l'université nantes angers le mans
COMMUNAUTÉ D'UNIVERSITÉS ET ÉTABLISSEMENTS

UNIVERSITÉ DE NANTES

Thèse de Doctorat

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Mémoire présenté en vue de l'obtention du
grade de Docteur de l'Université de Nantes
sous le label de L'Université Nantes Angers Le Mans

École doctorale : *Biologie-Santé*

Discipline : *Médecine*
Spécialité : *Immunologie*
Unité de recherche : *INSERM UMR1064*

Soutenue le 12 Novembre 2014
Thèse N° :

Autologous regulatory myeloid cell therapy in transplantation

JURY

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THANKS

First of all I would like to thank Dr Sylvaine You and Dr Francesc Borràs for having accepted to be my thesis reporters and Dr Ignacio Anegón for having examined and judged my work, and also for the opportunity he has given me to do my thesis in his laboratory.

I want to thank my follow-up thesis committee, Yves Delneste and Marc Gregoire, for their useful comments during those 3 years.

I also want to thank my thesis director, Dr Maria-Cristina Cuturi, for introducing me in the cell therapy world, and also for all her patience … it seems that finally all the efforts lead somewhere! I'm pleased to thank my co-director, Dr Marcelo Hill, for all his help and advice, even in the distance. Nos vemos pronto por Uruguay!

I would like to thank « the girls » for manuscript revision and good advices, Vanesa, Caro, Marion, Elodie P and Séverine B.

Merci à la petite équipe 1, Aurélie pour ton encadrement, tes conseils, la correction de ma thèse ainsi que les divers détournements du papier. Laurence (Hola!), merci pour ta gentillesse, ton sourire, toute ton aide désintéressée. Travailler à tes cotés a été une énorme chance et les manips de 16h partagées sont toujours plus agréables. Merci à Cédric pour les longues discussions scientifiques et la vision critique des résultats, et Lucile pour tout ce qu'on a partagé in and out of the lab. Manu, sans tes greffes ce travail n'aurait pas eu lieu, muchas gracias ! Et aussi à Claire pour toutes les injections, prises de sang… Et bien sûr, un grand merci à ceux qui sont partis de l'équipe 1 mais qui ont toujours une place dans nos cœurs : Mercedes, merci de m'avoir appris tout sur les TolDC, Gaëlle T, merci de toute ton aide au cours de cette thèse, scientifique et surtout non scientifique. Merci à toute la grande équipe 1, pour leur soutien pendant ces 3 années et les discussions autour des tables de réunion ou du golf ! Merci à Régis et Bernard pour leurs conseils scientifiques.

Je voudrais remercier de tout cœur à l'ensemble du labo, tous ces gens qui m'ont si bien accueillie dès mon arrivée en France, avec qui j'ai partagé des très bons moments et des grands rires. La thèse n'aurait pas été si agréable sans vous. Merci à mes copines (et copain) de bureau de m'avoir aidé à conserver la ligne :P Et merci aux gens de la Culture 2, pour dégager toujours plein d'énergie et de bonnes vibrations et être si disposés à aider les autres. Je tiens à citer Laurent T (« on travaille comme des moules ! »), Fred, Franck, Gaëlle B, Séverine R, Jérôme M, Reynald, Alison, Justine, Laure-Hélène, Gaëlle T, Alexis, Aurélie L, Coraline, Julien, Thomas et tous ceux et celles qui manquent, la liste serait interminable !

Merci à la meilleure équipe, « l'équipe 10 », qui n'apparait pas sur l'organigramme, mais sans laquelle rien n'aurait pas été pareil… Justine, ma copine de bureau, d'équipe, de thèse, de verres, de pleurs et de rires, merci d'avoir partagé avec moi ces 3 années, de m'avoir appris le français, d'avoir corrigé les fautes des mails et documents … merci d'être toi-même. Merci aux filles du bureau d'à côté, Elo et Séverine, pour tous les bons moments partagés au labo jusqu'à pas d'heure ou bien en dehors (aussi jusqu'à pas d'heure :P), les conseils et les bonnes discussions scientifiques. Merci à Marion, mi amiga y compañera, pour tous les cafés et les conversations profondes sur la vie et l'avenir. J'espère pouvoir regarder en arrière dans quelques années et rire de tout cela ! Merci pour m'avoir aidé à garder le calme dans les pires moments. Merci à Jason, le nouvel arrivant, pour ton écoute et ta motivation. Je te souhaite le meilleur pour ta thèse. Tout le reste de l'équipe 10, thésards ou pas, milles mercis pour avoir été toujours à mes côtés quand j'ai eu besoin d'aide ou d'une épaule sur laquelle pleurer. Merci à Angélique P, Alex, Sabrina, Michelle, Vanessa, Mélanie N, Laetitia L, Annie, PJ… Sans oublier ceux qui sont partis, mais qui m'ont laissé un très bon souvenir. Ma voisine de bureau, Amélie. Ma petite Laëtitia, merci pour ma première année de thèse incroyable, ton sourire et tes câlins :) I wish you the best in Australia. Pareil pour Antoine, Elo M et Gaëlle P, je vous souhaite le meilleur aux USA.

