor responses, we used the model of spontaneous melanoma driven by the RET oncogene (Kato et al., 1998). In this model, the primary uveal tumor disseminates early, but remains dormant for several weeks (Eyles et al., 2010). Then, MT/ret mice develop cutaneous metastases and finally distant (i.e., visceral, pulmonary and mediastinal adenopathy) metastases (Lengagne et al., 2004). As observed in the human melanoma pathology, a significant proportion of MT/ret mice spontaneously develops a vitiligo associated with a clinical benefit (Lengagne et al., 2004). This model is thus relevant to study the immune response to cancer all along the carcinogenesis process, from primary tumors to metastatic stages in presence or in absence of a concomitant auto-immune disease development.

The present study evaluates the impact of different immune cells on metastasic spread in MT/ret mice. Consistent with previous data obtained in melanoma patients (Viguier et al., 2004), we found that Treg cells accumulated in tumor-draining lymph nodes (TdLNs) of MT/ret mice. Treg depletion led to a decreased occurrence of cutaneous metastases associated with an increased percentage of mice developing vitiligo. Interestingly, $Ly6C^{high}$ monocytes, initially described as important cells during inflammatory and infectious processes, appeared to be critical effectors during the early phases of the anti-tumor response, in particular by killing disseminated malignant melanocytes and mediating vitiligo. Finally, altogether, our data suggest that Treg cells may favor tumor progression in part by inhibiting $Ly\text{-}6C^{high}$ monocyte recruitment/differentiation in the skin through an IL-10 dependent mechanism.

RESULTS

Decreased incidence of cutaneous metastases in MT/ret mice developing vitiligo

MT/ret mice were monitored from weaning to 6 months of age for the occurrence of primary melanoma, cutaneous metastases and vitiligo. Most of MT/ret mice display a primary tumor clinically detectable by 3 months of age (Table 1) that can even be detected microscopically as early as 10 days after birth (Eyles et al., 2010). Cutaneous metastases develop with time in nearly two thirds of 6-month old mice and vitiligo in one third. Interestingly, mice with vitiligo displayed less cutaneous metastases than mice without vitiligo (Fig. 1A, right panel), confirming our previous data showing that vitiligo is associated with clinical benefit (Lengagne et al., 2004). Next, we compared by multicolor flow cytometry the proportions of the various myeloid and lymphoid subsets (Fig. S1) recovered from spleens, and cervical lymph nodes (LNs that drained the primary tumor as well as facial cutaneous metastases) of 6 month old MT/ret mice as a function of whether or not they have developed vitiligo (Fig. 1B). We did not detect any significant difference. Moreover, the proportions of these subpopulations in secondary lymphoid organs of MT/ret mice were similar to those of age-matched control mice (Fig. 1B). The nature of hematopoietic cells infiltrating cutaneous metastases was also determined (Fig. 1B) and found not to be different both quantitatively ($CD45^+$ cells represented 3.9% ± 0.5 and 5.0% ± 0.5 of the cells recovered from metastases of mice with or without vitiligo respectively) and qualitatively (Fig. 1B) whether or not the mice displayed vitiligo. These last results suggest that the protection conferred by vitiligo against metastatic spreading would not rely on the quantity and nature of immune cells infiltrating settled tumors.

Treg cells accumulate in the spleen and TdLNs of non-vitiligo MT/ret mice

We then compared the proportion of $Foxp3^+$ cells among $CD4^+$ T cells in cutaneous metastases of MT/ret mice and in the secondary lymphoid organs of control mice (Fig. 2A, 2B). Unexpectedly, Treg cells were not more frequent within the tumor microenvironment. Moreover, there was no difference in the percentage of Treg cells in cutaneous metastases of MT/ret mice whether or not they displayed vitiligo. Interestingly, Treg cells accumulated both in proportion and absolute number (Fig. 2A, 2B; Fig. S2) in the spleen and cervical LNs of MT/ret mice without vitiligo when compared with either MT/ret mice developing vitiligo or control mice. More precisely, Treg cells from non-vitiligo MT/ret mice were more frequent in the spleen and cervical LNs whatever their clinical diagnosis (Fig. 2C). By contrast, the

proportion of Treg cells was increased in axillary LNs only in mice developing dorsal or distant metastases and, in mesenteric LNs only in mice developing distant metastases. The increase in the proportion of Treg cells observed in non-vitiligo MT/ret mice was thus restricted to tumor draining LNs (TdLNs) and the spleen.

Treg cells play a role in tumor spread and inhibit the onset of vitiligo

We then investigated the role of Treg cells in tumor spread and vitiligo occurrence. MT/ret mice were injected twice a week with the anti-CD25 depleting antibody, PC61, beginning 2 days after birth and diagnosed every week from weaning up to 6 months of age. Anti-CD25 treatment did not affect notably the occurrence of primary tumors (Table 1), but significantly delayed the development of cutaneous metastases (Fig. 2D). This reduced tumor spread correlated with a significant increase in the incidence of vitiligo (Fig. 2D). Treg cells may thus favor tumor spread through their influence on vitiligo.

We have recently shown that T-cell deficient MT/ret mice (RetCD3ε^{KO} mice) developed cutaneous metastases smaller than those from T-cell competent mice (Lengagne et al., 2011). Here, we further observed that T-cell deficiency correlated with a significant increase in the occurrence of vitiligo (Fig. 3A). Interestingly, RetCD3ε^{KO} mice with vitiligo developed less cutaneous metastases than non-vitiligo RetCD3ε^{KO} mice (Fig. 3A). Thus, like in MT/ret mice, vitiligo in RetCD3ε^{KO} mice conferred protection against metastatic spreading. To confirm that Treg cells were acting on tumor-cell dissemination, we reconstituted RetCD3ε^{KO} mice, five days after birth, with CD4^{+} T cells recovered from the spleen and cervical LNs of either C57BL/6 control mice or non-vitiligo tumor-bearing MT/ret mice and followed the development of clinical symptoms. Reconstituted RetCD3ε^{KO} mice displayed no significant difference in the incidence of primary tumors when compared to control RetCD3ε^{KO} mice (Table S1). Interestingly, they displayed an increased occurrence of cutaneous metastases correlated with a decrease in the incidence of vitiligo (Fig. 3B). More precisely, mice injected with CD4^{+} T cells from tumor-bearing MT/ret mice developed more cutaneous metastases and less vitiligo than those initially injected with wild-type CD4^{+} T cells, themselves developing with time a more dramatic phenotype than non-reconstituted RetCD3ε^{KO} mice (Table S1, Fig. 3B). Altogether, our results suggest that Treg cells may promote metastasis spread by inhibiting vitiligo development.

An innate immune population controls the development of cutaneous metastases and vitiligo

In order to determine which type of immune cells was responsible for the control of tumor spread and was inhibited by Treg cells in the MT/ret model, we used a depleting antibody strategy. First, MT/ret mice were injected with anti-CD8 antibody beginning 2 days after birth and followed every week from weaning up to 3 months of age for the development of tumors and vitiligo. $CD8^+$ T-cell depletion had no effect on the incidence of cutaneous tumors or vitiligo (Table 1, Fig. S3A). In line with our previous data (Eyles et al., 2010; Lengagne et al., 2008), the proportion of mice developing distant metastases increased after anti-CD8 treatment (Table 1), indicating that $CD8^+$ T cells, in our model, were not responsible for the control of cutaneous metastases, but were crucial for controlling visceral dissemination. A lack of implication of $CD8^+$ T cells in controlling tumor spread at the skin level in the MT/ret model fitted with the results obtained in $RetCD3\varepsilon^{KO}$ mice. Indeed, these latter mice developed a similar proportion of cutaneous metastases than MT/ret mice although they lacked $CD8^+$ T cells.

The development of vitiligo has been associated with a production of auto-antibodies directed against melanocyte antigens (Sandoval-Cruz et al., 2011). One could expect $CD3\varepsilon^{KO}$ mice inefficient in producing antibodies due to the absence of $CD4^+$ helper T cells in these mice. To test this hypothesis, sera from T-cell deficient or competent MT/ret mice were incubated with TIII cells, a cell line derived from a cutaneous metastases of a MT/ret mouse (Fig. 3C). Significant staining (MFI ratio > 2) revealing the presence of auto-antibodies was observed only when sera were derived from MT/ret mice and even in this case, only few animals (9 out of 54) were positive. Thus, B lymphocytes does not seem to be required for the development of vitiligo and to be involved in the control of cutaneous metastastic spread observed in $RetCD3\varepsilon^{KO}$ mice. Altogether, our data suggest that a cell population belonging to the innate immunity may play an important role in vitiligo development and in controlling tumor dissemination at the skin site.

$Ly6C^{high}$ monocytes play a key role in controlling tumor dissemination

To determine which innate immune cell subset was responsible for the control of tumor-cell dissemination at the skin level, we pursued our antibody depletion strategy. We depleted NK cells using the anti-NK1.1 antibody, PK136. No difference in the occurrence of primary tumors, cutaneous metastases or vitiligo was observed in treated MT/ret mice (Table 1, Fig.

S3B), suggesting no crucial role of NK cells in controlling tumor spread. Then, we assessed the role of cells from the myeloid lineage using the anti-Gr1 antibody. Gr1, a common epitope to Ly-6C and Ly-6G molecules, is expressed by granulocytes, inflammatory monocytes and a fraction of $CD4^+$ and $CD8^+$ T cells. In both MT/ret and RetCD3ε^{KO} mice, the treatment rapidly led to the death of an important proportion of our cohorts (Fig. 4A). Indeed, only 30.8% (9/23) of RetCD3ε^{KO} mice and 26.1% (6/23) of MT/ret mice were still alive after 3 months of treatment. The remaining mice displayed an increased occurrence of cutaneous metastases and a decrease in vitiligo incidence (Table 1). Mice displaying signs of poor health before 4 weeks of age were sacrificed and analyzed. Interestingly, 56.5% of these mice had cutaneous metastases and nearly all of them already displayed distant metastases (Fig. 4B). Thus, anti-GR1 depletion acts on tumor spread at both cutaneous and distant levels. As data were similar in T-cell competent and deficient mice, it seemed likely that granulocytes or/and inflammatory monocytes rather than T cells were involved in this process.

Next, we treated MT/ret mice with a depleting anti-Ly-6G antibody. Granulocyte-depleted mice displayed significantly both less cutaneous metastases and a higher incidence of vitiligo than untreated mice (Fig. 4C). A first hypothesis could be that, in our model, granulocytes exhibit pro-tumor properties, whereas inflammatory monocytes/DCs control tumor progression. Granulocyte depletion led to a rise in Ly-6C^{high} monocytes and inflammatory DCs in blood and spleen (Fig. 4D). Thus, depletion of granulocytes may act either directly through the depletion of key pro-tumor players and/or indirectly by increasing Ly6C^{high} monocyte egress from the bone-marrow. Inflammatory monocytes exit the bone marrow in response to CCL2 (Serbina and Pamer, 2006). Interestingly, a single injection of CCL2 rapidly led to transient increased proportions of both granulocytes and inflammatory monocytes in the blood (Fig. S4). Granulocyte depletion may thus increase the level of serum CCL2 and accordingly favor the accumulation of circulating monocytes that would mediate vitiligo and confer tumor protection. In line with this assumption, CCL2-treated MT/ret mice displayed a higher incidence of vitiligo and a more efficient tumor control than untreated mice (Fig. 4E). Interestingly, the occurrence of symptoms was quite similar after treatments with anti Ly-6G and CCL2 (Fig. 4C and 4E). Altogether, our results strongly suggest that Ly6C^{high} monocytes play a key role in controlling tumor-cell dissemination.

Ly6C^{high} monocytes accumulate in the skin of MT/ret mice with active vitiligo

To identify the immune population involved in the process of depigmentation in the MT/ret model, we then studied the nature of hematopoietic cells infiltrating skin biopsies from mice

with or without vitiligo. We hypothesized that actors mediating vitiligo may not remain in the skin once the skin has been purged of dormant malignant melanocytes. Accordingly, only inflammatory DCs were significantly more frequent in vitiliginous skin than in normal skin from 6 week-old mice (Fig. S5). Then, we studied 3 week-old mice with or without active ongoing vitiligo (Fig. 5). The proportion of Ly-6C^{high} monocytes was significantly higher in vitiliginous skin than in skin of MT/ret mice without vitiligo (Fig. 5A, 5B). This increase was mostly compensated by a decrease in the proportion of myeloid DCs (CD11b^{+} CD11c^{+}; Fig. 5A and Fig S6). Of note, the proportion of the subset of myeloid DCs expressing Ly-6C (named inflammatory DCs thereafter) was augmented in mice with ongoing vitiligo when compared to control mice or with MT/ret mice without vitiligo (Fig. 5C). The proportion of Tγδ cells was unaffected and although granulocytes seemed to be more frequent in the vitiliginous skin, this difference was not significant (Fig. S6). Thus, Ly-6C^{high} monocytes and inflammatory DCs accumulated in the skin of MT/ret mice with active vitiligo. Together with the results obtained by selectively depleting populations of hematopoietic cells (Table 1), these data strongly suggest that inflammatory monocytes are, in our model, the main actors of vitiligo development.

An increased proportion of Treg cells can be detected in the skin of MT/ret mice not developing vitiligo when compared to control mice or to MT/ret mice with ongoing vitiligo (Fig. 5D). Interestingly, the percentage of Ly-6C^{high} monocytes and inflammatory DCs among skin-derived cells inversely correlates with the proportion of Treg cells (Fig. 5E). More precisely, vitiliginous skin contains both less Treg cells and more inflammatory monocytes and DCs (Fig. 5E). Altogether, our results strongly suggest that Treg cells favor tumor spread by inhibiting the recruitment and/or differentiation of inflammatory monocytes in the skin and subsequent killing of dormant malignant melanocytes.

Treg cells may control innate immunity through an IL-10 dependent mechanism

Next, we investigated whether IL-10, a suppressive cytokine secreted by Treg cell, interferes with the anti-tumor activity of Ly6C^{high} monocytes. IL-10 neutralization *in vivo* through the administration of a blocking anti-IL-10 receptor antibody (anti-IL-10R) had no effect on the occurrence of primary tumors (Table 1). By contrast, the development of cutaneous metastases was significantly delayed in treated mice and this was correlated with an important increase in the occurrence of vitiligo (Fig. 6A). These curves were similar to those obtained after anti-CD25-treatment (Fig. 2D). Of note, IL-10 neutralization led to an important augmentation of the proportion of Treg cells in spleen and TdLNs of MT/ret mice displaying

or not vitiligo (Fig. 6B). Altogether, our data suggest that IL-10 derived from Treg cells may be crucial to suppress anti-tumor effectors of the innate immunity.

DISCUSSION

We reported for the first time a critical role of inflammatory (Ly-6C^{high}) monocytes in tumor immune surveillance. Our data indeed suggest that Ly-6C^{high} monocytes control tumor cells that have disseminated within the skin and subsequently promote vitiligo onset, an autoimmune disease associated to a significant delay in the spontaneous melanoma progression in our MT/ret mouse model as well as in the human pathology. Interestingly, we show that the accumulation of inflammatory monocytes is inversely correlated to the proportion of Treg cells in the skin of mice with ongoing vitiligo. Our results support a new role for Treg cells that may favor metastatic spread by inhibiting the recruitment of inflammatory monocytes in the skin and subsequent killing of dormant malignant melanocytes.

An accumulation of Treg cells is frequently recorded within the blood, spleen, TdLNs and tumor microenvironment of many tumor-bearing hosts (Zou, 2006). In particular, Treg cells are overrepresented in the peripheral blood of patients with metastatic melanoma compared to healthy donors, and are enriched in the primary tumor, TdLNs and metastatic lesions (Jacobs et al., 2012). In MT/ret mice (whether or not they displayed a concomitant vitiligo), we found that CD4^{+} T-cell infiltration remained marginal in cutaneous metastases and that Foxp3^{+} cells among these few CD4^{+} T cells were not more frequent than in the secondary lymphoid organs of their non-transgenic littermates. Treg cells may thus not be the main suppressor cells at the tumor site and other suppressive cells may hamper anti-tumor immunity locally in our model. In line with such an assumption, we have recently showed that type 2 macrophages infiltrate massively the most aggressive metastases (Lengagne et al., 2011), which is consistent with observations in human melanoma biopsies (Bronkhorst and Jager, 2012; Piras et al., 2005; Varney et al., 2005). In contrast, we found that the proportion of Treg cells among CD4^{+} T cells was greatly increased in the spleen and TdLNs of MT/ret mice without vitiligo, in agreement with the accumulation (recruitment and division) of Treg cells observed in TdLNs few days after tumor-cell implantation in several transplanted mouse tumor models (Darrasse-Jeze et al., 2009).

Immuno-suppression mediated by Treg cells is a dominant mechanism of tumor escape. In many mouse tumor models, CD25^{+} T-cell ablation before tumor transplantation enhances anti-tumor immunity, tumor clearance and survival of recipient mice (Nizar et al., 2010). In MT/ret mice, we found that Treg-cell depletion was associated with a significant decrease in the incidence of cutaneous metastasis. Our results contrast with those obtained by Umansky and colleagues (Kimpfler et al., 2009). Indeed, they reported that, in MT/ret mice,

anti-CD25 antibody treatment did not delay melanoma development. Failure of the treatment in their hands may result from the late depletion strategy they used. Indeed, they depleted Treg cells in adult MT/ret mice whereas we treated them from day 2 after birth. Thus, in the MT/ret mouse model, Treg cells may have a crucial suppressive role at early stages of spontaneous tumor development at the time when tumor cells disseminate from the primary tumor (Eyles et al., 2010). Treg-cell depletion from birth also induced a marked increase of the proportion of MT/ret mice developing vitiligo. In line with this result, Treg-cell frequency is decreased and/or their suppressive capacities impaired in vitiliginous or peri-lesional skin in humans (Ben Ahmed et al., 2012; Klarquist et al., 2010; Lili et al., 2012).

We have previously shown that strong melanoma specific $CD8^+$ T-cell responses were detected in the secondary lymphoid organs of MT/ret mice with melanoma-associated vitiligo and that mice developing vitiligo were fully protected towards a challenge with syngeneic melanoma cells by a $CD8^+$ T-cell dependent mechanism (Lengagne et al., 2004). Moreover, $CD8^+$ T cells specific for melanocyte differentiation antigens have been often detected in patients with vitiligo or melanoma-associated vitiligo (Le Gal et al., 2001; Ogg et al., 1998). Surprisingly, here, we found that $CD8^+$ T-cell depletion from birth had no impact on the onset of vitiligo as well as on the incidence of cutaneous metastases. A lack of implication of $CD8^+$ T cells in the etiology of vitiligo and in controlling metastatic spread at the skin level fits with the results obtained in $RetCD3\varepsilon^{KO}$ mice. Indeed, T-cell deficient MT/ret mice did not develop more aggressive cutaneous metastases than their T-cell competent littermates and still developed “protective vitiligo”. In MT/ret mice, increased T-cell reactivity against melanoma antigens would be thus a consequence of vitiligo rather than its cause. In line with this explanation, Byrne et al. have recently established that melanocyte destruction is crucial for inducing lasting melanoma specific $CD8^+$ T-cell mediated responses, thus illustrating that immune-mediated destruction of normal tissues can perpetuate adaptive immune responses to cancer (Byrne et al., 2011).

Our study provides new and unexpected insights into the mechanisms involved in the control of metastatic spread. Whereas $CD8^+$ T-cell or NK-cell depleted MT/ret mice survived over the 3-month period of diagnosis, the majority of MT/ret mice treated with anti-Gr1 antibody rapidly die and surviving mice exhibited an increased occurrence of both cutaneous and distant metastases. Treatments with anti-Ly-6G antibody or the chemokine CCL2, both resulting in a rise in circulating inflammatory monocyte and DC levels, are consistent with a key role for these innate immune cells in controlling tumor progression. Thus, in our model, inflammatory monocytes would play a more crucial role than T and NK cells in limiting

tumor spread. Distinct mechanisms of monocyte-mediated tumoricidal activity have been highlighted using melanoma-cell lines as target cells, related either on a direct recognition and cytotoxicity or on antibody-mediated lysis (te Velde and Figdor, 1992). Reactive oxygen intermediates and TRAIL have been involved in monocyte-mediated cytotoxicity *in vitro* (Griffith et al., 1999; Martin and Edwards, 1993). Inflammatory DCs also produce TNFα and iNOS and these 2 inflammatory mediators may exert direct tumoricidal activities. Additional work will be required to precise the effector mechanism(s) used by Ly-6C^{high} monocytes to kill disseminated tumor cells/melanocytes in our model.

We found that inflammatory monocytes accumulated in the skin of MT/ret mice with active vitiligo. Interestingly, the inverse correlation between the proportion of Treg cells and the percentage of Ly-6C^{high} monocytes and DCs among skin-derived cells suggest that Treg cells interfere with the anti-tumor properties of Ly-6C^{high} monocytes. Whereas it is clearly established that Treg cells potently interfere with tumor specific T cells (Antony et al., 2005; Shimizu et al., 1999), the impact of Treg cells on innate immune cells, in particular, on myeloid cells, has been clearly less investigated in the context of tumor development. Foxp3^{+} T cells have been shown to promote the death of DCs in TdLNs, subsequently limiting the onset of anti-tumor CD8^{+} T-cell responses (Boissonnas et al., 2010). Zhang et al. reported that continuous Treg-cell depletion in a liver cancer model resulted in the induction of myeloid derived suppressive cells (MDSC) which accelerated established tumor growth (Zhang et al., 2010). Treg cells also stimulate tumor-derived MDSC to express inhibitory B7-H1, B7-H3, and B7-H4 molecules and to produce IL-10 (Fujimura et al., 2012). Treg cells have been recently shown to limit, through their secretion of IL-10, inflammatory monocyte recruitment and subsequent differentiation into inflammatory DCs in the liver during *Trypanosoma* infection (Bosschaerts et al., 2010). Here, we found that the administration of an anti-IL-10R antibody resulted in a delayed development of cutaneous metastases and an increased occurrence of vitiligo. Thus, Treg-cell derived IL-10 may inhibit, in our model, monocyte recruitment and differentiation in the skin.

In MT/ret mice, tumor-cell dissemination occurs early in life as dormant cells can already be detected in 3 week-old mice (Eyles et al., 2010). Disseminated dormant cells may promote the trafficking of Ly-6C^{high} monocytes to the skin, their subsequent activation and differentiation into inflammatory DCs leading to the lysis of tumor cells and to the bystander destruction of normal melanocytes thereby causing depigmentation. Until now, monocytic cells have been rather considered as pro-tumoral cells that facilitate metastatic spread by promoting tumor-cell extravasation and dampen anti-tumor responses by differentiating into

pro-tumoral macrophages (Qian et al., 2011; Wolf et al., 2012). Our results rather suggest that inflammatory monocytes are the most efficient effectors in controlling disseminated melanoma cells in our model of spontaneous metastatic melanoma. Thus, depending on the tumor stage (primary tumors, disseminated malignant cells or established metastases), immune cells within the tumor microenvironment may display different properties, either pro- versus anti-tumoral, highlighting the importance of studying the anti-tumor response in the course of spontaneous tumor development.

MATERIALS AND METHODS

Mice

MT/ret$^{+/-}$ transgenic mice (called MT/ret later) that were on the C57BL/6 background expressed heterozygously the human Ret oncogene (Kato et al., 1998). They were used for experiments at different time points in the course of malignancy and age-matched non-transgenic MT/ret$^{-/-}$ littermates (called Ctrl later) were used as control mice. MT/ret mice were crossed with C57BL/6 CD3ε$^{-/-}$ mice (Malissen et al., 1995) to obtain RetCD3ε^{KO}. Clinical signs of MT/ret and RetCD3ε^{KO} mice were assessed weekly and development of primary tumors, facial or dorsal metastases and vitiligo was recorded. Mice were sacrificed at indicated times or when considered moribund (prostrated, bristly, skinny). All these mice were maintained in our SPF animal facility. All experiments were carried out in accordance with the guidelines of the French Veterinary Department.

Antibody and chemokine treatments

Purified anti-CD25 (PC61), anti-Ly-6G (1A8) and anti-CD8 (53.6.7) antibodies were obtained from BioXCell. Purified anti-Gr1 (RB6-8C5), anti-IL-10 receptor (1B1.2.1C4) and anti-NK1.1 (PK136) antibodies were obtained from hybridoma supernatants and purified using a G-protein coupled Sephadex bead column. CCL2 (MCP-1) chemokine was obtained from Peprotech. Treated mice were injected intra-peritoneously twice a week with either 200µg of antibodies or 400ng of CCL2. Cellular depletion was assessed using flow cytometric analysis of the blood.

Single cell suspension procedures from lymphoid organs, tumors and skin

Blood collection was performed to avoid contamination with peripheral blood mononuclear cells. Spleens and lymph nodes were mechanically dissociated, homogenized and passed through a nylon cell strainer (BD Falcon) in 5% FCS, 0.1% NaN3 (Sigma-Aldrich) in phosphate-buffered saline (PBS). For adoptive transfer, RPMI 1640 Glutamax (Gibco) supplemented with 10% fetal calf serum (FCS; Biochrom) was used instead. Cutaneous and distant metastases were mechanically dissociated and digested with 1mg/mL collagenase D and 0,1mg/mL DNase I (Roche, Mannheim, Germany) for 25 min at 37°C. Single cell suspensions were filtered washed in PBS 1X, 5% FCS, 0.5 mM EDTA, 0.1% sodium azide and resuspended in PBS 1X, 5% FCS, 0.1% sodium azide. For skin suspensions, ears were collected and split in dorsal and ventral lobe. Lobes were cut in small parts and digested with

0.4mg/mL liberase, 0.05mg/mL collagenase D and 0.1mg/mL DNase I (Roche, Mannheim, Germany). Suspensions were filtered, washed in PBS 1X, 5% FCS, 0.5 mM EDTA, 0.1% sodium azide and resuspended in PBS 1X, 5% FCS, 0.1% sodium azide.

Cell surface staining and flow cytometry

Cell suspensions were collected and dispensed into 96-well round-bottom microtiter plates (Greiner Bioscience; $6x10^6$ cells/well). Surface staining was performed by incubating the cells on ice, for 15 minutes per step, with Abs in 5% FCS (Biochrom), 0.1% NaN_3 (Sigma-Aldrich) phosphate-buffered saline. Each cell-staining reaction was preceded by a 15-minute incubation with purified anti-CD16/32 antibodies (FcγRII/III block; 2.4G2) obtained from hybridoma supernatants followed by labeling with specific Abs.

PE-conjugated anti-NK1.1 (PK136), anti-TcRγδ (GL3), PerCP-conjugated anti-CD4 (RM4-5), PerCP-Cy5.5 anti-NK1.1 (PK136), anti-CD45 (30F11), APC-conjugated anti-CD45 (30F11), FITC-conjugated anti-CD8 (53.6.7), anti-CD19 (1D3), anti-Ly-6G (1A8), PE-Cy7-conjugated anti-CD11c (HL3), streptavidin, APC-H7-conjugated anti-CD8 (53-6.7), Pacific Blue-conjugated anti-CD4 (RM4-5), biotinylated anti-Ly6C (AL-21), anti-TcRβ (H57-597) antibodies were obtained from BD Biosciences. APC-conjugated anti-CD11b (M1/70) antibody was obtained from eBioscience. Pacific Blue-conjugated streptavidin was obtained from Invitrogen.

Multi-color immunofluorescence was analyzed using a BD-FacsCalibur and BD-LSR2 cytometers (BD Biosciences). List-mode data files were analyzed using Cell Quest Pro and Diva softwares (BD Biosciences). Data acquisition was performed on the Cochin Immunobiology facility. For skin suspensions, incubation with Live/Dead Blue stain (Invitrogen), according to the manufacturer protocol, was performed prior to antibody staining.

Adoptive transfer of $CD4^+$ T cells

Cervical lymph nodes and spleen cells were incubated on ice for 20 minutes with a mixture of anti-CD8 (53-6.7), anti-CD11b (Mac-1), and anti-CD19 (1D3) antibodies, obtained from hybridoma supernatants, and then with magnetic beads coupled to anti-rat immunoglobulins (Dynal Biotech). Purified T-cell subsets were generally 95% to 97% pure. Purified $CD4^+$ T cells ($5x10^6$ cells) were injected intravenously into sex-matched RetCD3ε^{KO} mice at day 5 after birth.

Antibody titration by flow cytometry

Sera from MT/ret and RetCD3ε^{KO} mice were obtained following intra-cardiac blood collection. T1 melanoma cells (A cell line derived from a cutaneous tumor of a MT/ret mouse) were incubated with purified anti-CD16/32 antibodies (FcγRII/III block; 2.4G2) obtained from hybridoma supernatants and then incubated with the sera. Cells were labeled with Alexa 488 conjugated rat anti-mouse κ antibody from invitrogen. Cells submitted to the same protocol but without serum incubation were used as control.

Statistics

Data are expressed as mean ± SEM. The significance of differences between two series of results was assessed using the student's unpaired t test. The significance of differences between three or more series of results was assessed using the one-way ANOVA and comparison between sets of results was assessed using Tukey post-test. Comparison between incidence curves was performed using log-rank test. Correlation analyses were performed using Pearson's correlation test. Values of $p < 0.05$ were considered as statistically correlated. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). All statistical analyses were performed using Prism 5 software (GraphPad softwares).

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FIGURES

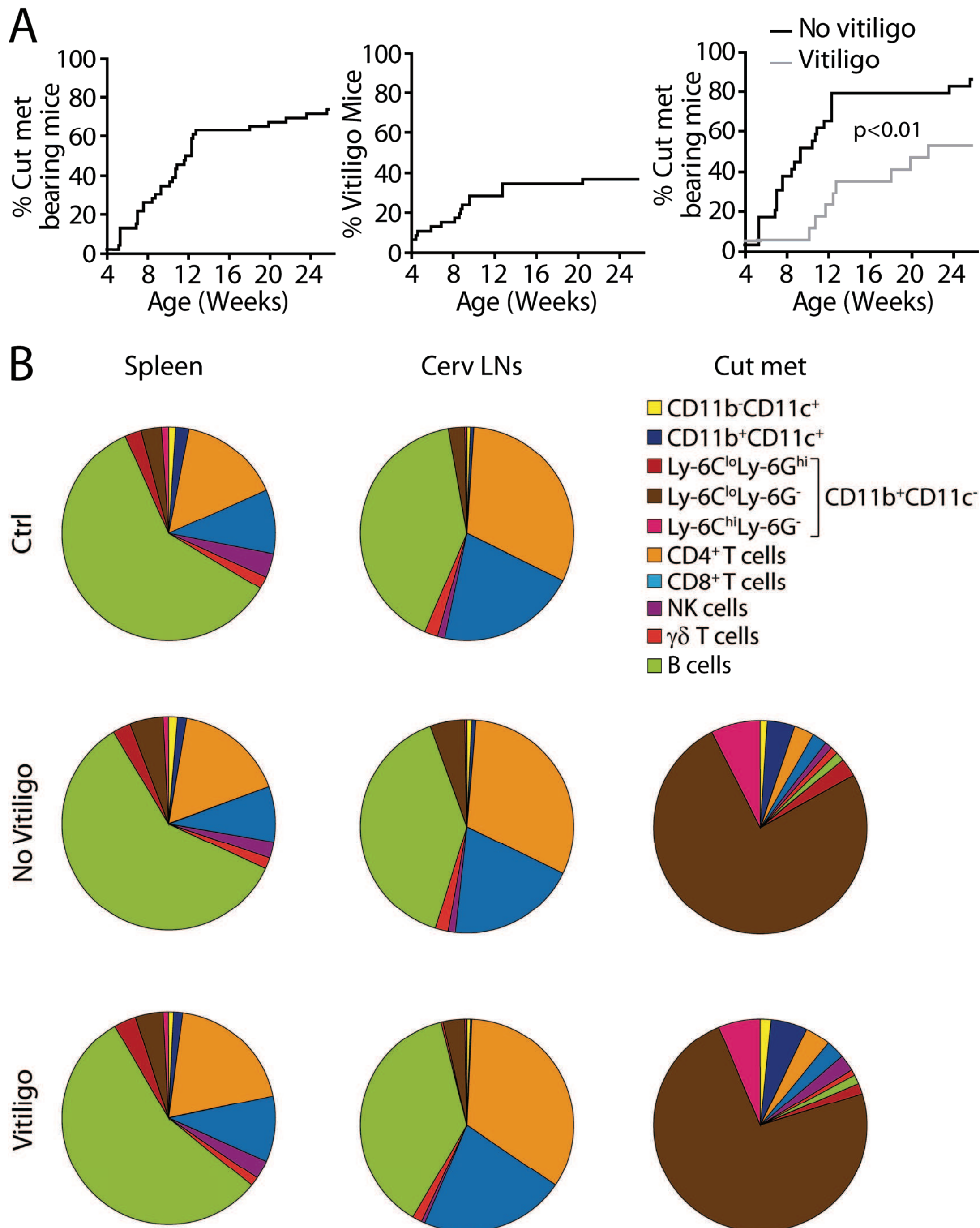

Figure 1: Characterization of the MT/ret mouse model of spontaneous melanoma. Diagnosis was performed weekly from weaning and over a 6-month period. (A) First panel: Incidence of cutaneous metastases (Cut met) in MT/ret mice (n = 46). Second panel: Vitiligo occurrence in MT/ret mice. Third panel: cutaneous metastasis incidence in MT/ret mice with (— ; n = 17) or without vitiligo (— ; n = 29). (B) Immune composition of the indicated tissues from 6-month old Ctrl or MT/ret mice with or without vitiligo. The different immune populations were defined as shown in FigS1. Proportions are shown within $CD45^{+}$ cells for cutaneous metastases. Statistical analyses were performed using log-rank test (A right panel).

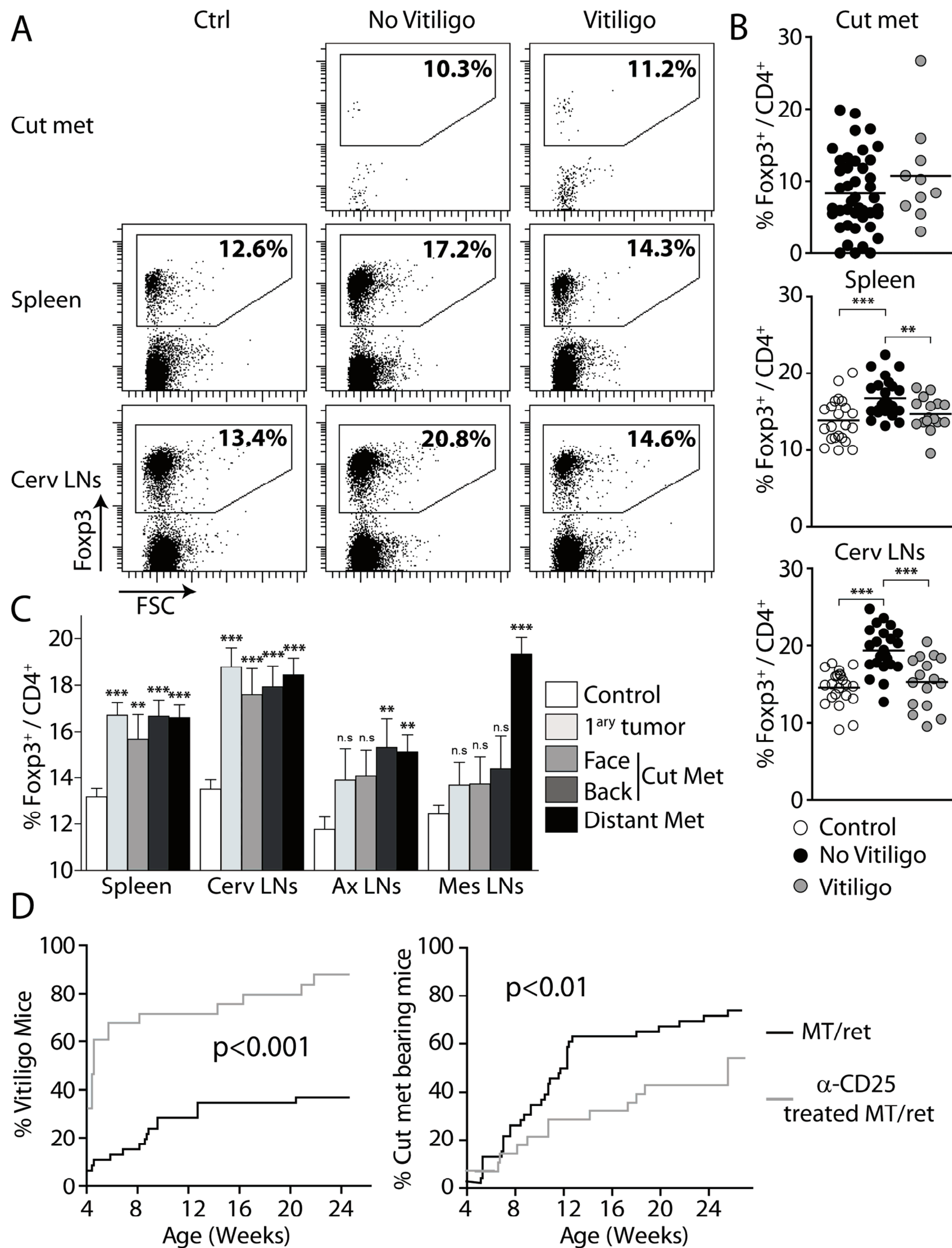

Figure 2: Treg cells accumulate in the spleen and draining lymph nodes of MT/ret mice not developing vitiligo. (A) Representative FSC/Foxp3 dot plots are shown for $CD45^+$ $CD4^+$ $CD8^-$ $TcR\beta^+$ cells from the indicated tissues of 6-month old Ctrl mice and MT/ret mice with or without vitiligo. (B) Percentage of Treg cells in cutaneous metastases (Cut met) of 6-month old MT/ret mice developing or not vitiligo and in the spleen and cervical lymph nodes (Cerv LNs) of Ctrl mice and MT/ret mice. (C) Percentage of Treg cells in the spleen, cervical (Cer), axillary (Ax) and mesenteric (Mes) lymph nodes of MT/ret mice non-displaying vitiligo according to their symptoms. (D) Incidence of cutaneous metastases and vitiligo in 6-month old MT/ret mice treated (▬ ; n=28) or not (▬; n=46) with anti-CD25 antibody. Statistical differences were assessed using either unpaired t test (B upper panel), one-way ANOVA with Tukey post-test comparison, (B bottom panels and C) or log-rank (D).

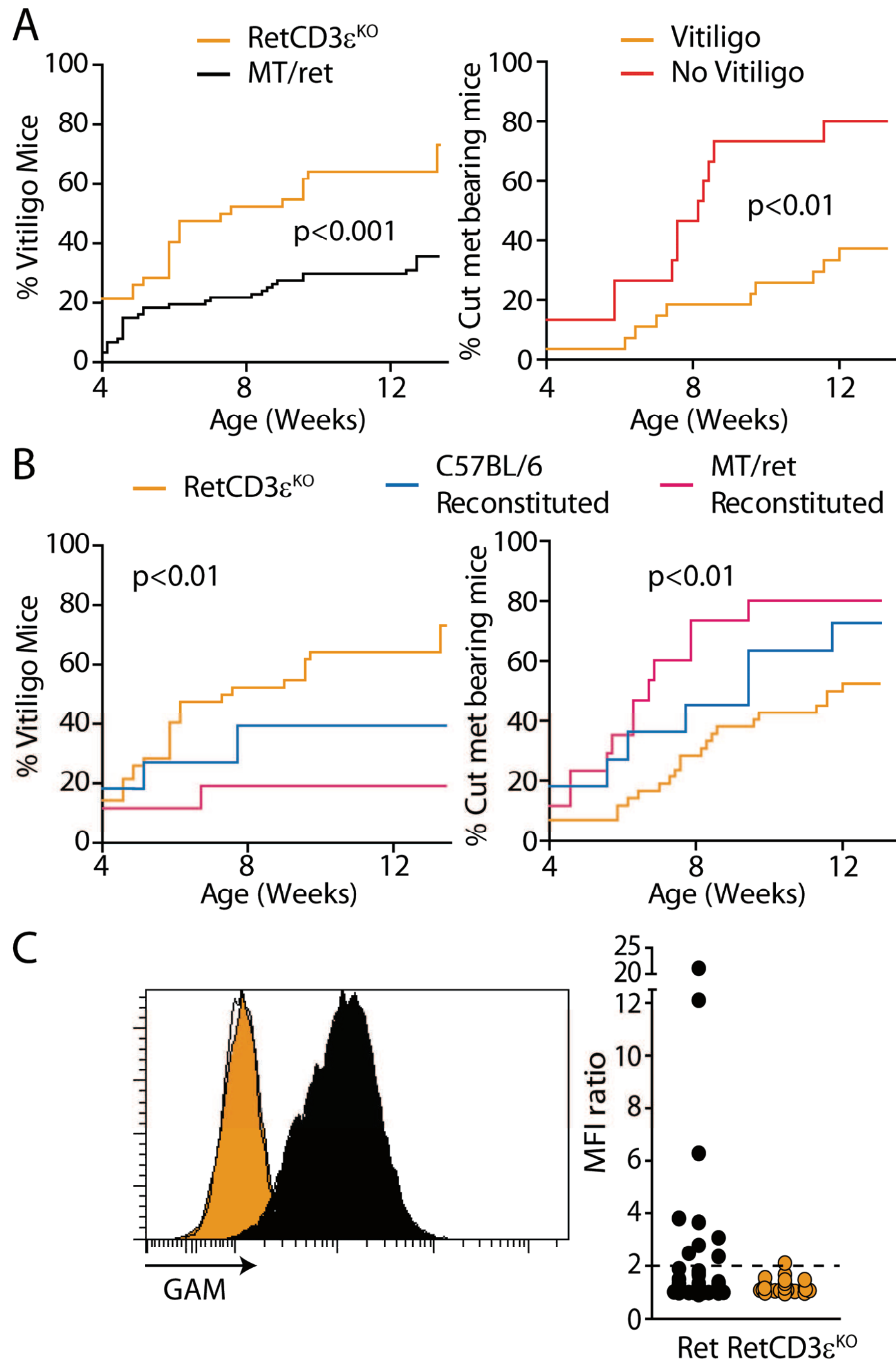

Figure 3: Increased incidence of protective vitiligo in RetCD3ε^{KO} mice. (A) Vitiligo occurrence in 3-month old MT/ret (▬ ; n= 87) and RetCD3ε^{KO} (▬ ; n=41) mice, and incidence of cutaneous metastases (Cut met) in RetCD3ε^{KO} mice with (▬) or without (▬) vitiligo. (B) Incidence of cutaneous metastases and vitiligo in RetCD3ε^{KO} mice (▬) or RetCD3ε^{KO} mice reconstituted with CD4^{+} T cells from either 3-month old MT/ret mice without vitiligo (▬; n=17) or C57BL/6 mice (▬ ; n=11). (C) Flow cytometry analysis of the presence of anti-tumor antibodies in the sera of 3-month old MT/ret and RetCD3ε^{KO} mice. First panel: representative histograms (empty histogram: staining with the secondary antibody only; grey histogram: example of a negative serum; black histogram: example of a positive serum). Second panel: quantification. MFI ratios were calculated by dividing the MFI obtained with a given serum by the MFI obtained with the secondary antibody only. Statistical analyses were performed using log-rank test (A, B).