Y como no, mil gracias a mis chilenos preferidos, Roberto y Sebastián. Vuestra llegada entre nosotros fue inesperada pero llena de cosas inolvidables. Muchas gracias por todos los momentos compartidos, entre experimentos y "chelas", vuestro buen humor, y todo el cariño que me habéis demostrado.

I mirant enrere, no podria mai oblidar els meus inicis a la ciencia, aquells professors d'universitat que van revetllar la meva passio per l'Immunologia (moltes gracies per fer tan bé el vostre treball, Francesc i Pilar), la meva primera estada a un laboratori de recerca : la Pilar, el meu exemple a seguir, el Roger, la Rosa (quantes discusions…), l'Edurne,… gent apassionada i capaços de transmetre aquesta passio als altres. I també l'Hospital Clinic, tota la gent que he conegut i amb qui he compartit els meus primers pasos a la ciencia, Jordi o "Mr. Cultius", Fina, Susana, Mireia, Carol, Rosa,… els meus companys Miguel, les Cristina's, Mario, Rafa, Emma, Vanesa (mil gracias por todo lo que has hecho por mi bunica), … I per ultim, voldria donar les gracies a la persona que més a cregut en mi, "tu vals per la ciencia", la frase que m'ha mantingut en aquest cami fins al dia d'avui. Manel, moltissimes gràcies per tot el que has fet per mi, des del dia que em vas "salvar" fins al dia que vas aceptar que marxés. Fins i tot ara, moltes gracies pels teus consells cientifics dels ultims mesos, per estar sempre disponible per mi, per haver-me ensenyat tot el que vaig aprendre durant 3 anys. Espero que els nostres camins es tornin a creuar en un futur.

I tota la gent amb qui he compartit estudis al llarg de la meva vida, començant pel Vidal, Deya, Laura, Esther, Cris, Noe, la meva nineta Cris, tant diferents al principi i tan avingudes al final, moltes gràcies per tot el teu suport. Els gloriosos anys de la UAB, els grups tan macos que vam formar i d'on he conservat molt bons amics, Laura i Ester, quanta motivacio i hores de biblioteca per arrivar fins

aqui... done! el super grup Alba, Roser, Loreto, Sandra, Alex, Rodri, Berni i Deib, con todas nuestras discusiones filosoficas y nuestro proyecto BioBar! I com oblidar, els companys de Màster amb qui he compartit tants moments, l'Immunocomplexe! Laura C, Raimon, Joan, Victor, Isaac, Laia... i una mencio molt especial per les Immunonenes, retroballes inesperades i els llaços més forts que he creat, Mariona, gràcies per fer que les coses no semblin tan complicades, Carol totes les xerrades i histories inospites, tots els moments més o menys durs, Eugènia, amb els nostres moments de desesperacio al Clinic, quant temps ha passat... i Anna, nina, a qui he ensenyat la importàancia de les abraçades :) el desti ha volgut donar-nos vàries segones oportunitats de coneixe'ns i creume quan et dic que m'en alegro de no haver-nos quedat amb la primera impressio. Moltes gràcies a totes per haver estat al meu costat i per tots els bons moments compartits, sovint amb unes braves i unes birres! Cada record de moment passat amb vosaltres em torna el somriure.

Sense oblidar tota la gent amb qui he compartit grans moments, els meus compis de pis a Barcelona, Charly, Ferràn i Imma, la meva cousin Natalia, quants cafès/tes/birres/discoteques..., Xavi, mi "gemelo", vaya tela! Como cambio mi vida cuando apareciste... y para bien! Et sans oublier non plus toutes les amitiés sur Nantes... Caro, merci de m'avoir si bien accueillie dès le J2 de mon arrivée et de m'avoir fait rencontrer les autres, merci pour les discussions scientifiques et les bons conseils, Gaëlle, Pascal (et Raphaëlle hihi), Loïc et Nancy, merci pour les bons moments partagés et pour ceux qui ne sont pas encore arrivés. Merci à Jérôme pour ton support à distance, je veux te voir et je vais te le démontrer ! And all international friends I have met outside the lab, Chiara, Teo, Clara, Nacho, Marco, Valerio,...

Y no podría acabar los agradecimientos sin nombrar a mi familia, que han estado siempre ahí para mí, animándome sin saber exactamente lo que hacía, pero creyendo en mi capacidad para hacer todo aquello que me proponga. Muchas gracias mama, papa, Pablo y Sombra (guau!). Las tietas y el tiet, Encarni, Angela, Belén y Jose Mari. Sin olvidar a la Super Abu, Lucia, el mayor ejemplo a seguir, siempre en mi corazón y al resto de la familia.

Et bien sûr, un grand merci à ma famille II, celle qui m'a accepté depuis mon arrivée, avec qui j'ai partagé des moments incroyables. Merci de m'avoir considérée un membre en plus parmi vous, Sylviane, Daniel, ma petite Audrey, merci pour ton amitié si précieuse, Charline et Damien.