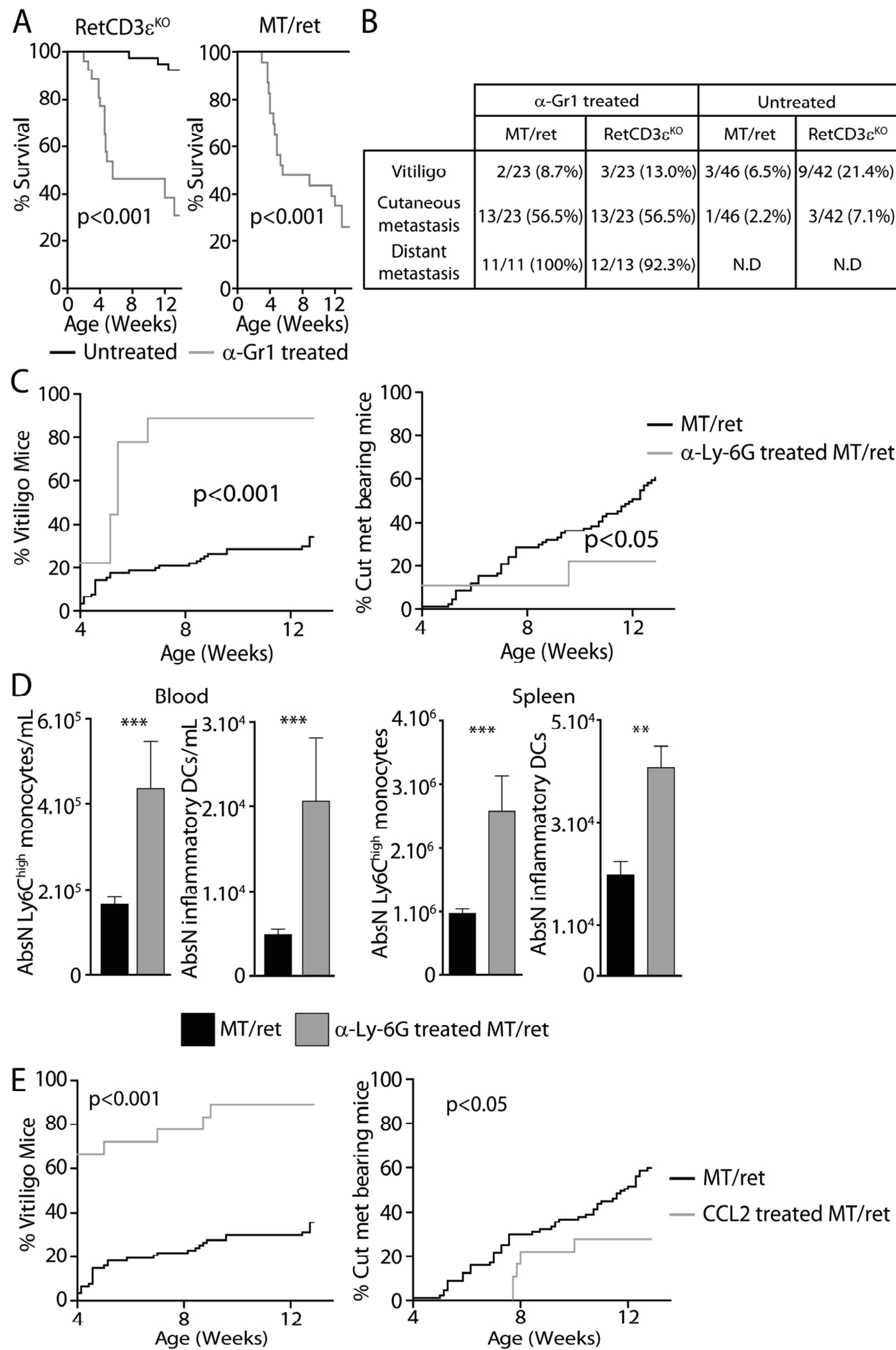

Figure 4: Ly-6C-expressing innate immune cells are involved in vitiligo development and control of tumor spread. (A) Survival curves of $RetCD3\varepsilon^{KO}$ (n=23) and MT/ret (n=23) mice treated (▬) or not (▬) with anti-Gr1 antibody. (B) Prevalence of vitiligo, cutaneous metastases and distant metastases in 4-week old, anti-Gr1 treated $RetCD3\varepsilon^{KO}$ and MT/ret mice. Results are expressed as the number of mice with the indicated symptoms over the total number of analyzed mice. Numbers in brackets represent the proportion of analyzed mice with the indicated symptoms. (C) Incidence of vitiligo and cutaneous metastases (Cut met) in 3-month old MT/ret mice treated (▬; n=9) or not (▬; n=87) with anti-Ly-6G antibody. (D) Absolute numbers of Ly-$6C^{high}$ monocytes and inflammatory DCs in the blood and spleen of 3-week old, anti-Ly-6G treated MT/ret mice compared to untreated ones. (E) Incidence of vitiligo and cutaneous metastases in 3-month old MT/ret mice treated (▬; n=18) or not (▬; n=87) with recombinant CCL2. Statistical analysis was performed using either log-rank test (A, C and E) or unpaired t-test (D).

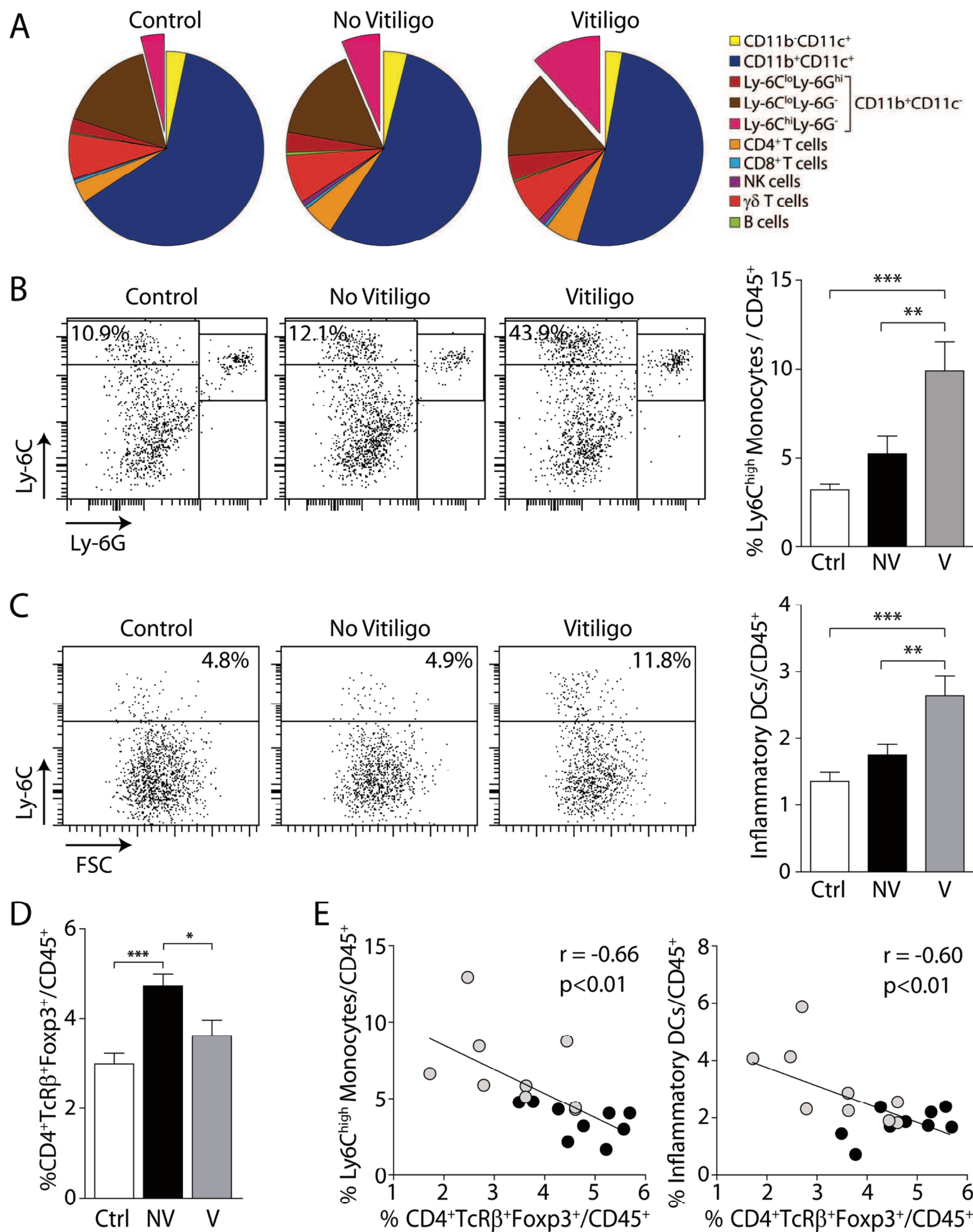

Figure 5: Ly-6C^{high} monocytes and inflammatory DCs accumulate in the skin of MT/ret mice with active vitiligo. (A) Characterization of immune cells infiltrating the skin of 3-week old Ctrl or MT/ret mice with or without vitiligo. The different immune populations were defined as shown in FigS1. Proportions are shown within CD45$^+$ cells. (B) Representative Ly-6G/Ly-6C dot plots are shown for CD45$^+$ CD11b$^+$ CD11c$^-$ NK1.1$^-$ cells. The proportion of Ly-6C^{high} monocytes (CD11b$^+$ CD11c$^-$ NK1.1$^-$ Ly-6G$^-$ Ly-6C^{high} cells) within CD45$^+$ cells was calculated and plotted (right panel) for 3-week old Ctrl or MT/ret mice with or without vitiligo. (C) Representative FSC/Ly-6C dot plots are shown for CD45$^+$ CD11b$^+$ CD11c$^+$ cells. The proportion of inflammatory DCs (CD11b$^+$ CD11c$^+$ Ly-6C^{high} cells) within CD45$^+$ cells was calculated and plotted (right panel) for 3-week old Ctrl or MT/ret mice with or without vitiligo. (D) Quantification of the Treg-cell infiltrate within CD45$^+$ cells in the skin of 3-week old Ctrl or MT/ret mice with or without vitiligo. (E) Correlation between the infiltration of Treg cells and that of Ly-6C^{high} monocytes (Left panel) or inflammatory DCs (Right panel) in the skin of 3-week old MT/ret mice with (●) or without vitiligo (●). Statistical analyses were preformed using either one-way ANOVA with Tukey post-test comparison. (B, C and D) or Pearson r test (E).

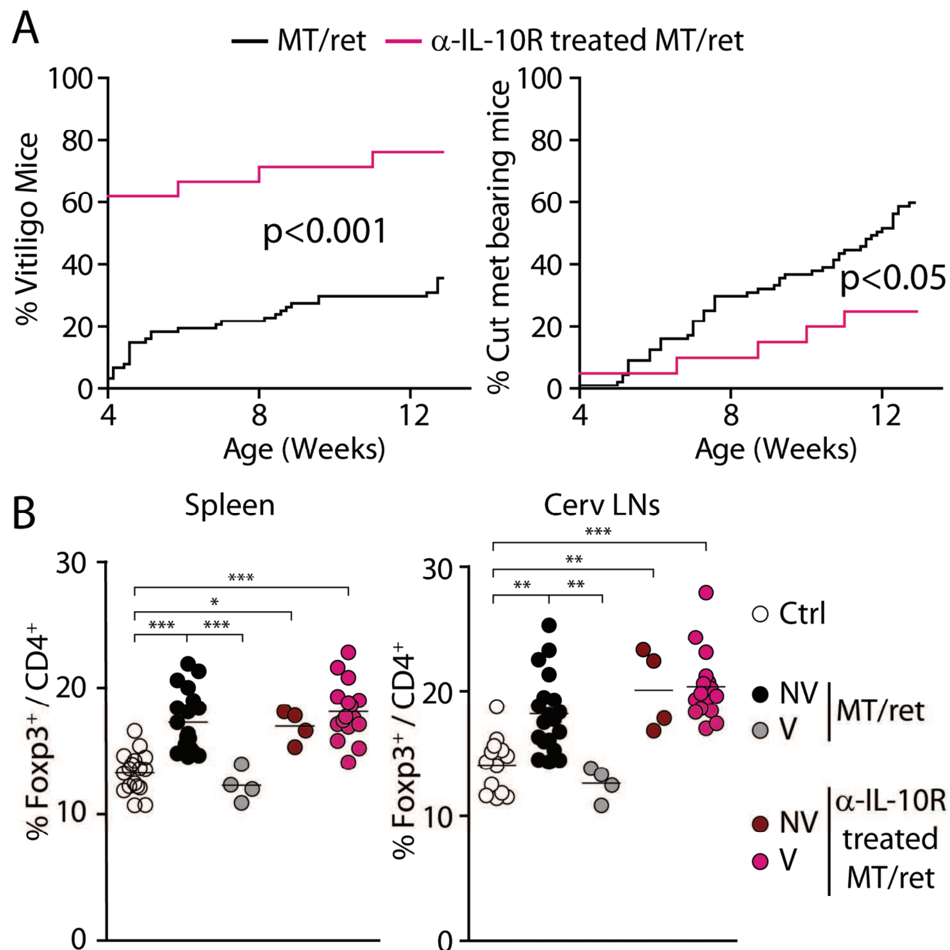

Figure 6: Effect of IL-10 receptor neutralization. (A) Incidence of cutaneous metastases (Cut met) and vitiligo in 3-month old MT/ret mice treated (▬; n=20) or not (▬; n=87) with anti-IL-10R antibody. (C) Percentage of Treg cells in the spleen and cervical lymph nodes of 3-month old Ctrl, MT/ret mice and anti-IL-10R treated MT/ret mice. Statistical analyses were performed using either log-rank test (A) or one-way ANOVA with Tukey post-test comparison (B).

Mice	MT/ret		MT/ret		MT/ret	MT/ret	MT/ret	MT/ret	MT/ret	MT/ret	RetCD3ε^{KO}	RetCD3ε^{KO}
Treatment	None		α-CD25		α-CD8	α-NK1.1	α-Gr1	α-Ly6G	CCL2	α-IL10R	None	α-Gr1
Age	3 months	6 months	3 months	6 months	3 months	3 months	3 months	3 months	3 months	3 months	6 months	3 months
incidence of 1^{ary} tumor	78/87 (89.7%)	43/46 (93.5%)	33/40 (82.5%)	25/28 (89.3%)	21/23 (91.3%)	12/14 (85.7%)	6/6 (100.0%)	8/9 (88.9%)	15/18 (83.3%)	17/20 (85.0%)	18/21 (85.7%)	8/9 (88.9%)
incidence of cut met[1]	52/87 (59.8%)	34/46 (73.9%)	9/40 (22.5%)	11/28 (39.3%)	13/23 (56.5%)	8/14 (57.1%)	6/6 (100.0%)	2/9 (22.2%)	5/18 (27.7%)	5/20 (25.0%)	16/21 (76.2%)	7/9 (77.8%)
incidence of distant met[2]	0/30 (0.0%)	4/41 (9.7%)	0/12 (0.0%)	0/28 (0.0%)	10/23 (43.5%)	2/14 (14.3%)	6/6 (100.0%)	0/9 (0.0%)	2/18 (11.1%)	0/20 (0.0%)	4/21 (19.1%)	7/9 (77.7%)
incidence of vitiligo	31/87 (35.6%)	17/46 (36.9%)	31/40 (77.5%)	24/28 (85.7%)	8/23 (34.8%)	6/14 (42.9%)	1/6 (16.7%)	8/9 (88.9%)	16/18 (88.9%)	16/20 (80.0%)	17/21 (80.9%)	3/9 (33.3%)

1: Cutaneous metastases 2: Distant metastases

Table 1: Summary of MT/ret mouse diagnosis in different settings. Prevalence of primary tumors, cutaneous metastases, distant metastases and vitiligo in MT/ret mice and RetCD3ε^{KO} mice as a function of mouse age and treatment. Results are expressed as the number of mice with the indicated symptoms over the total number of analyzed mice. Numbers in brackets represent the proportion of analyzed mice with the indicated symptoms.

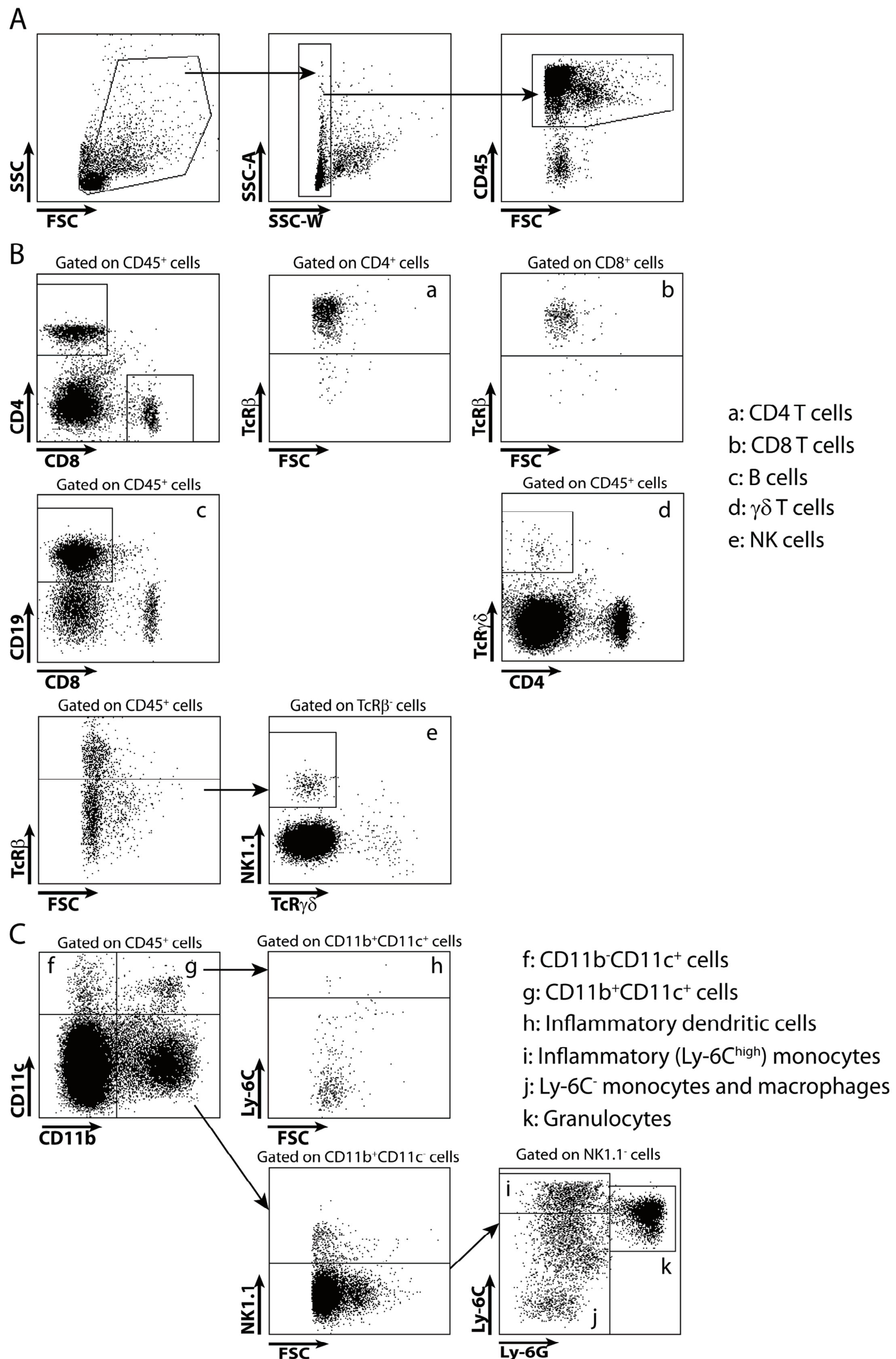

Figure S1: Identification of lymphoid and myeloid cell populations by flow cytometry. Representative dot plots showing (A) common gating strategy, identification of (B) lymphoid populations and (C) myeloid populations. Dot plots are shown for splenocytes of a 12-week old Ctrl mouse.

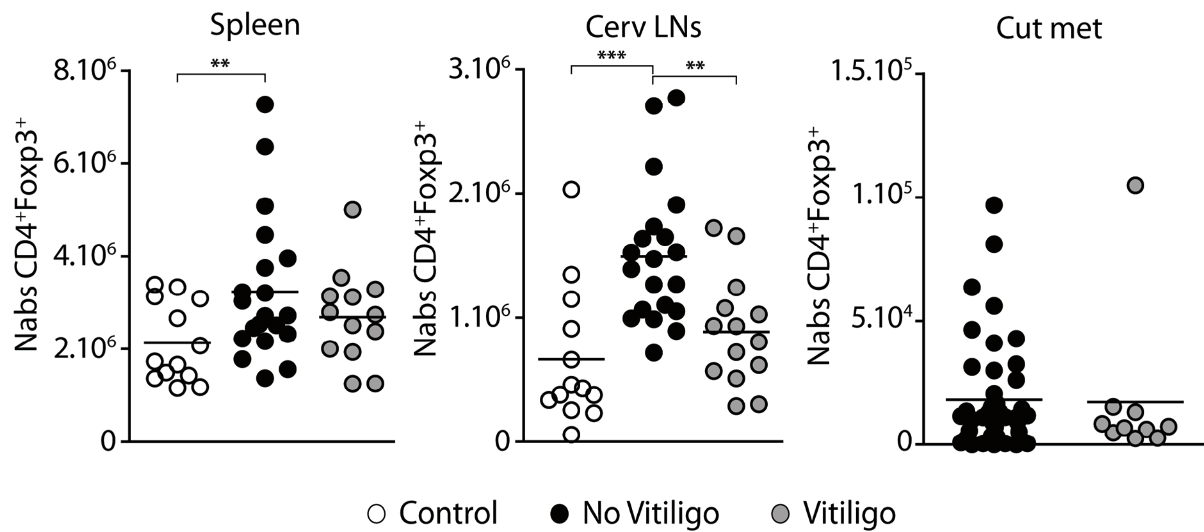

Figure S2: Accumulation of regulatory $CD4^+$ T cells in the spleen and cervical lymph nodes of non-vitiligo MT/ret mice. Absolute numbers of Treg cells ($CD4^+$ $CD8^-$ $Foxp3^+$ cells) in cutaneous metastases of 6-month old MT/ret mice developing or not vitiligo and in the spleen and cervical lymph nodes of Ctrl mice and MT/ret mice. Statistical analyses were performed using either one-way ANOVA with Tukey post-test comparison (left and middle panel) or unpaired t-test (right panel).

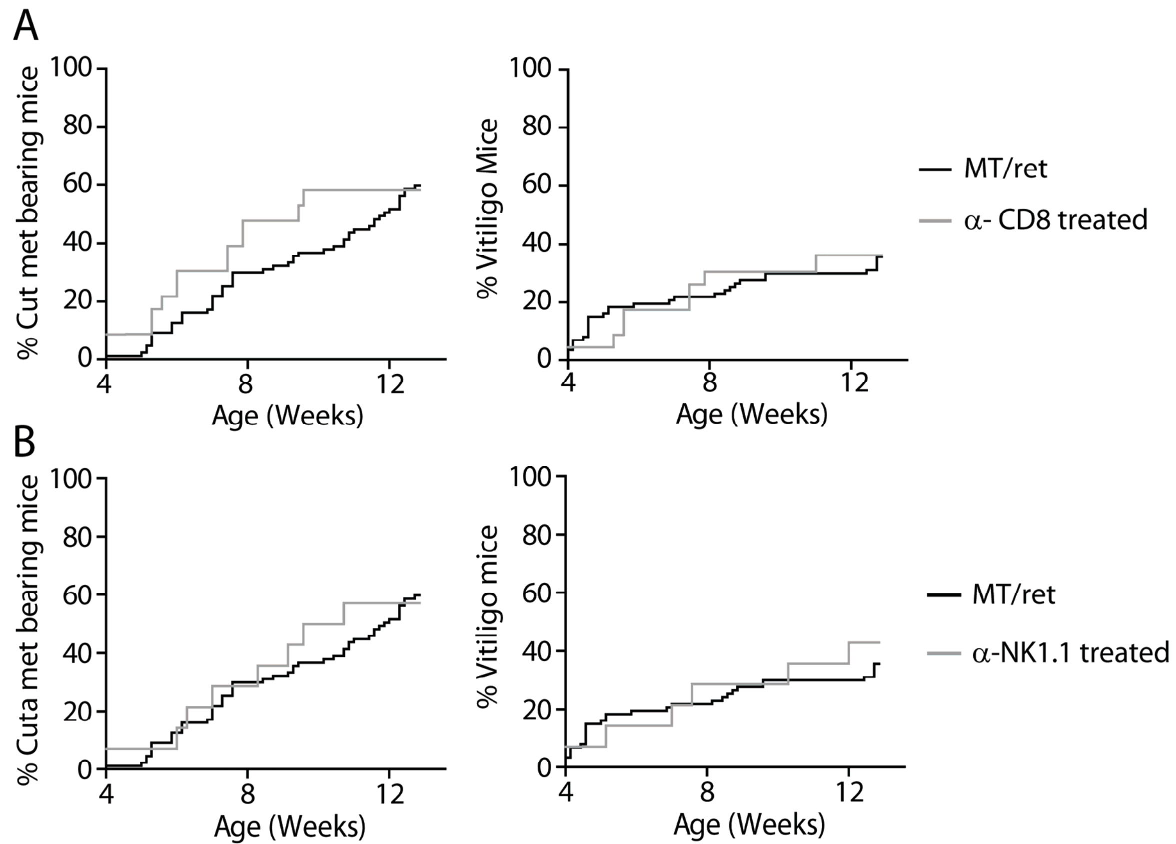

Figure S3: Effect of CD8^{+} T-cell or NK-cell depletion on the development of vitiligo and tumor spread at cutaneous level in MT/ret mice. Incidence of vitiligo and cutaneous metastases (Cut met) in MT/ret mice treated with (A) anti-CD8 antibody (n=20) or (B) anti-NK1.1 antibody (n=14). Statistical analyses were performed using log-rank test.

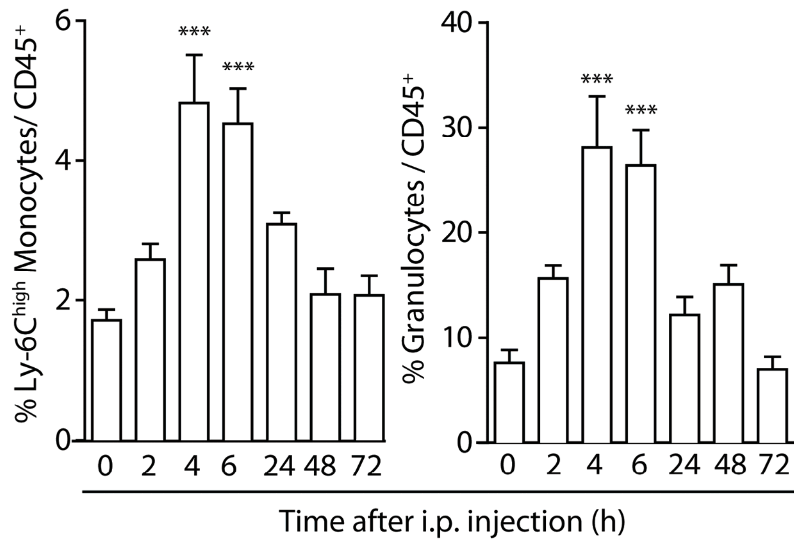

Figure S4: The proportion of circulating granulocytes and Ly-6C^{high} monocytes transiently increases after CCL2 injection. Proportion of Ly-6C^{high} monocytes (Left panel) and granulocytes (Right panel) in the blood of Ctrl mice 0, 2, 4, 6, 24, 48 and 72 hours after one intra-peritoneal injection of 400ng of CCL2. Statistical analyses were performed using one-way ANOVA with Tukey post-test comparison.

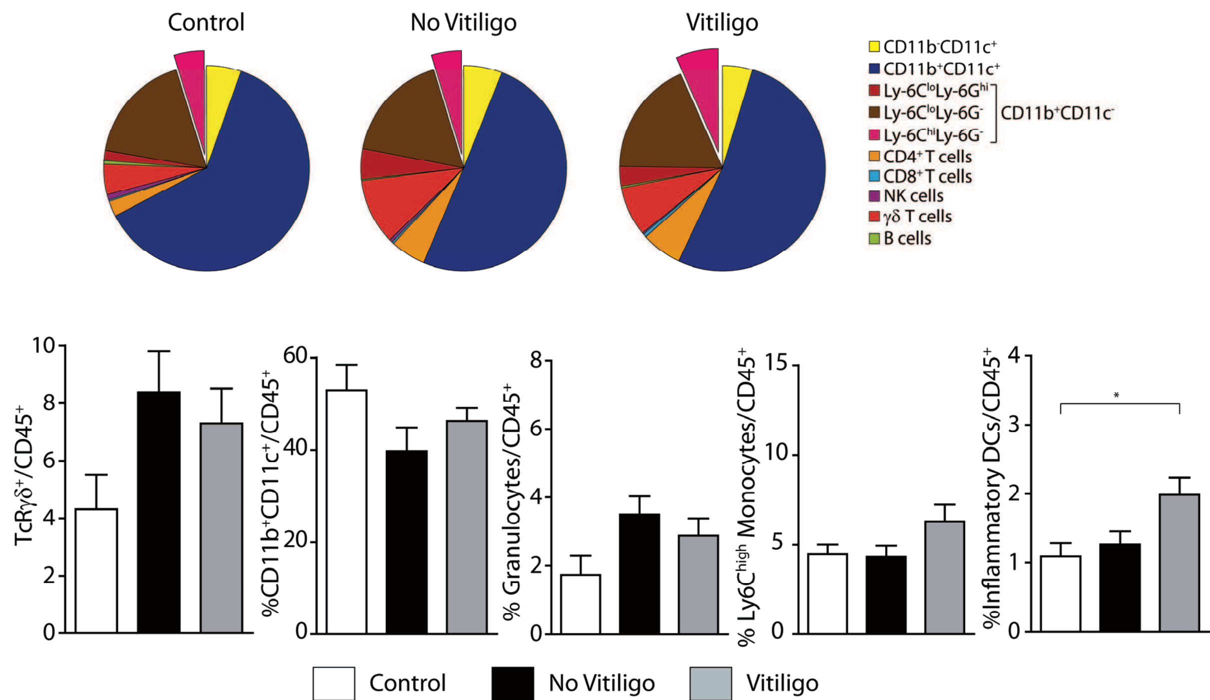

Figure S5: Characterization of immune cells infiltrating the skin of 6-week old MT/ret mice with or without vitiligo. (A) Characterization of immune cells infiltrating the skin of 6-week old Ctrl or MT/ret mice with or without vitiligo. The different immune populations were defined as shown in FigS1. Proportions are shown within $CD45^+$ cells. (B) Quantification of different immune-cell populations infiltrating the skin of 6-week old Ctrl or MT/ret mice with or without vitiligo. Statistical analyses were performed using one-way ANOVA with Tukey post-test comparison.

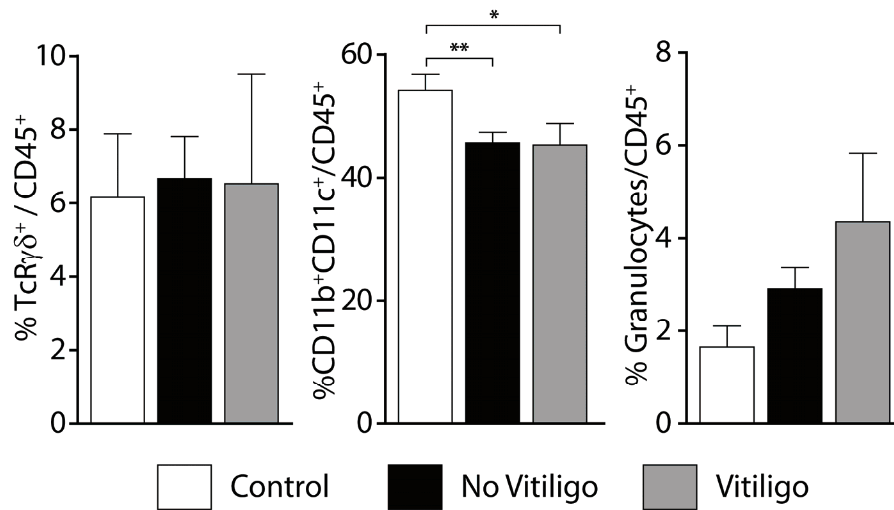

Figure S6: Quantification of various immune cells infiltrating the skin of 3-week old MT/ret mice with or without vitiligo. Quantification of different immune-cell populations infiltrating the skin of 3-week old Ctrl or MT/ret mice with or without vitiligo. Statistical analyses were performed using one-way ANOVA with Tukey post-test comparison.

12 week-old RetCD3ε^{KO} mice

Reconstitution	None	CD4 T cells (C57/BL6)	CD4 T cells (MT/ret)
incidence of 1^{ary} tumor	35/42 (83.3%)	10/11 (90.9%)	16/17 (94.1%)
incidence of cut met [1]	22/42 (52.4%)	8/11 (72.7%)	13/17 (76.5%)
incidence of distant met [2]	3/21 (14.3%)	3/11 (27.3%)	4/17 (23.5%)
incidence of vitiligo	31/42 (73.8%)	4/11 (39.4%)	3/17 (17.6%)

1: Cutaneous metastases 2: Distant metastases

Table S1: Diagnosis of reconstituted RetCD3ε^{KO} mice. Prevalence of primary tumors, cutaneous metastases, distant metastases and vitiligo in RetCD3ε^{KO} mice as a function of mouse age and treatment. Results are expressed as the number of mice with the indicated symptoms over the total number of analyzed mice. Numbers in brackets represent the proportion of analyzed mice with the indicated symptoms.

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DISCUSSION

A. Autour de l'immunosuppression dans le contexte tumoral :

L'avantage majeur d'un modèle de tumeurs spontanées, tel que le modèle MT/ret, est la possibilité d'analyser la réponse immunitaire anti-tumorale tout au long du processus de carcinogenèse, mais également à différentes localisations. Nous avons ainsi pu mettre en avant des rôles distincts des TAMs et des Tregs dans la suppression de la réponse anti-tumorale selon le compartiment étudié.

1) Les différences de répartition des TAMs et des Tregs révèlent différents niveaux d'immuno-suppression

1.1 Les TAMs s'accumulent dans les tumeurs

La très grande majorité des cellules immunitaires infiltrant les métastases cutanées dans le modèle MT/ret présentent un phénotype CD11b^{+} Gr1$^{faible/-}$ (Article 1 : Figure 2). L'anticorps anti-Gr1 reconnaissant un épitope commun à Ly-6C et Ly-6G, nous avons précisé ce phénotype par l'utilisation d'anticorps ciblant spécifiquement ces molécules. Les cellules myéloïdes du micro-environnement des métastases cutanées sont, en fait, dans leur majorité CD11b^{+} CD11c^{-} NK1.1^{-} Ly-6C$^{faible/-}$ Ly-6G^{-}, ce qui correspond à la définition phénotypique des TAMs (Article 2 : Figure 1B). De plus, les cellules myéloïdes présentes dans les métastases cutanées expriment des niveaux importants d'ARNm de F4/80, Mgl1 et Fizz1, marqueurs des M2 (Article 1 : Figure 3A). De façon concordante avec nos résultats, plusieurs études ont mis en évidence une accumulation de macrophages dans les tumeurs. Elle est, en effet, retrouvée dans les modèles TiRP de mélanome sporadique (Soudja 2010), MMTV-PyMT de carcinome mammaire (DeNardo 2009) et KP d'adénocarcinome pulmonaire (Cortez-Retamozo 2012). La proportion de TAMs dans le micro-environnement tumoral des souris MT/ret est corrélée à l'agressivité des tumeurs (Article 1 : Figure 2D). C'est également le cas dans les modèles MMTV-PyMT (DeNardo 2009), KP (Cortez-Retamozo 2012) et KPC (Bayne 2012) où une accumulation des TAMs est associée à un mauvais pronostic vital. Les études chez l'Homme viennent renforcer ces constatations. En effet, une forte infiltration des TAMs est corrélée à un mauvais pronostic vital dans les cancers de la thyroïde, du poumon, du foie ou du sein (Ruffell 2012). Dans le mélanome, le rôle pronostique des TAMs reste controversé. Il a été suggéré que la densité de TAMs dans les tumeurs primaires, révélée par

le marqueur CD68, n'a pas valeur de facteur pronostique (Piras 2005), mais une seconde étude utilisant la même technique démontre que plus les tumeurs sont épaisses et agressives, plus l'infiltration de TAMs est importante (Varney 2005). Nos résultats vont dans ce sens puisque nous démontrons que, dans le modèle MT/ret, l'infiltration de TAMs est plus importante dans les métastases cutanées les plus agressives (Article 1 : Figure 2D).

Les macrophages activés alternativement (M2), se différencient en réponse à l'IL-4 et l'IL-13, et sont impliqués dans les réponses de type Th2 (Mantovani 2002). Il a été suggéré que le contexte tumoral peut biaiser la différenciation des macrophages vers le type M2 (Mantovani 2010). Plusieurs études transcriptomiques (Biswas 2006, Ojalvo 2009) démontrent que les TAMs expriment beaucoup de gènes potentiellement immuno-suppresseurs. En outre, ils sont capables de supprimer la prolifération *in vitro* des LT effecteurs (Movahedi 2010). Nos résultats sur les métastases cutanées des souris MT/ret montrent que les TAMs présentent des niveaux d'expression très importants des gènes arg1, il10, fizz1 et mgl1 (Article 1 : Figure 3A). De plus, ils expriment de forts niveaux de F4/80 et d'IL-4Rα ainsi qu'une faible capacité de sécrétion d'IL-12 (Article 1 : Figure 3B). Enfin, ils suppriment la prolifération et la sécrétion d'IFNγ des LT CD8$^+$ (Article 1 : Figure 4). L'ensemble de ces données confirme que les TAMs retrouvés chez les souris MT/ret sont de type M2 et sont des acteurs importants de l'immuno-suppression dans le micro-environnement tumoral.

1.2 Les Tregs s'accumulent dans les ganglions drainants

Une accumulation de Tregs est retrouvée dans la rate et les ganglions cervicaux des souris MT/ret ne développant pas de vitiligo à l'âge de 6 mois (Article 2 : Figure 2A et B, Figure S2). Ceci est également vrai dès l'âge de 6 semaines dans les ganglions cervicaux et de 3 mois dans la rate (données non montrées). De plus, nous démontrons que cette accumulation est spécifique des ganglions drainants (Article 2 : Figure 2C). Ces résultats concordent avec ceux mis en avant aussi bien chez les patients atteints de mélanome (Viguier 2004) que dans des modèles murins de tumeurs transplantées (Hiura 2005) ou spontanées (Tien 2005). Ces données suggèrent également que les Tregs pourraient jouer un rôle dans la suppression des réponses anti-tumorales au niveau systémique en inhibant l'activation des effecteurs anti-tumoraux dans les ganglions drainants. Ce dernier point a déjà fait l'objet de nombreuses études démontrant que la déplétion des Tregs augmente l'activation des effecteurs anti-tumoraux dans les ganglions drainants (Tanaka 2002) ou bien que des LT CD8$^+$ transférés ne peuvent s'accumuler et proliférer dans les ganglions drainants si l'hôte n'a pas d'abord été

déplété en Tregs (Dercamp 2005). Nos résultats sur la déplétion des Tregs chez les souris MT/ret viennent confirmer ces données (Article 2 : Figure 2D).

1.3 Les Tregs s'accumulent dans la peau

Nos données montrent une accumulation de Tregs dans la peau des souris MT/ret de 3 semaines ne développant pas de vitiligo (Article 2 : Figure 5D). Dans ce modèle, la dissémination à partir de la tumeur primaire intervient très précocement, mais les cellules tumorales disséminées sont maintenues à l'état de dormance par le système immunitaire (Eyles 2010). Notre hypothèse est que ces cellules tumorales isolées recrutent ou induisent l'expansion locale des Tregs capables de supprimer la réponse effectrice *in situ* dans la peau. Les Tregs pourraient alors avoir des rôles distincts en fonction du stade de développement tumoral et de leur localisation. En accord avec ces résultats, il a été mis en évidence que les Tregs sont l'une des premières populations à migrer des ganglions à la peau, et réciproquement, lors d'une réponse immunitaire (Tomura 2010).

Nos résultats suggèrent que les Tregs inhibent la réponse des effecteurs anti-tumoraux dans la peau par un mécanisme dépendant de l'IL-10 (Article 2 : Figures 5E et 6). L'importance de l'IL-10 pour les fonctions suppressives des Tregs est longtemps restée sujette à débat (cf. chapitre B.4.1.1). Nos données démontrant une importance de l'IL-10 dans la peau concordent avec la démonstration que l'IL-10 est cruciale pour les fonctions suppressives des Tregs aux interfaces environnementales (Rubstov 2008). Nous ne pouvons cependant pas exclure la possibilité que les Tregs pourraient utiliser d'autres mécanismes (cf. Chapitre B.4).
De façon intéressante, il n'y a pas d'accumulation préférentielle des Tregs dans les métastases cutanées des souris MT/ret (Article 2 : Figure 2A et B) contrairement à ce qui est retrouvé dans de nombreux types de cancer (cf. tableau 7), mais pas dans le mélanome. Ceci suggère que les Tregs ne seraient pas les acteurs principaux de l'immuno-suppression dans les métastases cutanées alors qu'ils semblent l'être dans les ganglions drainants et localement dans la peau.

2) Comment expliquer ces différents niveaux d'inhibition des réponses immunitaires ?

Les Tregs et les TAMs semblent impliqués à différents niveaux de la régulation des réponses anti-tumorales. Cela peut s'expliquer par les différences fonctionnelles de ces deux types cellulaires.

2.1 Les Tregs, maîtres de la tolérance

Les ganglions drainants sont le premier site vers lequel les DCs migrent afin de présenter les antigènes au système immunitaire. Il s'agit donc du site critique pour la décision initiale entre activation du système immunitaire et tolérance. Les Tregs sont des cellules spécialisées dans l'inhibition des réponses immunitaires. Ils sont capables d'inhiber de nombreux types cellulaires grâce à des mécanismes divers (cf. chapitres B.4 et B.5). Dans le cadre du cancer, deux types cellulaires pourraient être leurs cibles privilégiées dans les ganglions drainants. Tout d'abord, il a été très largement démontré que les Tregs inhibent les LT $CD8^+$, considérés comme les principaux effecteurs anti-tumoraux du système immunitaire (cf. chapitre C.4.1.2). Ensuite, les Tregs peuvent lyser les DCs inhibant la présentation antigénique et donc indirectement l'activation des LT (Boissonnas 2010). De ce point de vue, l'accumulation des Tregs dans les ganglions drainants, à un moment où les animaux présentent des tumeurs établies, semble cohérente avec ce que nécessitent la croissance et la dissémination tumorale.

Nos résultats démontrent que les Tregs s'accumulent précocement dans la peau et inhibent les effecteurs anti-tumoraux du système immunitaire (Article 2 : Figure 5). Dans ce modèle, la tumeur primaire dissémine dès l'âge de 3 semaines et les cellules tumorales isolées sont maintenues en dormance par les LT $CD8^+$ pendant plusieurs semaines (Eyles 2010). Ces effecteurs pourraient donc être cruciaux, à ce moment précis, pour contrôler la dissémination tumorale. Leur inhibition par les Tregs concorde donc également avec les besoins des tumeurs pour leur croissance.

2.2 Les TAMs, couteau suisse des tumeurs

Les TAMs présentent des capacités immuno-suppressives de par leur expression de gènes à potentiel immuno-suppresseur et par leur capacité à limiter la prolifération des LT effecteurs *in vitro* (Gabrilovich 2009). Malgré tout, les TAMs inhibent moins la prolifération des LT que les Tregs (comparaison entre Article 1 : Figure 4B et Delpoux 2012 : Figure 1B en annexe). Néanmoins, les TAMs présentent d'autres capacités très importantes pour la croissance et la dissémination tumorale.

Nos données mettent en avant le rôle pro-tumoral des TAMs qui sont capables de potentialiser la croissance des cellules tumorales en co-culture (Article 1 : Figure 6). De plus, plusieurs études dans des modèles murins de tumeurs démontrent que les TAMs jouent un rôle important dans l'angiogenèse. Ils produisent des quantités importantes de VEGF et son ablation spécifique dans les macrophages conduit à la normalisation de la vascularisation des tumeurs (Stockmann 2008). En outre, les TAMs produisent du facteur de croissance placentaire qui stimule également l'angiogenèse dans les tumeurs (Rolny 2011). La sous-population de TAMs exprimant Tie2 est retrouvée très proche des vaisseaux sanguins des tumeurs et est cruciale pour l'angiogenèse (De Palma 2005). Il a été mis en évidence que cette localisation dépend de la production d'ANG2 par les cellules endothéliales qui attirent ainsi les TAMs via sa liaison avec Tie2 (Mazzieri 2011).

Les TAMs sont également importants dans les mécanismes d'invasion tumorale. Leur production d'EGF (Epidermal Growth factor) facilite l'invasion des cellules tumorales dans les tissus adjacents (Wyckoff 2004). De plus, les TAMs sécrètent beaucoup de molécules altérant la structure de la matrice extra-cellulaire telles que des métalloprotéases matricielles (Kessenbrock 2010), des sérines protéases et des cathepsines (Mason 2011), facilitant ainsi la dissémination tumorale.

2.3 Conclusion

L'ensemble de nos résultats met en avant plusieurs niveaux d'immuno-suppression dans le modèle MT/ret. D'une part, les Tregs semblent impliqués dans la suppression des réponses anti-tumorales aux localisations et aux moments où une forte inhibition des effecteurs anti-tumoraux est requise. D'un autre côté, les TAMs présentent, en plus de leurs capacités

immuno-suppressives, des fonctions importantes pour la croissance et la dissémination tumorale expliquant leur localisation dans le micro-environnement tumoral.

3) Perspectives

Les résultats que nous avons obtenus soulèvent plusieurs questions qui pourront être adressées à la suite de ce projet.

3.1 Concernant l'accumulation des TAMs dans le micro-environnement tumoral

- Quel est le mécanisme de recrutement des TAMs dans les métastases ?

La grande majorité des études s'est focalisée sur la caractérisation des chimiokines impliquées dans le recrutement des macrophages dans les tumeurs primaires et ont mis en évidence le rôle de CCL2 (Qian 2011) et CXCL12 (ou SDF-1) (Du 2008). Il serait intéressant d'étudier les mécanismes impliqués dans le recrutement des macrophages dans les métastases par l'analyse du micro-environnement chimiokinique des métastases. En effet, dans le modèle MT/ret, les macrophages ne représentent pas la population majoritaire infiltrant les tumeurs primaires (données non montrées), alors qu'ils infiltrent massivement les métastases. Il est majoritairement admis que l'accumulation de progéniteurs myéloïdes immatures issus de la moelle osseuse donne naissance aux MDSCs, puis aux TAMs (Gabrilovich 2012). Cependant, il a également été mis en évidence dans un modèle spontané que la rate pourrait être un réservoir important dont sont issus les TAMs (Cortez-Retamozo 2012). Nous pourrions étudier de quel compartiment cellulaire proviennent les TAMs dans le modèle MT/ret.

- Quelles stratégies thérapeutiques ciblant les TAMs pourraient être mises en place ?

Les TAMs semblent être une cible thérapeutique potentiellement intéressante. De plus, le fait que le modèle MT/ret récapitule bien l'histoire naturelle du mélanome en fait un bon modèle pour des essais pré-cliniques. Par exemple, nous pourrions, dans ce modèle, évaluer en détails les effets de l'utilisation des anticorps monoclonaux anti-VEGF ou anti-EGF déjà utilisés en clinique. En outre, le recrutement des TAMs pourrait être ciblé par l'utilisation d'antagonistes des récepteurs aux chimiokines CCL2 et/ou CXCL12. De plus, nous pourrions cibler les mécanismes immuno-suppresseurs des TAMs, par exemple grâce aux inhibiteurs de phosphodiestérases ciblant l'Arg1 et NOS2 (Serafini 2006).

3.2 Concernant l'accumulation de Tregs dans les ganglions drainants

Beaucoup de questions se posent concernant les Tregs dans les ganglions drainants :

- Quelle est l'origine de cette accumulation ?

Il a été démontré qu'il pourrait s'agir d'une prolifération de nTregs (cf. Chapitre C.2.2) ou d'une conversion de LT $CD4^+$ naïfs en iTregs (cf. Chapitre C.2.3). Nous émettons également l'hypothèse d'une domiciliation préférentielle. La prolifération et la génération *de novo* implique une activation cellulaire. Or, nos données phénotypiques ne montrent pas d'activation plus importante des Tregs provenant des souris MT/ret que des contrôles non transgéniques (CD5, CD25, CD44, CD69, CD62L, CD103, GITR, CTLA-4, ICOS, données non montrées), nous orientant donc vers l'hypothèse d'une domiciliation préférentielle. Pour tester cette hypothèse, nous pourrions bloquer l'activité de CD62L par l'administration d'un Ac spécifique à des souris MT/ret atteintes ou à des souris contrôles. Ce traitement empêchant l'entrée des LT dans les ganglions montrerait, si l'hypothèse est exacte, que les Tregs sont séquestrés dans les ganglions drainants des souris MT/ret alors que les contrôles se vident.

- Quelle est leur spécificité antigénique ?

La question de la spécificité antigénique des Tregs dans le contexte tumoral n'est pas encore totalement élucidée. Dans le contexte tumoral, les Tregs peuvent être spécifiques des antigènes tumoraux (cf. chapitre C3). Elucider la question de la spécificité antigénique des Tregs des souris MT/ret pourrait également donner des indications concernant l'origine de l'accumulation des Tregs. En effet, si ces cellules s'avèrent spécifiques de néo-antigènes tumoraux, il paraitrait alors probable qu'il s'agisse d'iTregs. La spécificité antigénique pourrait être testée *in vitro* à partir de Tregs triés et marqués au CFSE puis mis en culture avec des DCs chargées en peptides tumoraux.

- Quelles sont leurs cibles dans les ganglions drainants et par quels mécanismes les inhibent-ils ?

Les Tregs sont capables d'inhiber de nombreux effecteurs du système immunitaire par des mécanismes divers. Néanmoins, dans le contexte tumoral, la majorité des mécanismes et des cibles potentielles des Tregs n'a pas été étudiée. Il serait intéressant de déterminer quels sont les effecteurs inhibés par les Tregs dans les ganglions drainants. Par exemple, les capacités de présentation antigénique et de co-stimulation des DCs, la prolifération et les fonctions

effectrices des LT et NK ou encore les capacités de sécrétion de cytokines des macrophages sont-elles affectées par les Tregs ? Nous pourrions évaluer les fonctions de ces différents effecteurs suite au blocage des fonctions des Tregs, par exemple par l'utilisation d'Ac anti-IL-10R ou anti-TGFβ, ou à leur déplétion par anti-CD25 ou le denileukin diftitox. Nos résultats suggèrent fortement que les Tregs agissent par un mécanisme dépendant de l'IL-10 (Article 2 : Figure 6). Malgré tout, nous ne pouvons affirmer complètement que c'est bien l'IL-10 produite par les Tregs qui est importante. Afin de répondre à cette question nous pourrions utiliser des Tregs déficient en IL-10. L'utilisation d'un système Cre/lox est très efficace mais semble difficile à mettre en œuvre dans le modèle MT/ret. En revanche, le transfert de Tregs provenant de donneurs IL-10^{KO} dans des souris MT/ret CD3ε^{KO} pourrait permettre de répondre à cette question.

- Quel mécanisme dicte leur accumulation dans les ganglions drainants ?

L'accumulation de Tregs dans les ganglions drainants est retrouvée chez les animaux ne présentant qu'une tumeur primaire oculaire. Nous envisageons donc que ce soit la tumeur primaire qui dicte l'accumulation des Tregs. En effet, les tumeurs sont capables de sécréter des facteurs susceptibles de provoquer l'expansion ou l'induction des Tregs (cf. Chapitre C.2). Cependant, la majorité de ces facteurs agit au niveau local pour recruter ou expandre les Tregs du micro-environnement tumoral. Nous envisageons d'évaluer l'impact de ces médiateurs connus, mais également de nouveaux, à distance dans les ganglions. Nous pourrions également étudier le rôle des DCs, macrophages ou MDSCs qui pourraient provoquer l'accumulation des Tregs dans les ganglions drainants. En effet, il a été démontré que l'IL-10 produite par les macrophages de la lamina propria est nécessaire pour maintenir l'activité des Tregs et empêcher la colite auto-immune (Murai 2009). Ceci soulève la possibilité que l'IL-10 produite par les macrophages de type 2 présents dans les ganglions dans le contexte tumoral puisse favoriser l'activité des Tregs.

B. Autour de la balance entre réponse régulatrice et réponse anti-tumorale :

La grande majorité des réactions immunitaires est régie par une balance entre deux effets antagonistes. On peut citer, par exemple, la régulation de l'activité des NK via des signaux activateurs et inhibiteurs antagonistes ou encore la contraction des LT $CD8^+$ après leur expansion et leur réponse. Les réactions observées dans le cadre tumoral ne semblent pas déroger à cette règle.

1) Immunité anti-tumorale et autoimmunité : Le vitiligo associé au mélanome

Un lien important existe entre immunité anti-tumorale et autoimmunité. Ceci est principalement du au recoupement important entre les antigènes exprimés par les tumeurs et leur contrepartie dans les tissus sains. Le meilleur exemple de ce lien est l'association entre le mélanome et le vitiligo. Le vitiligo est une destruction autoimmune des mélanocytes qui est de bon pronostic chez les patients atteints de mélanome. De plus, son incidence est augmentée par les immunothérapies favorisant la réponse lymphocytaire T anti-mélanome. Nos résultats démontrent également que, dans le modèle MT/ret, le vitiligo est de bon pronostic (Article 2 Figure 1A). En 1971, Milton suggère que les régressions spontanées, parfois observées dans le mélanome et le vitiligo qui leur est associé, sont liées à une réponse immunitaire active (Milton 1971). De façon concordante avec cette hypothèse, un infiltrat lymphocytaire a été observé dans les tumeurs et les zones dépigmentées des patients présentant des régressions spontanées (Smith 1965).

A l'heure actuelle, deux mécanismes majeurs sont proposés pour expliquer l'origine du vitiligo. D'un côté, des Ac spécifiques de la tyrosinase, de TRP-1 et de TRP-2 ont été retrouvés dans le sérum des patients atteints d'un vitiligo associé au mélanome (Ram 2007). De plus, il y a une corrélation entre le niveau des anticorps sériques dirigés contre les mélanocytes et l'importance du vitiligo (Norris 1988) et ces anticorps sont capables de lyser les mélanocytes sains et transformés *in vitro* (Naughton 1986). Cependant, ces dernières observations ont été faites chez des patients atteints de vitiligo mais pas de mélanome. D'un autre côté, les LT $CD8^+$ dans le vitiligo ont été mis en évidence au sein des lésions dépigmentées et sont capables de lyser des mélanocytes sains et transformés *in vitro* (Oyarbide-Valencia 2006). Les mêmes clones sont retrouvés dans les tumeurs et autour des

zones dépigmentées (Becker 1999). Enfin, les LT infiltrant les lésions dépigmentées sont des LT $CD8^+$ reconnaissant aussi bien les mélanocytes sains que transformés (Le Gal 2001). Réciproquement, le rôle du vitiligo dans l'établissement d'une bonne réponse anti-tumorale a récemment été mis en avant dans un modèle de mélanome transplanté (Byrne 2011). En effet, cette étude démontre que dans ce modèle expérimental, les souris développant un vitiligo sont capables de mettre en place une réponse CD8 mémoire plus importante et qui se maintient beaucoup plus longtemps que les animaux ne développant pas de vitiligo. En outre, ces résultats corroborent ceux précédemment publiés par notre équipe indiquant que les fonctionnalités des LT $CD8^+$, chez les souris MT/ret développant un vitiligo, sont augmentées (Lengagne 2004). L'ensemble de ces études établit le vitiligo comme étant le reflet d'une bonne réponse immunitaire anti-tumorale. Dans notre modèle, les souris développent spontanément un vitiligo associé au mélanome dans environ 30% des cas (Article 2 : Figure 1A). De plus, ce vitiligo est corrélé à un retard et une baisse d'incidence des métastases cutanées (Article 2 : Figure 1A).

La génération de souris MT/retCD3ε^{KO} dépourvues de LT nous a permis d'observer que ces animaux développent dans une proportion plus importante que les animaux immuno-compétents, (Article 2 : Figure 3A) un vitiligo corrélé à un meilleur pronostic (Article 1 : Figure 6). De plus, chez les souris spécifiquement déplétées en LT $CD8^+$, l'incidence de vitiligo et de métastases cutanées est inchangée par rapport aux souris non traitées (Article 2 : Figure S3A). Nos résultats montrent également qu'il n'y a pas d'auto-anticorps spécifiques des mélanocytes dans le sérum des souris MT/ret CD3ε^{KO} (Article 2 : Figure 3C). L'ensemble de ces données suggère qu'une population de l'immunité innée serait responsable du vitiligo et du contrôle de la dissémination tumorale dans le modèle MT/ret. Beaucoup d'études ont soulevé la question d'autres mécanismes responsables de l'apparition d'un vitiligo associé ou pas au mélanome. En effet, une infiltration de NK (Durham-Pierre 1995), de mastocytes (Aroni 2010) ou encore d'éosinophiles (Anbar 2009) a été mise en évidence dans les lésions dépigmentées. De plus, il a été démontré une augmentation de l'angiogenèse (Aroni 2010) et du taux sérique des cytokines IL-6, IL-8, GM-CSF, TNFα (Yu 1997) et MIF (Serarslan 2009) chez les patients atteints de vitiligo. L'ensemble de ces résultats suggèrent que le vitiligo pourrait également provenir de l'immunité innée.

2) Les monocytes Ly-6C^{fort} : un nouvel effecteur anti-tumoral important

Nos résultats permettent d'identifier les monocytes Ly-6C^{fort} ainsi que les DCs inflammatoires comme étant des effecteurs anti-tumoraux importants dans le contrôle de la dissémination tumorale (Article 2 : Figure 4). Ces deux types cellulaires n'avaient, jusqu'à présent, pas été identifiés comme effecteurs anti-tumoraux.

2.1 Généralités sur les monocytes Ly-6C^{fort} et les DCs inflammatoires

Il existe deux sous-populations de monocytes définis, à l'origine, en fonction de leur expression de Gr-1, puis de Ly-6C (Geissmann et Jung 2003). Les monocytes Ly-6C^{-} patrouillent dans les vaisseaux sanguins et sont rapidement recrutés dans les tissus lors d'une infection (Auffray 2007). Les monocytes Ly-6C^{fort} expriment des niveaux importants de CCR2 et CD62L ainsi que de faibles niveaux de CX3CR1 et sont les équivalents murins des monocytes CD14^{+} humains (Geissmann et Jung 2003). Ils sont recrutés dans les tissus et les ganglions inflammés et produisent de grandes quantités de TNFα et d'IL-1, ce qui leurs a valu d'être appelés monocytes inflammatoires (Sunderkötter 2004). Les monocytes Ly-6C^{fort} sont capables de renouveler les macrophages et les DCs résidents du poumon (Landsman 2007) et de la peau (Ginhoux 2006). Ils peuvent également se différencier en DCs inflammatoires productrices de TNFα (Serbina 2008), capables de capturer les antigènes dans les tissus et de migrer dans les organes lymphoïdes (Serbina 2003). Bien que l'origine des DCs inflammatoires est longtemps restée sujette à débat, l'identification récente du facteur de transcription Ztbt46 spécifique des DCs conventionnelles et de leur lignage a permis de démontrer que les DCs inflammatoires n'expriment pas ce facteur de transcription et ne sont donc pas issues des DCs conventionnelles (Satpathy 2012 et Meredith 2012). Dans nos expériences, nous avons défini les monocytes Ly-6C^{fort} comme étant CD11b^{+}CD11c^{-}NK1.1^{-} Ly-6G^{-}Ly-6C^{fort} et les DCs inflammatoires CD11b^{+}CD11c^{fort}Ly-6C^{fort} (Article 2 : Figure S1).

2.2 Les monocytes Ly-6C^{fort} et les DCs inflammatoires dans les infections

Les monocytes Ly-6C^{fort} ont principalement été étudiés dans les infections bactériennes par *Listeria Monocytogenes* (*Lm*). A la suite de cette infection, les monocytes Ly-6C^{fort} sortent massivement de la moelle osseuse par un mécanisme dépendant de CCR2 (Tsou 2007), sont recrutés dans les tissus où ils se différencient en DCs inflammatoires. Les DCs inflammatoires

sécrètent alors des quantités importantes de TNFα, NO et radicaux libres qui sont capables de lyser les bactéries. Les souris déficientes en CCR2 présentent une réduction drastique du nombre de monocytes Ly-6C^{fort} et de DCs inflammatoires et combattent mal l'infection par *Lm* (Serbina 2003). Les mécanismes de recrutement des monocytes Ly-6C^{fort} ne sont pas encore complètement élucidés, mais une étude suggère que leur recrutement dans la peau se fait via l'axe CCR6/CCL20 (Le Borgne 2006). Les monocytes Ly-6C^{fort} et les DCs inflammatoires sont également importants dans le contrôle d'autres infections bactériennes telles que *Brucella melitensis* (Copin 2007) ou encore les infections parasitaires par *Toxoplasma gondii* (Robben 2005) et *Trypanosoma brucei* (Bosschaerts 2010). De plus, ils sont impliqués dans la réparation des dommages de la moelle épinière (Shechter 2009).

2.3 Les monocytes Ly-6C^{fort} dans le contexte tumoral

Il est remarquable de constater qu'aucun rôle anti-tumoral n'avait jusqu'ici été attribué aux monocytes Ly-6C^{fort}. Ceci peut être principalement du à deux constatations. D'une part, les monocytes Ly-6C^{fort} sont phénotypiquement indissociables des M-MDSCs et la majorité des études ne se focalise que sur ces dernières. D'un autre côté, l'inflammation dans le contexte tumoral est majoritairement vue comme délétère de par les propriétés initiatrices de tumeur de l'inflammation chronique. Nos résultats mettent en avant qu'une population myéloïde du système immunitaire semble avoir des propriétés anti-tumorales importantes. Nous avons tout d'abord identifié cette population comme exprimant le Gr-1 (Article 2 : Figure 4A et B), ce qui regroupe les monocytes Ly-6C^{fort}, les granulocytes et une partie des LT. Ce traitement a été réalisé à la fois chez les souris MT/ret et MT/ret CD3ε^{KO} afin d'exclure le rôle éventuel des LT. De ces expériences, nous concluons que les cellules responsables du contrôle de la dissémination tumorale sont, soit les granulocytes, soit les monocytes Ly-6C^{fort}. L'identification des monocytes Ly-6C^{fort} comme responsables du contrôle de la dissémination tumorale et du vitiligo provient de la déplétion des granulocytes en ciblant spécifiquement Ly-6G. Les souris déplétées en granulocytes montrent une incidence de vitiligo augmentée corrélée à un meilleur contrôle de la dissémination métastatique (Article 2 : Figure 4C). La première conclusion que nous tirons de cette expérience est que les granulocytes ne semblent pas responsables du vitiligo et du contrôle métastatique. Ensuite, la protection observée chez les animaux déplétés en granulocytes pourrait s'expliquer de plusieurs façons. Ils pourraient tout d'abord posséder des capacités pro-tumorales. La déplétion des granulocytes pourrait, de plus, rendre disponible une quantité plus importante de chimiokines, telles que CCL2,

impliquées dans la sortie de la moelle osseuse des monocytes Ly-6C^{fort} et des granulocytes. Enfin leur déplétion pourrait provoquer une activation des précurseurs myéloïdes de la moelle osseuse afin de compenser le manque de granulocytes, conduisant à une génération plus importante de monocytes Ly-6C^{fort} et de DCs inflammatoires. L'augmentation du nombre de monocytes Ly-6C^{fort} et de DCs inflammatoires dans le sang et la rate des souris traitées par anti-Ly-6G (Article 2 : Figure 4D) semble confirmer les deux dernières hypothèses, ce qui vient également renforcer l'idée d'un rôle anti-tumoral de ces cellules. Afin de confirmer ceci, nous avons décidé de provoquer une augmentation du nombre de monocytes Ly-6C^{fort} chez les souris MT/ret grâce à l'injection de la chimiokine CCL2 (Article 2 : Figure S4) tel que cela est décrit dans la littérature (Combadière 2008). Ce traitement entraîne une augmentation importante de l'incidence de vitiligo corrélée à un meilleur contrôle de la dissémination métastatique (Article 2 : Figure 4E), confirmant l'implication des monocytes Ly-6C^{fort}. En outre, les monocytes Ly-6C^{fort} et les DCs inflammatoires sont retrouvés en nombre augmenté dans la peau des souris développant un vitiligo (Article 2 : Figure 5A, B et C). L'ensemble de ces données démontre que les monocytes Ly-6C^{fort} et les DCs inflammatoires sont des effecteurs anti-tumoraux efficaces. Dans le modèle MT/ret, ils peuvent être considérés comme les acteurs majeurs de la protection contre la dissémination tumorale puisque leur déplétion conduit à une mortalité importante des souris et à une dissémination métastatique cutanée et distante beaucoup plus forte que chez les animaux dépourvus de LT ou encore déplétés en LT $CD8^+$ ou lymphocytes NK (Article 2 : Figure 4A et B).

3) La balance entre réponses anti-tumorales et régulatrices : Un phénomène omniprésent dans le contexte tumoral

De nombreux exemples de cellules immunes pouvant être anti-tumorales ou pro-tumorales en fonction de l'environnement existent dans le système immunitaire. Beaucoup d'entre eux semblent avoir un rôle important dans la réponse anti-tumorale.

3.1 Polarisation : Cellules anti-tumorales contre cellules régulatrices

La majorité des cellules du système immunitaire possède une contrepartie régulatrice. L'exemple le mieux caractérisé à l'heure actuelle est, sans nul doute, celui des LT conventionnels et Tregs. Ces dernières années, beaucoup d'études se sont penchées sur la contrepartie régulatrice d'autres cellules du système immunitaire et ont évalué leur impact

dans le contexte tumoral. Comme nous l'avons vu précédemment, les macrophages possèdent également ces deux fonctionnalités (Mantovani 2002). En accord avec ces résultats, nous avons montré que les TAMs peuvent être plus ou moins polarisés en M1 ou M2 en fonction des conditions environnementales (Article 1). En 2009, il a été mis en évidence une différence de polarisation entre granulocytes neutrophiles N1, présentant une activité anti-tumorale importante, et N2, pro-tumoraux et assimilables aux PMN-MDSCs (Fridlender 2009). Le même type de polarisation a été observé et impliqué dans la suppression des réponses anti-tumorales pour les NK (Zhang 2006) et les LB (DiLillo 2009).

Nos résultats mettent en avant que les monocytes Ly-6C^{fort} sont des effecteurs anti-tumoraux très efficaces (Article 2). Or, ces cellules étant phénotypiquement indissociables des M-MDSCs, nous émettons l'hypothèse qu'il s'agit du même type cellulaire, mais à des états de polarisation différents. La majorité des études sur les cellules myéloïdes dans le contexte tumoral se fait soit dans des modèles spontanés, mais à des stades tardifs lorsque les tumeurs sont établies, soit dans des modèles transplantés qui peuvent également être assimilés à des tumeurs établies. Le fait que nous étudions des stades très précoces du développement tumoral permet d'expliquer que nous ayons pu mettre en évidence cette différence de polarisation. Dans le modèle MT/ret, l'augmentation de MDSCs dans la rate est beaucoup moins forte que celle observée dans la majorité des modèles transplantés (Article 1 : Figure 1C). De plus, les MDSCs ne représentent qu'une faible proportion des cellules immunitaires infiltrant les métastases cutanées (Article 2 : Figure 1B). Ces deux derniers point démontrent que, dans le modèle MT/ret, les MDSCs ne semblent pas être des acteurs majeurs de l'immunosuppression que ce soit au niveau systémique ou dans les métastases cutanées, ce qui pourrait également expliquer pourquoi nous avons mis en avant les monocytes Ly-6C^{fort} comme anti-tumoraux. Alors que dans notre modèle, l'injection de CCL2 dès la naissance provoque une augmentation de la proportion de vitiligo associé à une baisse de l'incidence de métastases cutanées (Article 2 : Figure 4E), dans le modèle PyMT, sa neutralisation améliore le pronostic des animaux (Qian 2011). Cette étude, qui parait aller à l'encontre de nos résultats, vient en fait les renforcer. En effet, il est intéressant de remarquer que ces travaux ont été faits chez des animaux présentant des tumeurs mammaires et/ou des métastases pulmonaires établies. CCL2 joue alors un rôle important dans le recrutement des monocytes Ly-6C^{fort} présentant des capacités pro-tumorales. Le stade d'avancement tumoral semble donc jouer un rôle important dans les capacités pro- ou anti-tumorales des monocytes Ly-6C^{fort}, suggérant ainsi l'existence de différents états de polarisation de ces cellules.

3.2 Comment les effecteurs anti-tumoraux du système immunitaire acquièrent-ils des fonctions suppressives ?

De nombreuses études démontrent d'ores et déjà que la balance entre cellules effectrices et contreparties régulatrices est influencée par l'environnement. Nos résultats montrent que l'absence de LT influence les TAMs du micro-environnement tumoral qui sécrètent alors plus d'IL-12 (Article 1 : Figure 5B), suppriment moins la réponse médiée par les LT $CD8^+$ (Article 1 : Figure 5C) et inhibent la croissance tumorale (Article 1 : Figure 6). Les LT semblent donc jouer un rôle dans la polarisation des TAMs d'un type M1 vers un type M2. Ces constatations sont renforcées par une étude dans le modèle PyMT de cancer du sein où il a été montré le rôle des Th2 producteurs d'IL-4 dans cette polarisation vers un phénotype M2 (DeNardo 2009). Les LB peuvent également participer à la polarisation des macrophages vers un phénotype pro-tumoral (de Visser 2005). En effet, les LB diminuent la production de TNFα, IL-1β et CCL3 par les TAMs et augmentent leur sécrétion d'IL-10 (Wong 2010). En outre, les MDSCs diminuent la production d'IL-12 par les TAMs (Sinha 2007). Finalement, les cellules tumorales amènent également les macrophages à produire des niveaux accrus de molécules immunosuppressives (Hagemann 2006).

C'est également l'environnement qui dicte la polarisation en cellules régulatrices pour les autres cellules de l'immunité mises en jeu lors du développement tumoral. Ainsi, la polarisation des neutrophiles de N1 vers N2 est influencée par le TGFβ (Fridlender 2009). La polarisation des NK est, quand à elle, influencée par l'IL-4, l'IL-10 et l'IL-13 (Zhang 2006). Nous émettons l'hypothèse qu'il en soit de même pour les monocytes Ly-$6C^{fort}$ et les M-MDSCs par des mécanismes qui restent à déterminer.

4) Perspectives

4.1 Concernant les monocytes Ly-6C^{fort} et les DCs inflammatoires

- Par quels mécanismes les monocytes Ly-6C^{fort} et les DCs inflammatoires agissent-ils ?

Les monocytes Ly-6C^{fort} et DCs inflammatoires sont capables de produire de grandes quantités de TNFα, de NO et d'espèces réactives oxygénées (ROS) (Auffray 2009). Le TNFα a été identifié dans un premier temps par son activité nécrosante sur les tumeurs (Carswell 1975). Depuis, son impact dans le contexte tumoral reste controversé en raison de son implication dans l'inflammation chronique. De la même façon que pour le TNFα, l'activité anti-tumorale du NO (Sonveaux 2009) et des ROS (Liou 2010) reste sujette à débat. En effet, des concentrations de NO et de ROS modérées peuvent promouvoir l'inflammation chronique, activer des oncogènes, induire l'hypoxie ou encore faciliter l'infiltration des TAMs. Cependant, de fortes concentrations comme celles que les monocytes Ly-6C^{fort} et les DCs inflammatoires sont capables de sécréter peuvent conduire à la lyse des cellules tumorales.

Afin d'évaluer l'impact de ces différentes molécules, nous envisageons tout d'abord une approche *in vitro*. La co-culture des monocytes Ly-6C^{fort} avec une lignée tumorale issue des souris MT/ret pourrait permettre, par l'utilisation d'Ac bloquant anti-TNFα ou d'inhibiteurs du NO et des ROS, de mettre en évidence le mécanisme utilisé par les monocytes Ly-6C^{fort}. Dans un second temps, la co-culture pourrait être réalisée avec des monocytes Ly-6C^{fort} issus de souris déficientes en TNFα (Xu 2007), pour l'enzyme NOS2 (MacMicking 1997) à l'origine de la production de NO dans les monocytes Ly-6C^{fort} et DCs inflammatoires ou encore UCP2 (Kuhla 2010) qui présente une production de ROS très importante. Une approche *in vivo* pourra également être mise en place par la neutralisation du TNFα grâce à l'Enbrel (récepteur au TNFa soluble entrant en compétition avec le récepteur membranaire endogène) (Grounds 2005) ou encore croisement des souris MT/ret avec déficientes pour le TNFα, NOS2 ou UCP2.

- Comment les monocytes Ly-6C^{fort} sont recrutés dans la peau ?

Dans le modèle MT/ret, les cellules tumorales disséminent rapidement et pourraient sécréter des chimiokines capables de recruter les monocytes Ly-6C^{fort}. Il a été mis en évidence que CCL2 est important pour le recrutement des monocytes Ly-6C^{fort} dans les tumeurs (Qian

2011). De plus, une étude suggère que leur recrutement dans la peau se fait via l'axe CCR6/CCL20 (Le Borgne 2006). L'analyse de la sécrétion de chimiokines par les cellules tumorales disséminées pourrait nous permettre d'évaluer l'impact de celles qui sont connues, mais également de mettre en évidence le rôle de nouvelles chimiokines.

- Comment l'IL-10 des Tregs influence les monocytes Ly-6C^{fort} et les DCs inflammatoires ?

Nos résultats suggèrent que les Tregs inhibent les monocytes Ly-6C^{fort} et les DCs inflammatoires par un mécanisme dépendant de l'IL-10 (Article 2 : Figure 6). Au cours d'une infection parasitaire, il a été démontré que l'IL-10 peut jouer à plusieurs niveaux. En inhibant la sécrétion de CCL2, l'IL-10 empêche la sortie des monocytes Ly-6C^{fort} de la moelle osseuse et peut-être leur recrutement. L'IL-10 inhibe également la différenciation des monocytes Ly-6C^{fort} en DCs inflammatoires (Bosschaerts 2010). L'analyse de l'infiltrat immunitaire de la peau des souris traitées par anti-IL-10R pourrait nous permettre de mettre en évidence à quel niveau l'IL-10 intervient.

4.2 Quels signaux dictent la polarisation des monocytes Ly-6C^{fort} en M-MDSCs ?

Il existe plusieurs études qui se sont penchées sur la polarisation des cellules myéloïdes effectrices en régulatrices, mais aucune ne s'est encore intéressée aux monocytes Ly-6C^{fort}. Comme nous l'avons vu précédemment, la polarisation des macrophages d'un type M1 vers M2 peut-être due aux LT (Article 1), plus précisément aux Th2 (DeNardo 2009). De même, le TGFβ dicte la polarisation des N1 en N2 (Fridlender 2009). La source de cette cytokine n'a pas été identifiée. Il pourrait s'agir de la tumeur, mais également des Tregs. Il a été montré chez l'homme que les Tregs poussent les monocytes inflammatoires à se différencier en cellules suppressives en inhibant leur réactivité au lipopolysaccharide (LPS), et en augmentant leur expression de CD206 et de CD163 (Tiemessen 2007). Nous pourrions évaluer si les Tregs sont responsables de la polarisation des monocytes Ly-6C^{fort} en M-MDSCs et déterminer quels mécanismes sont mis en jeu au cour de ce processus.

4.3 Quels facteurs influencent la dominance entre réponse anti-tumorale et réponse régulatrice ?

Nos résultats mettent en évidence une balance dans la dominance entre réponse anti-tumorale et régulatrice. Il semble que si les Tregs prennent l'ascendant sur les monocytes Ly-6C^{fort}, la réponse suppressive sera dominante conduisant à plus de métastases. En revanche, si ce sont les monocytes Ly-6C^{fort} qui sont dominants, la réponse anti-tumorale se mettra en place conduisant à un contrôle des métastases et au développement d'un vitiligo. Un tel phénomène a déjà été mis en évidence entre Tregs et LT conventionnels dans un modèle de mélanome transplanté (Darrasse-Jèze 2009). Les auteurs postulent que cette balance repose sur le statut naïf ou mémoire des Tregs et des LT conventionnels. Dans le modèle MT/ret, aucune différence phénotypique des Tregs entre les souris développant ou non un vitiligo n'a été mise en évidence (données non montrées), notamment concernant le CD44. Le statut mémoire des Tregs ne semble donc pas expliquer leur dominance. La tumeur pourrait influencer cette balance de dominance par les molécules qu'elle sécrète. Ainsi, l'inflammation chronique accompagnée d'un recrutement et/ou d'une induction concomitante de Tregs et du maintien de l'immaturité des progéniteurs myéloïdes pourrait amener à l'induction d'un micro-environnement suppresseur. En revanche, des conditions inflammatoires aigües pourraient suffire à passer outre l'inhibition des Tregs. L'analyse du profil de sécrétion des tumeurs primaires, mais également des cellules tumorales disséminées, pourrait nous permettre de mettre en avant des différences expliquant les rapports de dominance entre Tregs et monocytes Ly-6C^{fort}.

CONCLUSION

A. Bilan scientifique

La figure 16 schématise le bilan scientifique et les perspectives à court terme de ce projet. Nos résultats suggèrent pour la première fois un rôle des monocytes Ly-6C^{fort} dans le contrôle tumoral via la lyse de ces dernières ou encore le maintien de la dormance des cellules tumorales disséminées. En conséquence, nous proposons de les ajouter à la liste des acteurs immunitaires directement impliqués lors des phases d'élimination et d'équilibre de la théorie de l'immuno-éditing. De plus, nous mettons en évidence leur inhibition par les Tregs, ce qui n'avait pas non plus été décrit précédemment. Ceci nous pousse à suggérer de prendre plus en compte l'impact des Tregs sur d'autres populations immunitaires que les LT dans le contexte tumoral.

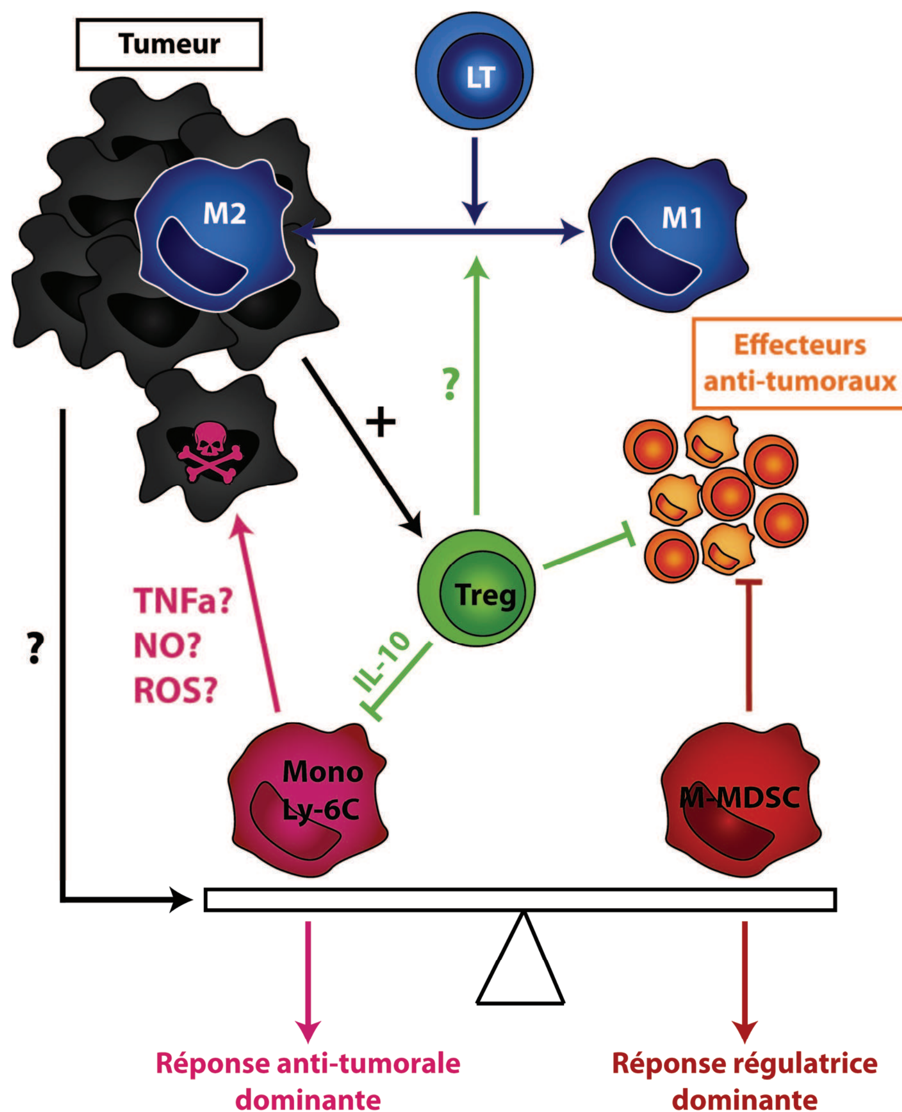

Figure 16 : Schéma récapitulatif des travaux réalisés et des perspectives à court terme

B. Bilan personnel

Beaucoup de doctorants s'insurgent contre l'utilisation du terme « étudiant » pour les qualifier. Au début de ma thèse, je faisais partie de ceux là, tant il est vrai qu'il est difficile de trouver sa place dans ce statut étrange. Je comprends maintenant en quoi « étudiant » est un terme particulièrement adapté. Lorsque l'on débute une thèse, la motivation principale est bien souvent de mener un projet, d'apporter de nouveaux résultats. En effet, le quotidien est surtout composé de techniques, de mise à profit de ses connaissances et d'en acquérir de nouvelles. Après un master, ces aspects ne posent plus vraiment de problème, il est alors facile de considérer sa formation comme terminée. On ne mesure pas forcément que l'apport principal d'une thèse ne se situe, selon moi, ni au niveau technique, ni au niveau des connaissances.

J'ai effectué mes travaux de thèse en quatre ans, cinq si l'on compte mon année de master. Ce n'est, je pense, qu'à la fin de ma troisième année que j'ai pris conscience de ce que j'ai réellement appris pendant cette période. Apprendre à mener un projet de thèse, c'est aussi apprendre à mener tous les autres qui viendront ensuite et à se forger un esprit scientifique pour appréhender les bonnes questions de la bonne façon. Ce processus prend du temps et ne peut, à mon sens, que s'acquérir auprès d'un professeur. C'est sûrement en cela que nous restons bien des étudiants. Dans le climat actuel, où la performance et la compétitivité priment, j'espère que ceux qui me suivront pourront encore prendre le temps de faire ce travail.

Comme je l'ai mentionné à la fin de l'avant-propos, la thèse a beau être un accomplissement personnel important, elle n'en reste pas moins que le début. En cela, je resterai probablement un étudiant toute ma vie.

ANNEXES

Article 3 :

Foxp3-independent loss of regulatory CD4$^+$ T-cell suppressive capacities induced by self-deprivation

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Arnaud Delpoux, Maud Poitrasson-Rivière, Armelle Le Campion, Arnaud Pommier, Philippe Yakonowsky, Sébastien Jacques, Frank Letourneur, Clotilde Randriamampita, Bruno Lucas et Cédric Auffray

A la périphérie, l'expression de Foxp3 est considérée comme suffisante au maintient des capacités suppressives des lymphocytes T CD4$^+$ régulateurs. En effet, chez des chimères hématopoïétiques où l'expression du CMH de classe II est restreinte à l'épithélium thymique, les lymphocytes T CD4$^+$ régulateurs périphériques perdent leurs capacités suppressives. De plus, les lymphocytes T CD4$^+$ régulateurs récupérés 5 jours après transfert dans un hôte n'exprimant pas les molécules de classe II du CMH ne sont pas capables d'inhiber la prolifération des lymphocytes T CD4$^+$ conventionnels à la fois *in vitro* et *in vivo*. La perturbation des interactions entre molécules du CMH de classe II et le récepteur des lymphocytes T conduit rapidement à l'altération du phénotype, de la capacité de réponse aux stimuli, de la production d'IL-10 et de la signature transcriptionnelle des lymphocytes T CD4$^+$ régulateurs. De façon intéressante, la privation de ces interactions n'affecte pas l'expression de Foxp3, indiquant que la reconnaissance du soi par les lymphocytes T CD4$^+$ régulateurs induit une signature transcriptionnelle et des caractéristiques fonctionnelles uniques ne reposant pas sur l'expression de Foxp3.

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Foxp3-independent loss of regulatory CD4+ T-cell suppressive capacities induced by self-deprivation

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In the periphery, Foxp3 expression is considered sufficient to maintain natural regulatory CD4+ T-cell suppressive function. In this study, we challenge this model. Indeed, in mouse chimeras in which major histocompatibility complex (MHC) class II expression is restricted to the thymus, peripheral regulatory CD4+ T cells lack suppressive activity. In addition, regulatory CD4+ T cells recovered 5 days after transfer into recipient mice lacking expression of MHC class II molecules (self-deprived) are unable to inhibit the proliferative response of conventional CD4+ T cells both in vitro and in vivo. Disruption of TCR/MHC class II interactions rapidly leads to alterations in the regulatory CD4+ T-cell phenotype, the ability to respond to stimulation and to produce interleukin-10, and the transcriptional signature. Interestingly, self-deprivation does not affect Foxp3 expression indicating that in regulatory CD4+ T cells, self-recognition induces unique transcriptional and functional features that do not rely on Foxp3 expression.

Keywords: Autoreactivity • Foxp3 • Regulatory T cells

Supporting Information available online

Introduction

Naturally occurring regulatory CD4+ T (Treg) cells are important for the maintenance of self-tolerance in the periphery. In particular, they are key players in the prevention of various autoimmune and inflammatory disorders. Natural Treg cells arise in the thymus where T-cell receptor (TCR) signals lead to interleukin (IL)-2 sensitivity enhancement in developing thymocytes. Then, IL-2 signaling induces Foxp3 expression that, in turn, strengthens Treg-cell lineage stability [1, 2]. Foxp3 expression is then important to maintain a distinct transcriptional program required for their suppressive function [3–5].

Recent studies have clearly established that the TCR has an instructive role in inducing commitment of developing thymocytes into the Treg-cell lineage [6, 7]. More precisely, Treg-cell development would be instructed by TCRs with high avidity for self-peptides bound to major histocompatibility complex (MHC) class II molecules (self). Indeed, the proportion of Treg cells is increased when TCR transgenic T cells are forced to see their cognate antigens in the thymus [1, 2, 8, 9]. This model is further supported by the observation that there is a limited amount of

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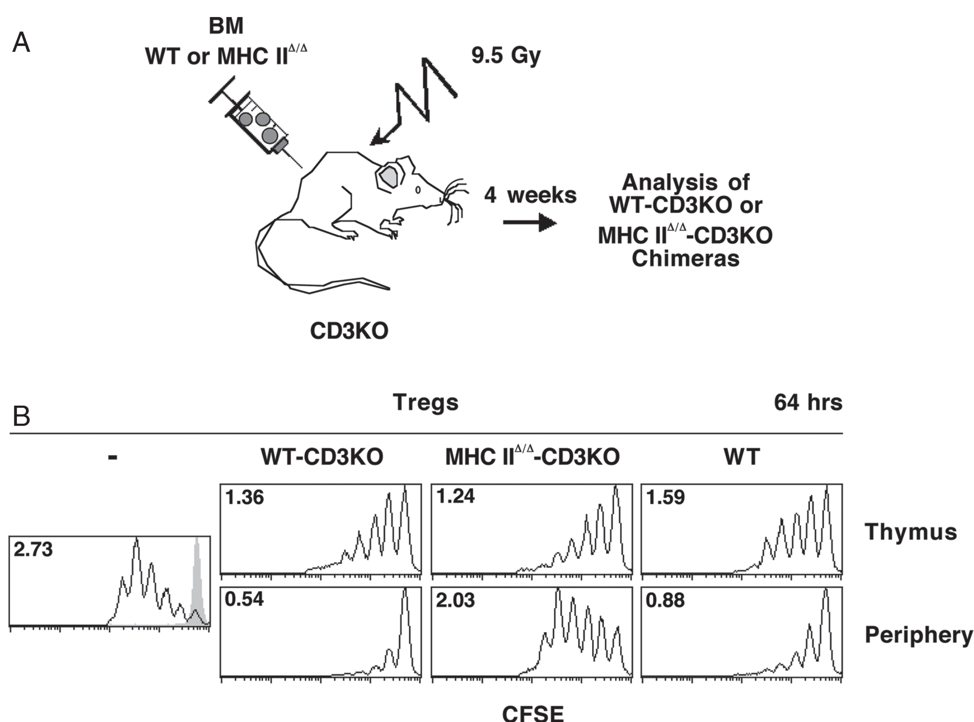

Figure 1. Major histocompatibility complex (MHC) II$^{\Delta/\Delta}$-CD3KO chimeras lack functional Treg cells in the periphery. CD3KO mice were lethally irradiated and their immune system reconstituted with wild-type (WT) (WT-CD3KO chimeras) or MHC II$^{\Delta/\Delta}$ (MHC II$^{\Delta/\Delta}$-CD3KO chimeras) bone marrow (BM) cells. Chimeras were analyzed 28 days after BM-cell transfer. (A) Diagram illustrating the experimental model. (B) CD4$^+$ CD25$^+$ (Treg) cells were purified from the periphery or the thymus of chimeras and WT mice. CD4$^+$ CD25$^-$ (Tconv) cells were purified from lymph nodes of WT mice, labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE), and cultured alone (–) or together with the indicated Treg cells at a 1/1 Treg/Tconv cell ratio. CFSE fluorescence histograms of Tconv cells (CFSE$^+$) are shown 64 h after the beginning of culture. Histograms in the absence of anti-CD3 stimulation are also shown (left, filled histograms). Values correspond to the average number of cell divisions undergone by Tconv cells in response to anti-CD3 stimulation during the culture period. The histograms shown were generated from one experiment but are representative of three individual experiments.

overlap (10–20%) between the TCR sequences expressed within the conventional CD4$^+$ T (Tconv) cell and the Treg-cell repertoire [10]. Interestingly, overlapping is more important when the Treg-cell repertoire was compared with the repertoire of pathogenic autoreactive effector T cells [11].

After migrating to the periphery, Treg cells still interact with self. Indeed, based on autoimmune ovarian disease and prostatitis models, Tung and colleagues [12, 13] have determined that continuous interactions with self are required to allow Treg cells to accumulate in the draining lymph-nodes. More recently, Lathrop et al. [14] have confirmed that the Treg-cell TCR repertoire varies by anatomical location in the periphery. Finally, Darrasse-Jèze et al. [15] have obtained interesting data showing that MHC class II-expressing dendritic cells are required to maintain Treg-cell numbers in the periphery. Altogether, these data strongly suggest that natural Treg cells are submitted to continuous interactions with self in the periphery.

In this study, we investigated whether self-deprivation (induced in our experimental settings by the nonexpression of MHC class II molecules in the periphery) would alter peripheral Treg-cell suppressive capacities. By using two complementary mouse experimental models, we show that self-deprived Treg cells lack suppressive activity. Interestingly, self-deprivation does not affect Foxp3 expression indicating that in Treg cells, self-recognition induces unique transcriptional and functional features that do not rely on Foxp3 expression.