And last, but not least, I would like to thank with all my heart the person who has stood me (and by me) every day of those 3 years. Merci de m'avoir comprise, conseillée, respectée, gâtée, nourrie, aimée. Merci d'avoir été à la merci de mes horaires, de mes humeurs, d'avoir partagé des pleurs et des rires, de m'avoir rappelé qui je suis chaque fois que je me suis perdue. Moltes gràcies, tu has fet de mi una millor persona. Y gracias también por todos los momentos que nos quedan por vivir. Nico, you're my person.

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ABREVIATIONS

ADCC	Antibody Dependent Cellular Cytotoxicity
AGM	Aorta-Gonads-Mesonephros
AHR	Aryl Hydrocarbon Receptor
AI	Autoimmune
AICD	Activation Induced Cell Death
AIRE	AutoImmune Regulator
APC	Antigen Presenting Cell
APECED	AI PolyEndocrinopathy-Candidiasis-Ectodermal Dystrophy
ATDC	Autologous Tolerogenic Dendritic Cell
ATG	Anti-Thymocyte Globulin
BcR	B cell Receptor
BM	Bone marrow
CCL	Chemokine (C-C motif) Ligand
CCR	C-C chemokine Receptor
CD	Cluster of Differentiation
cDC	conventional Dendritic Cell
CDP	Common DC precursor
CDR	Complementarity Determining Regions
CFSE	Carboxyfluorescein Succinimidyl Ester
CLP	Common Lymphoid Progenitor
CMLP	Common Myeloid Lymphoid Progenitor
cMoP	common Monocyte Precursor
CMP	common Myeloid Progenitor
CNI	Calcineurin inhibitors
CSF-1	Colony Stimulating Factor-1
cTEC	cortical Thymic Epitelial Cell
CTLA-4	Cytotoxic T-Lymphocyte-Associated protein 4
DAMP	Damage-Associated Molecular Pattern
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagle Medium
DN	Double Negative
DNA	Deoxyribonucleic Acid
DNT	Double Negative T cells
DP	Double Positive
DST	Donor-Specific Transfusion
EAE	Experimental autoimmune encephalomyelitis
EBI3	Epstein-Barr virus Induced gene 3
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal Calf Serum
FDA	Food and Drug Administration
FDC	Follicular Dendritic Cells
FGL-2	Fibrinogen-Like Protein 2
FLT3	Fms-Like Tyrosine Kinase 3
FLT3L	Fms-Like Tyrosine Kinase 3 Ligand

FoxP3	Forkhead box P3
GC	Germinal Center
G-CSF	Granulocyte colony-stimulating factor
GITR	Glucocorticoid-Induced TNFR family Related protein
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
GMP	Granulocyte Macrophage Precursors
GVHD	Graft Versus Host Disease
HEPES	acide 4-(2-hydroxyéthyl)-1-pipérazine éthane sulfonique
HLA	Human Leukocyte Antigen
HO-1	Hemeoxigenase-1
HSC	Hematopoietic Stem Cells
IC	Immunocomplex
ICAM-1	Intercellular Adhesion Molecule-1
ICSBP	Interferon consensus sequence binding protein
iDC	immature Dendritic Cell
IDO	indoleamine 2 3-dioxygenase
IFNγ	Interferon γ
IL	Interleukin
ILC	Innate Lymphoid Cells
ILT	Immunoglobulin-like transcript
infDC	inflammatory Dendritic Cell
iNOS	inducible Nitric Oxide Synthase
IRF-8	interferon regulatory factor-8
IS	Immunosuppressor
KI	Knock-in
KIR	Killer cell Ig-like Receptor
KLF4	Kruppel-like factor 4
KO	Knock-out
LC	Langerhans Cells
L-NMMA	NG-monomethyl-L-arginine
LPS	Lipopolysaccharide
Ly6G	Lymphocyte antigen 6G
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage Colony-Stimulating Factor
MDP	Macrophage-Dendritic cell Progenitor
MDSC	Myeloid-derived Suppressor Cells
mH	minor Histocompatibility
MHC	Major Histocompatibility
MIC	MHC class I-related chain
MMF	Mycophenolate mofetil
MPP	Multipotent progenitor
MPS	Mononuclear Phagocyte System
MSC	Mesenchymal Stem Cells
Mreg	regulatory macrophages
mTEC	medullar Thymic Epitelial Cell
NADPH	nicotinamide adenine dinucleotide phosphate
NK	Natural Killer
NFAT	Nuclear Factor of Activated T cells
NF-kβ	nuclear factor kappa-light-chain-enhancer of activated B cells