Results

Self-deprived Treg cells are not functional

To assess the role of interactions with self in the suppressive capacities of peripheral Treg cells, we first studied mouse bone-marrow (BM) chimeras in which MHC class II expression was restricted to the thymus (Fig. 1A). When MHC class II expressing BM cells were injected, the resulting chimeras (WT-CD3KO chimeras) displayed a pattern of MHC class II molecule expression similar to that of unmanipulated wild-type (WT) mice. In chimeras generated by injecting BM cells that did not express MHC class II molecules into recipient mice (MHC II$^{\Delta/\Delta}$-CD3KO chimeras), MHC class II molecule expression was mostly restricted to radioresistant thymic epithelial cells [16]. In these chimeras, CD4$^+$ T cells, notably Treg cells, were produced in the thymus even more efficiently than in

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WT-CD3KO chimeras, due to the lack of efficient thymic negative selection by BM-derived antigen-presenting cells (APCs) in the thymic medulla [16]. Four weeks after BM cell transfer, the proportion of peripheral $CD4^+$ T cells expressing Foxp3 was more important in WT-CD3KO chimeras than in MHC $II^{\Delta/\Delta}$-CD3KO chimeras (26.3 ± 0.8% in WT-CD3KO chimeras versus 16.8% ± 1.0 in MHC $II^{\Delta/\Delta}$-CD3KO chimeras).

We compared the in vitro suppressive capacities of thymic and peripheral Treg cells from both types of chimeras. Thymic Treg cells from MHC $II^{\Delta/\Delta}$-CD3KO chimeras inhibited Tconv-cell proliferation to a similar extent than thymic Treg cells from WT-CD3KO chimeras or from WT mice (Fig. 1B). By contrast, peripheral Treg cells from MHC $II^{\Delta/\Delta}$-CD3KO chimeras failed to efficiently suppress the proliferative response of Tconv cells to anti-CD3 stimulation. Thus, in MHC $II^{\Delta/\Delta}$-CD3KO chimeras, Treg cells are losing their functional characteristics when migrating from the thymus (MHC II^+) to the periphery (MHC II^-).

Then, we transferred large numbers of total T cells from the periphery of WT mice into $CD3\varepsilon^{-/-}$ recipient mice lacking or not MHC class II molecule expression (CD3KO-MHC $II^{\Delta/\Delta}$ or CD3KO-MHC II^+ recipient mice, respectively; Fig. 2A). Five days later, peripheral Treg cells were purified and their suppressive capacities tested in vitro (Fig. 2B–F).

As soon as 16 h after the beginning of culture, Treg cells from MHC II^+ recipient mice or from WT mice inhibited the expression of late-activation markers by Tconv cells (increase in cell size and expression of CD25; Fig. 2B). By contrast, Treg cells from CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice were not able to suppress the activation of Tconv cells in response to anti-CD3 stimulation in vitro. These results were further confirmed when the suppressive capacities of Treg cells from CD3KO-MHC $II^{\Delta/\Delta}$ and CD3KO-MHC II^+ recipient mice were studied 64 h after the beginning of the coculture (Fig. 2C). Indeed, at that time point, Treg cells from CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice failed to efficiently suppress the proliferative response of Tconv cells to anti-CD3 stimulation. The lack of efficient inhibition of Tconv-cell activation and proliferation by Treg cells from CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice was found to apply for various Treg/Tconv-cell ratios (Fig. 2D). These results were found to be highly statistically significant when data from six independent experiments were pooled (Fig. 2E). In the above experiments, Treg cells were purified as $CD25^+$ $CD4^+$ T cells using magnetic beads (see Materials and methods and Supporting Information Fig. 1A). To exclude the possibility that contaminants in Treg cells purified from CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice (activated $CD25^+$ $Foxp3^-$ $CD4^+$ T cells for example) might explain our results, we repeated the suppression assay using Foxp3-GFP (where GFP is green fluorescent protein) mice and isolating Treg cells by flow cytometry sorting. Using this protocol, whatever the origin of Treg cells, purity was above 99% (Supporting Information Fig. 1B). Highly purified Treg cells from CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice were still not able to suppress the response of Tconv cells in response to anti-CD3 stimulation in vitro (Supporting Information Fig. 1C). Interestingly, whatever their origin and suppressive capacities, Treg cells exhibited a stable phenotype, still expressing Foxp3 and CD25 after 3 days of coculture (Supporting Information Fig. 1D).

We injected large numbers of T cells in order to fill the periphery and to limit lymphopenia-induced proliferation (LIP) of injected T cells. Indeed, we have previously shown that when more than 50 × 10^6 $CD4^+$ T cells were transferred into T-cell deficient recipient mice, their LIP was largely reduced [17]. However, although limited, lymphopenia-induced Treg-cell activation and proliferation still existed to a certain extent in CD3KO-MHC II^+ recipient mice and led to improvement of their suppressive capacities as shown by their enhanced capacities to control Tconv-cell proliferation when compared with Treg cells purified directly from WT mice (Fig. 2D and E, Supporting Information Fig. 1).

The nonfunctionality of Treg cells from CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice was not due to increased cell death of these cells in our culture assay. Indeed, for all Treg/Tconv-cell ratios tested, 16 h after the beginning of culture, only slight differences in the proportion of Treg cells can be observed in the culture wells whatever the origin of Treg cells (Supporting Information Fig. 2).

To test whether this loss of function was definitive or could be reversed, Treg cells from both types of recipient mice were precultured for 2 days in the presence of anti-CD3 and APC, with or without IL-2, before testing their suppressive capacities (Fig. 2F). After preactivating them in the presence or absence of IL-2, Treg cells from CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice were as efficient as Treg cells from CD3KO-MHC II^+ recipient mice in suppressing the response of Tconv cells to anti-CD3 stimulation (Fig. 2F). Moreover, Treg cells from CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice did not recover their suppressive capacities when precultured for 2 days with IL-2 alone (Supporting Information Fig. 3). TCR signaling is thus necessary and sufficient to reverse the loss of suppressive function observed when Treg cells cannot interact with self.

Altogether, our data suggest that continuous interactions with self are required for maintaining the suppressive capacities of Treg cells. However, in CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice, $CD4^+$ Tconv cells are also not receiving any TCR signals, which may result in environmental changes such as diminished in vivo IL-2 levels that may explain our data. To exclude this possibility, CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice were injected daily with 2 × 10^5 IU of IL-2 (Fig. 3). As described previously [18], WT mice injected 3 consecutive days with 2 × 10^5 IU of IL-2 exhibited increased proportion of Treg cells in the periphery and their Treg cells expressed significantly higher CD25 surface amounts but unchanged Foxp3 levels when compared with those of Treg cells from untreated mice (Fig. 3A). Treg cells from IL-2-treated CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice were still completely inefficient in inhibiting CD25 expression on $CD4^+$ Tconv cells 16 h after the beginning of culture, and were only able to inhibit slightly the proliferation of $CD4^+$ Tconv cells 64 h after the beginning of culture (Fig. 3B). In CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice, increased production of IL-2 by injected $CD8^+$ Tconv cells may compensate for

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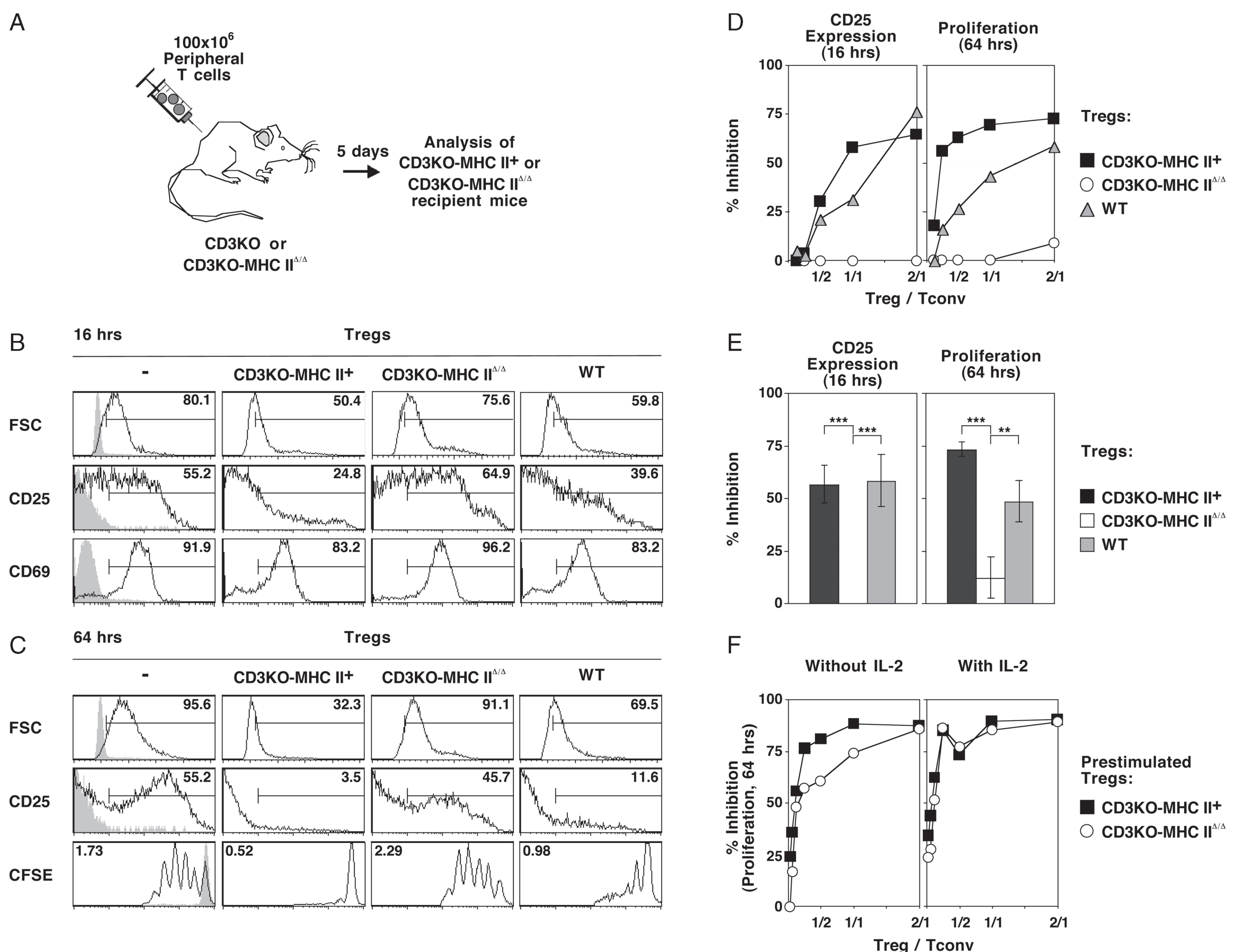

Figure 2. Self-deprived Treg cells are not functional in vitro. T cells purified from the periphery (lymph nodes + spleen) of WT mice were injected into CD3KO mice (CD3KO-MHC II^{+} recipients) or into CD3KO-MHC II$^{\Delta/\Delta}$ mice (CD3KO-MHC II$^{\Delta/\Delta}$ recipients). Five days later, the suppressive capacities of Treg cells purified from the periphery of recipient mice were analyzed. (A) Diagram illustrating the experimental model. (B) Tconv cells were purified from lymph nodes of WT mice, labeled with CFSE, and cultured alone (–) or together with the indicated Treg cells in the presence of soluble anti-CD3 and antigen-presenting cells (APCs). Forward Scatter (FSC), CD25, and CD69 histograms of Tconv cells (CFSE^{+}) are shown 16 h after the beginning of culture for a 1/1 Treg/Tconv ratio. (C) FSC, CD25, and CFSE histograms of Tconv cells (CFSE^{+}) are shown 64 h after the beginning of culture for a 1/1 Treg/Tconv ratio. Histograms in the absence of anti-CD3 stimulation are shown as controls (left, filled histograms). (D) Inhibition indexes (inhibition of Tconv-cell CD25 expression was calculated after a culture period of 16 h; inhibition of Tconv-cell proliferation was estimated 64 h after the initiation of culture) are shown for various Treg/ Tconv-cell ratios (2, 1, 1/2, 1/4, 1/8). (E) The same inhibition indexes are shown for a 1/1 Treg/ Tconv-cell ratio as means ± SEM values of six independent experiments. (F) Treg cells from the indicated mice were stimulated for 2 days with soluble anti-CD3 and APCs in the presence or absence of interleukin (IL)-2. Then, they were tested for their suppressive abilities. Data are shown as inhibition indexes of Tconv-cell proliferation after a culture period of 64 h are shown for various Treg/ Tconv-cell ratios (2, 1, 1/2, 1/4, 1/8, 1/16, 1/32) and are representative of two individual experiments. $^{**}p < 0.01$, $^{***}p < 0.001$, Student's unpaired *t*-test.

"self-depriving" CD4^{+} Tconv cells (Fig. 3C). Moreover, as described previously [19], peripheral CD4^{+} Treg cells from MHC II$^{\Delta/\Delta}$ mice (that are presumably selected on MHC class I molecules) were fully suppressive in vitro in spite of the virtual absence of CD4^{+} Tconv cells in these mice (Fig. 3D). Thus, in contrast with self-deprived Treg cells, CD4^{+} Tconv-cell deprived Treg cells are functional.

Finally, we tested whether self-deprived Treg cells were also lacking suppressive capacities in vivo. CD4^{+} Tconv cells (CD45.1) were injected alone or together with Treg cells (CD45.2) from CD3KO-MHC II^{+}, CD3KO-MHC II$^{\Delta/\Delta}$ recipient mice or WT mice at a 1/1 ratio (Fig. 4A). Two weeks later, absolute numbers of CD4^{+} T cells recovered from the periphery of injected mice were estimated. As previously described [20], with such a ratio, control Treg cells significantly inhibited the initial expansion of CD4^{+} Tconv cells (Fig. 4B). In lines with our in vitro data, Treg cells from CD3KO-MHC II^{+} recipient mice were more efficient than Treg cells purified directly from WT mice in inhibiting

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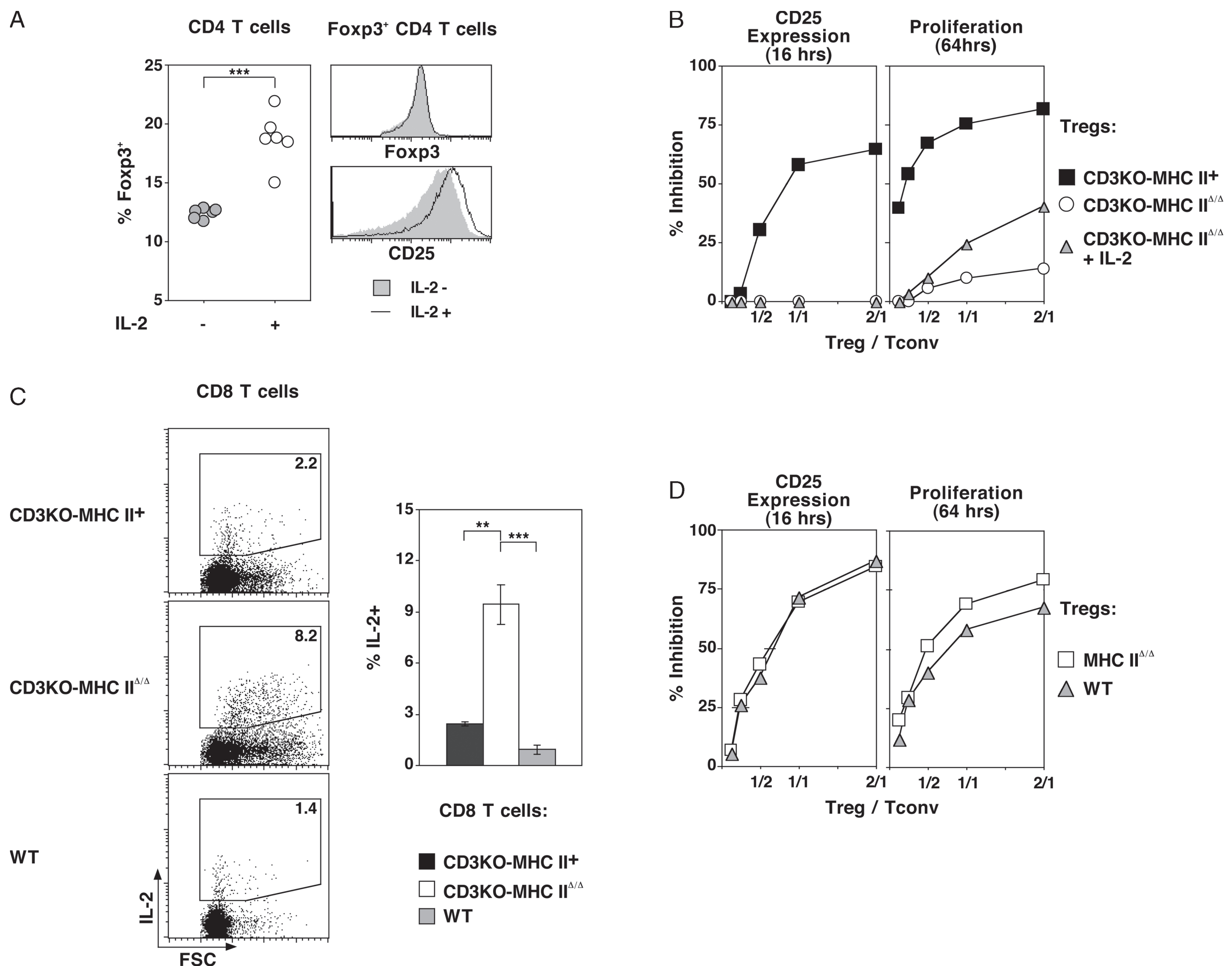

Figure 3. Decreased IL-2 production by Tconv cells does not account for self-deprived Treg-cell loss of suppressive function. (A) WT mice were treated 3 days with daily i.p. injections of 200,000 IU of recombinant human IL-2. Twenty-four h after the last injection, peripheral cells of treated and control mice were recovered and stained for CD4, $CD8_\alpha$, CD25, and Foxp3 expression. The proportion of Foxp3-expressing cells among $CD4^+$ $CD8_\alpha{}^-$ cells was estimated. Foxp3 and CD25 fluorescence histograms of peripheral $CD4^+$ $CD8_\alpha{}^-$ $Foxp3^+$ from IL-2-treated (IL-2+) or -untreated (IL-2 –) WT mice are shown. Each symbol represents a single mouse (left). The histograms shown in the right part were generated from the data for one mouse, but are representative of six mice from two individual experiments. (B) CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice were daily injected or not with IL-2. Treg cells were purified from the periphery of CD3KO-MHC II^+, CD3KO-MHC $II^{\Delta/\Delta}$, and IL-2-treated CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice. Tconv cells were purified from lymph nodes of WT mice, labeled with CFSE, and cultured alone or together with the indicated Treg cells at various Treg/ Tconv-cell ratios, in the presence of soluble anti-CD3 and APCs. Data are shown as inhibition indexes for various Treg/ Tconv-cell ratios (2, 1, 1/2, 1/4, 1/8) and are representative of two independent experiments. (C) Peripheral cells from CD3KO-MHC II^+, CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice and WT mice were cultured for 2 h in the presence of phorbol myristate acetate (PMA), ionomycin, and brefeldin A. They were then stained for the surface expression of CD25, CD4, and $CD8_\alpha$, and finally for intracellular IL-2. Representative FSC/IL-2 fluorescence dot-plots are shown. Percentages of IL-2-producing cells among $CD4^-$ $CD8\alpha^+$ T cells are expressed as means ± SEM values of six mice for two independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$, Student's unpaired *t*-test. (D) Treg cells were purified from the periphery of MHC $II^{\Delta/\Delta}$ and WT mice. Tconv cells were purified from lymph nodes of WT mice, labeled with CFSE, and cultured alone or together with the indicated Treg cells in the presence of soluble anti-CD3 and APCs. Data are shown as inhibition indexes for various Treg/ Tconv-cell ratios (2, 1, 1/2, 1/4, 1/8) and are representative of two independent experiments.

Tconv-cell proliferation in vivo. Interestingly, no significant difference was observed whether $CD4^+$ Tconv cells were injected alone or together with Treg cells from CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice (Fig. 4B). This loss of function did not correlate with decreased numbers of Treg cells (Fig. 4C). Thus, self-deprived Treg cells are not functional both in vitro and in vivo.

Increased response to TCR stimulation of Treg cells deprived of self-recognition in vivo

Treg cells require antigen stimulation via their TCR to exert their suppressive function in vitro [21, 22]. Several studies have demonstrated that TCR contacts with self amplify naive T-cell

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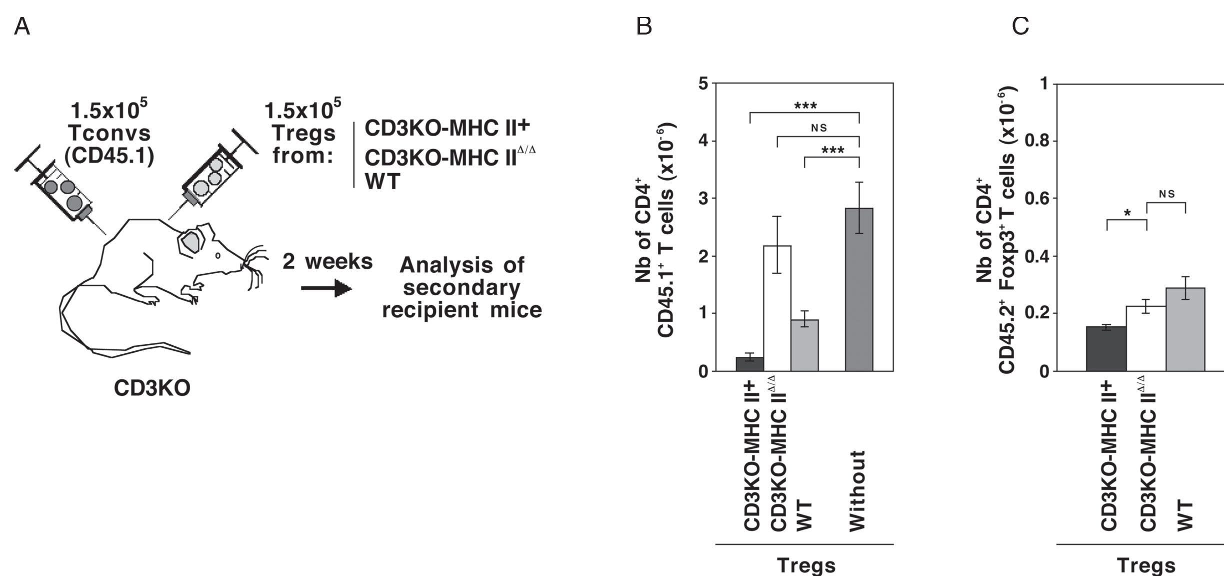

Figure 4. Self-deprived Treg cells are not functional in vivo. Purified lymph node $CD4^+$ T cells from C57BL/6 CD45.1 mice were stained for CD44 and CD25 expression and naive $CD4^+$ Tconv cells were sorted by flow cytometry. Naive $CD4^+$ Tconv cells (CD45.1) were injected alone or together with Treg cells (CD45.2) from CD3KO-MHC II^+, CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice or WT mice. Two weeks later, secondary recipient mice were sacrificed and peripheral cells were stained for CD4, $CD8_\alpha$, T-cell receptor (TCR)-β, CD45.1, CD45.2, and Foxp3 expression. (A) Diagram illustrating the experimental model. (B) The absolute numbers of $CD45.1^+$ $CD4^+$ T cells in the periphery (pooled lymph node and spleen cells) were estimated. (C) The absolute numbers of $CD45.2^+$ $Foxp3^+$ $CD4^+$ T cells in the periphery (pooled lymph node and spleen cells) were estimated. (B, C) Data are expressed as means ± SEM of $n = 10$ mice per group pooled from two individual experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, Student's unpaired *t*-test.

responsiveness to foreign antigens [23]. Thus, Treg cells recovered from CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice may be less responsive to anti-CD3 stimulation than Treg cells from WT or CD3KO-MHC II^+ recipient mice and this may explain their severely impaired suppressive capacities in vitro. To test this hypothesis, we compared the response of Treg cells from CD3KO-MHC II^+, CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice or WT mice to anti-CD3 stimulation (Fig. 5). As already described [24], we found that, after TCR ligation, Treg cells mobilized intracellular calcium stores less efficiently than Tconv cells (Fig. 5A). Surprisingly, Treg cells from CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice displayed markedly increased TCR-induced calcium flux in comparison with Treg cells from CD3KO-MHC II^+ recipient mice or WT mice (Fig. 5A and B). Accordingly, a higher proportion of Treg cells from CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice upregulated CD69 in response to anti-CD3 stimulation and resulting $CD69^+$ cells expressed higher surface amounts of this activation marker when compared with that of Treg cells from CD3KO-MHC II^+ recipient mice or WT mice (Fig. 5C and D). Such an increased expression of CD69 by self-deprived Treg cells in response to stimulation was also observed when Treg cells were cocultured with Tconv cells (at a 1/1 ratio, after 16 h of culture, % $CD69^+$ cells among Treg cells = 43 ± 10 for CD3KO-MHC II^+ recipient mice, 79 ± 6 for CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice, and 61 ± 8 for WT mice).

Finally, 2 days after the beginning of culture, Treg cells from CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice (analysis was restricted to Foxp3-expressing cells) proliferated to a similar extent than Tconv cells (Fig. 5E and F). Although responding to anti-CD3 stimulation more efficiently than did Treg cells from WT mice, Treg cells from CD3KO-MHC II^+ recipient mice cycled significantly less than self-deprived Treg cells (Fig. 5E).

Thus, in contrast with naive T cells, self-deprivation did not lead to a defect in Treg-cell ability to be activated by TCR signals. On the contrary, their response was found to be augmented and comparable with the response of Tconv cells in terms of calcium mobilization, CD69 upregulation and proliferation.

Self-deprivation alters the phenotype of Treg cells and their ability to produce IL-10

We compared the phenotype of self-deprived Treg cells with the phenotype of Treg cells from CD3KO-MHC II^+ recipient mice or WT mice. Numerous molecules are differentially expressed by Treg cells and Tconv cells (Fig. 6A, Supporting Information Fig. 4A). Expression of several of them including CD39, CD103, glucocorticoid-induced tumor necrosis factor receptor (GITR), and CTLA-4 was found to be unaffected by self-deprivation (Supporting Information Fig. 4B). In contrast, self-deprived Treg cells overexpressed PDL1 and CD25 when compared with Treg cells from CD3KO-MHC II^+ recipient mice or WT mice. CD25 upregulation was even more pronounced 10 days after transfer (Fig. 6B). Interestingly, Treg cells from CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice exhibited lower levels of CD73 than Treg cells from CD3KO-MHC II^+ recipient mice or WT mice. CD73 is an ectoenzyme that

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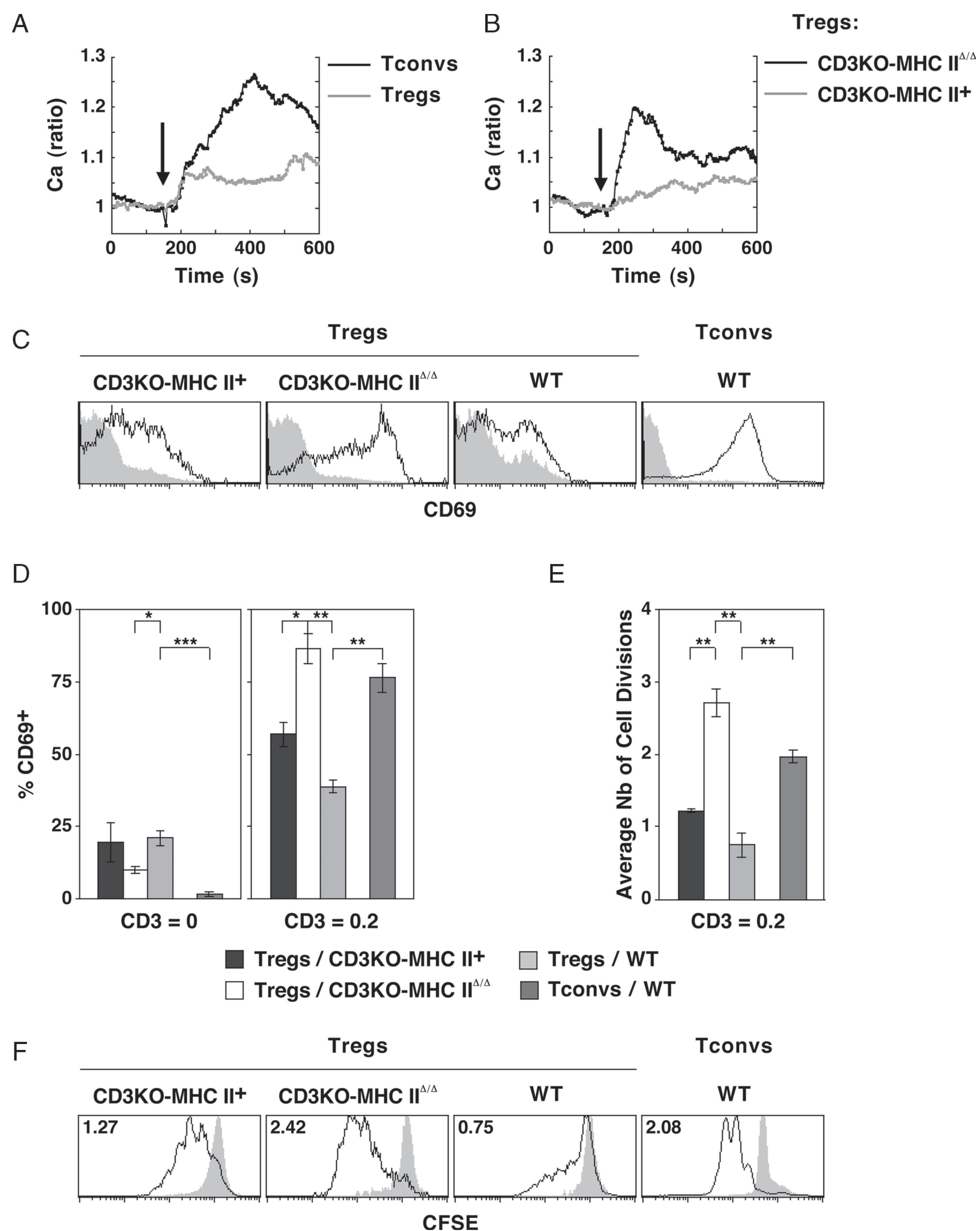

Figure 5. Efficient response of self-deprived Treg cells to TCR stimulation. (A) Example of calcium (Ca) mobilization after anti-CD3 stimulation (+150 s, arrow) in CD4$^+$ CD25$^+$ Treg cells and Tconv cells from WT mice. (B) Example of the average Ca response measured in Treg cells from CD3KO-MHC II$^+$ or CD3KO-MHC II$^{\Delta/\Delta}$ recipient mice. (C) Purified Treg cells and Tconv cells were stimulated in the presence of soluble anti-CD3 and APCs. CD69 fluorescence histograms are shown 16 h after the beginning of culture. Histograms in the absence of anti-CD3 stimulation are shown as controls (filled histograms). (D) The percentage of CD69$^+$ cells among Treg cells or Tconv cells was estimated after 16 h of culture. (E) Purified CFSE-labeled Treg cells and Tconv cells were stimulated in the presence of soluble anti-CD3 and APCs. After 48 h of culture, cells were stained for CD4, CD25, and Foxp3 expression, and the CFSE dilution induced by stimulation was estimated. The average number of cell divisions for each subset was then calculated. For Treg cells, analysis was restricted to Foxp3-expressing cells. (F) CFSE histograms are shown 48 h after the beginning of culture (For Treg cells, analysis was restricted to Foxp3-expressing cells). Histograms in the absence of anti-CD3 stimulation are shown as controls (filled histograms). (A–C, F) The histograms shown were generated from the data for one mouse, but are representative of mice from three individual experiments. (D, E) Results are expressed as means ± SEM values of three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, Student's unpaired t-test.

catalyzes the generation of adenosine and its activity has been associated with Treg-cell suppressive capacities in vitro [25]. CD73 surface amounts on self-deprived Treg cells decreased over time. Indeed, CD73 surface expression on Treg cells was nearly completely lost 10 days after transfer into MHC II deficient mice (Fig. 6B). Other molecules have been shown to be involved in the in vitro Treg-cell suppressive capacities such as galectin-1, IL-35 (EBI3 + IL-12p35), transforming growth factor (TGF)-β, and

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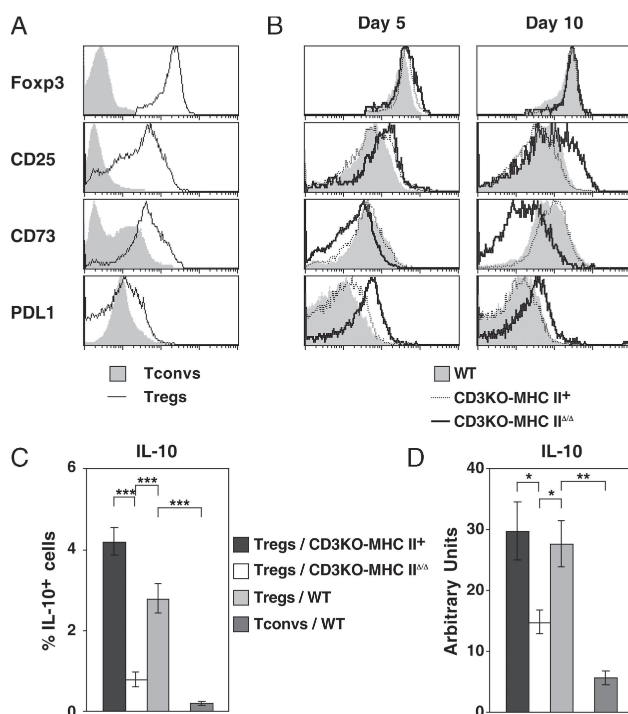

Figure 6. Altered phenotype and cytokine production of self-deprived Treg cells. (A) Foxp3, CD25, CD73, and PDL1 fluorescence histograms of peripheral CD4$^+$ CD8$_\alpha^-$ Foxp3$^+$ (Treg cells; solid line histogram) and CD4$^+$ CD8$_\alpha^-$ Foxp3$^-$ (Tconv cells; filled histogram) cells are shown for WT mice. (B) The expression of these markers is shown for peripheral Treg cells from CD3KO-MHC II$^+$ (dotted line histogram) and CD3KO-MHC II$^{\Delta/\Delta}$ (bold line histogram) recipient mice 5 and 10 days after transfer as well as from WT mice (filled histogram). The histograms shown were generated from the data for one mouse, but are representative of three individual experiments with three mice per group. (C) Peripheral cells were cultured for 2 h in the presence of PMA, ionomycin, and brefeldin A. They were then stained for the surface expression of CD25, CD4, and CD8$_\alpha$, and finally for intracellular IL-10. Percentages of cytokine-producing cells among Treg cells (CD25$^+$) are shown for CD3KO-MHC II$^+$ and CD3KO-MHC II$^{\Delta/\Delta}$ recipient mice 5 days after transfer, and WT mice. Data obtained with Tconv cells (CD25$^-$) purified from the periphery of WT mice are also shown. Data are mean ± SEM values of nine mice pooled from three independent experiments. (D) The transcript levels of IL-10 were analyzed by qRT-PCR in the indicated CD4$^+$ T-cell subsets (5 days after transfer for recipient mice) and are shown as mean ± SEM values of relative expression. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, Student's unpaired *t*-test.

granzyme B [25]. Expression of these molecules was not modified (or augmented) by self-deprivation (Supporting Information Fig. 4C).

It has been shown that decreased Foxp3 expression in the periphery causes defective suppressive function of Treg cells and their conversion into effector cells, which contribute to, rather than inhibit, autoimmune diseases [26, 27]. Here, we found that Foxp3 expression was not affected by self-deprivation. Indeed, Treg cells from CD3KO-MHC II$^+$, CD3KO-MHC II$^{\Delta/\Delta}$ recipient mice as well as from WT mice expressed similar amounts of Foxp3 (Fig. 6B). Thus, self-deprivation leads to multiple alterations in the phenotype of Treg cells with up- or downregulation of key suppressor molecules without affecting Foxp3 expression.

Then, we assessed the ability of Treg cells from CD3KO-MHC II$^+$ and CD3KO-MHC II$^{\Delta/\Delta}$ recipient mice to produce the anti-inflammatory cytokine, IL-10 (Fig. 6C). Interestingly, Treg cells from CD3KO-MHC II$^{\Delta/\Delta}$ recipient mice produced less IL-10 than Treg cells from CD3KO-MHC II$^+$ recipient mice or WT mice. Similar differences were observed at the mRNA level (Fig. 6D). Thus, lack of self-recognition events alters both the phenotype and cytokine production of Treg cells.

Self-deprivation alters Treg-cell transcriptional signature

To further compare self-deprived Treg cells with the fully functional Treg cells from CD3KO-MHC II competent mice, we obtained Affymetrix gene expression profiles from CD4$^+$ CD25$^+$ TCR$^+$ cells directly isolated from the periphery of CD3KO-MHC II$^+$ or CD3KO-MHC II$^{\Delta/\Delta}$ recipient mice by flow cytometry sorting (Fig. 7). A total of 563 Affymetrix targets (representing 547 genes) were significantly differentially expressed (at a 1.5-fold cutoff) between the two types of Treg cells (263 overexpressed and 300 underexpressed in Treg cells from CD3KO-MHC II$^{\Delta/\Delta}$ recipient mice, respectively, Supporting Information Fig. 5A).

Hill et al. have recently published a list of 603 Affymetrix targets (corresponding to 490 genes) defined as representing the peripheral Treg-cell transcriptional signature [28]. Comparison of our gene list with this Treg-cell signature revealed an overlap of 50 genes (14 genes downregulated in Treg cells when compared with Tconv cells; 36 upregulated; Fig. 7A and B). Interestingly, among the 14 genes defined by Hill et al. as downregulated in Treg cells, 11 (79%) were upregulated in Treg cells from CD3KO-MHC II$^{\Delta/\Delta}$ recipient mice. Similarly, 32 of the 36 genes (89%) that are normally upregulated in Treg cells were downregulated in self-deprived Treg cells. These results were confirmed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) (Fig. 7C, Supporting Information Fig. 5B). Indeed, all of the 11 genes normally downregulated in Treg cells when compared with Tconv cells, and found to be more transcribed in CD3KO-MHC II$^{\Delta/\Delta}$ than in CD3KO-MHC II$^+$ recipient mice were upregulated in self-deprived Treg cells nearly to the levels observed in Tconv cells from WT mice. Gene expression profiles were also obtained from peripheral Treg cells of MHC II$^{\Delta/\Delta}$-CD3KO and WT-CD3KO chimeras. Interestingly, microarray analysis of Treg cells from chimeras and adoptive transfers revealed overlapping results (Pearson's correlation: $p < 0.0001$; Supporting Information Fig. 5C). In particular, the expression of several genes of the Treg-cell signature (such as pde3b, atp8b4, and klrd1) was affected in both experimental models by self-deprivation. Thus, part of the Treg-cell transcriptional signature is abolished by self-deprivation.

The expression of approximately one-third of the 490 genes of the Treg-cell signature correlates with Foxp3 expression or exhibits the strong negative correlation expected for genes repressed

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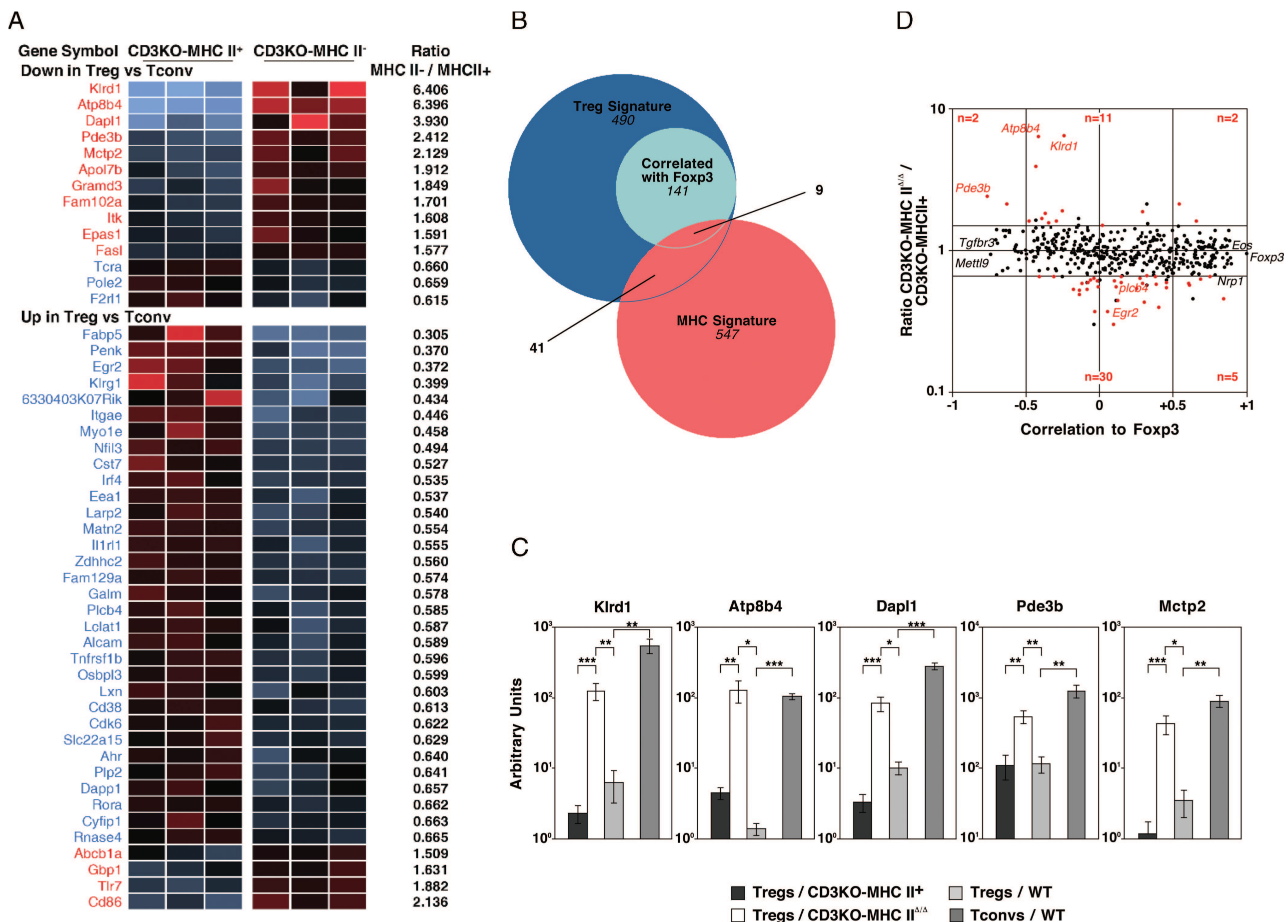

Figure 7. Gene expression profiling of self-deprived Treg cells. Treg cells were isolated by fluorescence-activated cell sorter (FACS) from the periphery of CD3KO-MHC II^{+} and CD3KO-MHC II$^{\Delta/\Delta}$ recipient mice, 5 days after transfer. Total mRNA was isolated, amplified, biotin labeled, purified, and hybridized to Affymetrix mouse genome arrays. (A) Expression pattern of Affymetrix targets differentially expressed (± 1.5-fold change) between Treg cells from CD3KO-MHC II$^{\Delta/\Delta}$ and CD3KO-MHC II^{+} recipient mice that have been identified as genes of the Treg-cell transcriptional signature by Hill et al. [28]. The Z-score normalized induction (red) or repression (blue) is shown for each Affymetrix target. (B) Overlapping between Foxp3-dependent genes of the Treg-cell signature and the self-deprived Treg-cell signature. (C) The transcript levels of a panel of the genes presented in (A) were analyzed by qRT-PCR in the indicated CD4^{+} T-cell subsets. Mean ± SEM values of relative expression are shown for indicated genes and were calculated from six mice per group pooled from two independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, Student's unpaired *t*-test. (D) Scatter plot of the changes induced by self-deprivation versus the correlation values to Foxp3 for genes of the common Treg-cell signature. Red dots correspond to genes significantly differentially expressed between Treg cells from CD3KO-MHC II$^{\Delta/\Delta}$ and CD3KO-MHC II^{+} recipient mice.

by Foxp3 (Pearson's correlation coefficient > 0.5 or < –0.5 as calculated in [28]). Interestingly, the transcription of only nine of these 141 genes was affected by self-deprivation (Fig. 7B). More precisely, we did not find any clear correlation between the Treg-cell genes affected by self-deprivation and Foxp3 (Fig. 7D). Similar results were obtained in the BM chimeras experimental model (Supporting Information Fig. 5D). Accordingly, the expression of several genes of the Treg-cell signature known to be strongly correlated with Foxp3 (such as Foxp3 itself) was unaffected by self-deprivation (Fig. 7D, Supporting Information Fig. 5D). Thus, continuous interactions with self induce unique transcriptional and functional signatures in Treg cells that do not rely on Foxp3 expression.