NKT	Natural Killer T
NLR	NOD-like receptors
NO	Nitric Oxide
OVA	Ovalbumin
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PC	Plasma Cells
PD-1	Programmed Death-1
PD-L1	Programmed Death-Ligand 1
pDC	Plasmacytoid Dendritic Cell
p-MB	progenitors-myeloid/B cell
p-MT	progenitors-myeloid/T cell
PRR	Pattern Recognition Receptor
pTreg	peripheral regulatory T cell
RMC	Regulatory Myeloid Cell
RNI	Reactive Nitrogen Intermediates
RORγτ	retinoic acid-related orphan receptor γτ
ROS	reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute 1640
SLO	Secondary Lymphoid Organ
SnPP	tin (Sn) protoporphryin-IX
SP	Single Positive
SRTR	Scientific Registry of Transplant Recipients
STAT	Signal Transducer and Activator of Transcription
TAIC	Transplant Acceptance-Inducing Cells
TAP	Transporter associated with Antigen Processing
Tc	cytotoxic T cell
TcR	T-cell Receptor
TF	Transcription Factor
Tfh	T follicular helper
TGFb	Transforming growth factor beta
Th	helper T cell
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
tolDC	tolerogenic Dendritic Cell
Treg	regulatory T cell
tTreg	thymic regulatory T cell
VitD3	Vitamin D3

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INTRODUCTION

PART I. IMMUNE SYSTEM: COMPONENTS AND RESPONSES

In the course of evolution, eukaryote organisms, to face pathogen invasion, have developed a complex network of tissues, cells and molecules called the immune system. Importantly, the immune system of vertebrates is composed of two types of responses named innate immune response and adaptive immune responses that tightly cooperate to protect efficiently the host from external threaten.

When confronted with a challenge, like a pathogenic invasion, the innate immune response is the first one to be triggered. Natural protective barriers, such as skin and mucosae, represent the first line of defense, isolating the organism from the exterior. Moreover, the innate immune system is also composed of cells that are characterized by an expression of germline-encoded receptors, called pattern recognition receptors (PRRs) that recognize conserved molecules exclusive to microorganisms, called pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). When PRRs recognize PAMPs or DAMPs, innate cells are activated, which implies cellular changes that notably lead to the secretion of molecules specialized in the communication between cells allowing the recruitment of other cell types. Within cell populations from the innate immune system, there are phagocytic cells (monocytes/macrophages and granulocytes), cytotoxic cells (mainly Natural Killer (NK) cells but also γδ T cells), the recently discovered innate lymphoid cells (ILCs) and dendritic cells (DC), which play a key role by initiating the adaptive immune response. Indeed, dendritic cells can recognize PAMPs, internalize fragments of pathogens and then present them to cells from the adaptive immune response.

Even though the adaptive immune response needs more time to be set up, this late response is specific of the pathogenic agent and thus very efficient at eliminating the menace. The main difference with innate immune system is that cells from the adaptive immune system, called lymphocytes, express receptors that recognize a broad range of specific antigens, each lymphocyte bearing a single type of receptor with a unique specificity. After antigen recognition, lymphocytes undergo clonal expansion, rapidly proliferating and polarizing towards the secretion of different types of soluble mediators, depending on the type of menace. This activation allows the organization of an optimal response leading to the elimination of the pathogen. One important feature of the adaptive immune response is immunological memory, which implies a quickest response the second time an organism is invaded by the same pathogen.

It is noteworthy to highlight that both, innate and adaptive immune responses are tightly linked, as signals coming from innate immune cells help to trigger adaptive immunity. Depending on the soluble mediators that cells from the innate immunity secrete, the adaptive immunity will be modulated towards one or other type of effector response. Moreover, cells from the adaptive immune

system will then activate and/or modulate the functional properties of the innate immune cells and, thus, improve their efficiency.

Cells from the adaptive immune system are derived from a common lymphoid progenitor, whereas most of the cells from the innate immune system are comprised in the myeloid lineage. According to the Akashi-Kondo-Weissman scheme of hematopoietic differentiation (Kondo et al. 1997; Akashi et al. 2000), immune cells originate from a unique progenitor, called hematopoietic stem cell (HSC) in the bone marrow. HSC differentiates into multipotent progenitor (MPP), which are considered the branching point between the two main cellular lineages of the immune system: myeloid lineage (which derives from common myeloid progenitor, CMP) and lymphoid lineage (deriving from common lymphoid progenitor, CLP).

1 LYMPHOID CELLS

1.1 Origin and development of lymphocytes

Lymphocytes are the main players of the adaptive immune system. CLP can give rise to pro-T and pro-B cells that will then develop into mature T and B cells and can also generate NK cells (Kondo et al. 1997). Interestingly, a study from Jacobsen's laboratory have described an alternative developmental pathway to generate myeloid and lymphoid cells by identifying a macrophage/T cell/B cell restricted progenitor (Adolfsson et al. 2005). Therefore according to this model, some myeloid progenitors can develop either by the classical CMP pathway or via a lympho-myelomonocytic pathway.

The existence of CLP has been challenged by a study from Y. Katsura and colleagues (Katsura 2002). Indeed, by using a multi-lineage progenitor assay, they have described the T and B lymphocyte precursor as being a common myeloid lymphoid progenitor (CMLP) instead of CLP. Their data have shown that CMLP can give rise to two branches, either progenitors-myeloid/T cell (p-MT) or progenitors-myeloid/B cell (p-MB) and are found in mouse fetal liver, whereas progenitors-T cell/B cell (p-TB), which would be the common precursor of T and B lymphocytes, the so called CLP, were not found (**Figure 1**). Other studies found out that T cell lineage commitment occurs pre-thymically in fetal liver (Kawamoto et al. 1999) and that pre-T cells seem to emerge earlier in ontogeny than pre-B cells, clearly indicating independence of T and B cell developmental pathways (Kawamoto et al. 2000).

Early lymphoid and myeloid lineage development pathways are driven and tightly regulated by a balance of activation and repression of expression of multiple transcription factors (TF).

Notably, PU.1 and Ikaros are two TF that play a key role in that process. Indeed, it has been described that PU.1 repression causes inhibition of B and myelomonocytic cell development, as well as differentiation defects in T cells and DCs, as CLP and CMP progenitors are absent (Laiosa et al. 2006). Ikaros, is expressed at the HSC level and promote lymphoid cell fates by gene transcription modeling. Ikaros absence severely affects lymphoid lineage development, as well as some cells from the myeloid lineage (Georgopoulos et al. 1994).

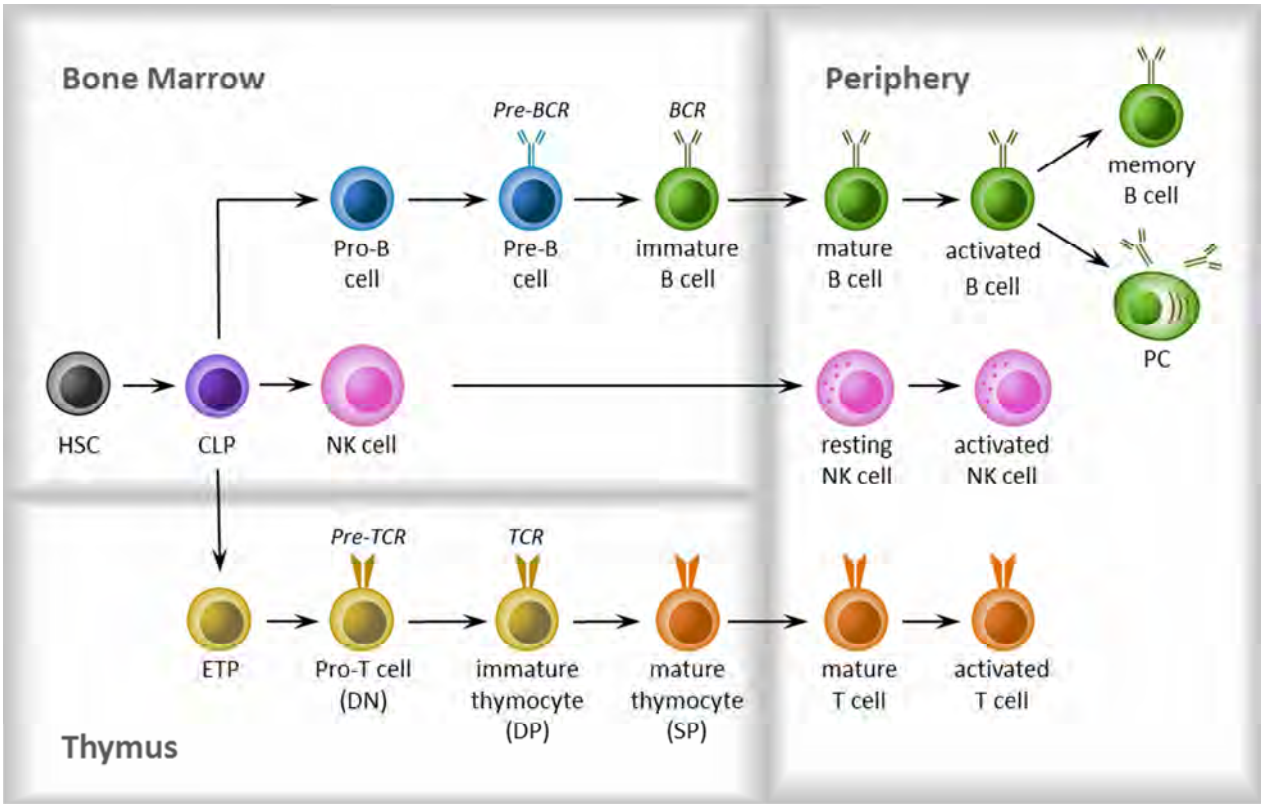

Figure 1. Lymphoid cell development. Lymphoid lineage differentiates from HSC via CLP, and give rise to mature B cells, NK cells and mature T cells. HSC, Hematopoietic Stem Cell, CLP, Common Lymphoid Progenitor, DN, Double Negative, DP, Double Positive, SP, Single Positive.

1.2 Cell types

1.2.1 T lymphocytes

T lymphocytes must respond to a huge number of potential foreign antigens but at the same time must not respond against self-antigens expressed in host tissues. To be able to fulfill their functions, during their development in the thymus, T cells get through selection processes to test their specific clonal surface receptor that recognize each antigen in the context of self MHC (Major Histocompatibility Complex) molecules.