Discussion

In the periphery, Foxp3 expression is required to maintain Treg-cell suppressive capacities. Indeed, decreased Foxp3 expression in the periphery causes defective suppressive function of Treg cells and their conversion into effector cells, which contribute to, rather than inhibit, autoimmune diseases [26, 27, 29]. Recent data suggest strongly that IL-2 may play a role in the maintenance of peripheral Treg-cell suppressive capacities by promoting sustained expression of Foxp3 [18, 30, 31]. In the present article, we show that continuous interactions with self are required for maintaining Treg-cell suppressive function in the periphery. Indeed, peripheral Treg cells from mouse chimeras in which MHC

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class II expression is restricted to the thymus lack suppressive activity. Similarly, Treg cells recovered 5 days after transfer into recipient mice lacking expression of MHC class II molecules are unable to inhibit the proliferative response of Tconv cells to anti-CD3 stimulation. By contrast to IL-2, TCR-dependent stabilization of peripheral Treg-cell suppressive function does not appear to rely on Foxp3 expression. Indeed, we found that Foxp3 expression is unaffected by self-deprivation. Moreover, among the genes of the Treg-cell signature in which expression is altered by self-deprivation, very few are known to be directly or indirectly controlled by Foxp3. In many aspects, self-deprived Treg cells share more functional and phenotypic similarities with Tconv cells than non-"self-deprived" Treg cells do. Indeed, several genes of the Treg-cell signature are similarly expressed by Tconv cells and self-deprived Treg cells. Moreover, self-deprived Treg cells mobilize intracellular calcium stores and upregulate CD69 as efficiently as Tconv cells do in response to anti-CD3 stimulation. Finally, the ability of self-deprived Treg cells to produce the anti-inflammatory cytokine, IL-10, is similar to that observed for Tconv cells. Thus, continuous interactions with self induce unique transcriptional and functional signatures in Treg cells that do not rely on Foxp3 expression.

Altogether, our data suggest strongly that Foxp3 expression, although necessary, is not sufficient to maintain the suppressive function of peripheral Treg cells. Such a conclusion may seem contradictory to previous data showing that ectopic Foxp3 expression is sufficient to induce suppressive capacities in Tconv cells [3–5]. In two of these studies, naive T cells were stimulated with anti-CD3 and IL-2 and transduced with a retrovirus expressing Foxp3 [3, 5]. Infected cells were thus receiving strong TCR signals while acquiring Foxp3 expression. In the last study, Khattri et al. used Foxp3 transgenic mice [4]. All T cells from these mice expressed Foxp3 and showed suppressor activity in vitro. Interestingly, although T cells from Foxp3 transgenic mice expressed higher amounts of Foxp3 than WT Treg cells, they were less efficient than the latter cells to control the proliferation of naive T cells in response to anti-CD3 stimulation. Thus, suppressor activity is not strictly correlated to Foxp3 expression level. One explanation could be that most T cells from Foxp3 transgenic mice are in fact naive T cells forced to express Foxp3, and that, as all naive T cells, they have only a limited affinity for self and subsequently receive only weak TCR signals.

Interruption of Tconv-cell contact with self-peptide MHC ligands leads to a rapid decline on signaling and response sensitivity to foreign stimuli [23]. In the present article, we show that in contrast with Tconv cells, self-deprivation does not lead to a defect in Treg-cell ability to be activated by TCR signals. On the contrary, their response was found to be augmented in terms of calcium mobilization, CD69 upregulation and proliferation. Recent data show that Treg cells deficient for the expression or expressing inactive forms of key molecules of the TCR signaling pathway exhibit defective suppressive function in vitro [32–36]. Nevertheless, in these studies, it was not possible to determine precisely at which step TCR signals were important. Indeed, it is now well established that engagement of their TCR during the in vitro assay is required for allowing Treg cells to suppress the activation of responder T cells [21, 22]. Thus, the defective in vitro suppressive function of Treg cells with an impaired TCR signaling pathway may result either from inefficient integration of TCR signals resulting from continuous interactions with self in vivo, or from a defective response to anti-CD3 stimulation in vitro. Our experimental model allows us to discriminate between these two possibilities. Indeed, in the present article, we show that self-deprived Treg cells, although they respond well to anti-CD3 stimulation, lack suppressive function in vitro.

In the thymus, strong TCR signals lead to IL-2 sensitivity enhancement in developing thymocytes. Then, IL-2 signaling induces Foxp3 expression that, in turn, strengthens Treg-cell lineage stability [1, 2]. These three successive events are now well recognized to be important steps of thymic Treg-cell development. Then, Foxp3 expression is considered as sufficient to maintain natural Treg-cell suppressive function in the periphery. Recent data suggest that IL-2 is important to stabilize Foxp3 expression in peripheral Treg cells [18, 30, 31]. Our study places on firm ground the importance of continuous interactions with self in maintaining Treg-cell suppressive capacities in the periphery. Thus, the three actors leading to Treg-cell generation in the thymus still act in concert in the periphery to allow maintenance of their suppressive function.

Materials and methods

Mice

C57BL/6 mice were obtained from Harlan. C57BL/6 CD45.1 mice, C57BL/6 MHC II$^{\Delta/\Delta}$ mice [37, 38], C57BL/6 CD3$_{\varepsilon}^{-/-}$ mice (CD3KO [39]), and CD3$_{\varepsilon}$/MHC II$^{\Delta/\Delta}$ double-deficient mice (CD3KO-MHC II$^{\Delta/\Delta}$ mice [40]) were maintained in our own animal facilities (Cochin Institute, Paris, France) under specific pathogen-free conditions in agreement with current European legislation on animal care, housing, and scientific experimentation. C57BL/6 Foxp3-GFP mice were obtained from Dr. B. Malissen [34]. All experiments were performed in compliance with French Ministry of Agriculture regulations for animal experimentation (number 75–562).

Adoptive transfer of BM cells

BM chimeras were generated as previously described [16].

Adoptive transfer of T cells

Peripheral cells were incubated on ice with anti-CD11b (Mac-1) and anti-CD19 (1D3) antibodies, and then with magnetic beads coupled to anti-rat immunoglobulin (Dynal Biotech). Purified

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T-cell subsets were usually 90–95% pure. We injected 100×10^6 purified T cells i.v. into each recipient mouse.

IL-2 treatment

When indicated, mice were treated with daily i.p. injections of 200,000 IU of recombinant human IL-2 (Proleukin, Novartis).

In vitro suppression assay

Thymic and peripheral $CD4^+$ T cells were purified as previously described [40] and labeled with phycoerythrin (PE)-conjugated anti-CD25 antibodies (clone PC61, BD Biosciences). $CD25^+$ T cells were then positively selected using magnetic-activated cell sorting (MACS) anti-PE microbeads (Miltenyi Biotech). After magnetic bead purification based on CD25, the percentage of Foxp3-expressing cells among $CD4^+$ T cells was around 95% for WT mice, 90% for WT-CD3KO and MHC $II^{\Delta/\Delta}$-CD3KO chimeras, and at least 75% for CD3KO-MHC II^+ and CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice. $CD4^+$ $CD25^-$ cells purified from WT mice were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes). A total of 5×10^4 $CD4^+$ $CD25^-$ CFSE-labeled cells were then cultured alone or together with various numbers of $CD4^+$ $CD25^+$ cells purified from WT mice, BM chimeras, CD3KO-MHC $II^{\Delta/\Delta}$ or CD3KO-MHC II^+ recipient mice, in the presence of soluble anti-CD3 antibodies (145–2C11; 0.2 μg/mL) and APCs (25×10^4 irradiated splenocytes from CD3KO-MHC $II^{\Delta/\Delta}$ mice).

Preculture assays consisted in culture of various numbers of $CD4^+$ $CD25^+$ cells for 2 days in the presence of soluble anti-CD3 antibodies (0.2 μg/mL), 25×10^4 APC (irradiated splenocytes from CD3KO-MHC $II^{\Delta/\Delta}$ mice), and in the presence or absence of human recombinant IL-2. Culture medium was then washed away and 5×10^4 $CD4^+$ $CD25^-$ CFSE-labeled cells were added per well with soluble anti-CD3 antibodies and 15×10^4 APCs. In all protocols, cells were recovered, stained, and analyzed by flow cytometry, 16–64 h after the beginning of culture.

In vivo suppression assay

Purified lymph node $CD4^+$ T cells from C57BL/6 CD45.1 mice were stained for CD44 and CD25 expression and naive $CD4^+$ Tconv cells, flow cytometry sorted as $CD44^{-/low}$ $CD25^-$ cells. A total of 1.5×10^5 naive $CD4^+$ Tconv cells (CD45.1) were injected alone or together with 1.5×10^5 Treg cells (CD45.2) from the indicated mice.

Flow cytometry

Cell surface and intracellular staining were performed as previously described [40].

In vitro Treg-cell activation

For calcium measurements, T cells were loaded with 0.5 μM Fura-2/AM (Molecular Probes) for 15 min at 37°C. T cells were stimulated with anti-CD3 antibody (145–2C11; 10 μg/mL). Images were acquired at 37°C every 5 s on a Nikon microscope, with a 20× objective. Cells were excited alternatively at 350 and 380 nm and emissions at 510 nm were used to measure Ca variations with Metafluor software (Molecular devices). Ca levels are represented as a 350/380 fluorescence intensity ratio normalized to the ratio at t0. For studying CD69 expression and proliferation in response to stimulation, Treg cells were cultured alone in the presence of soluble anti-CD3 antibodies (0.2 μg/mL) and 25×10^4 APCs.

Microarray

Treg cells from CD3KO-MHC II^+ and CD3KO-MHC $II^{\Delta/\Delta}$ recipients as well as from WT-CD3KO and MHC $II^{\Delta/\Delta}$-CD3KO chimeras were enriched as described above and flow cytometry sorted as $CD4^+$ $TCR\beta^+$ $CD25^+$ cells. Total RNA was extracted using the RNeasy Mini kit (QIAGEN). A total of 100 ng of total RNA was reversed transcribed following the Genechip Whole transcript (WT) Sense Target labeling assay kit (Affymetrix). The cDNA obtained was then purified, fragmented, and hybridized to GeneChip® murine Gene (Affymetrix) at 45°C for 17 h. Statistical analysis was then performed with MEV software (TIGR, Rockville MD, USA). Data discussed in this publication have been deposited in the Gene Expression Omnibus at http://www.ncbi.nlm.nih.gov/geo/ (accession number GSE27153).

qRT-PCR analysis

Total RNA was isolated from flow cytometry sorted cells as described above and reverse transcribed with SuperScript™ III Reverse Transcriptase (Invitrogen) using 100 ng of Random Hexamers. Quantitative PCR analysis was performed using Power SYBR® Green PCR Master Mix (Applied Biosystems) and a real-time PCR system (ABI7300; Applied Biosystems) according to standard PCR conditions. For quantitative calculations, results were normalized to hprt expression. Primers used are listed in Supporting Information Table 1.

Calculations

The average number of cell divisions in response to anti-CD3 stimulation was calculated as follows. First, we estimated the CFSE dilution factor (f) due to stimulation: f = CFSE mean fluorescence intensity (MFI) in absence of stimulation divided by CFSE MFI in presence of stimulation. Then, as the intracellular amount of CFSE is halved during each cell cycle, the average number of cell

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divisions (A) was calculated with the following formula: A = $LOG_2(f)$. Inhibition indexes were calculated as follows:

Proliferation Inhibition = 100 × (([A($CD4^+$ Treg cells = 0) – A($CD4^+$ Treg cells = +))/A($CD4^+$ Treg cells = 0) in which A is the average number of cell divisions in response to anti-CD3 stimulation calculated as explained above.

CD25 Expression Inhibition = 100 × (%$CD25^+$ among $CD4^+$ Tconv cells ($CD4^+$ Treg cells = 0) – %$CD25^+$ among $CD4^+$ Tconv cells ($CD4^+$ Treg cells = +))/%$CD25^+$ among $CD4^+$ Tconv cells ($CD4^+$ Treg cells = 0).

Statistics

Data are expressed as mean ± SEM, and the significance of differences between two series of results was assessed using the Student's unpaired *t*-test. Values of $p < 0.05$ were considered significant (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **LIP**: lymphopenia-induced proliferation · **qRT-PCR**: quantitative reverse transcriptase PCR · **self-deprivation**: lack of MHC class II expression

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SUPPORTING INFORMATION

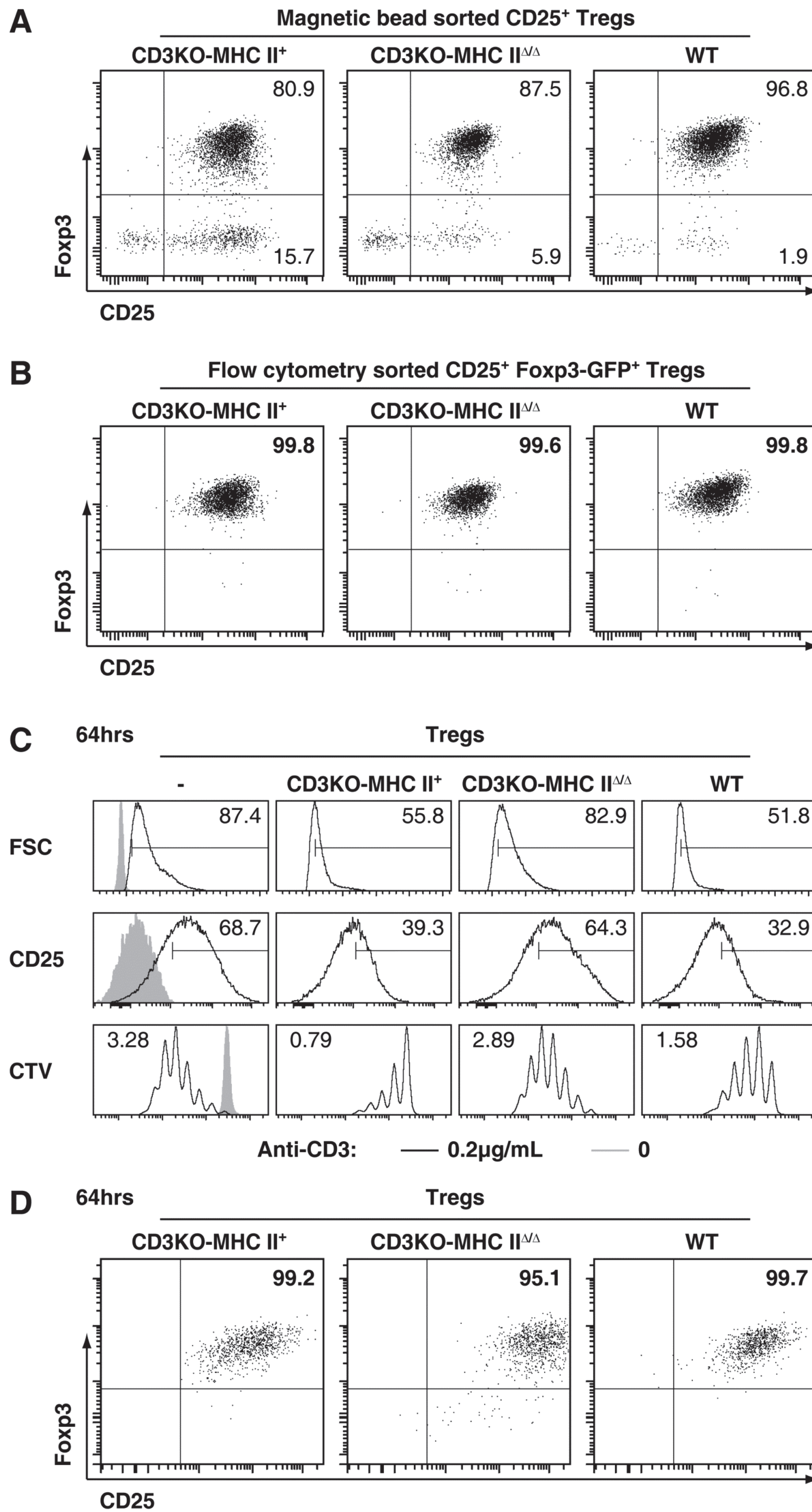

Figure S1. Highly purified self-deprived Treg cells are not functional in vitro.

T cells purified from the periphery (lymph nodes + spleen) of C57BL/6 Foxp3-GFP mice were injected into CD3KO mice (CD3KO-MHC II^{+} recipients) or into CD3KO-MHC $II^{\Delta/\Delta}$ mice (CD3KO-MHC $II^{\Delta/\Delta}$ recipients). (A) 5 days later, Treg cells were purified from the periphery of recipient mice as $CD25^{+}$ $CD4^{+}$ T cells using magnetic beads. Treg cells were also purified from lymph-nodes of C57BL/6 Foxp3-GFP mice. Representative CD25 / Foxp3-GFP fluorescence dot-plots for gated $CD4^{+}$ T cells are shown. (B) Treg cells were further purified by flow cytometry as $CD25^{+}$ Foxp3-GFP^{+} cells. Representative CD25 / Foxp3-GFP fluorescence dot-plots are shown. (C) The suppressive capacities of these highly purified Treg cells were then analyzed. Tconv cells (GFP^{-} $CD4^{+}$ T cells) were purified from lymph nodes of C57BL/6 Foxp3-GFP mice, labeled with Cell Trace Violet (CTV) and cultured alone (-) or together with the indicated Treg cells at a 1/1 Treg/Tconv-cell ratio. FSC, CD25 and CFSE histograms of Tconv cells (CTV^{+}) are shown 64 hours after the beginning of culture. For CFSE histograms, numbers correspond to the average number of cell divisions undergone by Tconv cells in response to anti-CD3 stimulation during the culture period. (D) Representative CD25 / Foxp3-GFP fluorescence dot-plots for gated Treg cells (CTV^{-}) are shown 64 hours after the beginning of culture. Data are representative of two individual experiments.

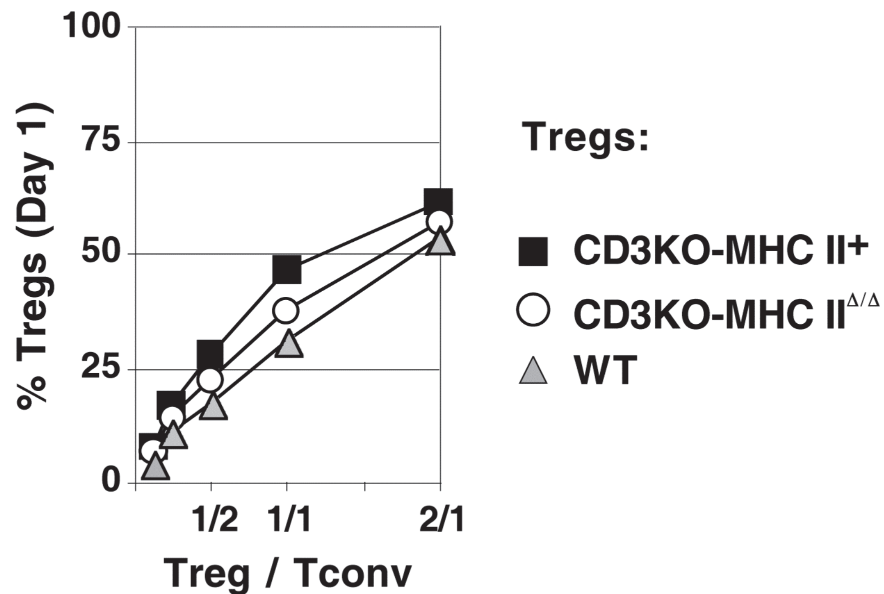

Figure S2. Unaltered survival of self-deprived Treg cells in culture.

Treg cells were purified from the periphery of CD3KO-MHC II^+ and CD3KO-MHC $II^{\Delta/\Delta}$ recipient mice 5 days after transfer. Treg cells and Tconv cells were also purified from lymph-nodes of WT mice. Tconv cells were labeled with CFSE and cultured together with the indicated Treg cells at various Treg/Tconv-cell ratios, in the presence of soluble anti-CD3 and APC. 16 hours after the beginning of culture, cells were stained for CD4 and CD8α expression and the proportion of Treg cells ($CD4^+$ $CD8\alpha^-$ $CFSE^-$) cells determined.

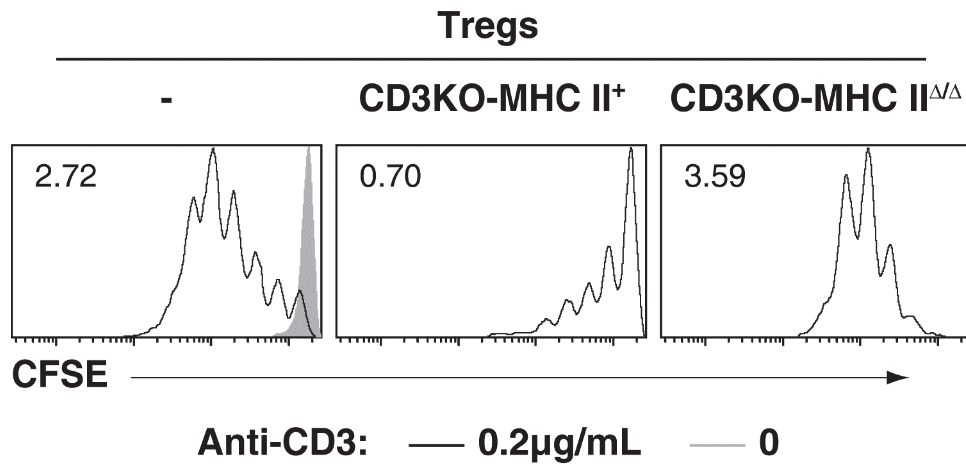

Figure S3. Il-2 is not sufficient to restore the suppressive function of self-deprived Treg cells.
T cells purified from the periphery (lymph nodes + spleen) of WT mice were injected into CD3KO mice (CD3KO-MHC II^+ recipients) or into CD3KO-MHC $II^{\Delta/\Delta}$ mice (CD3KO-MHC $II^{\Delta/\Delta}$ recipients). 5 days later, Treg cells were purified from the periphery of recipient mice as $CD25^+$ $CD4^+$ T cells using magnetic beads. Treg cells from the indicated mice were cultured for 2 days with IL-2 and APCs. Then, they were tested for their suppressive abilities. Briefly, $CD4^+$ $CD25^-$ cells (Tconv cells) were purified from lymph nodes of WT mice, labeled with CFSE and cultured alone (-) or together with the indicated Treg cells at a 1/1 Treg/Tconv-cell ratio. CFSE fluorescence histograms of Tconv cells ($CFSE^+$) are shown 64 hours after the beginning of culture. Histograms in the absence of anti-CD3 stimulation are shown (filled histograms). Numbers correspond to the average number of cell divisions undergone by Tconv cells in response to anti-CD3 stimulation during the culture period. Data are representative of two individual experiments.

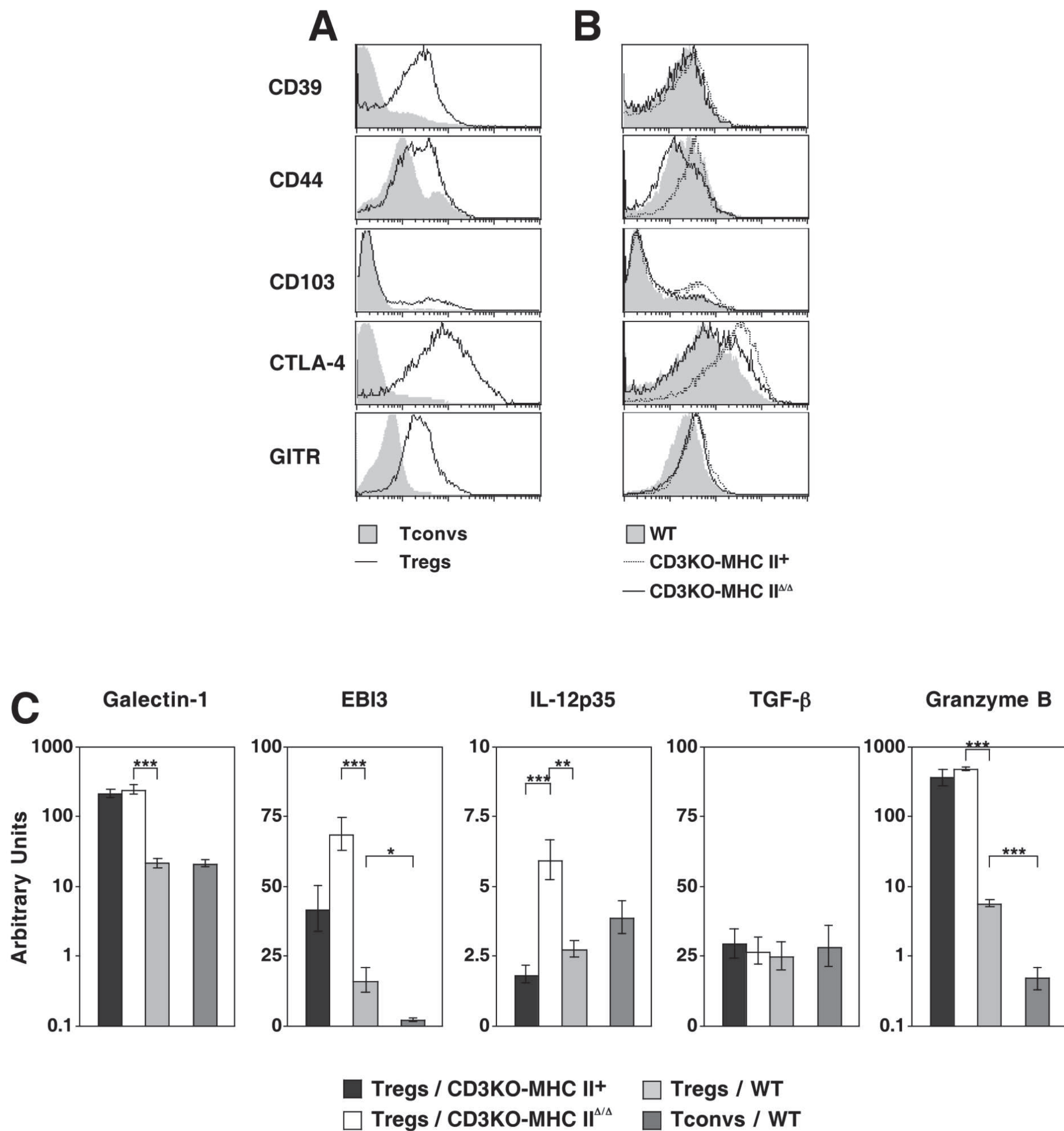

Figure S4. Phenotypic analysis of self-deprived Treg cells.

The periphery of CD3KO-MHC II^{+} and CD3KO-MHC II$^{\Delta/\Delta}$ recipient mice was harvested 5 days after transfer. Periphery of WT mice was also recovered. (A) CD39, CD44, CD103, CTLA-4 and GITR fluorescence histograms of peripheral CD4^{+} CD8$_{\alpha}^{-}$ Foxp3^{+} (Treg cells; plain line) and CD4^{+} CD8$_{\alpha}^{-}$ Foxp3^{-} (Tconv cells; filled) cells are shown for WT mice. (B) The expression of these same markers is shown for peripheral Treg cells of CD3KO-MHC II^{+} recipient mice, (dotted line), CD3KO-MHC II$^{\Delta/\Delta}$ (bold line) and WT mice (filled) 5 days after transfer. The histograms shown were generated from the data for one mouse, but are representative of three individual experiments with three mice per group. (C) The transcript levels of Galectin-1, EBI3, IL-12p35, TGF-β and Granzyme B were analyzed by qRT-PCR in the indicated CD4^{+} T cell subsets. Mean values ± SEM of relative expression are shown for indicated genes. (* $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

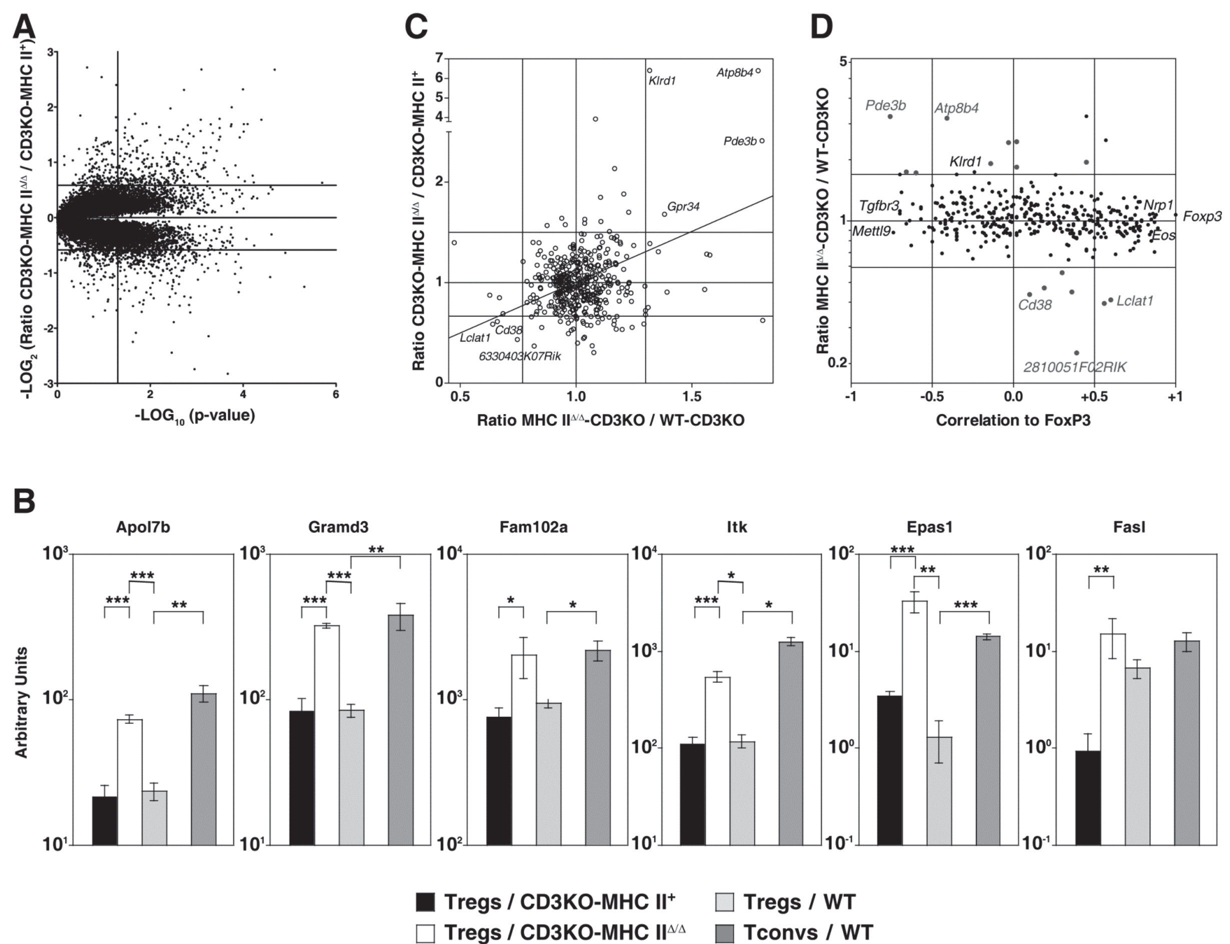

Figure S5. Self-deprived Treg cells from MHC II∆/∆-CD3KO chimeras exhibit altered transcriptional signature.

(A) "Volcano plot" representation (Log_2(fold change) versus Log_{10}(t test p value)) between Tregs from CD3KO-MHC II∆/∆ and CD3KO-MHC II+ recipient mice. (B) The transcript levels of a panel of the genes presented in Figure 7A were analyzed by qRT-PCR in the indicated CD4+ T cell subsets. Mean ± SEM values of relative expression are shown for indicated genes. (* $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). (C) Microarray analysis of Treg cells from chimeras and adoptive transfers revealed overlapping results. Analysis was restricted to genes of the common Treg-cell signature. (D) Any clear correlation can be observed between the Treg-cell genes affected by self-deprivation in the chimera experimental model and Foxp3. Scatter plot of the changes induced by self-deprivation versus the correlation values to Foxp3 for genes of the common Treg-cell signature. Red dots corresponds to genes significantly differentially expressed between Treg cells from MHC II∆/∆-CD3KO and WT-CD3KO chimeras.

Genes	sequence 5'->3'
Apol7b forward	*5'-CCTTGAGTGAAGCCCTGGGTAAGA-3'*
Apol7b reverse	*5'-CAGTTTCACCCGAGGATAAGCAT-3'*
Atp8b4 forward	*5'-TGCCATCAATATCGGTTATGCC-3'*
Atp8b4 reverse	*5'-TTCCTGAGTTCTTCTCGCACTTCC-3'*
Dapl1 forward	*5'-GTGCCATCACAAATGTCGCCAAGA-3'*
Dapl1 reverse	*5'-GTGCCGTGTGAACTGTCGCTGGA3'*
Ebi3 forward	*5'-TTGTGGCTGAGCGAATC-3'*
Ebi3 reverse	*5'-GCGGAGTCGGTACTTGA-3'*
Epas1 forward	*5'-TTGGGAAGAAGAGCAAAGACGTGT-3'*
Epas1 reverse	*5'-GCCGACTTGAGGTTGACAGT-3'*
Fam102a forward	*5'-GAAGATGGACAGAGCCCTAGTGTG-3'*
Fam102a reverse	*5'-GGGCAGTCTTCCTGACAGTACCAA-3'*
Fasl forward	*5'-AGGAGTGTGGCCCATTTAACAG-3'*
Fasl reverse	*5'-TCACTCCAGAGATCAGAGCGGTTC-3'*
Gramd3 forward	*5'-TCTGTGTGCGGACACTTAGAGA-3'*
Gramd3 reverse	*5'-TGAAATCCAGAGGCAGAGACGAAG-3'*
Gzmb forward	*5'-CTCCACGTGCTTTCACCAAA-3'*
Gzmb reverse	*5'-AGGATCCATGTTGCTTCTGTAGTTAG-3'*
Hprt forward	*5'-GCTGGTGAAAAGGACCTCT-3'*
Hprt reverse	*5'-CACAGGACTAGAACACCTGC-3'*
Il10 forward	*5'-GGTTGCCAAGCCTTATCGGA-3'*
Il10 reverse	*5'-ACCTGCTCCACTGCCTTGCT-3'*
Il-12p35 forward	*5'-CCTTGCCCTCCTAAACCAC-3'*
Il-12p35 reverse	*5'-TTTCGGGACTGGCTAAGACA-3'*
Itk forward	*5'-CCCTGGTCATTGCCTTGTACGAC-3'*
Itk reverse	*5'-TCTCGGAGCTGTCCAGCAGGTAGT-3'*
Klrd1 forward	*5'-CAGTCCAAGCAAAAGCGTTTCT-3'*
Klrd1 reverse	*5'-CCGTGGACCTTCCTTGTCTATACC-3'*
Lgals1 forward	*5'-GTCGCCAGCAACCTGAATCTC-3'*
Lgals1 reverse	*5'-GGGCATTGAAGCGAGGATTG-3'*
Mctp2 forward	*5'-CGACAAGACTCACGGCAATGACGA-3'*
Mctp2 reverse	*5'-TGGTGAGGAGGTACGCGAAGG-3'*
Pde3b forward	*5'-CCGGGACATTCCATATCAC-3'*
Pde3b reverse	*5'-ATCTGCTTTGGTTTCCGTTTCA-3'*
Tgfb1 forward	*5'-CGCAACAACGCCATCTATGA-3'*
Tgfb1 reverse	*5'-GCACTGCTTCCCGAATGTCT-3'*

Table S1: primers

Article 4 :

IL-2 and IL-7 determine the homeostatic balance between the regulatory and conventional CD4$^+$ T-cell compartments during peripheral T-cell reconstitution

The Journal of Immunology, 2012, 189

Armelle Le Campion*, Arnaud Pommier*, Arnaud Delpoux, Laurence Stouvenel, Cédric Auffray, Bruno Martin*, Bruno Lucas*

Durant les dernières décennies, les facteurs influençant la survie et l'homéostasie des lymphocytes T conventionnels ont été identifiés. L'IL-7 et le signal TcR permettent la survie des lymphocytes T CD4$^+$ et CD8$^+$ naïfs chez des souris reconstituées et leur prolifération dans un environnement lymphopénique, alors que la survie e la prolifération homéostatique des lymphocytes T CD4$^+$ et CD8$^+$ mémoires reposent sur une combinaison entre l'IL-7 et l'IL-15. En revanche, les facteurs régissant la prolifération des lymphocytes T CD4$^+$ régulateurs induite par la lymphopénie sont beaucoup moins connus. Nous avons évalué ici l'impact des facteurs « classiques » influençant la prolifération tel que l'IL-2, l'IL-7 et le signal TcR. L'ensemble de nos résultats suggère qu'alors que la prolifération des lymphocytes T CD4$^+$ conventionnels est étroitement liée au taux d'IL-7, la prolifération des lymphocytes T CD4$^+$ régulateurs repose sur l'IL-2. La capacité de l'IL-7 à provoquer la prolifération des lymphocytes T CD4$^+$ conventionnels avec une expansion concomitante minime de lymphocytes T CD4$^+$ régulateurs ouvre la possibilité de traitements des patients souffrant de lymphopénie particulièrement dans le cadre d'infections virales chroniques et d'immunothérapies anti-tumorales.

IL-2 and IL-7 Determine the Homeostatic Balance between the Regulatory and Conventional CD4$^+$ T Cell Compartments during Peripheral T Cell Reconstitution

Armelle Le Campion,[1] Arnaud Pommier,[1] Arnaud Delpoux, Laurence Stouvenel, Cédric Auffray, Bruno Martin,[2] and Bruno Lucas[2]

Work over the last decades has led to the identification of the factors that influence the survival and homeostasis of conventional T cells. IL-7 and TCR signaling promote the survival of naive CD4$^+$ and CD8$^+$ T cells in lymphoreplete mice and their proliferation in a lymphopenic environment, whereas survival and homeostatic proliferation of memory CD4$^+$ and CD8$^+$ T cells crucially depend on a combination of IL-7 and IL-15. In contrast, there is little information regarding the factors driving the proliferation of regulatory CD4$^+$ T cells in response to lymphopenia. In this study, we investigated whether regulatory CD4$^+$ T cell proliferation in response to lymphopenia was guided by classical homeostatic resources, such as IL-2, IL-7, or TCR–MHC interactions. Altogether, our data suggest that, although homeostatic proliferation of conventional naive CD4$^+$ T cells is closely related to IL-7 levels, the proliferation of regulatory CD4$^+$ T cells in response to lymphopenia appears to be primarily controlled by IL-2. The capacity of IL-7 to augment conventional T cell proliferation with minimal concomitant regulatory T cell expansion may be clinically exploitable in the treatment of patients with lymphopenia, especially in the case of chronic viral diseases or cancer immunotherapy. *The Journal of Immunology*, **2012, 189: 000–000.**

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The size of the peripheral T cell pool is notably constant, despite continuous output from the thymus, turnover of existing cells, and clonal expansion of Ag-specific cells in the course of an immune response (1). This process is achieved through several homeostatic mechanisms that regulate both cell survival and proliferation. Environmental factors that regulate these responses vary, depending on the T cell subset and on the nature, naive or memory, of the T cell (2). The precise identification of these factors regulating T cell homeostasis appears to be crucial for the development of new strategies and clinical trials for future immunotherapy, such as cancer or antiviral therapies, or in the context of autoimmunity and lymphoproliferative diseases.

Work over the last decades established a critical role for cytokines in the maintenance and homeostatic proliferation of memory T cells (2). More precisely, it was shown that survival and homeostatic proliferation of memory CD8$^+$ and CD4$^+$ T cells crucially depend on a combination of IL-7 and IL-15 (3-7). However, optimum memory CD4$^+$ T cell function may depend on interactions with MHC II molecules (8). In contrast, it was shown that disruption of MHC class I molecule–TCR interactions did not affect self-renewal, function, or survival of memory CD8$^+$ T cells (9).

Numerous studies have led to the identification of the factors that influence the survival and homeostasis of naive T cells (10). In physiological settings, IL-7 and TCR signaling promote the survival and normal function of naive CD4$^+$ and CD8$^+$ T cells (11–13). In a lymphopenic environment, the same cues promote the proliferation of these cells (14, 15). More precisely, naive CD4$^+$ T cells can be divided into two subsets as a function of their behavior after transfer into lymphopenic mice (16, 17). On one hand, a small proportion of the initially injected CD4$^+$ T cells expands strongly in response to interactions with self-peptides or commensal bacterium-derived peptides presented by MHC class II molecules. IL-7 is not required for this process, which is called "spontaneous proliferation" (18). On the other hand, the vast majority of injected naive T cells cycles slowly in response to the great availability of IL-7 in lymphopenic environments (19). TCR signaling can also synergize with IL-7 to enhance this latter process, which is called "homeostatic T cell proliferation" (18).

It is well established that IL-2 is essential for regulatory CD4$^+$ T cell survival in the periphery (20–22). Accordingly, defective IL-2 signaling leads to spontaneous lymphoproliferative and autoimmune diseases in mice and humans because of the impaired development and function of these cells (23). Surprisingly, there is little information regarding the factors driving the proliferation of regulatory CD4$^+$ T cells in response to lymphopenia. Cozzo et al. (24) and Hsieh et al. (25) proposed that this process is dependent on interactions with self-peptide/MHC complexes, but the role of IL has not been clearly addressed.

In the present study, we investigated whether regulatory CD4$^+$ T cell proliferation in response to lymphopenia was guided by "classical" homeostatic resources, such as IL-2, IL-7, or TCR–MHC interactions. We show that proliferation of regulatory CD4$^+$

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The online version of this article contains supplemental material.

Abbreviations used in this article: CTv, CellTrace violet; LN, lymph node; MFI, mean fluorescence intensity.

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T cells in response to lymphopenia is not controlled by IL-7 levels but rather requires IL-2 production by conventional CD4⁺ T cells. This result is of importance, because several clinical trials of recombinant human IL-7 are ongoing in the settings of acquired immunodeficiency, cancer, and chronic viral infection.

Materials and Methods

Mice

Q:9 C57BL/6 mice (CD45.2) were obtained from Harlan Laboratories. CD45.1 C57BL/6 mice, C57BL/6 CD3$\varepsilon^{-/-}$ mice, were maintained in our own animal facilities under specific pathogen-free conditions. C57BL/6 CD3$\varepsilon^{-/-}$ mice (26) were crossed with MHC II$^{\Delta/\Delta}$ mice (27) to obtain CD3ε/MHC II double-deficient mice (CD3$\varepsilon^{-/-}$ II$^{\Delta/\Delta}$ mice) (13). C57BL/6 Foxp3-GFP reporter mice were initially provided by Dr. Bernard Malissen (Centre Q:10 d'Immunologie de Marseille-Luminy, France) (28, 29) and maintained in our own animal facilities. Experiments were carried out in accordance with the guidelines of the French Veterinary Department.

Cell suspensions

Peripheral and mesenteric lymph nodes (LNs) and spleen tissue were homogenized and passed through a nylon cell strainer (BD Falcon) in RPMI 1640 Glutamax (Life Technologies), supplemented with 10% FCS (Biochrom) for adoptive transfer (LNs only), or in 5% FCS, 0.1% NaN3 (Sigma-Aldrich) in PBS for flow cytometry (pooled LN and spleen cells - periphery).

Adoptive transfer of CD4⁺ T cells

LN cells (pooled superficial cervical, axillary, brachial, inguinal, and mesenteric LNs) were incubated on ice for 20 min with a mixture of anti-CD8 (53-6.7), anti-CD11b (Mac-1), anti-GR1 (8C5), and anti-CD19 (1D3) Abs, obtained from hybridoma supernatants, and then with magnetic beads coupled to anti-rat Igs (Dynal Biotech). Purified T cell subsets were generally 95–97% pure. When indicated, CD4⁺ T cells were labeled with 5 μM CFSE (Molecular Probes) before injection. In some experiments (Fig. 6), regulatory GFP-Foxp3⁺ CD4⁺ T cells from C57BL/6 Foxp3-GFP reporter mice were FACS sorted in a MoFlo XDP flow cytometer (Beckman Coulter). Regulatory GFP-Foxp3⁺ CD4⁺ T cells were then labeled with a 5 μM CellTrace violet proliferation kit (Invitrogen), according to the manufacturer's guidelines, before injection. Purified CD4⁺ T cells (5×10^6 cells) and/or regulatory CD4⁺ T cells (1×10^6 cells) were injected i.v. into sex-matched lymphopenic recipient mice.