The majority of lymphocytes committed to the thymocyte lineage originate in bone marrow and enter the thymus, where they initiate a process of gene rearrangement of α and β chains of the T cell receptor (TcR). At this stage, in the cortex of the thymus, thymocytes are $CD4^+CD8^+$ double-positive (DP) cells. DP cells express a pre-TcRαβ. If their pre-TcRαβ does not appropriately interact with a MHC/self-peptide complex expressed by thymic epithelial cortical cells (cTECs), DP thymocytes do not receive survival signals. They can either rearrange the TcR α locus in order to generate a new pre-TcRαβ with a different affinity or die by apoptosis. This process is called **positive selection**. Only around 5% of DP cells are able to bind the complex mildly which induces DP maturation to the stage of single positive (SP) thymocytes, either expressing CD4 or CD8 co-receptor chain (Murphy et al. 2008).

Positive selected cells migrate to the medullar part of the thymus, where they get through a second selective process, called **negative selection**: it consists on the clonal deletion of cells displaying high affinity to self-peptide/self-MHC complexes. Those complexes are presented by thymic epithelial medullar cells (mTECs) and by thymic dendritic cells. Therefore, the vast majority of autoreactive T cells is already eliminated at the thymus, and never reache the periphery (Murphy et al. 2008).

The ectopic expression of tissue-specific antigens in the thymus is possible thanks to the intrathymic genetic regulation by the expression of the TF AIRE (AutoImmune Regulator) by mTECs and the presentation of extrathymic antigens uptaken by thymic DCs. The genetically defined lack of functionality of AIRE in humans is responsible of the Autoimmune PolyEndocrinopathy-Candidiasis-Ectodermal Dystrophy (APECED) syndrome, which is characterized by high levels of autoreactive T cells in periphery, leading to autoimmune disorders (Aaltonen et al. 1994).

Only T lymphocytes who have overcome positive and negative selection processes are allowed to circulate in periphery. This represents about 3% of the initial number of thymocytes. Once in the periphery, T lymphocytes recirculate until they reach T cell zones of secondary lymphoid organs. T lymphocytes stay in SLO (Secondary Lymphoid Organ) until they are activated by an APC (Antigen Presenting Cell) expressing MHC/peptide complex that will be specifically recognized by their TcR.

A small subset of T lymphocytes (1-5%) does not display αβTcR at their surface. Instead, they display an invariant TcR composed of two chains, γ and δ. Their development can be thymic dependent or independent. γδ-T lymphocytes are not restricted by MHC molecules and are able to recognize soluble proteins and other molecules. They are widespread located within epithelial-rich tissues, such as skin, intestine and reproductive tract (Carding & Egan 2002).

In parallel to "ordinary" naive T lymphocytes selection, another cell type displaying regulatory functions (Treg) is also selected. The function of these thymus-derived naturally arising Treg (tTreg) cells is to neutralize the response of autoreactive T lymphocytes which escape the thymic selection process in a self-antigen specific manner; therefore, tTregs express TcRs which are specific for self-antigens. The question that arises then is: how do tTregs escape negative selection? Multiple models have been proposed. The most widely accepted consists in a two phase selection. Concerning $CD4^+$ Treg cells, tTregs would originate from DP thymocytes which have been positive selected to be $CD4^+$ SP thymocytes. In the first place, the high avidity of $CD4^+$ SP thymocytes for self-MHC/self-peptides complex would induce a high surface expression of CD25, the α-chain of IL-2 receptor. Then, IL-2 would bind its receptor in the surface, leading to the expression of FoxP3 (Forkhead box P3) TF (Lio & Hsieh 2008; Hsieh et al. 2012). FoxP3 is the hallmark TF of Tregs, responsible of Tregs development and suppressive function. FoxP3 binds to promoters of genes involved in T cells regulatory function while repressing the transcription of genes normally transcribed following T cell stimulation (Marson et al. 2007).

a. Effector T lymphocytes

As already mentioned, most of the T lymphocytes carrying TcRαβ are classified in two main subtypes, depending on the co-receptor they express at the stage of SP cells: $CD3^+CD4^+$ T lymphocytes or $CD3^+$ $CD8^+$ T lymphocytes. A small percentage of TcRαβ T lymphocytes do express neither CD4 nor CD8 co-receptors (called double negative (DN) lymphocytes) or express both, CD4 and CD8 (called double positive (DP) lymphocytes).

When a T lymphocyte encounters an APC at secondary lymphoid organs, TcR/MHC interaction transduces activation signals towards the APC. The activated APC activates T lymphocytes back. T cell activation implies changes in their cytokine production profile that are responsible of the outcome of the response.