In vivo treatment with anti–IL-7R and/or anti–IL-2–blocking Abs and IL-2 immune complexes

In the experiments depicted in Figs. 4 and 6, mice were injected i.p. every 2 d, beginning at the time of cell transfer, with anti–IL-2 Abs (S4B6 and JES6-1A12, 200 μg each/mouse; Bio X Cell) and/or anti–IL-7Rα Ab (A7R34, 200 μg/mouse) obtained from hybridoma supernatants. In some experiments (Fig. 5), mice were injected i.p. every 2 d with IL-2/anti–IL-2 complexes, beginning at the time of cell transfer. IL-2/anti–IL-2 complexes were made, as previously described (30), by mixing 2 μg recombinant mouse IL-2 (0.5 μg/mouse; PeproTech) with 10 μg anti–IL-2 Ab (clone S4B6, 2.5 μg/mouse; Bio X Cell).

Cell surface staining and flow cytometry

Cell suspensions were collected and dispensed into 96-well round-bottom microtiter plates (Greiner Bioscience; 6×10^6 cells/well). Surface staining was performed by incubating the cells on ice, for 15 min/step, with Abs in 5% FCS (Biochrom), 0.1% NaN_3 (Sigma-Aldrich) in PBS. Each cell-staining reaction was preceded by a 15-min incubation with purified anti-CD16/32 Abs (FcγRII/III block, 2.4G2) obtained from hybridoma supernatants.

Peridin chlorophyll protein-conjugated anti-CD4 Ab (RM4-5), FITC-conjugated anti-CD25 Ab (7D4), PE cyanin 7-conjugated anti-CD3 Ab (145-2C11), biotinylated anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD25 (PC61), allophycocyanin-H7–conjugated anti-CD8α (53-6.7), Pacific Blue-conjugated anti-CD4 (RM4-5), allophycocyanin-conjugated anti-CD45.1 (A20), and allophycocyanin-conjugated streptavidin were obtained from BD Biosciences. Allophycocyanin Alexa Fluor 750-conjugated anti-CD8α (53-6.7), biotinylated anti-CD127 (A7R34) Abs were Q:11 obtained from eBioscience. Pacific Blue-conjugated streptavidin was obtained from Invitrogen.

For intranuclear Foxp3 staining, cells were fixed and permeabilized with the eBioscience Foxp3 staining buffer set and then stained with PE-conjugated anti-Foxp3 Ab (FJK-16s). Four- and seven-color immunofluorescence analyses were carried out with a FACSCalibur flow cytometer and a BDLSRII flow cytometer, respectively (BD Biosciences). List-mode data files were analyzed with CellQuest and Diva software (BD Biosciences).

In vitro culture assay

LN cells were incubated on ice for 20 min with anti-CD8 (53-6.7), anti-CD11b (Mac-1), anti-GR1 (8C5), and anti-CD19 (1D3) Abs, obtained from hybridoma supernatants, and then with magnetic beads coupled to anti-rat Ig (Dynal Biotech). Purified CD4⁺ T cells were labeled with biotinylated anti-CD25 (clone PC61) Ab. Then, CD25⁺ and CD25⁻ T cells were separated using MACS streptavidin MicroBeads (Miltenyi Biotec). CD25⁺ CD4⁺ T cells (50×10^3) and CD25⁻ CD4⁺ T cells (50×10^3) were cultured separately in the presence or absence of recombinant human IL-2 (10 ng/ml) or recombinant mouse IL-7 (10 ng/ml) (both from R&D Systems).

Calculations

The average number of cell cycles was calculated as follows. First, we estimated the CFSE dilution factor (f): CFSE mean fluorescence intensity (MFI) of nondivided cells (cycle 0) divided by CFSE MFI of the entire CFSE⁺ cell subset. Then, because the intracellular amount of CFSE is halved during each cell cycle, the average number of cell cycles (A) was calculated using the following formula: $A = \log_2(f)$.

Statistics

Data are expressed as mean ± SEM, and the significance of differences between two series of results was assessed using the Student unpaired or paired t test. Values of $p < 0.05$ were considered significant.

Results

IL-7 alone does not drive homeostatic proliferation of regulatory CD4⁺ T cells in response to lymphopenia

Our study was conducted by transferring 5×10^6 CD4⁺ T cells from C57BL/6 mice, labeled with CFSE, into lymphopenic C57BL/6 CD3$\varepsilon^{-/-}$ mice lacking or not lacking MHC class II molecule expression. No cell division was detected for the first 2 d after transfer (Fig. 1A). Two weeks later, in both recipients, F1 some recovered cells had undergone a limited number of divisions, whereas the intracytoplasmic dye had totally disappeared from others. Thus, as previously described by us and other investigators (13, 16–18), we confirmed that CD4⁺ T cells from normal C57BL/6 mice can be divided into two subsets with respect to their behavior after transfer into lymphopenic mice. The first subset (CFSE⁺ CD4⁺ T cells), corresponding to the vast majority of transferred CD4⁺ T cells, cycles very slowly; this process is called "homeostatic T cell proliferation." The second subset (CFSE⁻ CD4⁺ T cells) is generated by the strong expansion of a small proportion of injected CD4⁺ T cells (31). This process is termed "spontaneous proliferation" (17, 18) and requires interactions with MHC molecules (13).

To study the homeostatic proliferation of conventional and regulatory CD4⁺ T cells, we focused on CFSE⁺ CD4⁺ T cells. As previously reported (13), we observed a similar proliferation pattern of conventional CD4⁺ T cells (CFSE⁺ Foxp3⁻ CD4⁺) in both recipient mice (Fig. 1A, 1B). This slow and limited proliferation of naive CD4⁺ T cells is known to result directly from the greater availability of IL-7 in lymphopenic environments (19, 32, 33). In contrast, although regulatory CD4⁺ T cells (CFSE⁺ Foxp3⁺ CD4⁺) were able to proliferate even more strongly than were their conventional CD4⁺ T cell counterparts after transfer into lymphopenic MHC class II-expressing recipient mice, this proliferating capacity appeared to be strongly compromised in mice lacking the expression of MHC class II molecules (Fig. 1A, 1B). Consequently, the proportion of regulatory CD4⁺ T cells among

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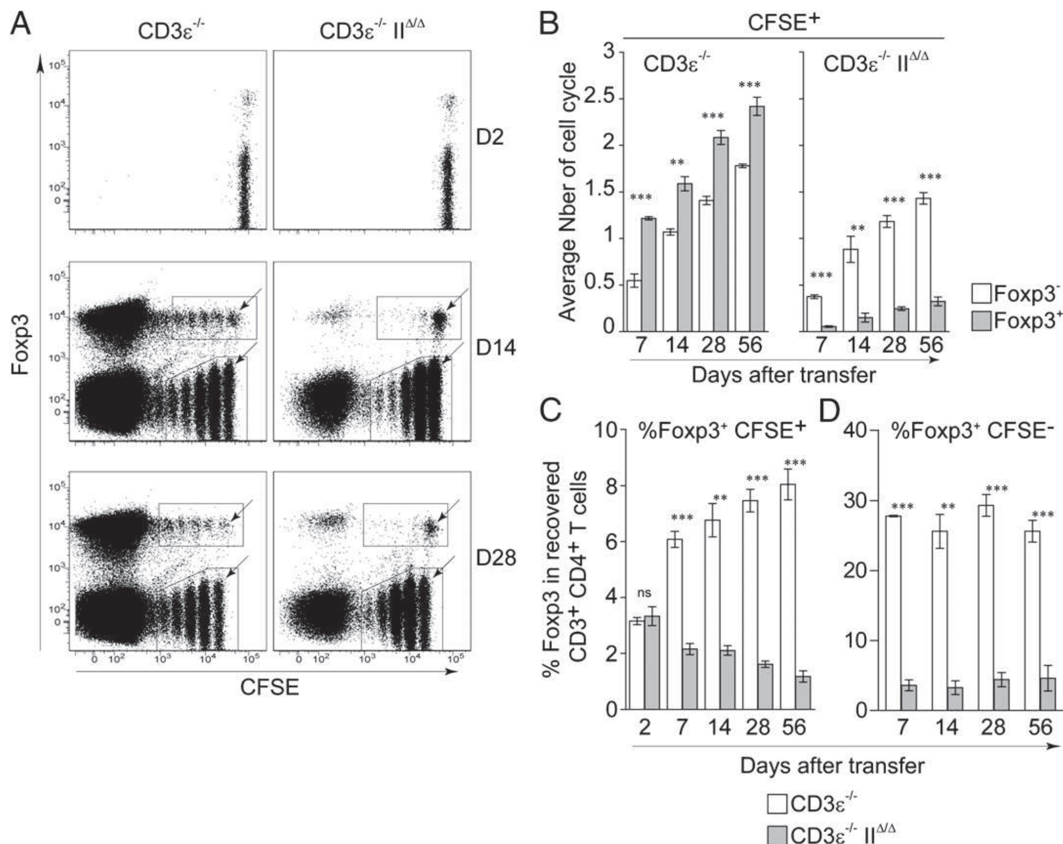

FIGURE 1. Homeostatic proliferation of regulatory CD4+ T cells in response to lymphopenia requires MHC class II molecule expression. CFSE-labeled LN CD4+ T cells (5×10^6) from C57BL/6 mice were injected i.v. into C57BL/6 CD3ε$^{-/-}$ mice and C57BL/6 CD3ε$^{-/-}$ MHC II$^{\Delta/\Delta}$ mice. At various times after transfer, LNs and spleen were recovered and pooled, and single-cell suspensions were prepared. (**A**) Foxp3/CFSE dot plots of CD4+ CD3+ CD8− T cells from representative recipient mice 2, 14, and 28 d after transfer. Arrows in each dot plot indicate nondivided conventional and Foxp3+ regulatory CD4+ T cells. (**B**) Average number of cell cycles undergone by CFSE+ Foxp3+ CD4+ CD3+ CD8− T cells and CFSE+ Foxp3− CD4+ CD3+ CD8− T cells recovered from C57BL/6 CD3ε$^{-/-}$ and C57BL/6 CD3ε$^{-/-}$ MHC II$^{\Delta/\Delta}$ recipient mice at various times after transfer. Results are expressed as means ± SEM for three independent experiments. (**C**) Proportion of CFSE+ CD3+ CD4+ CD8− T cells expressing Foxp3 at various times after transfer. Results are expressed as means ± SEM for three independent experiments. (**D**) Proportion of CFSE− CD3+ CD4+ CD8− T cells expressing Foxp3 at various times after transfer. Results are expressed as means ± SEM for three independent experiments.

CFSE+ CD4+ T cells decreased with time after transfer into CD3ε$^{-/-}$ mice lacking the expression of MHC class II molecules (Fig. 1C), reflecting, in this setting, a progressive dysregulation of the homeostatic balance between regulatory and conventional CD4+ T cells.

Lymphopenia-induced T cell spontaneous proliferation is thought to strictly depend on interactions with MHC molecules (13). Accordingly, very few CFSE− cells were generated at early time points after transfer into mice lacking MHC class II molecules (Fig. 1A, Supplemental Fig. 1). We showed previously that these cells are, in fact, responding to MHC class I molecules (13). Interestingly, these rare cells included, in proportion, less regulatory CD4+ T cells than when recipient mice were expressing MHC class II molecules (Fig. 1D).

Thus, the greater availability of IL-7 found in lymphopenic environments is not able to induce homeostatic proliferation of regulatory CD4+ T cells by itself. As proposed by Cozzo et al. (24) and Hsieh et al. (25), our data suggest that, in contrast with their conventional CD4+ T cell counterparts, homeostatic T cell proliferation of regulatory CD4+ T cells in response to lymphopenia requires both IL-7 and TCR signaling.

We then compared the expression level of IL-7Rα (CD127) on conventional and regulatory CFSE+ CD4+ T cells (Fig. 2). Park et al. (34) clearly demonstrated that high consumption of IL-7 led to a marked downregulation of its receptor, CD127. Thus, the study of CD127 expression on T cells in the course of peripheral T cell reconstitution may help us to evaluate whether IL-7 is consumed by these cells. Two days after their transfer into both CD3ε$^{-/-}$ and CD3ε$^{-/-}$ II$^{\Delta/\Delta}$ recipient mice, CD127 expression was strongly decreased in conventional CD4+ T cells (Fig. 2A). This downregulation remained stable for 1 mo in MHC class II-expressing CD3ε$^{-/-}$ recipient mice, whereas it rapidly returned to control levels in CD3ε$^{-/-}$ II$^{\Delta/\Delta}$ recipient mice (Fig. 2B). This latter result may indicate that TCR signaling boosts IL-7 consumption and subsequent IL-7R downregulation. In contrast, at all studied time points, CD127 was not downregulated at the cell surface of regulatory CD4+ T cells in either group of recipient mice, indicating a weak consumption of IL-7 by these cells.

Taken together, these data suggest that, in contrast to their conventional CD4+ T cell counterparts, homeostatic proliferation of the regulatory CD4+ T cell pool in response to lymphopenia is independent of IL-7.

The extent of both homeostatic and spontaneous proliferations of regulatory CD4+ T cells in response to lymphopenia requires IL-2 rather than IL-7

IL-2 was shown to be crucial for regulatory CD4+ T cell homeostasis in the periphery. Moreover, IL-2 allows regulatory CD4+ T cells to proliferate in vitro in response to anti-CD3 Ab stimulation. Thus, we decided to compare, in vitro and in vivo, the

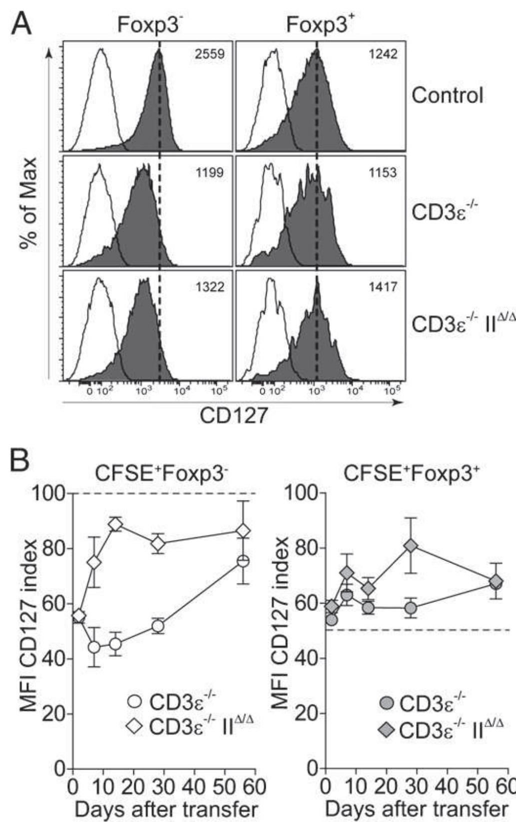

FIGURE 2. CD127 expression on conventional and regulatory $CD4^+$ T cells undergoing homeostatic proliferation in response to lymphopenia. CFSE-labeled LN $CD4^+$ T cells (5×10^6) from C57BL/6 mice were injected i.v. into C57BL/6 $CD3\varepsilon^{-/-}$ mice and C57BL/6 $CD3\varepsilon^{-/-}$ MHC $II^{\Delta/\Delta}$ mice. At various times after transfer, LNs and spleen were recovered and pooled, and single-cell suspensions were prepared. (**A**) CD127 fluorescence graphs (filled graphs) of $CFSE^+$ $Foxp3^+$ $CD4^+$ $CD3^+$ $CD8^-$ T cells and $CFSE^+$ $Foxp3^-$ $CD4^+$ $CD3^+$ $CD8^-$ T cells recovered 2 d after transfer. CD127 fluorescence graphs of $CD4^+$ T cells from control C57BL/6 mice are also shown. The open graphs represent isotype-stained cells. Number in each graph represents CD127 MFI. (**B**) CD127 expression of the indicated transferred cells at various times after transfer. Results are expressed as MFI index (CD127 MFI of the indicated recovered $CD4^+$ T cells/CD127 MFI of conventional $CD4^+$ T cells [$Foxp3^-$] from control C57BL/6 mice $\times$ 100). The dashed line represents the MFI index of $Foxp3^-$ (*left panel*) and $Foxp3^+$ (*right panel*) $CD4^+$ T cells from control C57BL/6 mice. Results are expressed as means $\pm$ SEM for two independent experiments with at least three mice per group and per day.

effect of IL-2 and IL-7 on the survival and lymphopenia-induced proliferation of conventional and regulatory $CD4^+$ T cells.

First, to ascertain whether IL-2 and/or IL-7 promote, in vitro, the survival of conventional and/or regulatory $CD4^+$ T cells, $CD25^-$ and $CD25^+$ $CD4^+$ T cell subsets from C57BL/6 mice were cultured separately for 3 d, in the presence or absence of IL-2 or IL-7 (Fig. 3). As shown in Fig. 3A, in the absence of TCR stimulation, IL-7, but not IL-2, allowed the maintenance of conventional $CD4^+$ T cell numbers. In contrast, we observed that the survival of regulatory $CD4^+$ T cells in this setting was mainly dependent on the presence of IL-2. Nevertheless, we found that IL-7 as well significantly supported the survival of these cells in vitro, although to a lesser extent than IL-2 (Fig. 3A). Thus, regulatory $CD4^+$ T cells, although they express lower surface levels of CD127 than conventional $CD4^+$ T cells, could respond to IL-7. In addition, we verified that the proportion of $Foxp3^+$ $CD4^+$ T cells in the $CD25^+$ $CD4^+$ T cell culture remained stable over time, and that we could

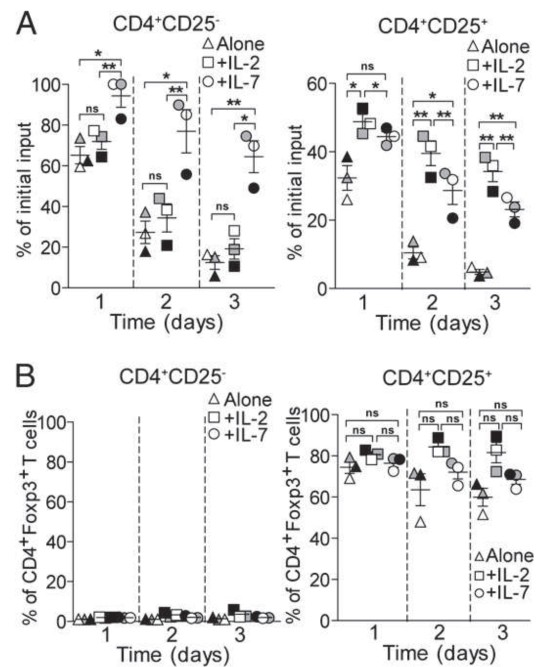

FIGURE 3. In vitro survival of regulatory $CD4^+$ T cells depends mainly on IL-2 in the absence of TCR stimulation. Purified LN $CD25^-$ and $CD25^+$ $CD4^+$ T cells from C57BL/6 mice were cultured separately for 3 d with medium alone or in the presence of IL-2 or IL-7. (**A**) Absolute numbers of recovered $CD4^+$ T cells 1, 2, and 3 d after the onset of culture. (**B**) Proportion of $CD4^+$ T cells expressing Foxp3 1, 2, and 3 d after the onset of culture. Black-, gray-, and white-filled symbols represent individual experiments.

detect only few Foxp3-expressing cells in the $CD25^-$ $CD4^+$ T cell culture (Fig. 3B).

We next investigated the respective role of IL-2 and IL-7 in the proliferation of regulatory $CD4^+$ T cells observed in MHC class II-expressing lymphopenic recipient mice. To address this question, 5×10^6 CFSE-labeled $CD4^+$ T cells from C57BL/6 mice were injected into $CD3\varepsilon^{-/-}$ mice that were treated or not with anti–IL-7R, anti–IL-2, or both blocking Abs (Fig. 4). Fourteen days after transfer, homeostatic proliferation of regulatory $CD4^+$ T cells was clearly reduced in mice treated with anti–IL-2 Ab (Fig. 4A, 4B). Accordingly, anti–IL-2 Ab treatment resulted in a decrease of the proportion of regulatory $CD4^+$ T cells among $CFSE^+$ $CD4^+$ T cells (Fig 4C). In contrast, we observed that administration of anti–IL-7R Abs preferentially affected the homeostatic proliferation of conventional $CD4^+$ T cells (Fig. 4A, 4B). Consequently, this latter treatment resulted in a strong increase in the proportion of regulatory $CD4^+$ T cells among $CFSE^+$ $CD4^+$ T cells (Fig. 4C). Anti–IL-7R Abs have a weak, although significant, effect on the homeostatic proliferation of regulatory $CD4^+$ T cells.

As previously reported by many groups, IL-7 deprivation had only a modest and nonsignificant effect on lymphopenia-induced T cell spontaneous proliferation (Fig. 4D). In contrast, IL-2 deprivation resulted in a strong decrease in the proportion of regulatory $CD4^+$ T cells among $CFSE^-$ $CD4^+$ T cells. Thus, IL-2 synergizes with TCR signals to increase the magnitude of regulatory $CD4^+$ T cell spontaneous proliferation in response to lymphopenia.

Altogether, our results strongly suggest that, during immune reconstitution, the balance between the regulatory and the conventional $CD4^+$ T cell compartments is closely related to IL-2 and IL-7 levels in vivo.

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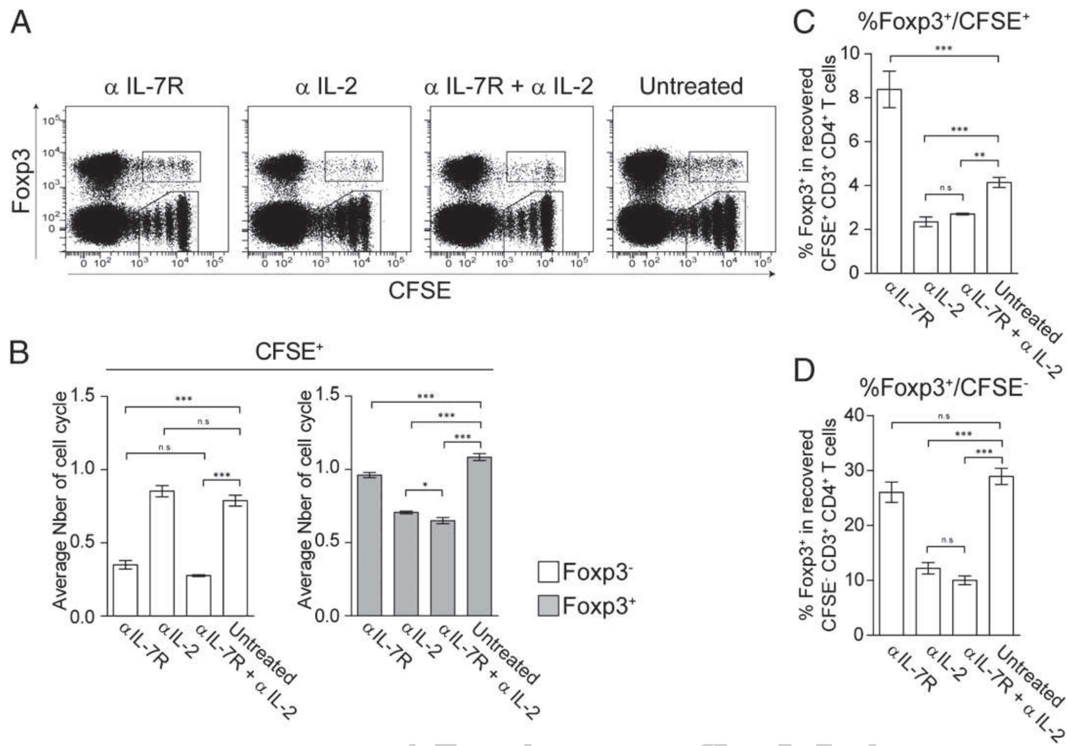

FIGURE 4. Homeostatic proliferation of regulatory CD4⁺ T cells in response to lymphopenia relies mainly on IL-2. CFSE-labeled LN CD4⁺ T cells (5×10^6) from C57BL/6 mice were injected i.v. into lymphopenic C57BL/6 CD3$\varepsilon^{-/-}$ mice treated or not with anti–IL-2, anti–IL-7Rα, or both blocking Abs. Fourteen days after transfer, LNs and spleen were recovered and pooled, and single-cell suspensions were prepared. (**A**) Foxp3/CFSE dot plots of CD4⁺ CD3⁺ CD8⁻ T cells from representative recipient mice 14 d after transfer. (**B**) Average number of cell cycles of CFSE⁺ Foxp3⁻ CD4⁺ CD3⁺ CD8⁻ T cells and CFSE⁺ Foxp3⁺ CD4⁺ CD3⁺ CD8⁻ T cells 14 d after transfer. Results are shown as means ± SEM for three independent experiments. (**C**) Proportion of recovered CFSE⁺ CD3⁺ CD4⁺ T cells expressing Foxp3 14 d after transfer. Results are shown as means ± SEM for two independent experiments with at least three mice/group. (**D**) Proportion of recovered CFSE⁻ CD3⁺ CD4⁺ T cells expressing Foxp3 14 d after transfer. Results are shown as means ± SEM for two independent experiments with at least three mice/group.

IL-2 alone is sufficient to drive regulatory T cell homeostatic proliferation

A total of 5×10^6 CFSE-labeled CD4⁺ T cells was injected into CD3$\varepsilon^{-/-}$ II$^{\Delta/\Delta}$ mice treated or not with IL-2/anti–IL-2 complexes (35). Untreated MHC II-expressing CD3$\varepsilon^{-/-}$ recipient mice were also studied in parallel (Fig. 5). Proliferation of transferred CD4⁺ T cells was analyzed 14 d after transfer. Administration of IL-2–agonist complexes partially restored the homeostatic proliferation of regulatory CD4⁺ T cells injected into lymphopenic mice lacking expression of MHC class II molecules, whereas the homeostatic proliferation of conventional CD4⁺ T cells remained unchanged (Fig. 5A). More precisely, we observed a 6-fold increase in the average number of divisions of regulatory CD4⁺ T cells when CD3$\varepsilon^{-/-}$ II$^{\Delta/\Delta}$ mice were treated with IL-2/anti–IL-2 complexes (Fig. 5B). Consequently, this treatment resulted in a significant increase in the proportion of regulatory CD4⁺ T cells among CFSE⁺ CD4⁺ T cells (Fig 5C). Thus, ILs are able to drive the homeostatic proliferation of both conventional and regulatory CD4⁺ T cells independently of TCR signaling. However, although the homeostatic proliferation of conventional CD4⁺ T cells is closely related to IL-7 levels, the proliferation of regulatory CD4⁺ T cells appears to be primarily controlled by IL-2.

As noted above (Fig. 1), in the absence of MHC class II molecules, very few regulatory T cells were contained within CFSE⁻ CD4⁺ T cells recovered from the periphery of recipient mice 14 d after transfer. IL-2 treatment led to a significant increase in the proportion (Fig. 5D) and absolute number (Supplemental Fig. 2) of regulatory T cells among CFSE⁻ CD4⁺ T cells, confirming that IL-2 plays a role in the extent of regulatory CD4⁺ T cell spontaneous expansion in response to lymphopenia.

Regulatory CD4⁺ T cells receive help from conventional CD4⁺ T cells to undergo homeostatic proliferation in response to lymphopenia

Because we noticed that IL-2 was a crucial factor in driving the homeostatic proliferation of regulatory CD4⁺ T cells in response to lymphopenia, we speculated that such a process requires assistance from conventional T cells. To address this hypothesis directly, 1×10^6 FACS-sorted, regulatory CD4⁺ T cells from C57BL/6 Foxp3-GFP CD45.2 mice, labeled with CellTrace violet (CTv), were injected alone or with 5×10^6 conventional CD4⁺ T cells from C57BL/6 CD45.1 mice into CD45.1 CD3$\varepsilon^{-/-}$ mice. Proliferation of transferred regulatory CD45.2⁺ GFP⁺ CD4⁺ T cells was analyzed 14 d after transfer (Fig. 6). When injected alone, regulatory CD4⁺ T cells underwent homeostatic proliferation. This proliferation required MHC class II molecule expression and was strongly decreased by IL-2 deprivation (Fig. 6A, 6B). Interestingly, spontaneous proliferation of regulatory CD4⁺ T cells in response to lymphopenia was completely abolished in the absence of MHC class II molecule expression. As described by Duarte et al. (36), when injected alone, some regulatory CD4⁺ T cells lost Foxp3 expression and underwent spontaneous proliferation in response to lymphopenia that induced CTv complete dilution and their accumulation as CTv⁻ GFP⁻ CD45.2⁺ CD4⁺ T cells. These converted cells may provide the IL-2 required for the homeostatic proliferation of regulatory CD4⁺ T cells. Interestingly, regulatory CD4⁺ T cell homeostatic proliferation was significantly increased when regulatory CD4⁺ T cells were coin-

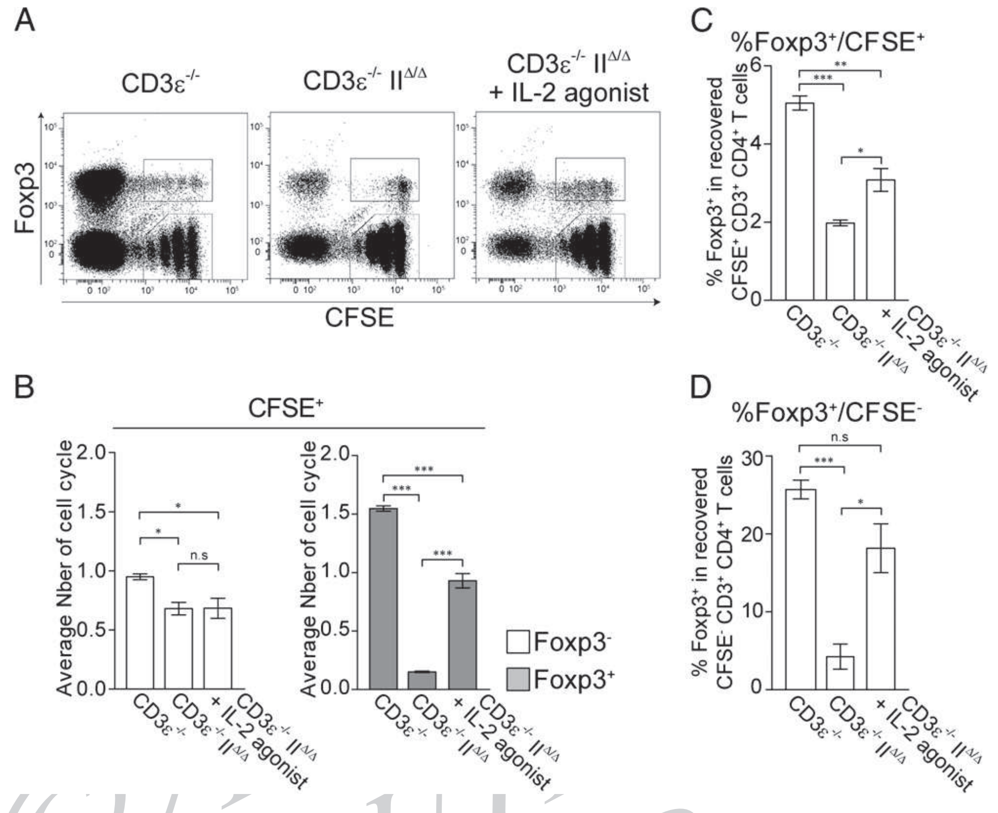

FIGURE 5. IL-2 is able to induce the proliferation of regulatory CD4+ T cells independently of self-recognition. CFSE-labeled LN CD4+ T cells (5×10^6) from C57BL/6 mice were injected into C57BL/6 CD3ε$^{-/-}$ mice and C57BL/6 CD3ε$^{-/-}$ II$^{\Delta/\Delta}$ mice treated or not treated with IL-2/anti–IL-2 complexes. Fourteen days after transfer, LNs and spleen were recovered and pooled, and single-cell suspensions were prepared. (**A**) Foxp3/CFSE dot plots of CD4+ CD3+ CD8− T cells recovered from representative recipient mice 14 d after transfer. (**B**) Average number of cell cycles of CFSE+ Foxp3+ CD4+ CD3+ CD8− T cells and CFSE+ Foxp3− CD4+ CD3+ CD8− T cells 14 d after transfer. (**C**) Proportion of CFSE+ CD3+ CD4+ CD8− T cells expressing Foxp3. Results are shown as means ± SEM for two independent experiments with at least three mice/group. (**D**) Proportion of CFSE− CD3+ CD4+ CD8− T cells expressing Foxp3. Results are shown as means ± SEM for two independent experiments with at least three mice/group.

jected with conventional T cells (Fig. 6C, 6D). Thus, the presence of conventional T cells at the moment of the transfer significantly increased the extent of regulatory T cell homeostatic proliferation, strongly suggesting that conventional CD4+ T cells are providing the resources (IL-2) required for this process.

Discussion

Several situations lead to T cell lymphopenia, such as chemotherapy, radiotherapy, and viral infections. In the last decade, several groups, including our own, have tried to decipher the mechanisms that could help to replenish the peripheral T cell pool after a lymphopenic episode. On one hand, it was shown that the

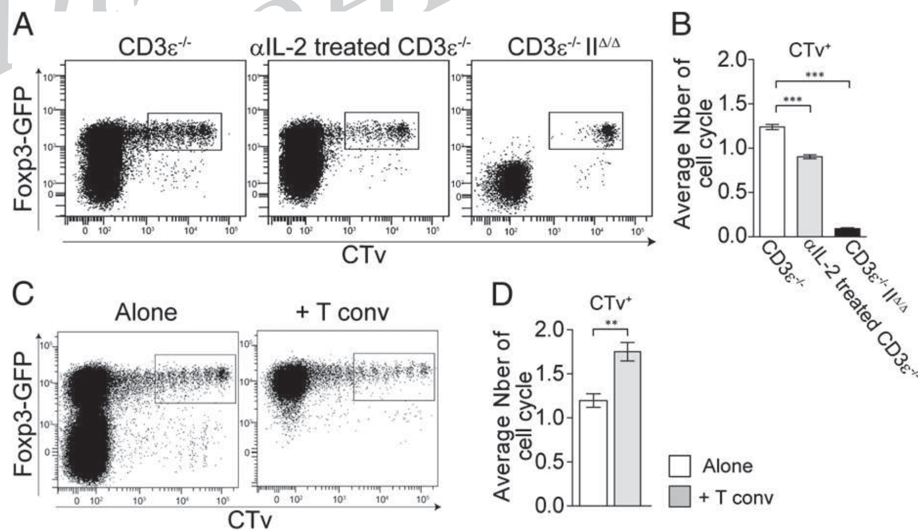

FIGURE 6. Regulatory CD4+ T cells need help from conventional CD4+ T cells to undergo homeostatic proliferation in response to lymphopenia. A total of 1×10^6 Foxp3-GFP+ CD4+ T cells from the LNs of C57BL/6 Foxp3-GFP mice was FACS sorted, labeled with CTv, and injected i.v. into lymphopenic C57BL/6 CD3ε$^{-/-}$ II$^{\Delta/\Delta}$ mice and C57BL/6 CD3ε$^{-/-}$ mice treated or not treated with IL-2–anti-blocking Abs. (**A**) Foxp3-GFP/CTv dot plots of CD4+ CD3+ CD8− T cells recovered from representative recipient mice 14 d after transfer. (**B**) Average number of cell cycles of recovered CTv+ Foxp3-GFP+ CD4+ CD3+ CD8− T cells, 14 d after transfer. Results are shown as means ± SEM for two independent experiments with at least three mice/group. A total of 1×10^6 Foxp3-GFP+ CD4+ T cells from the LNs of C57BL/6 Foxp3-GFP CD45.2 mice was FACS sorted, labeled with CTv, and injected i.v. alone or with 5×10^6 CD25− CD4+ T cells from normal C57BL/6 CD45.1 mice into lymphopenic CD45.1 C57BL/6 CD3ε$^{-/-}$ mice. Fourteen days after transfer, LNs and spleen were recovered and pooled, and single-cell suspensions were prepared. (**C**) Foxp3-GFP/CTv dot plots of CD45.2+ CD4+ CD3+ CD8− T cells recovered from representative recipient mice 14 d after transfer. (**D**) Average number of cell cycles of recovered CTv+ Foxp3-GFP+ CD45.2+ CD4+ CD3+ CD8− T cells 14 d after transfer. Results are shown as means ± SEM for two independent experiments with at least three mice/group.

bulk of naive T cells proliferate slowly in response to lymphopenia. Such a process has been termed "homeostatic proliferation," and it relies on both TCR signaling and the greater availability of IL-7 found in lymphopenic environments (13, 37, 38); these are the two main factors ensuring naive T cell survival in a nonlymphopenic environment (10). On the other hand, in the case of profound lymphopenia, some naive $CD4^+$ T cells are able to proliferate and expand strongly, a process known as spontaneous proliferation, which relies primarily on interactions with MHC class II molecules (18, 31).

In contrast, little is known about the factors mediating the proliferation of regulatory $CD4^+$ T cells in response to lymphopenia. We show that IL-7 is not involved in the spontaneous proliferation of both regulatory and conventional $CD4^+$ T cells in response to lymphopenia. Furthermore, although IL-7 is crucial for naive $CD4^+$ T cell homeostatic proliferation in response to lymphopenia, it has minimal impact on the homeostatic proliferation of regulatory $CD4^+$ T cells. These results agree with previous data showing that the maintenance of the regulatory $CD4^+$ T cell pool in the periphery does not require IL-7 (39). Nevertheless, as shown by us (Fig. 3) and other investigators (40), IL-7 is able to significantly increase regulatory $CD4^+$ T cell survival in vitro. Thus, although regulatory $CD4^+$ T cells express very low surface levels of IL-7R, they are still able to integrate IL-7–mediated signals. However, the concentrations of IL-7 used in in vitro assays (10–50 ng/ml) are higher than the concentrations reached, in vivo, in a lymphopenic environment [~50 pg/ml in the serum of T cell-deficient mice (19)]. Interestingly, after injection into lymphopenic recipient mice expressing or not expressing MHC class II molecules, IL-7R surface levels on conventional $CD4^+$ T cells decreased to the levels observed on regulatory $CD4^+$ T cells but not to lower levels. Altogether, our results suggest that IL-7R expression on regulatory $CD4^+$ T cells may be too low to enable them to respond to the IL-7 levels reached in a lymphopenic environment. In line with this hypothesis, it was shown that regulatory $CD4^+$ T cells are able to proliferate in vivo in response to the high levels of IL-7 reached in IL-7–transgenic mice or in mice injected with high concentrations of exogenous IL-7/anti–IL-7 complexes (41, 42).

The data presented in this article demonstrate that IL-2, a key cytokine for the development of regulatory $CD4^+$ T cells in the thymus and their homeostasis in the periphery, is also crucial to drive their proliferation in response to lymphopenia. More precisely, the extent of both spontaneous and homeostatic proliferation of regulatory $CD4^+$ T cells in response to lymphopenia is strongly diminished by blocking IL-2 through injection of anti–IL-2 Abs. Such a conclusion may seem contradictory to previous data showing that IL-2 neutralization does not affect the lymphopenia-induced proliferation of regulatory $CD4^+$ T cells (20). However, in this study, the investigators injected few $CD4^+$ T cells (3×10^5) into lymphopenic recipients and analyzed their proliferation quite soon after transfer (4 d). In fact, they were only studying lymphopenia-induced spontaneous proliferation of regulatory T cells (homeostatic proliferation has not started after only 4 d) and, with such a protocol, the T cell compartment was far from being replenished at that time point. In the current study, by injecting 5×10^6 $CD4^+$ T cells, reconstitution was already completed 1 wk after transfer (Supplemental Fig. 1), and we studied the effect of IL-2 neutralization 1 wk later (Fig. 4). It may be that IL-2 plays a role in increasing regulatory T cell half-life once the T cell compartment has been filled, thus allowing their accumulation with time rather than being required for the proliferation process itself. Such a hypothesis fits with our previous study suggesting that IL-2 is not absolutely required for regulatory T cell spontaneous proliferation in response to lymphopenia (31), as well as with previous data demonstrating that, after reconstitution of the peripheral T cell pool, the number of regulatory T cells is indexed to the number of IL-2–producing cells (43, 44).

Cozzo et al. (24) and Hsieh et al. (25), using TCR-transgenic regulatory $CD4^+$ T cells, proposed that self-peptides drive the proliferation of regulatory $CD4^+$ T cells in response to lymphopenia. Our data showing that regulatory T cells do not undergo homeostatic proliferation after transfer into T cell-deficient mice lacking the expression of MHC class II molecules support such a conclusion. However, as proposed by Carneiro et al. (45), our data may reflect, in part, an indirect role for interactions with MHC class II molecules. Indeed, such interactions could also be required to allow conventional $CD4^+$ T cells to produce IL-2 that, in turn, drives the homeostatic proliferation of regulatory $CD4^+$ T cells. Accordingly, our data strongly suggest that regulatory $CD4^+$ T cells receive help from conventional $CD4^+$ T cells to undergo homeostatic proliferation in response to lymphopenia. Moreover, IL-2 is able to drive regulatory $CD4^+$ T cell homeostatic proliferation independently of interactions with MHC class II molecules. Indeed, injection of IL-2–agonist complexes partially restores the homeostatic proliferation of regulatory $CD4^+$ T cells transferred into recipient mice deficient for the expression of MHC class II molecules. Thus, interactions with MHC class II molecules are required for the bulk of regulatory T cells to proliferate slowly in response to lymphopenia; however, it is difficult to determine whether such interactions act directly by stimulating regulatory T cells, indirectly by promoting IL-2 production by conventional T cells, or both.

Altogether, our data suggest that, although the homeostatic proliferation of conventional $CD4^+$ T cells in response to lymphopenia is closely related to IL-7 levels, both the homeostatic and spontaneous proliferation of regulatory $CD4^+$ T cells appear to be primarily controlled by IL-2. Accordingly, IL-2 therapy in lymphopenic patients leads to increases in regulatory-like CD4 T cell counts at the expense of the conventional T cell compartment, leading to altered responses to pathogens (46–48). Conversely, administration of IL-7 in humans induces expansion of naive and memory T cell subsets (49–51) and, in some clinical trials, a relative decrease in the percentage of regulatory $CD4^+$ T cells was observed (52). Thus, on one hand, the capacity of IL-7 to augment conventional T cell proliferation with minimal concomitant regulatory T cell expansion may be clinically exploitable in the treatment of patients with lymphopenia, especially in the case of chronic viral diseases (53) or cancer immunotherapy (54). On the other hand, increased systemic IL-7 levels during lymphopenia may lead to an imbalance between the conventional and regulatory T cell compartments at the expense of regulatory T cells and may exacerbate deleterious immune reactions, such as graft-versus-host disease (55) or autoimmunity (56–58).

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Disclosures

The authors have no financial conflicts of interest.

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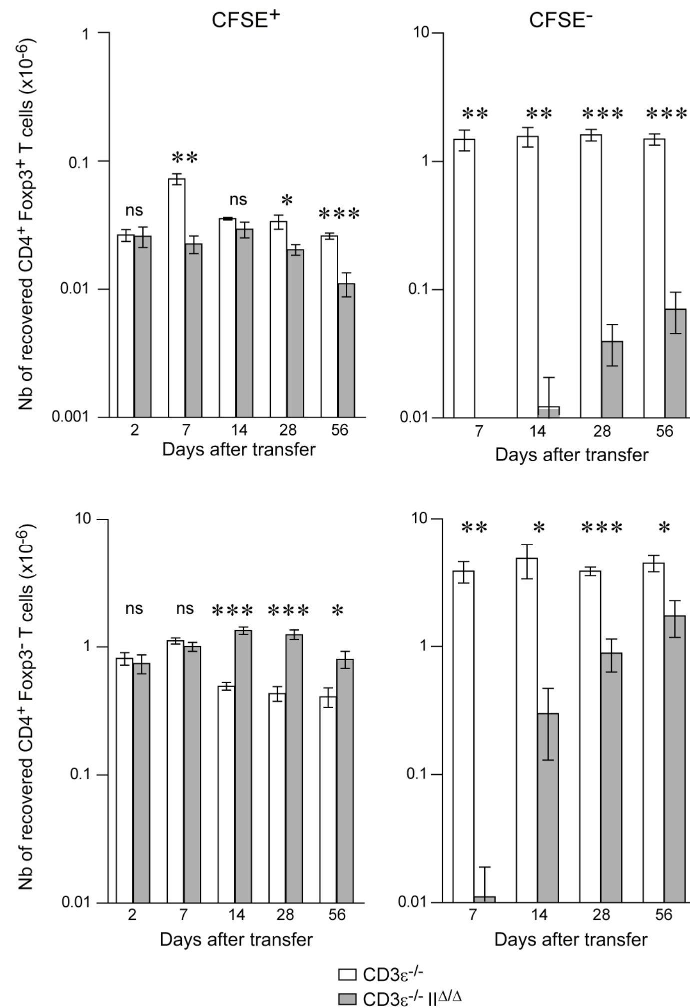
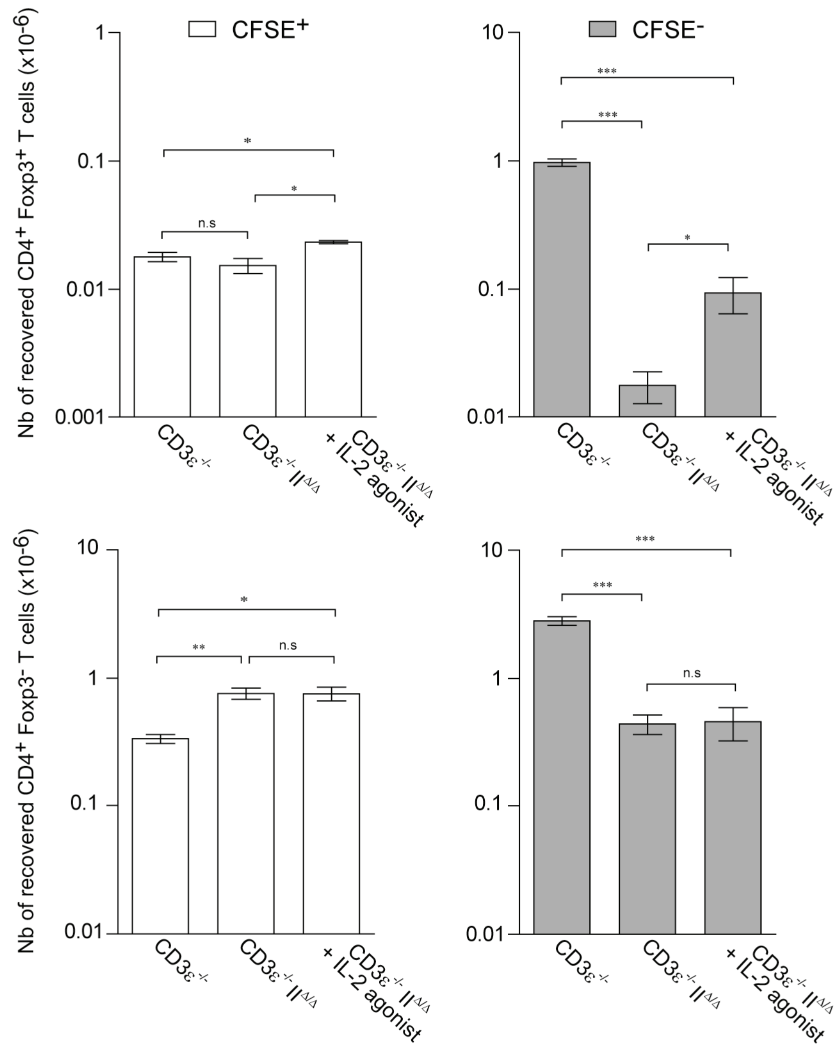


- Supplemental Figure 1 -

Supplemental Figure 1: **Absolute numbers of conventional and regulatory CD4$^+$ T-cell subsets after transfer into lymphopenic mice lacking or not lacking MHC class II molecule expression**

CFSE-labeled lymph node CD4$^+$ T cells (5 x 10^6) from C57BL/6 mice were injected intravenously into C57BL/6 CD3ε$^{-/-}$ mice and C57BL/6 CD3ε$^{-/-}$ MHC II$^{\Delta/\Delta}$ mice. At various times after transfer, lymph nodes and spleen were recovered and pooled, and single-cell suspensions were prepared. Absolute numbers of recovered CFSE$^+$ Foxp3$^+$ CD4$^+$ CD3$^+$ CD8$^-$ T cells, CFSE$^+$ Foxp3$^-$ CD4$^+$ CD3$^+$ CD8$^-$ T cells, CFSE$^-$ Foxp3$^+$ CD4$^+$ CD3$^+$ CD8$^-$ T cells and CFSE$^-$ Foxp3$^-$ CD4$^+$ CD3$^+$ CD8$^-$ T cells from indicated recipient mice 2, 7, 14 and 28 days after transfer.



- Supplemental Figure 2 -

Supplemental Figure 2: Absolute numbers of conventional and regulatory CD4$^+$ T-cell subsets after transfer into lymphopenic mice lacking or not lacking MHC class II molecule expression, treated or not treated with IL-2-anti-IL-2 complexes

CFSE-labeled lymph node CD4$^+$ T cells (5 x 10^6) from C57BL/6 mice were injected into C57BL/6 CD3ε$^{-/-}$ mice and C57BL/6 CD3ε$^{-/-}$ II$^{\Delta/\Delta}$ mice treated or not treated with IL-2-anti-IL-2 complexes. 14 days after transfer, lymph nodes and spleen were recovered and pooled, and single-cell suspensions were prepared. Absolute numbers of recovered CFSE$^+$ Foxp3$^+$ CD4$^+$ CD3$^+$ CD8$^-$ T cells, CFSE$^+$ Foxp3$^-$ CD4$^+$ CD3$^+$ CD8$^-$ T cells, CFSE$^-$ Foxp3$^+$ CD4$^+$ CD3$^+$ CD8$^-$ T cells and CFSE$^-$ Foxp3$^-$ CD4$^+$ CD3$^+$ CD8$^-$ T cells from indicated recipient mice 14 days after transfer.

Article 5 :

Differentiation into induced regulatory T cells: all naïve CD4 T cells are not created equal

Article soumis pour publication dans “Nature communication”

Bruno Martin*, Cédric Auffray*, Arnaud Delpoux, Arnaud Pommier, Céline Charvet, Philippe Yakonowsky, Hubert de Boysson, Alexandra Audemard, Bernard Malissen and Bruno Lucas

Lors de leur activation, les lymphocytes T CD4$^+$ naïfs se différencient en de multiplent types de lymphocytes T CD4$^+$ auxiliaires caractérisés par leuir production de cytokines. A l’heure actuelle, il est considéré que l’engagement vers un lignage donné est majoritairement dépendant des conditions environnementales dans lesquelles les lymphocytes T CD4$^+$ naïfs se trouvent. Nous mettons en question ici ce modèle basé sur la supposée homogénéité du compartiment lymphocytes T CD4$^+$ naïfs. Tout d’abord, les lymphocytes T CD4$^+$ naïfs peuvent être subdivisés en deux sous-populations en fonction de leur expression de Ly-6C. De plus, ces deux sous-populations ne sont pas intrinsèquement capables de se différencier de la même façon en lymphocytes T CD4$^+$ régulateurs induits. Enfin, l’analyse phénotypique et les expériences de transfert adoptif révèlent que l’expression de Ly-6C est dépendante de la reconnaissance du soi, définisant Ly-6C comme un nouveau marqueur de la réactivité au soi des lymphocytes T CD4$^+$ naïfs. L’ensemble de nos réultats montrent que les lymphocytes T CD4$^+$ naïfs ayant la plus forte avidité pour le soi sont enclins à se différencier en lymphocytes T CD4$^+$ régulateurs induits.

Differentiation into induced regulatory T cells: all naïve CD4 T cells are not created equal

by

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ABSTRACT

Upon activation, naïve CD4 T cells (CD4 T_N cells) differentiate into a variety of T-helper-cell subsets characterized by specific cytokine production. Currently, lineage commitment is considered to depend mostly on the environmental context to which CD4 T_N cells are exposed. Here, we challenge this model based on the supposed homogeneity of the CD4 T_N-cell compartment. First, peripheral CD4 T_N cells can be subdivided into two subsets according to Ly-6C expression. Second, the two newly defined subsets (Ly6C$^-$ and Ly6C$^+$ CD4 T_N cells) are not equal in their intrinsic ability to commit into the induced regulatory T-cell (iTreg) lineage. Finally, phenotypic analysis and adoptive transfer experiments revealed that Ly-6C expression depended on self-recognition, defining Ly-6C marker as a new sensor of CD4 T_N-cell self-reactivity. Altogether, our results show that CD4 T_N cells with the highest avidity for self are prone to differentiate into iTreg cells.

INTRODUCTION

In normal young adult mice, the peripheral CD4 T-cell compartment is composed of almost 80% of naïve T cells. The remaining 20% are distributed between regulatory and effector/memory phenotype CD4 T cells. Following activation by antigen presenting cells (APCs) in the periphery, naïve CD4 T cells (CD4 T_N cells) can differentiate into a variety of well documented T-helper (T_H) cell subsets, such as T_H1, T_H2, T_H17 or induced regulatory T (iTreg) cells, characterized by their cytokine production profiles and specific effector functions. For instance, T_H1 cells are characterized by their production of interferon (IFN)-γ, a potent activator of cell-mediated immunity[1,2]. T_H2 cells are characterized by production of interleukin (IL)-4, IL-5, and IL-13, which are potent activators of B-cell immunoglobulin production [1,2]. In addition, T_H17 cells are characterized by IL-17A, IL-17F and IL-22 secretion and are described to play an important role in anti-microbial responses[3,4]. Finally, iTreg cells produce TGF-β and share phenotypic and functional characteristics with natural regulatory T cells that play a crucial role in maintaining peripheral self-tolerance [5]. Identification of T-bet, GATA-3, RORγt/RORα, and Foxp3 as lineage-defining transcription factors solidified the categorization into T_H1, T_H2, T_H17 and iTreg effector cell subsets[6-10]. Additional T-helper cell subsets have also been described such as IL-10 producing type 1 regulatory T cells[11], IL-9 producing T_H9 cells[12] or T_{FH} cells[13], but their precise role, *in vivo*, still need to be better defined.

Currently, lineage commitment leads to a broad diversity of effector cell functions and is considered to depend mostly on the immunological context in which CD4 T_N cells are immersed at the time of their activation. Indeed, the differentiation decision appears predominantly governed by extrinsic factors such as cytokines or environmental signals. Specifically, *in vitro* culture assays and *in vivo* models have been used to establish that IL-12, IL-4, TGF-β alone or TGF-β in combination with pro-inflammatory cytokines such as IL-6, are crucially required for T_H1, T_H2, iTreg or T_H17 cell differentiation respectively[14-18]. This commonly accepted model implicitly posits that the CD4 T_N cells on which the various cytokines act constitute an homogeneous population of cells. However, intrinsic heterogeneity within the CD4 T_N-cell pool such as TCR affinity for antigen or sensitivity to co-stimulatory signals and cytokines should be taken into account when considering, *in vivo*, CD4 T_N-cell differentiation[19].

In light of these considerations, we decided to study whether intrinsic properties of CD4 T_N cells, in association with the cytokine environment in which they are immersed, could

contribute to helper CD4 T-cell commitment. Altogether, our results suggest strongly that CD4 T_N cells with the highest avidity for self, those receiving the strongest tonic signaling among the CD4 T_N-cell compartment, have a biased commitment toward the iTreg-cell lineage.

RESULTS

CD4 T_N cells can be subdivided into two subsets on the basis of Ly-6C expression

All along this study, CD4 T_N lymphocytes were defined as $Foxp3^-$ $CD25^-$ $CD44^{-/low}$ $CD4^+$ $CD8\alpha^-$ $TCR\beta^+$ cells. (Supplementary Fig. 1a). Whereas the CD4 T_N-cell compartment is commonly considered as homogenous, we observed, as previously described[20,21], that CD4 T_N cells from the secondary lymphoid organs of C57BL/6 Foxp3-GFP mice (peripheral and mesenteric lymph nodes and spleen) can be subdivided into 2 subsets according to Ly-6C expression (Fig. 1a, b). This applied also to CD4 T_N cells from bone marrow, Peyer's patches, blood and peritoneal cavity. Although the proportion of CD4 T_N cells varied greatly between these organs, in all of them, about two-thirds of CD4 T_N cells expressed Ly-6C (Supplementary Fig. 1b). In contrast, Ly-6C$^+$ CD4 T_N cells were indeed almost completely absent in the thymus.

Both Ly-6C$^-$ and Ly-6C$^+$ CD4 T_N cells from pLNs exhibited a true naïve phenotype illustrated by their high expression of CD45RB, CD62L and CD127 and absent expression of CD69 (Fig. 1c). According to their T_N-cell phenotype, both subsets were not able to produce IL-2, IL-4, IL-10, IL-17 or IFN-γ in response to stimulation (Fig. 1d). By contrast, memory-phenotype CD4 T cells (CD4 T_{Mem} cells) produced all of these cytokines and, as expected, regulatory CD4 T cells (CD4 T_{Reg} cells) synthesized IL-10. Finally, we observed that a similar proportion of Ly-6C$^-$ and Ly-6C$^+$ CD4 T_N cells produced TNF-α. Altogether, our data suggest that Ly-6C expression reveals phenotypic heterogeneity within the peripheral CD4 T_N-cell pool, dividing this compartment into two subsets.

Ly-6C$^-$ and Ly-6C$^+$ CD4 T_N cells exhibit distinct pathogenicity and effector commitment *in vivo*

To test whether the phenotypic heterogeneity of the CD4 T_N-cell compartment might reveal differential expansion/differentiation potentials, highly purified Ly-6C$^-$ or Ly-6C$^+$ CD4 T_N cells were transferred into T-cell deficient recipients (Fig. 2a, Supplementary Fig. 2). T-cell compromised animals injected with CD4 T_N cells are known to rapidly develop inflammatory bowel disease (IBD)[22,23]. Interestingly, mice injected with Ly-6C$^-$ CD4 T_N cells developed with time a more severe wasting disease than those injected with Ly-6C$^+$ CD4 T_N cells (Fig. 2b). This correlated with higher histological colitis scores (determined 7 weeks after transfer by examination of colon sections) when mice were injected with Ly-6C$^-$ CD4 T_N cells (Fig.

2c). Surprisingly, contrasting with clinical observations, Ly-6C$^-$ CD4 T_N cells expanded to a lesser extent than their Ly-6C$^+$ cell counterparts when transferred into T-cell deficient recipients (Fig. 2d). Differences were not only quantitative as, in this setting, Ly-6C$^-$ CD4 T_N cells gave rise to a far more greater proportion of iTreg cells as compared with Ly-6C$^+$ CD4 T_N cells (Fig. 2e). In addition, whereas Ly-6C$^+$ CD4 T_N-cell transfer led to higher proportions of IFNγ-producing CD4 T cells in secondary lymphoid organs, the differentiation of Ly-6C$^-$ CD4 T_N cells resulted in far greater proportions of newly generated IL-17$^+$ CD4 T cells (Fig. 2f). Interestingly, the proportion of IL-17$^+$ CD4 T cells was definitely correlated with the proportion of newly generated iTreg cells (Fig. 2g).

Newly generated iTreg cells promote T_H17-cell differentiation

We hypothesized that, in our model, Treg-cell generation might promote T_H17 CD4 T cell development as recently suggested in other settings[24,25]. To test this assumption, we performed co-transfer experiments in which Ly-6C$^-$ CD4 T_N cells from CD45.2 mice were co-injected with Ly-6C$^+$ CD4 T_N cells from CD45.1 mice into T-cell deficient recipients (Fig. 3a). T-cell deficient mice injected with Ly-6C$^-$ or Ly-6C$^+$ CD4 T_N cells alone were used in parallel as control mice (Supplementary Fig. 3a).

Mice co-injected with both Ly-6C$^-$ and Ly-6C$^+$ CD4 T_N cells developed a wasting disease and colitis as severe as mice injected with Ly-6C$^-$ CD4 T_N cells alone (Supplementary Fig. 3b, c). When both cell subsets were injected together, Ly-6C$^-$ CD4 T_N cells still gave rise to a higher proportion of newly differentiated iTreg cells than their Ly-6C$^+$ cell counterparts (Fig. 3b). By contrast, in all secondary lymphoid organs studied, neither expansion nor T_H17/T_H1 differentiation appeared significantly different between both transferred cell subsets (Fig. 3b, c). This last result suggests that newly differentiated iTreg cells regulate the expansion potential of transferred cells and determine the T_H17/T_H1-cell differentiation balance. We then analyzed the correlations between the ability of Ly-6C$^-$ CD4 T_N cells to differentiate into iTreg cells and the capacity of co-injected Ly-6C$^-$ and Ly-6C$^+$ CD4 T_N cells to give rise to IL-17-producing CD4 T cells (Supplementary Fig. 3d). Interestingly, the proportion of IL-17-producing CD4 T cells arising from initially injected Ly-6C$^-$ and Ly-6C$^+$ CD4 T_N cells similarly correlated with the percentage of iTreg cells generated from CD45.2$^+$ Ly-6C$^-$ CD4 T_N cells. Finally, we conducted iTreg cell depletion experiments. More precisely, Ly-6C$^-$ CD4 T_N cells were injected into T-cell deficient recipients treated or not with anti-CD25 Ab (Fig. 3d). Anti-CD25 Ab treatment resulted in reduction of colitis severity and complete inhibition

of T_H17-cell development. Taken together, all these data strongly suggest that, in our experimental model, iTreg-cell differentiation promotes T_H17-cell generation.

Ly-6C$^-$ CD4 T_N cells differentiate more effectively into iTreg cells than Ly-6C$^+$ CD4 T_N cells *in vitro*

Ly-6C$^-$ CD4 T_N cells are more efficient in differentiating into iTreg cells than Ly-6C$^+$ CD4 T_N cells *in vivo* when transferred into a lymphopenic environment. To define whether this characteristic feature was an intrinsic property of Ly-6C$^-$ CD4 T_N cells, we performed iTreg-cell polarization assays *in vitro*. Purified Ly-6C$^-$ or Ly-6C$^+$ CD4 T_N cells were stimulated with anti-CD3 and anti-CD28 coated antibodies in the presence of graded doses of TGFβ1. For suboptimal doses of exogenous TGFβ1, Ly-6C$^-$ CD4 T_N cells gave rise to a 2-fold higher proportion of iTreg cells than Ly-6C$^+$ CD4 T_N cells did (Fig. 4a). Two groups have recently described that suboptimal activation of CD4 T_N cells in the absence of exogenous TGFβ led to their conversion into iTreg cells[26,27]. In this setting, a more efficient conversion was also observed in Ly-6C$^-$ CD4 T_N-cell cultures (Fig. 4b). Taken together, our data strongly suggest that Ly-6C$^-$ CD4 T_N cells have an intrinsic ability to efficiently differentiate into iTreg cells.

Ly-6C expression by CD4 T_N cells is acquired in the periphery and is modulated by interactions with MHC class II molecules

We then decided to determine where and when Ly-6C expression was acquired by CD4 T_N cells. To follow thymic output and specifically identify recent thymic emigrants in secondary lymphoid organs, we used RAG2p-GFP transgenic mice, in which GFP expression is driven by the recombination activating gene 2 promoter (RAG2p). The multicopy transgene generates a bright GFP signal during thymic differentiation that remains detectable in the periphery throughout the first few days after their migration[28]. Whereas most CD4 T_N single-positive thymocytes were Ly-6C$^-$, about 40% of CD4 T_N recent thymic emigrants (GFP^{hi}) in lymph-nodes already expressed Ly-6C molecule, suggesting that the acquisition of Ly-6C expression by CD4 T_N cells occurs very rapidly after their exit from the thymus (Fig. 5a). Furthermore, the proportion of Ly-6C$^+$ cells among peripheral CD4 T_N lymphocytes increased with GFP brightness decay, indicating that acquisition of Ly-6C expression occurred in a rapid and gradual manner over time.

Then, we injected Ly-6C$^-$ CD4 T_N single-positive thymocytes from CD45.2 mice into CD45.1 mice expressing or lacking MHC class II molecule expression (Fig. 5b). As expected, Ly-6C$^-$ CD4 T_N single-positive thymocytes gave rise to both Ly-6C$^-$ and Ly-6C$^+$ CD4 T_N cells after transfer into MHC class II-competent recipients. More surprisingly, all CD45.2$^+$ CD4 T cells recovered from MHC class II-deficient recipients were expressing Ly-6C, suggesting that the down-modulation of Ly-6C at the surface of CD4 T_N cells was either dependent on the strength of self-recognition or that survival of Ly-6C$^-$ CD4 T_N cells was highly compromised in the absence of MHC class II molecule expression.

To address these issues, peripheral Ly-6C$^-$ or Ly-6C$^+$ CD4 T_N cells from CD45.2 mice were transferred into CD45.1 mice expressing or lacking MHC class II molecule expression (Fig. 5c). The survival of both Ly-6C$^-$ and Ly-6C$^+$ CD45.2$^+$ CD4 T_N cell subsets was affected by MHC class II molecule deprivation excluding the hypothesis of a preferential disappearance of Ly-6C$^-$ CD4 T_N cells in MHC class II-deficient recipients. Ly-6C expression by CD45.2$^+$ CD4 T_N cells was also assessed. First, we noticed that Ly-6C$^-$ and Ly-6C$^+$ phenotypes were stable over time after transfer into MHC class II-competent recipients. By contrast, almost all peripheral CD45.2$^+$ CD4 T_N cells recovered from MHC class II-deficient recipients expressed Ly-6C whether or not they were expressing it before transfer. Altogether, these results strongly suggest that the down-modulation of Ly-6C expression by CD4 T_N cells was highly dependent on continuous interactions with MHC Class II molecules.

Self-recognition reinforces the ability of CD4 T_N cells to differentiate into iTreg cells

Our data suggest that Ly-6C surface levels on CD4 T_N cells might predict their propensity to interact with peripheral self-MHC class II molecules. To address this issue, we analyzed the expression by peripheral Ly-6C$^-$ and Ly-6C$^+$ CD4 T_N cells of surface molecules such as the TCR itself, CD4 or CD5 which expression on naïve T cells is modulated by their ability to interact with self. In particular, CD5 expression has been clearly shown to be adjusted to reflect TCR contact with self[29,30]. In fact, the more a CD4 T_N cell interacts with self, the more this cell expresses CD5. Consistent with our hypothesis, we observed that Ly-6C$^-$ CD4 T_N cells exhibited significantly higher density of CD5 and lower levels of the TCRβ chain and CD4 compared to their Ly-6C$^+$ cell counterparts (Fig. 6a).

We then determined whether self-recognition might play a role in the differential ability of Ly-6C$^-$ and Ly-6C$^+$ CD4 T_N cells to differentiate into iTreg cells. First, we studied the expression of CD5 and Ly-6C by peripheral monoclonal CD4 T_N cells from AND and

Marylin TCR-transgenic mice. CD5 expression by AND CD4 T cells has been described as bright when compared to polyclonal CD4 T cells whereas Marylin CD4 T cells have been shown to express slightly lower CD5 surface levels than polyclonal CD4 T cells suggesting that AND CD4 T cells have a higher avidity for self than marylin CD4 T cells[31,32]. As expected, AND CD4 T cells expressed far higher density of CD5 than Marylin CD4 T cells (Fig. 6b). In agreement with our assumption that Ly-6C non-expression might reflect the magnitude of CD4 T_N cell self-reactivity, all Marylin CD4 T cells were expressing high surface amounts of Ly-6C whereas the majority of AND CD4 T cells did not (Fig. 6b). Finally, in lines with our experiments with polyclonal CD4 T_N cells, we observed that AND CD4 T cells differentiated more efficiently than Marilyn CD4 T cells into iTreg cells *in vitro* (Fig. 6c).

Second, we adoptively transferred Ly-6C$^-$ and Ly-6C$^+$ CD4 T_N cells from CD45.2 mice into CD45.1 recipients expressing or lacking MHC class II molecules (Fig. 6d). Three weeks after transfer, CD45.2$^+$ CD4 T_N cells were purified from the periphery of recipient mice and assessed for their ability to differentiate into iTreg cells *in vitro*. For suboptimal doses of TGFβ1, cells purified from MHC II-competent recipients initially injected with Ly-6C$^-$ CD4 T_N cells gave rise to a 2-fold higher proportion of iTreg cells than cells derived from MHC class II-deficient recipients initially injected with the same cells or from MHC II-competent recipients initially injected with Ly-6C$^+$ CD4 T_N cells (Fig. 6d, e). Altogether, our data strongly suggest that the ability of a naïve T cell to commit into the iTreg-cell lineage pathway upon *in vitro* stimulation is shaped by its capacity to interact with self in its original environment.

Gene expression profiling of Ly-6C$^-$ and Ly-6C$^+$ CD4 T_N cells

To further compare Ly-6C$^-$ and Ly-6C$^+$ CD4 T_N cells, we obtained Affymetrix gene expression profiles from both CD4 T_N-cell subsets directly isolated from peripheral LNs of C57BL/6 Foxp3-GFP mice (Fig. 7). Only few genes were significantly differentially expressed between the two types of CD4 T_N cells (Fig. 7a). Interestingly, we found that transcription of several genes characteristically expressed in Treg cells such as Ctla4, Folr4, Cd200 or Il2rb (CD122) were up-regulated in Ly-6C$^-$ CD4 T_N cells when compared to Ly-6C$^+$ CD4 T_N cells. By comparing CD4 T-cell effectors with naïve CD4 T cells, Wei et al. have recently defined the transcriptional signature of the main CD4 T_H-cell subsets such as *ex vivo* peripheral Treg cells and *in vitro* induced Treg cells, T_H1, T_H2 and T_H17 cells[33]. Comparison

of our gene list with these cell signatures revealed that the differences in gene expression observed between the two types of CD4 T_N cells only correlated significantly with the *ex vivo* Treg-cell and *in vitro* induced Treg-cell signatures (Fig. 7B). More precisely, the expression of 26 genes out of the 73 differentially transcribed between Ly-6C$^-$ and Ly-6C$^+$ CD4 T_N cells were found to be part of the transcriptional signature of peripheral Treg cells. In line with the correlation observed between the Ly-6C$^-$ CD4 T_N-cell signature and the Treg-cell signature, 16 out of 17 of the genes defined by Wei et al. as up-regulated in Treg cells were also up-regulated in Ly-6C$^-$ CD4 T_N cells when compared with Ly-6C$^+$ CD4 T_N cells (Fig. 7c). Similarly, 7 out of 9 of the genes that are normally down-regulated in Treg cells were also down-regulated in Ly-6C$^-$ CD4 T_N cells.

We then validated these results at the protein level by flow cytometry (Fig. 7d). Expression levels of CD122, FolR4, CD200, CD73 and ICOS by Ly-6C$^-$ and Ly-6C$^+$ CD4 T_N cells were analyzed and compared to that observed in regulatory CD4 T cells. As expected, we found that all these cell-surface molecules were highly expressed by Treg cells. In line with our microarray analysis, Ly-6C$^-$ CD4 T_N cells were also expressing higher amounts of CD122, FolR4, CD200, CD73 and ICOS than their Ly-6C$^+$ CD4 T_N cell counterparts at the protein level. More precisely, Ly-6C$^-$ CD4 T_N cells seemed to exhibit an intermediate phenotype between Ly-6C$^+$ CD4 T_N cells and regulatory CD4 T cells. Since we noticed that Ly-6C$^-$ CD4 T_N cells are receiving more TCR signals from self-recognition than their Ly-6C$^+$ cell counterparts, we thus wondered whether autoreactivity might shape the phenotype of Ly-6C$^-$ CD4 T_N cells. Ly-6C$^-$ CD4 T_N cells were subdivided into 2 subsets according to CD5 expression (Fig. 7e). Using this gating strategy, we observed that the Ly-6C$^-$ CD4 T_N cells expressing the highest level of CD5 were also expressing the highest levels of CD122, FolR4, CD200, CD73 and ICOS. Thus, the more a cell receives signals from self-recognition, the more this cell expresses high levels of molecules known to be overexpressed by Treg cells. Altogether, our results indicate that self-recognition induces the expression of regulatory T cell markers by Ly-6C$^-$ CD4 T_N cells.

DISCUSSION

Over the last decades, the CD4 T_N cells have been commonly considered as a homogenous T cell compartment. In the present paper, we found that Ly-6C expression splits this compartment into positive and negative cells with one third of peripheral CD4 T cells lacking this marker. Expression of Ly-6C is acquired in the periphery as all thymocytes are Ly-6C$^-$. More precisely, analysis of RAG2p-GFP mice and adoptive transfer experiments have allowed us to show that thymocytes rapidly gained Ly-6C expression upon migration to the periphery. Importantly, the expression of Ly-6C by peripheral CD4 T_N cells is modulated by their ability to interact with self. First, thymocytes transferred into MHC class II-deficient recipients all acquired Ly-6C whereas part of them remained negative after transfer into MHC class II-competent recipients. Second, the expression of Ly-6C by peripheral CD4 T_N cells is stable over time only in a MHC class II$^+$ environment. Finally, Ly-6C$^-$ CD4 T_N cells significantly expressed more CD5 (which expression has been clearly shown to reflect self-reactivity in naïve T cells[29,30]) than Ly-6C$^+$ CD4 T_N cells. Thus, Ly-6C can be considered as a previously unrecognized sensor of T-cell self-reactivity in CD4 T_N cells with Ly-6C$^-$ cells being more "autoreactive" and integrating more signals from self-recognition than their Ly-6C$^+$ cell counterparts. Together with CD5 and nur-77[29,30,34], Ly-6C expression may help discriminating between lowly and highly autoreactive cells in the CD4 T cell compartment. Ly-6C has 2 advantages over the 2 previously described sensors of self-reactivity. It is expressed at the cell surface unlike the transcription factor nur-77 and its expression in CD4 T_N cells is bimodal unlike CD5 and nur-77 allowing a clear dichotomization of the CD4 T_N-cell compartment.

Repeated subthreshold TCR stimulations are triggered within secondary lymphoid organs where T cells interact with self-peptides while scanning the surface of dendritic cells. Stefanova et al. have first shown that interruption of CD4 T_N cell contact with self leads to a rapid decline on signaling and response sensitivity to foreign stimuli[35]. These data were further confirmed by several elegant studies[36-38]. Recently, using CD5 expression to discriminate between T_N cells interacting strongly (CD5^{hi}) or not (CD5^{lo}) with self, several groups have shown that TCR contact with self in the periphery causes CD8, but not CD4, T_N cells to be hypersensitive to IL-2, IL-7 and IL-15[39-41]. Altogether, these results strongly suggest that self-recognition and the resulting tonic signaling in T_N cells not only allow their survival but also increase quantitatively their responsiveness towards their cognate antigens for both CD4 and CD8 T_N cells and towards interleukins in the case of CD8 T_N cells[42].

However, it has not been yet addressed whether self-recognition qualitatively affects the response of T_N cells to stimulation. Here, we show that the ability of a CD4 T_N cell to differentiate into an iTreg cell upon apropriate stimulation is increased by self-recognition perceived by this cell prior to its activation. Indeed, the most self-reactive CD4 T_N cells (Ly-6C$^-$ CD4 T_N cells) are the cells that differentiate the more efficiently into iTreg cells both *in vitro* and *in vivo*. Moreover, disruption of interactions with MHC class II molecules decreases the commitment efficacy of Ly-6C$^-$ CD4 T_N cells toward the iTreg-cell lineage. Indeed, the iTreg-cell polarization potential of Ly-6C$^-$ CD4 T_N cells parked for 3 weeks in MHC class II-deficient recipients dropped to the level observed in Ly-6C$^+$ CD4 T_N cells which own potential is not affected by MHC deprivation. Thus, self-recognition affects not only quantitatively but also qualitatively the response of CD4 T_N cells to their cognate antigens.

Our findings are not contradictory to the present main theory assuming that CD4 T_N-cell differentiation upon stimulation depends on extrinsic factors such as the cytokines present in their environment at the time of their activation but they extend it by showing that intrinsic factors, such as the avidity of the TCR for self, also play a role in CD4 T_N-cell fate determination. 15 years ago, several studies have proposed that the strength of TCR signal at the time of antigen recognition would play a role in the Th1/Th2 lineage choice[43,44]. More recently, Gottschalk et al. have shown that a low dose of a strong agonist resulted in maximal induction of iTreg cells[45]. Thus, differentiation of CD4 T_N cells into iTreg cells upon stimulation nonetheless relies on extrinsic factors such as the presence of TGFβ in their environment but also on intrinsic factors such as the avidity of the TCR they express for both self and foreign ligands.

Upon activation, a CD4 T_N cell can differentiate into a great variety of effector cells including T_H1, T_H2, T_H17 or iTreg cells. The present theory assumes that the choice made by CD4 T_N-cells to commit into one of these lineage pathways would be mainly governed by extrinsic factors such as the cytokines released in the environment at the time of their activation. Our data extend this theory by showing that self-reactivity enhances the potential of CD4 T_N cells to differentiate into iTreg cells. Indeed, our data clearly show that the more a CD4 T_N cell is autoreactive, the more this cell would differentiate efficiently into an iTreg cell upon appropriate stimulation. In our experimental *in vivo* setting, injection of Ly-6C$^-$ CD4 T_N cells into T-cell deficient recipients leads to enhanced pathogenicity when compared to the transfer of Ly-6C$^+$ CD4 T_N cells. As demonstrated recently in other settings[24,25], we show that iTreg cells also promote Th17-cell development in the IBD experimental model. As Ly-6C$^-$ CD4 T_N

cells differentiate efficiently into iTreg cells after transfer into T-cell deficient recipients, the observed concomitant rise in Th17 cell production may exacerbate the disease. However, another explanation may be that Ly-6C$^-$ CD4 T_N cells induce a more severe IBD than their Ly-6C$^+$-cell counterparts because of an increased frequency of pathogenic precursors in this subset due to its high self-reactivity. In other settings, in particular in lymphoreplete animals, the enhanced ability of the most autoreactive CD4 T_N cells to commit to the iTreg-cell lineage pathway may represent a previously undescribed mechanism of self-tolerance. This process would diminish the risk of the most self-reactive CD4 T cells to become deleterious effector cells through responding to bacteria, viruses or parasites. Indeed, such a mechanism would allow the organism to augment its own protection during each infectious episode, especially by avoiding molecular mimics to induce novel, potent and self-peptide reactive CD4 T cell effectors when T cell responses raised against the pathogen cross-react with self-peptides.

ONLINE METHODS

Mice

C57BL/6 mice (CD45.2) were obtained from Charles River Laboratories. C57BL/6 CD45.1 mice, C57BL/6 CD3$^{-/-}$ mice, C57BL/6 MHC II$^{\Delta/\Delta}$ CD45.1 mice, C57BL/6 AND TCR-transgenic RAG2$^{-/-}$ mice were maintained in our own animal facilities, under specific pathogen-free conditions. C57BL/6 Marilyn TCR-transgenic RAG2$^{-/-}$ mice were provided by Dr. Emmanuel Donnadieu, Institut Cochin, Paris, France. C57BL/6 Foxp3-GFP CD45.2 mice were initially obtained from Dr. Bernard Malissen, Centre d'Immunologie de Marseille-Luminy, France [1]. They were then crossed with C57BL/6 CD45.1 mice to generate C57BL/6 Foxp3-GFP CD45.1 mice. C57BL/6 RAG2p-GFP reporter mice [2] were provided by Dr. Antonio Bandeira, Unité du Développement des Lymphocytes, Institut Pasteur, Paris, France. Experiments were carried out in accordance with the guidelines of the French Veterinary Department.

Cell suspensions

Peripheral lymph nodes (pLNs), mesenteric lymph nodes (mLNs), spleen and thymus were homogenized and passed through a nylon cell strainer (BD Falcon) in RPMI 1640 Glutamax (Gibco) supplemented with 10% fetal calf serum (FCS; Biochrom) for adoptive transfer (LNs only), or in 5% FCS, 0.1% NaN3 (Sigma-Aldrich) in phosphate-buffered saline, for flow cytometry.

Adoptive transfer of CD4 T_N cells

CD4 T cells were purified from LNs (pooled superficial cervical, axillary, brachial, inguinal, and mesenteric LNs) or thymi of C57BL/6 Foxp3-GFP mice by incubating cell suspensions on ice for 20 minutes with a mixture of anti-CD8 (53-6.7), anti-CD11b (Mac-1) and anti-CD19 (1D3) Abs obtained from hybridoma supernatants, and then with magnetic beads coupled to anti-rat immunoglobulins (Dynal Biotech). Purified CD4 T cells were then labeled with biotinylated anti-Ly-6C (AL21), PE-conjugated anti-CD25 (PC61), APC-conjugated anti-CD44 (IM7), all from BD biosciences, and Pacific Blue-conjugated streptavidin (Invitrogen). Ly-6C$^-$ and Ly-6C$^+$ CD4 T_N cells were flow cytometry sorted as GFP$^-$ CD25$^-$ CD44$^{-/lo}$ cells using a FACS-ARIA3 flow cytometer (BD Biosciences), and injected intravenously into sex-matched recipient mice.

Cell surface staining and flow cytometry

Cell suspensions were collected and dispensed into 96-well round-bottom microtiter plates (Greiner Bioscience; $6x10^6$ cells/well). Surface staining was performed by incubating the cells on ice, for 15 minutes per step, with Abs in 5% FCS (Biochrom), 0.1% NaN_3 (Sigma-Aldrich) phosphate-buffered saline. Each cell-staining reaction was preceded by a 15-minute incubation with purified anti-CD16/32 antibodies (FcγRII/III block; 2.4G2) obtained from hybridoma supernatants. For determination of intracellular cytokine production, cells were stimulated with 0.5 µg/ml PMA, 0.5 µg/ml ionomycin, and 10 µg/ml BrefeldinA (all Sigma) for 2 hrs at 37°C. Cells were then stained for surface markers, fixed in 2% paraformaldehyde in PBS, and permeabilized with 0.5% saponin, followed by labeling with specific cytokine Abs.

PerCP-conjugated anti-CD4 (RM4-5), PE-conjugated anti-CD5 (53-7.3), anti-CD25 (PC61), anti-CD45.1 (A20), anti-CD69 (H1.2F3), anti-TCRβ (H57-597), anti-FolR4 (TH6), anti-CD122 (TM-β1), anti-IL-2 (JES6-5H4), anti-IL-4 (11B11), anti-IL-10 (JES5-16E3), anti-IL-17A (TC11-18H10), APC-conjugated anti-CD44 (IM7), anti-TCRβ (H57-597), anti-IFN-γ (XMG1.2), streptavidin, FITC-conjugated anti-TNF-α (MP6-XT22), PE-Cy7-conjugated anti-Ly-6C (AL-21), streptavidin, PerCP-Cy5.5-conjugated anti-CD45.2 (104), APC-H7-conjugated anti-CD8 (53-6.7), Pacific Blue-conjugated anti-CD4 (RM4-5), Alexa Fluor 700–conjugated anti-Ly-6C (AL-21), biotinylated anti-Ly6C (AL-21), anti-CD5 (53-6.7), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD45RB (16A), anti-CD62L (MEL14), were obtained from BD Biosciences. PE-conjugated anti-CD45.2 (104), anti-CD200 (OX90), APC-conjugated anti-ICOS (C398.4A), PE-Cy5-conjugated anti-CD25 (PC61.5), Alexa Fluor 700–conjugated anti-CD45.2 (104), PerCP-Cy5.5-conjugated anti-TCRβ (H57-597) and biotinylated anti-CD127 (A7R34), anti-CD73 (TY/11.8) were obtained from eBioscience. Pacific Blue-conjugated streptavidin was obtained from Invitrogen.

Multi-color immunofluorescence was analyzed using a BD-LSR2 cytometer (BD Biosciences). List-mode data files were analyzed using Diva software (BD Biosciences). Data acquisition and cell sorting were performed on the Cochin Immunobiology facility.

Colitis scoring

For the colitis model, colons were removed and fixed in PBS containing 10% formaldehyde. Five-micrometer paraffin-embedded sections were cut and stained with H&E and then blindly analyzed. Each segment was given a score of 0–4: grade 0, no significant changes; grade 1, minimal scattered mucosal inflammatory cell infiltrates, with or without minimal epithelial hyperplasia; grade 2, mild scattered to diffuse inflammatory cell infiltrates, sometimes

extending into the submucosa and associated with erosions, with mild to moderate epithelial hyperplasia and mild to moderate mucin depletion from goblet cells; grade 3, moderate inflammatory cell infiltrates that were sometimes transmural, with moderate to severe epithelial hyperplasia and mucin depletion; grade 4, marked inflammatory cell infiltrates that were often transmural and associated with crypt abscesses and occasional ulceration, with marked epithelial hyperplasia, mucin depletion, and loss of intestinal glands.

iTreg-cell differentiation *in vitro* assay

Flow-cytometry sorted Ly-6C$^-$ and Ly-6C$^+$ CD4 T_N cells from LNs of C57BL/6 Foxp3-GFP mice were stimulated for 4 days with immobilized anti-CD3 Ab (clone 145.2C11; 4µg/ml; obtained from hybridoma supernatants) and 4µg/ml anti-CD28 Ab (37.51; eBioscience) antibodies, in the presence of graded concentrations of exogenous recombinant-human TGFβ1 (Invitrogen).

Suboptimal T-cell activation *in vitro* assay

Flow-cytometry sorted Ly-6C$^-$ and Ly-6C$^+$ CD4 T_N cells from LNs of C57BL/6 Foxp3-GFP were stimulated for 3 days with irradiated splenocytes from C57BL/6 CD3ε$^{-/-}$ mice in the presence of graded concentrations of soluble anti-CD3 Ab (145.2C11). These experiments were conducted in the absence of addition of exogenous cytokines.

Microarray

CD4 T cells from LNs of C57BL/6 Foxp3-GFP mice were enriched as described above. Then, Ly-6C$^-$ and Ly-6C$^+$ CD4 T_N cells were flow-cytometry sorted as CD4$^+$ CD8α$^-$ TCRβ$^+$ GFP$^-$ CD25$^-$ CD44$^{-/lo}$ cells using a FACS-ARIA3 flow cytometer. Total RNA was extracted using the RNeasy Mini kit (QIAGEN). RNA quality was validated with Bioanalyzer 2100 (using Agilent RNA6000 nano chip kit). Experimental and analytical part of the microarray analysis was performed according to the MIAME standards. Amplified, fragmented and biotinylated sense-strand DNA targets were synthesized from 100 ng total RNA according to the manufacturer's protocol (Genechip Whole transcript (WT) Sense Target labelling assay kit (Affymetrix)) and hybridized to a mouse gene 1.0 ST array (Affymetrix). The stained chips were read and analyzed with a GeneChip Scanner 3000 7G and Expression Console software (Affymetrix). Raw data (.cel files) were then processed and normalized using the quantile normalization method in RMA with R package (Bioconductor). Statistical analysis was then performed with MEV software (TIGR). Microarrays were performed on the Cochin Genom'ic facility. Data discussed in this publication have been deposited in the Gene Expression

Omnibus at http://www.ncbi.nlm.nih.gov/geo/ (accession number GSE37336).

Statistics

Data are expressed as mean ± SEM, and the significance of differences between two series of results was assessed using the student's unpaired t test. Values of $p < 0.05$ were considered significant. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Correlation analyses were performed using Pearson's correlation test. Values of $p < 0.05$ were considered as statistically correlated. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

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AUTHOR CONTRIBUTIONS

B.M., C.A. and B.L. designed experiments. B.M., C.A., A.D. A.P., C.C., P.Y., H.B., and A.A. did the experiments. B.M., C.A., B.M. and B.L wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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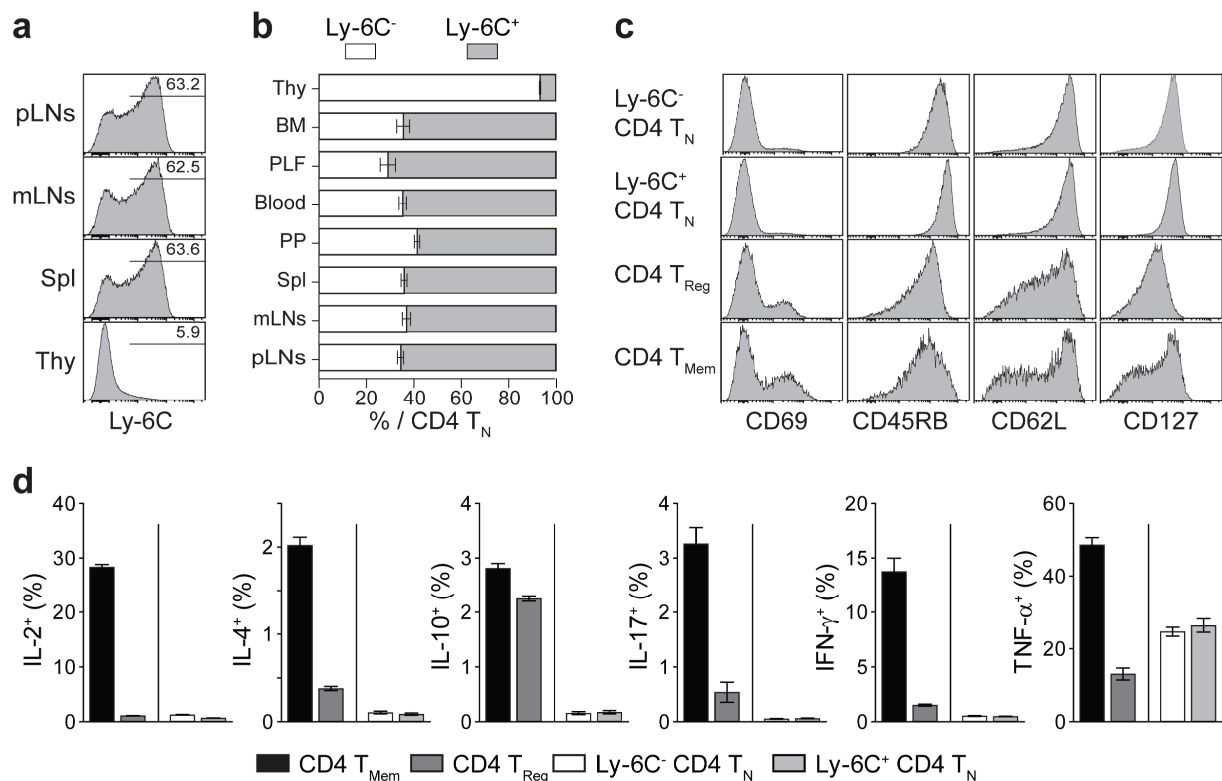


Figure 1: CD4 T_N cells can be subdivided into two subsets on the basis of Ly-6C molecule expression

(a) Ly-6C fluorescence histograms of CD4 T_N cells ($CD4^+$ $CD8\alpha^-$ $TCR\beta^+$ $Foxp3^-$ $CD25^-$ $CD44^{-/lo}$ T cells) recovered from peripheral LN (pLNs), mesenteric LN (mLNs), spleen (Spl) and thymus (Thy) are shown for a representative C57BL/6 Foxp3-GFP mouse. Numbers in each histogram represent the percentage of Ly-$6C^+$ cells among CD4 T_N cells. (b) The proportions of Ly-$6C^-$ and Ly-$6C^+$ cells among CD4 T_N cells recovered from the thymus, bone marrow (BM), peritoneal lavage fluid (PLF), blood, Peyer's patches (PP), spleen, mLNs and pLNs of C57BL/6 mice were calculated. Results are shown as means ± SEM for 6 mice from 2 independent experiments. (c) CD69, CD45RB, CD62L and CD127 fluorescence histograms of pLNs Ly-$6C^-$ CD4 T_N cells, Ly-$6C^+$ CD4 T_N cells, regulatory CD4 T cells (CD4 T_{Reg}) and memory CD4 T cells (CD4 T_{Mem} as defined in supplementary Figure 1a) are shown for a representative C57BL/6 Foxp3-GFP mouse. The histograms are representative of at least 6 mice from 3 individual experiments. (d) The proportions of IL-2, IL-4, IL-10, IL-17, IFN-γ or TNF-α producing cells among memory, regulatory, naïve Ly-$6C^-$ and naïve Ly-$6C^+$ CD4 T cells recovered from pLNs of C57BL/6 Foxp3-GFP mice were estimated after a pulse stimulation with PMA and ionomycin. Results are shown as means ± SEM for 6 mice from 2 independent experiments.