Classically, effector $CD8^+$ T lymphocytes have been defined by their ability to lyse virus-infected target cells and to produce high levels of IFNγ (Interferon γ), acting as cytotoxic cells. Later, it became clear that $CD8^+$ T cells were able to produce a variety of different cytokines. Depending on the cytokinic microenvironment present during primary stimulation, $CD8^+$ effector T lymphocytes can differentiate into type 1 (Tc1) or type 2 (Tc2) cytokine producing cells. **Tc1** secrete IFNγ and IL-2 and are the main $CD8^+$ cytolytic subset. They express the TF T-bet. **Tc2** secrete IL-4, IL-5, IL-6 and

IL-10 and express the TF GATA-3 (reviewed in Carter & Dutton 1996). More recently, it has been shown that $CD8^+$ T lymphocytes can also differentiate into IL-17 secreting cells (**Tc17**) (Hamada et al. 2009). The majority of Tc17 also secrete TNF (Tumor Necrosis Factor) and IL-2 but they contain very few cells that secrete IFNγ or granzyme B, and do not exhibit cytolytic activity. They express the TF RORγτ (retinoic acid-related orphan receptor γτ). They play an important role in protection against viruses and recruit neutrophils into the affected sites (Hamada et al. 2009). Those different cytokine secretion profiles can drive T responses towards different outcomes.

Our current knowledge about $CD4^+$ T lymphocytes differentiation is a little more complex than the one about $CD8^+$ T lymphocytes (schematized in **Figure 2**). In 1986, a two subset model of $CD4^+$ T helper (Th) cells was proposed, based on the different cytokine secretion pattern (namely Th1 and Th2) (Mosmann et al. 1986). In the recent years, this model has been expanded to include new Th subsets. Effector $CD4^+$ T lymphocytes are called T helper cells, as they do not eliminate pathogens or infected cells directly but instead they recruit or activate other cell types in different ways, depending on their polarization. Naive $CD4^+$ T lymphocytes (also called Th0) can differentiate into **Th1** cells in the presence of IL-12 (mainly secreted by DCs, macrophages and other APC) and IFNγ (produced by NK cells, NKT (Natural Killer T) cells or other T lymphocytes) (Hsieh et al. 1993). Interaction of the mentioned cytokines with their receptors at the T cell surface activate STAT (Signal Transducer and Activator of Transcription) molecules (mainly STAT-4 and STAT-1 respectively) leading to the expression of T-bet TF in Th0 cells. T-bet is essential for Th1 differentiation. Once expressed, T-bet inhibits the expression of other factors that would deviate the response towards alternative polarization states (Szabo et al. 2000). Th1 lymphocytes mainly secrete IFNγ, IL-2 and TNFα, favoring proliferation and maturation of $CD8^+$ T lymphocytes, recruitment and activation of macrophages and NK cells and promoting B lymphocytes switch towards IgG2a (IgG1 and IgG3 in humans) (Stevens et al. 1988). Therefore, Th1 lymphocytes have a basic pro-inflammatory/cytotoxic profile.

Polarization towards **Th2** profile takes place in the presence of IL-4 and other cytokines such as IL-2. IL-4 ligation activates STAT-6, which induces GATA-3 (trans-acting T cell specific transcription factor 3) expression, a hallmark of Th2 polarization. Due to GATA-3 TF expression, Th2 cells transcribe and secrete IL-4, IL-5 and IL-13, while inhibiting the differentiation towards a Th1 profile (Yagi et al. 2011; Zhu et al. 2006). They also secrete the anti-inflammatory cytokine IL-10. Those cytokines favor B lymphocytes activation and induce an IgG1 and IgE immune response (IgG2 in humans) (Rizzo et al.1995).

In the early 2000, another pivotal type of Th cells was found to be the cause of most autoimmune disorders. This subpopulation, called **Th17**, is induced mainly by IL-21 and IL-23, but also by TGFβ (Transforming growth factor beta) and IL-6 (Korn et al. 2007) through the expression

of RORγτ transcription factor (Ivanov et al. 2006). They secrete pro-inflammatory cytokines such as IL-17a, IL-17f, IL-21 and IL-22. Th17 cells play an important role in immune responses against extracellular antigens (Weaver et al. 2007) and against fungi in mucosa (Zhou & Littman 2009).

Other T helper subpopulations have been described, although their roles are less characterized. TGFβ associated to IL-4 leads to **Th9** differentiation, which are potent IL-9 producers (Schmitt et al. 1994;Dardalhon et al. 2008; Veldhoen et al. 2008; Schmitt et al. 1994). They also produce IL-10 and IL-21, although their role is not clear (Kaplan et al. 2011). TGFβ induces the expression of PU.1 TF, essential for Th9 development (Chang et al. 2010; Ramming et al. 2012), as well as STAT6, IRF4 and GATA3. They have been shown to participate in inflammatory processes in autoimmune models (Jäger et al. 2009), as well as to play a role in asthma (Erpenbeck et al. 2003). IL-23 and IL-6 polarize naive CD4$^+$ T lymphocytes towards a **Th22** phenotype. Th22 cells are dependent on AHR (Aryl Hydrocarbon Receptor) TF and mainly produce IL-22. They play an important role in mucosal immune defense by the secretion of anti-microbial peptides (Aujla et al. 2008) as well as in some inflammatory and autoimmune diseases (N. Zhang et al. 2011). Finally, combination of IL-6 and IL-21 induce **Tfh (T follicular helper)** lymphocytes, which are dependent on Bcl-6 (B-cell lymphoma protein 6) transcription factor (Ma et al. 2012). They are key elements in the formation of germinal centers in lymph nodes, where they play a major role in activation and differentiation of B lymphocytes towards plasma cells through IL-21 secretion (Vinuesa et al. 2005).