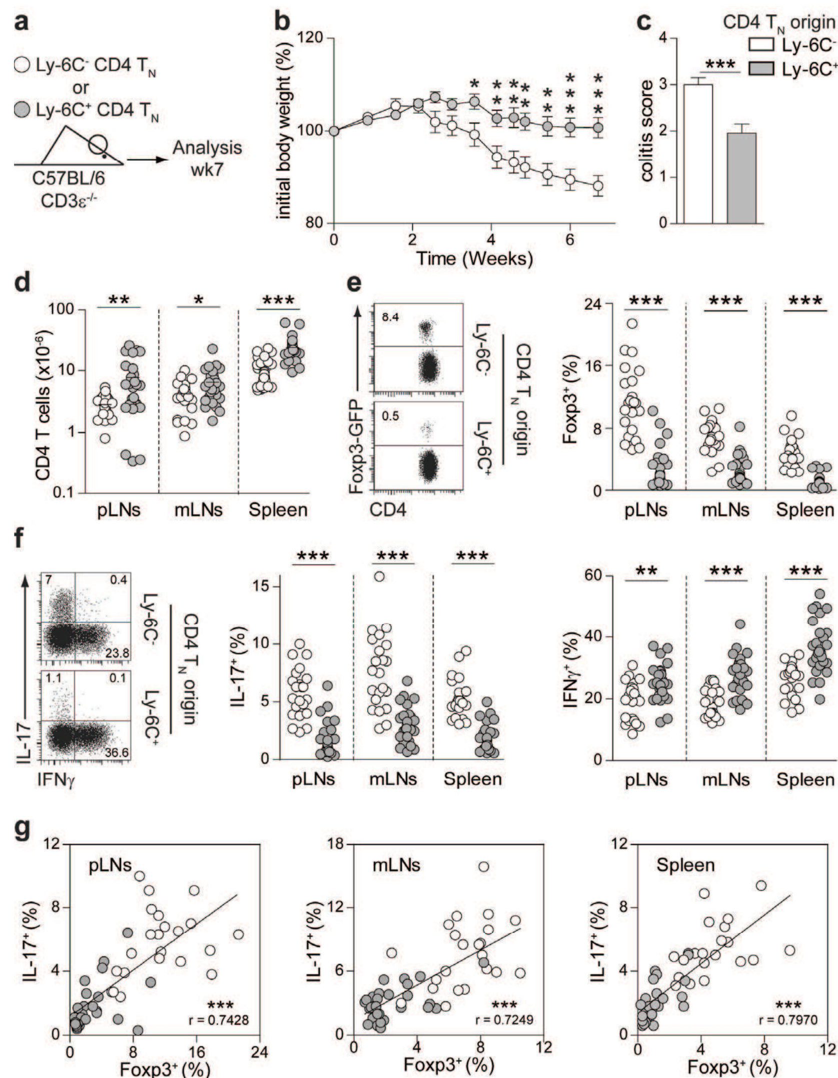


Figure 2: Differential pathogenicity of Ly-6C$^-$ and Ly-6C$^+$ CD4 T$_N$ cells after transfer into T-cell deficient recipients

0.5x10^6 flow-cytometry sorted Ly-6C$^-$ or Ly-6C$^+$ CD4 T$_N$ cells from C57BL/6 Foxp3-GFP mice were injected i.v. into C57BL/6 CD3ε$^{-/-}$ mice. Seven weeks after transfer, pLNs, mLNs and spleen were recovered separately. (a) Diagram illustrating the experimental model. (b) Body weight of recipient mice was monitored up to 7 weeks post-transfer and percentages of initial body weight were calculated and plotted. (c) Colitis scores of recipient mice were assessed 7 weeks after transfer. (d) Absolute numbers of CD4 T cells recovered from pLNs, mLNs and spleen of recipient mice. (e) Foxp3/CD4 dot-plots for gated CD4$^+$ CD8α$^-$ CD3$^+$ T cells recovered from pLNs of representative recipient mice and proportion of Foxp3$^+$ cells among CD4$^+$ CD8α$^-$ CD3$^+$ T cells recovered from pLNs, mLNs and spleen of recipient mice. (f) IL-17/IFNγ dot-plots for gated CD4$^+$ CD8α$^-$ CD3$^+$ T cells recovered from pLNs of representative recipient mice and proportion of IL-17- and IFNγ–producing CD4$^+$ CD8α$^-$ CD3$^+$ T cells recovered from pLNs, mLNs and spleen of recipient mice. (g) Correlation between the proportion of Foxp3-expressing and IL-17-producing cells among CD4$^+$ CD8α$^-$ CD3$^+$ T cells recovered from pLNs, mLNs and spleen of recipient mice. (b, c) Results are shown as means ± SEM for at least 6 mice per group per experiment, from 4 independent experiments. (d-g) Each dot represents an individual mouse.

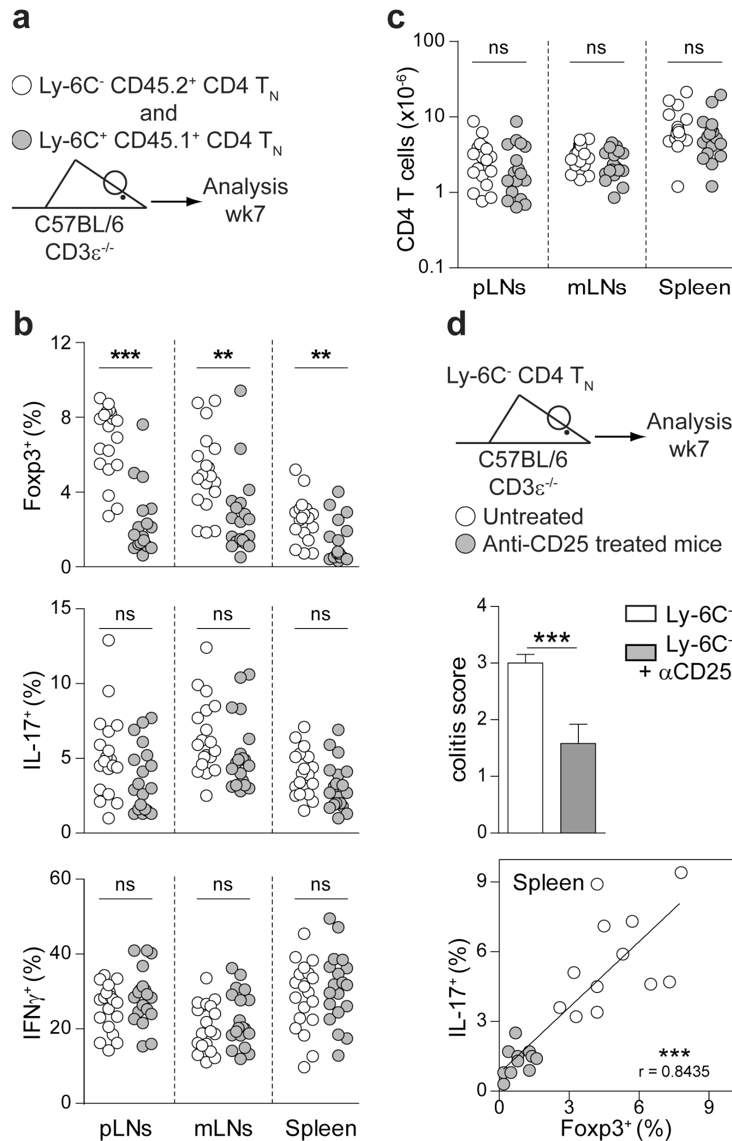


Figure 3: Newly generated iTreg cells promote T_H17-cell differentiation after CD4 T_N-cell transfer into T-cell deficient recipients

(a-c) 0.25x10^6 Ly-6C$^-$ CD4 T_N cells from C57BL/6 Foxp3-GFP CD45.2 mice were injected i.v. into C57BL/6 CD3ε$^{-/-}$ mice together with 0.25x10^6 Ly-6C$^+$ CD4 T_N cells from C57BL/6 Foxp3-GFP CD45.1 mice. Seven weeks after transfer, pLNs, mLNs and spleen were recovered separately and analyzed. (a) Diagram illustrating the experimental model. (b) Proportions of CD45.1$^+$ or CD45.2$^+$ CD4 T cells expressing Foxp3 or producing IL-17 or IFNγ recovered from pLNs, mLNs and spleen of recipient mice. (c) Absolute numbers of CD45.1$^+$ or CD45.2$^+$ CD4 T cells recovered from pLNs, mLNs and spleen of recipient mice. (d) 0.5x10^6 Ly-6C$^-$ CD4 T_N cells from C57BL/6 Foxp3-GFP mice were injected i.v. into C57BL/6 CD3ε$^{-/-}$ mice treated or not with anti-CD25 Ab. A diagram illustrating the experimental model, the colitis scores assessed 7 weeks after transfer (results are shown as means ± SEM for at least 10 mice from 2 independent experiments) and the correlation between the proportions of Foxp3-expressing and IL-17-producing cells among CD4$^+$ CD8α$^-$ CD3$^+$ T cells recovered from the spleen of recipient mice are shown. (b-d) Each dot represents an individual mouse.

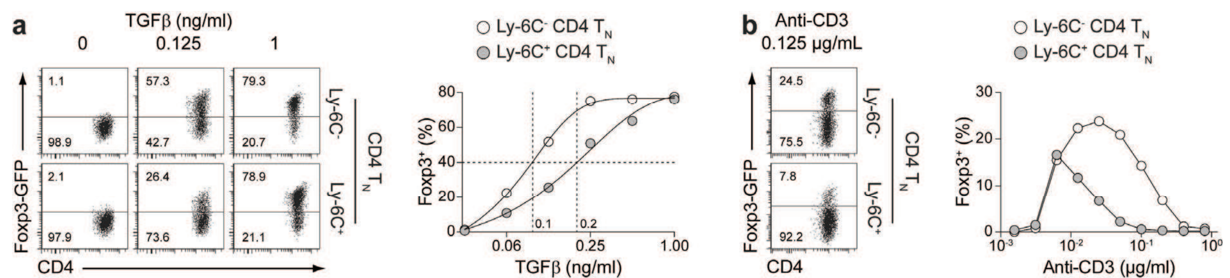


Figure 4: Ly-6C$^-$ CD4 T_N cells differentiate more efficiently into iTreg cells than Ly-6C$^+$ CD4 T_N cells *in vitro*

(a) Flow-cytometry sorted LNs Ly-6C$^-$ or Ly-6C$^+$ CD4 T_N cells from C57BL/6 Foxp3-GFP mice were stimulated for 4 days with coated anti-CD3 and anti-CD28 Abs in the presence of graded concentrations of TGFβ1. Representative Foxp3/CD4 dot-plots for gated CD4 T cells and the proportion of Foxp3$^+$ cells among CD4 T cells are shown as a function of TGFβ1 concentration. (b) Flow-cytometry sorted LNs Ly-6C$^-$ and Ly-6C$^+$ CD4 T_N cells from C57BL/6 Foxp3-GFP mice were cultured for 3 days with irradiated splenocytes from C57BL/6 CD3ε$^{-/-}$ mice in the presence of graded concentrations of soluble anti-CD3 Ab. Representative Foxp3/CD4 dot-plots for gated CD4 T cells and the proportion of Foxp3$^+$ cells among CD4 T cells are shown as a function of anti-CD3 Ab concentration.

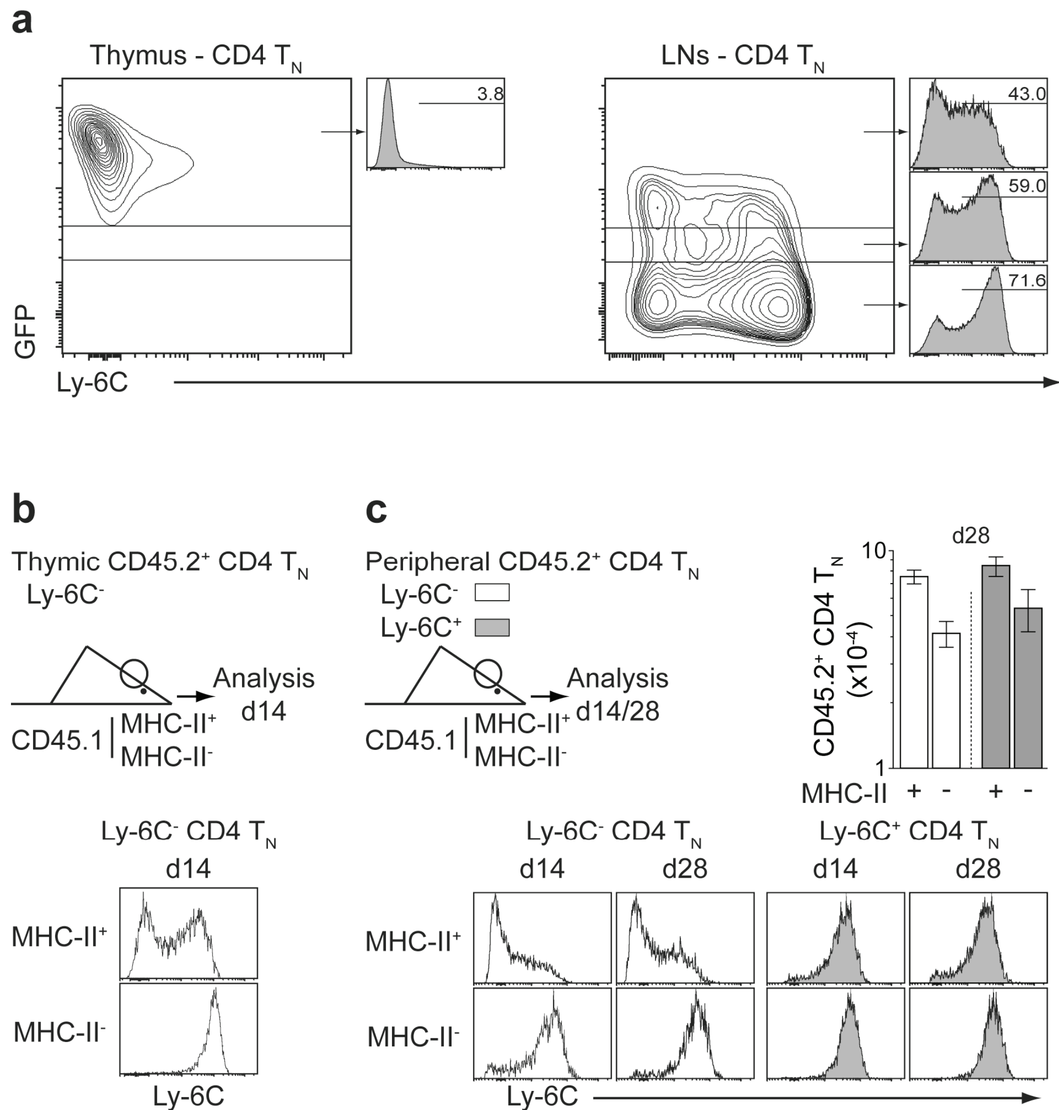


Figure 5: Ontogeny of Ly-6C⁻ and Ly-6C⁺ CD4 T_N cells

(a) GFP/Ly-6C contour-plots and Ly-6C fluorescence histograms on gated CD4 T_N cells recovered from the thymus and LNs of a representative C57BL/6 RAG2p-GFP reporter mouse. Numbers in each histogram represent the percentage of Ly-6C$^+$ cells among the indicated CD4 T_N cell subsets. (b) 1x10^6 thymic Ly-6C$^-$ CD4 T_N cells from C57BL/6 Foxp3-GFP CD45.2 mice were injected i.v. into C57BL/6 CD45.1 mice lacking (MHC-II$^-$) or not lacking (MHC-II$^+$) MHC class II molecule expression. A diagram illustrating the experimental model and Ly-6C fluorescence histograms on gated CD45.2$^+$ CD4 T_N cells recovered from LNs of recipient mice 14 days after transfer are shown. (c) 1x10^6 Ly-6C$^-$ or Ly-6C$^+$ CD4 T_N cells from LNs of C57BL/6 Foxp3-GFP CD45.2 mice were injected i.v. into CD45.1 C57BL/6 mice lacking or not lacking MHC class II molecule expression. A diagram illustrating the experimental model is shown. The absolute numbers of CD45.2$^+$ CD4 T_N cells recovered from the periphery (LNs + spleen) of recipient mice 28 days after transfer are shown as means ± SEM. Ly-6C fluorescence histograms of CD45.2$^+$ CD4 T_N cells recovered from LNs of recipient mice 14 and 28 days after transfer are shown.

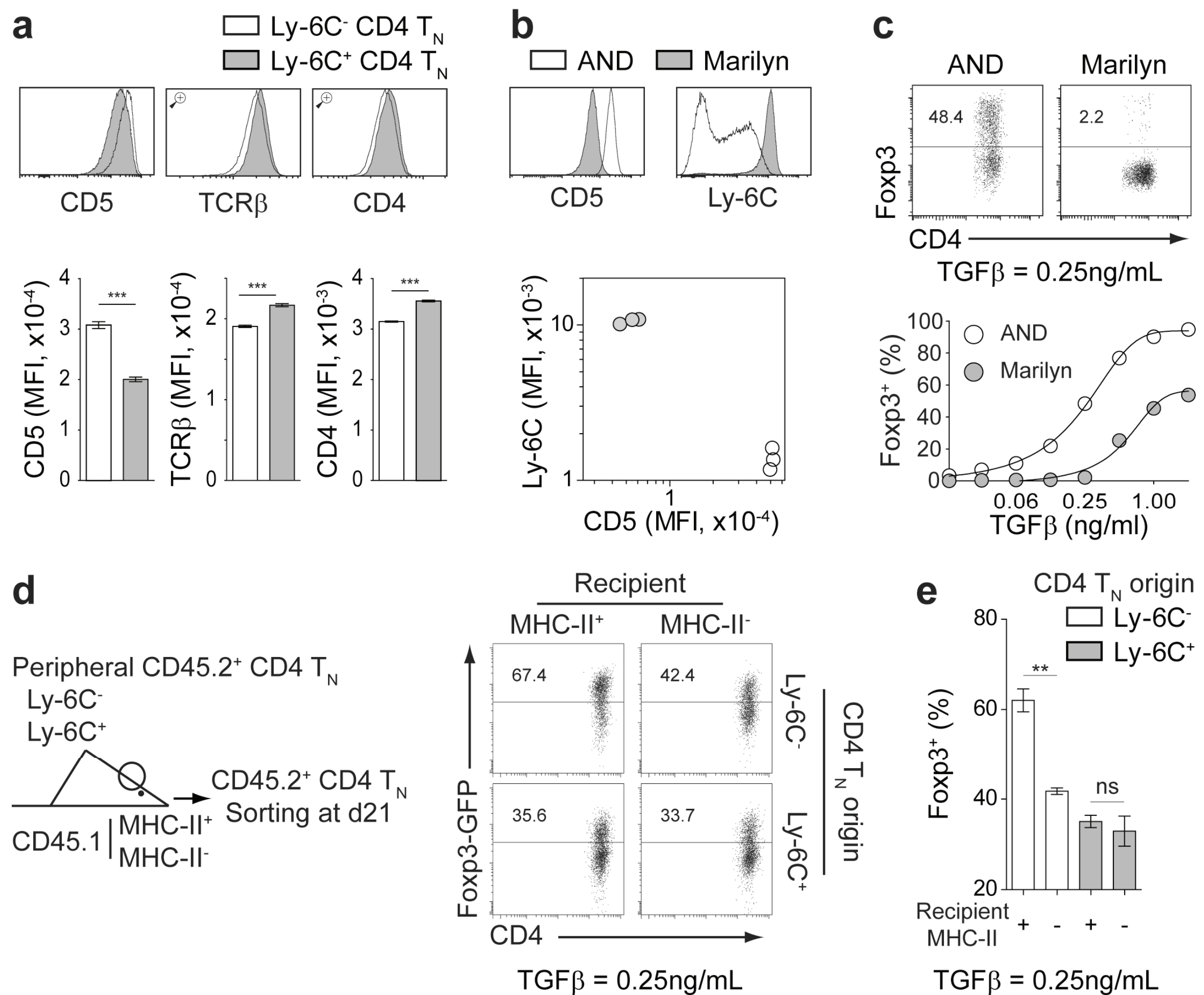


Figure 6: Ly-6C molecule expression as a new sensor of CD4 T_N cell self-reactivity

(a) CD5, TCRβ and CD4 fluorescence histograms on gated Ly-6C$^-$ and Ly-6C$^+$ CD4 T_N cells from LNs of a representative C57BL/6 Foxp3-GFP mouse. Mean Fluorescence Intensities (MFI) are shown as means ± SEM for at least 6 mice from 2 independent experiments. (b) CD5 and Ly-6C fluorescence histograms and graph representing Ly-6C MFI as a function of CD5 MFI on gated CD4 T_N cells from LNs of AND (solid line/white) and Marylin (filled histogram/gray) TCR-transgenic mice. Each dot represents an individual mouse. (c) CD4 T_N cells from LNs of AND and Marylin TCR-transgenic mice were stimulated for 4 days with coated anti-CD3 and anti-CD28 Abs in the presence of graded concentrations of TGFβ1. Representative Foxp3/CD4 dot-plots for gated CD4 T cells and the proportion of Foxp3$^+$ cells among CD4 T cells are shown as a function of TGFβ1 concentration. (d) $5x10^6$ Ly-6C$^-$ or Ly-6C$^+$ CD4 T_N cells from LNs of C57BL/6 Foxp3-GFP CD45.2 mice were injected i.v. into CD45.1 C57BL/6 mice lacking or not lacking MHC class II molecule expression (MHC-II$^-$ and MHC-II$^+$, respectively). CD45.2$^+$ CD4 T_N cells were then FACS-sorted 21 days after transfer from the periphery of recipient mice and stimulated for 4 days with coated anti-CD3 and anti-CD28 Abs in the presence of 0.25ng/ml of TGFβ1. A diagram illustrating the experimental model is shown. Representative Foxp3/CD4 dot-plots for gated CD4 T cells are shown as a function of recipient mice and CD4 T_N cells origin. (e) Proportion of Foxp3$^+$ cells among CD4 T cells as a function of recipient mice and CD4 T_N cells origin are shown as means ± SEM for at least 6 mice from 2 independent experiments.

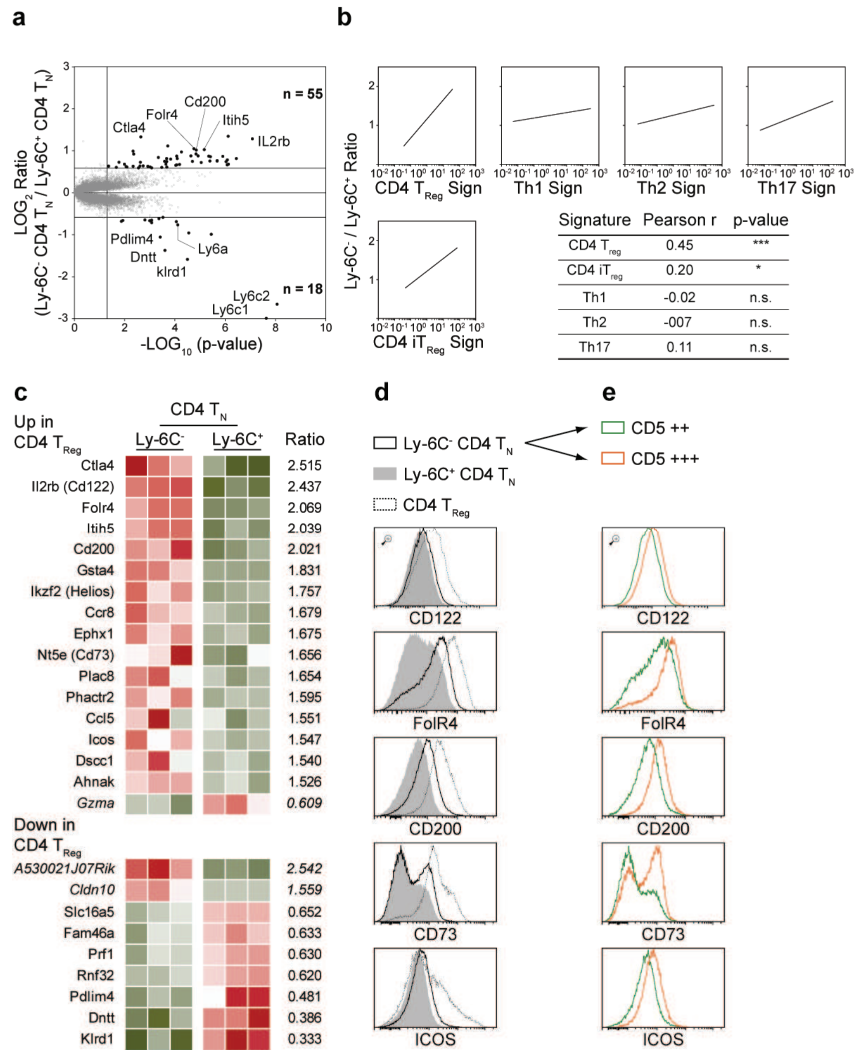
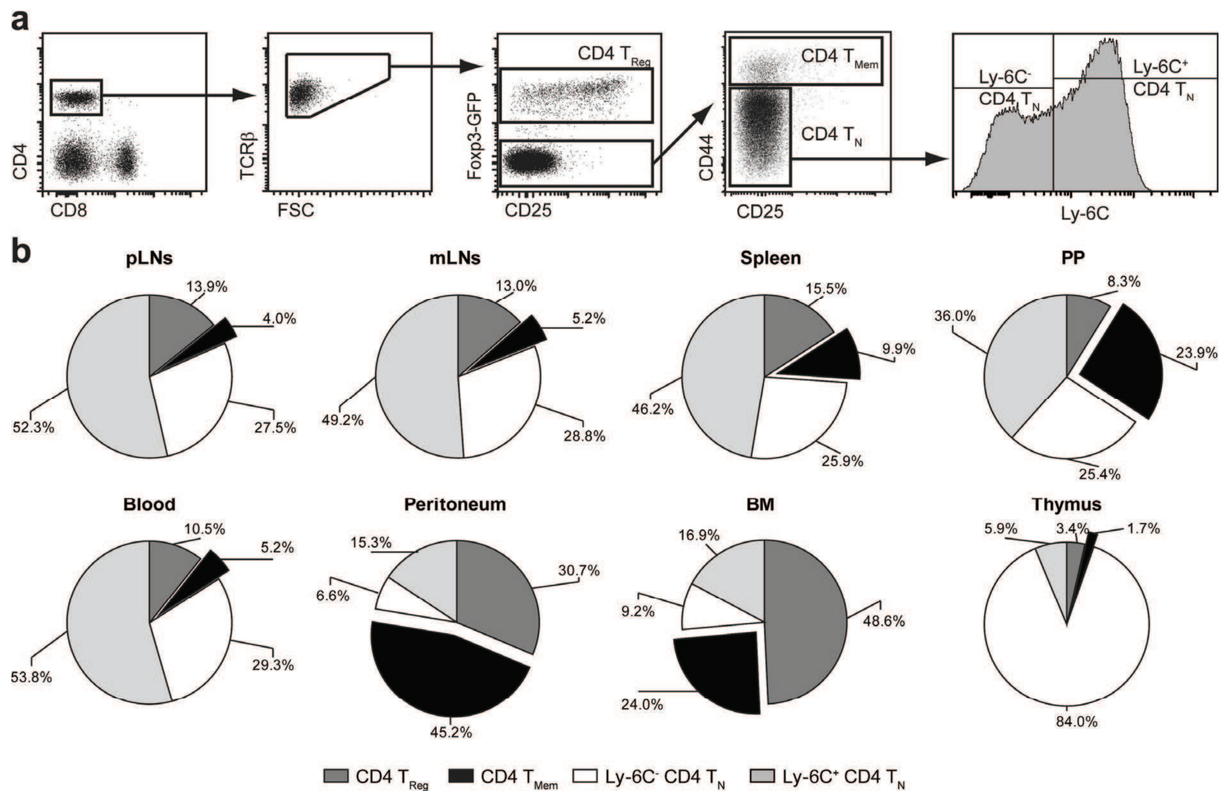


Figure 7: Gene expression profiling of Ly-6C$^-$ and Ly-6C$^+$ CD4 T_N cells

(a) "Volcano plot" representation (Log_2 (ratio) versus Log_{10} (t test p value)) between Ly-6C$^-$ CD4 T_N cells and Ly-6C$^+$ CD4 T_N cells from C57BL/6 Foxp3-GFP mice. Genes expressed >1.5-fold higher or lower in Ly-6C$^-$ CD4 T_N cells compared to Ly-6C$^+$ CD4 T_N cells with a *P* value of <0.05 are highlighted in black. The number of genes up- or down-regulated (1.5-fold cutoff) for each comparison is indicated. (b) Ratio versus ratio representation comparing gene expression ratio between Ly-6C$^-$ CD4 T_N cells and Ly-6C$^+$ CD4 T_N cells (1.3-fold cutoff with a *P* value of <0.05; 133 genes) with *ex vivo* peripheral Treg, *in vitro* induced Treg, T_H1, T_H2 and T_H17 cell signature that have been identified by Wei et al. (ratio of CD4 T_H-cell subsets to naïve CD4 T cells). (c) Expression pattern of Affymetrix targets differentially expressed (± 1.5 fold change, with a *P* value of <0.05) between Ly-6C$^-$ CD4 T_N cells and Ly-6C$^+$ CD4 T_N cells that have been identified as genes of the Treg-cell transcriptional signature by Wei et al. The Z-score normalized induction (red) or repression (green) is shown for each Affymetrix target. (d) CD122, FolR4, CD200, CD73 and ICOS fluorescence histograms on gated Ly-6C$^-$ CD4 T_N cells (solid line histogram), Ly-6C$^+$ CD4 T_N cells (filled histogram) and CD4 T_{Reg} cells (dotted line histogram) recovered from LNs of a representative C57BL/6 Foxp3-GFP mouse. (e) Ly-6C$^-$ CD4 T_N cells were subdivided into 2 subsets according to CD5 expression and the 2 newly defined subsets were then analyzed. CD122, FolR4, CD200, CD73 and ICOS fluorescence histograms of CD5^{++} Ly-6C$^-$ CD4 T_N cells (green line) and CD5^{+++} Ly-6C$^-$ CD4 T_N cells (red line) recovered from LNs of a representative C57BL/6 Foxp3-GFP mouse.

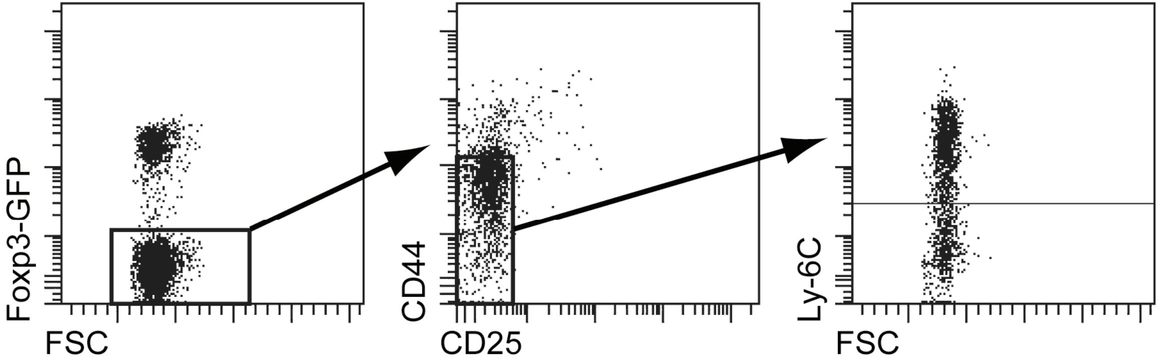
Supplementary figure 1:

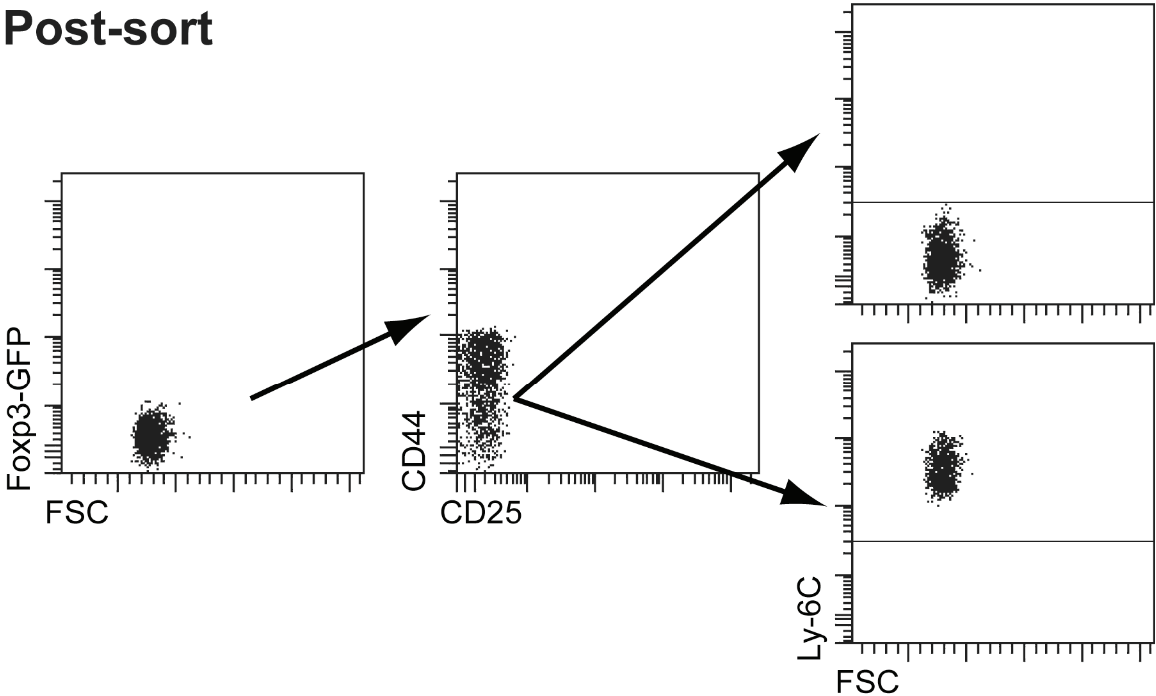


Supplementary Figure 1: Gating strategy used to discriminate peripheral CD4 T cell subsets

(a) Dot plots illustrating the gating strategy used to define peripheral naïve CD4 T cells (CD4 T_N) as $Foxp3^-$ $CD25^-$ $CD44^{-/low}$ $CD4^+$ $CD8\alpha^-$ $TCR\beta^+$ cells, regulatory CD4 T cells (CD4 T_{Reg}) as $Foxp3^+$ $CD4^+$ $CD8\alpha^-$ $TCR\beta^+$ cells and memory CD4 T cells (CD4 T_{Mem}) as $Foxp3^-$ $CD44^{hi}$ $CD4^+$ $CD8\alpha^-$ $TCR\beta^+$ cells and Ly-6C fluorescence histograms on gated CD4 T_N cells recovered from peripheral LNs of a representative C57BL/6 Foxp3-GFP mouse. (b) Pie charts illustrating the proportions of CD4 T_{Reg}, CD4 T_{Mem}, Ly-$6C^-$ and Ly-$6C^+$ CD4 T_N cells recovered from peripheral LN (pLNs), mesenteric LN (mLNs), spleen, Peyer's patches (PP), blood, peritoneum, bone marrow (BM) and thymus of a representative C57BL/6 Foxp3-GFP mouse.

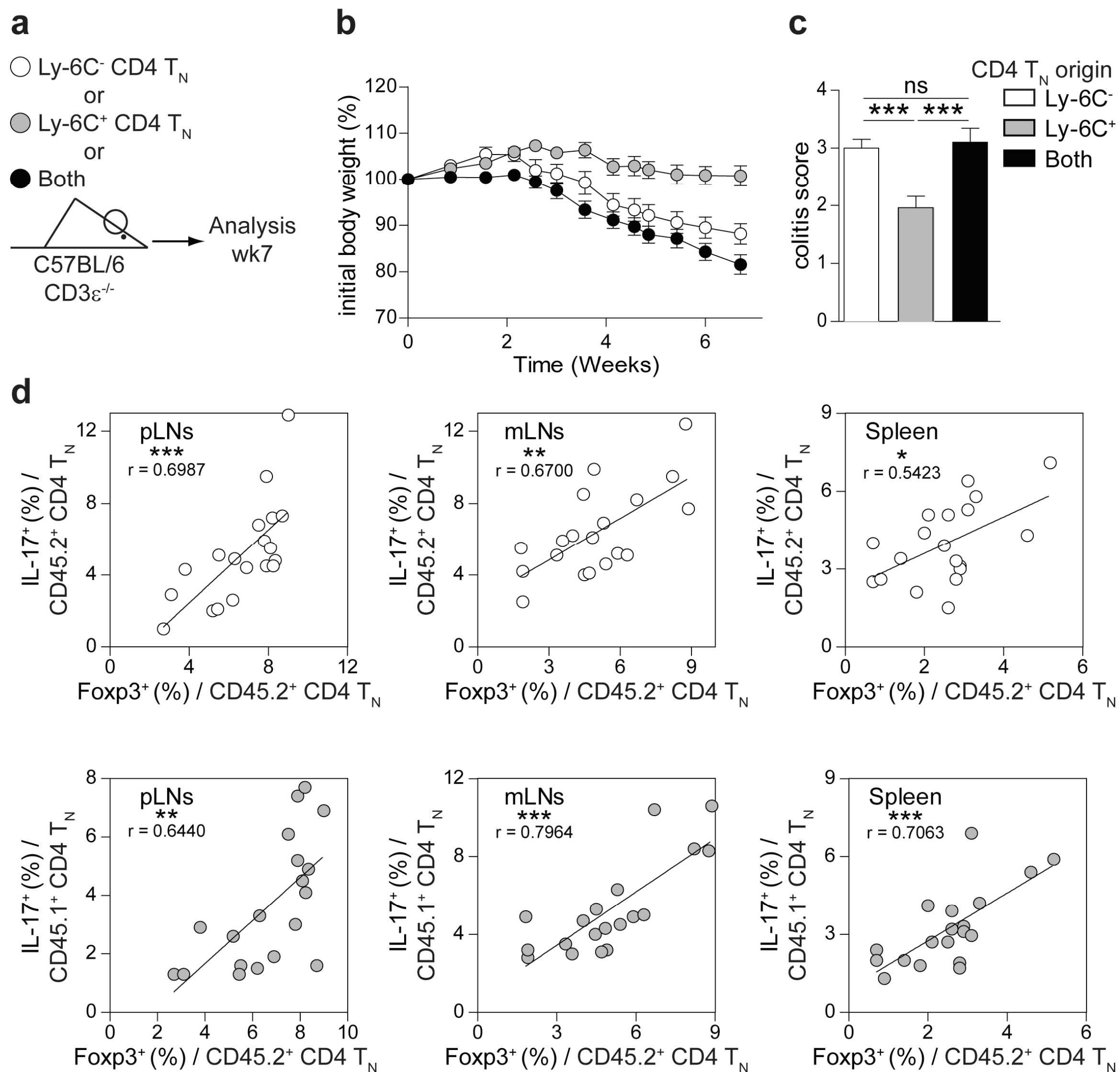
Supplementary figure 2:





Supplementary Figure 2: Flow-cytometry sorting strategy
Dot plots illustrating the gating strategy used to sort by flow-cytometry Ly-6C$^-$ and Ly-6C$^+$ CD4 T_N cells from LNs of C57BL/6 Foxp3-GFP mice. Pre- and post-sort analyses are shown.

Supplementary figure 3:



Supplementary Figure 3: Co-transfer of conventional naïve Ly-6C$^-$ and Ly-6C$^+$ CD4 T cells into lymphopenic mice induce as severe wasting disease and colitis as transfer of Ly-6C$^-$ CD4 T_N cells alone

Flow-cytometry sorted Ly-6C$^-$ CD4 T_N cells from C57BL/6 Foxp3-GFP CD45.2 mice and Ly-6C$^+$ CD4 T_N cells from C57BL/6 Foxp3-GFP CD45.1 mice were either injected i.v. separately (0.5x10^6 cells) or co-injected (0.25x10^6 cells of each subset) into lymphopenic C57BL/6 CD3ε$^{-/-}$ mice. (a) Diagram illustrating the experimental model. (b) Body weight of recipient mice was monitored up to 7 weeks post-transfer and percentages of initial body weight were calculated, plotted and shown as means ± SEM. (c) Colitis scores of recipient mice were assessed 7 weeks after transfer. Results are shown as means ± SEM. (d) Correlation between the proportion of Foxp3-expressing cells among CD45.2$^+$ CD4$^+$ CD8α$^-$ CD3$^+$ T cells and proportions of IL-17-producing cells among CD45.2$^+$ or CD45.1$^+$ CD4$^+$ CD8α$^-$ CD3$^+$ T cells recovered from pLNs, mLNs and spleen of recipient mice. Each dot represents an individual mouse.

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RÉSUMÉ

La génération et/ou le recrutement de cellules immuno-suppressives fait parti des mécanismes majeurs utilisés par les tumeurs afin d'échapper aux réponses anti-tumorales du système immunitaire. Parmi les cellules capables d'inhiber les réponses anti-tumorales, les lymphocytes T $CD4^+$ régulateurs et les macrophages de type II tiennent un rôle de premier ordre dans le contexte tumoral. Au cours de ma thèse, j'ai pu étudier l'impact de ces deux populations dans la suppression des réponses immunitaires anti-tumorales dans le modèle MT/ret de mélanome spontané métastatique.

L'ensemble de nos résultats met en avant plusieurs niveaux d'immuno-suppression dans le modèle MT/ret. D'une part, les lymphocytes T $CD4^+$ régulateurs, de par leur localisation dans les ganglions drainants et dans la peau, semblent impliqués dans la suppression des réponses anti-tumorales aux localisations et aux moments où les tumeurs nécessitent une forte inhibition des effecteurs anti-tumoraux. D'un autre côté, les macrophages de type II présentent, en plus de leurs capacités immuno-suppressives, des fonctions importantes pour la croissance et la dissémination tumorale justifiant leur localisation dans le micro-environnement tumoral.

Dans un second temps, nos données suggèrent pour la première fois un rôle des monocytes Ly-$6C^{fort}$ dans le contrôle tumoral via la lyse de ces dernières ou encore le maintien de la dormance des cellules tumorales disséminées. En conséquence, nous proposons de les ajouter à la liste des acteurs immunitaires directement impliqués lors des phases d'élimination et d'équilibre de la théorie de l'immuno-éditing. De plus, nous mettons en évidence leur inhibition par les lymphocytes T $CD4^+$ régulateurs, ce qui n'avait pas non plus été décrit précédemment. Ceci nous pousse à suggérer de prendre plus en compte l'impact des lymphocytes T $CD4^+$ régulateurs sur d'autres populations immunitaires que les lymphocytes T dans le contexte tumoral.