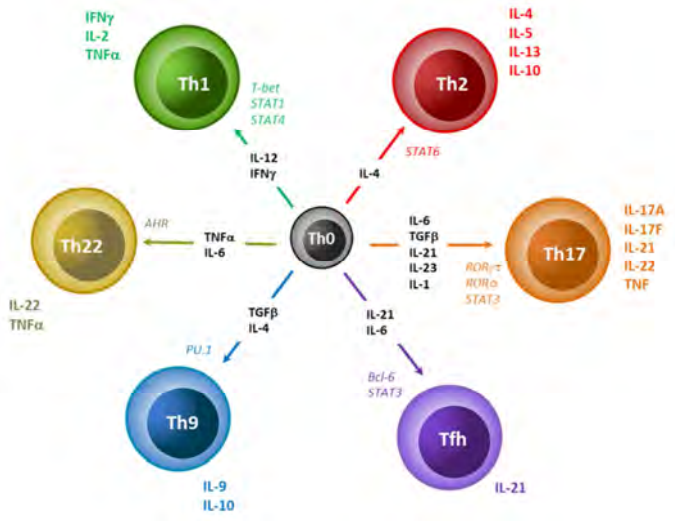

Figure 2. CD4$^+$ T lymphocyte polarization. Naive CD4$^+$ Th0 lymphocytes differentiate into effector populations depending on factors present in the environment. Different effector T cells produce a diversity of cytokines.

b. Regulatory T cells

A wide range of regulatory T cells have been described since the late 60's. Generally speaking, Tregs can be classified depending on their origin as thymic Tregs (tTregs) (before called natural T regs, nTregs) or peripheral Tregs (pTregs) (before called induced Tregs, iTregs). tTregs are generated in the thymus by escaping negative selection, whereas pTregs are generated in periphery, from conventional T lymphocytes, after their activation under a tolerogenic microenvironment. Both, tTregs and pTregs can be $CD4^+$, $CD8^+$ or double negative T cells.

The most extensively studied Treg subset is **$CD4^+$ Treg cells**, more accurately $CD4^+CD25^+FoxP3^+$ Treg cell subset. They also express CTLA-4 (Cytotoxic T-Lymphocyte-Associated protein 4) and GITR (Glucocorticoid-Induced TNFR family Related protein) and in humans they are $CD127^-LAG3^+$. They can be both, tTregs and pTregs. B. Hall described them for the first time in a rat transplant model, induced after cyclosporine treatment (Hall et al. 1985; Hall et al. 1990) but their existence was not widely accepted until 1995, when S. Sakaguchi's team found out that adoptive transfer of $CD4^+CD25^-$ and $CD4^+CD25^+$ T lymphocytes in a thymus-lacking mouse, suppressed the progression of autoimmunity. On the contrary, when $CD4^+CD25^-$ T lymphocytes were transferred alone, the disease developed (Sakaguchi et al. 1995). Thus, the suppressive function of Treg cells has been highlighted and extensively studied. The suppressive mechanisms used by Treg cells include cell to cell contact, cytolysis, IL-2 deprivation or secretion of inhibitory cytokines, like IL-10 or TGFβ (Vignali et al. 2008). tTregs and pTregs are phenotypically indistinguishable. Helios TF has recently been described as being specifically expressed by tTregs, therefore being a potential marker to distinguish between both origins (Thornton et al. 2010), although it is still being controversial (Himmel et al. 2013).

Other $CD4^+$ regulatory T cell subsets have also been described. Tr1 cells that are generated from T lymphocytes and activated in an antigen-specific manner in the presence of IL-10 (Groux et al. 1996; Groux et al. 1997). Tr1 also express CD25 and CTLA-4, but lack FoxP3 expression. They produce high quantities of IL-10 and TGFβ, which mediate their regulatory functions (Roncarolo et al. 2001). Recently, CD49b and LAG-3 co-expression by CD4+ T cells have been found to be specific markers identifying the Tr1 population and enabeling their isolation both in mouse and human (Gagliani et al. 2013). Th3 cells that are also induced after antigenic stimulation, mediate their regulatory functions mainly through TGFβ. They express CD25, CTLA-4 and FoxP3 (Weiner 2001). They are important in mucosal immunity. Th3 regulatory T cells are involved in IgA class switch and inhibit Th1 and Th2 responses. A less well described Treg population in rats is the naturally occurring $CD4^+CD45RC^{low}$ Treg cells, which do not proliferate and do not produce cytokines in response to alloantigens (Xystrakis, Bernard, et al. 2004). In mice, $CD4^+CD45RB^{low}$ T cells were also shown to display immunorregulatory properties (Read et al. 1998).

$CD8^+$ Treg cells are less extensively characterized than $CD4^+$ Treg cells, even if their discovery dates from the 70's (McCullagh 1970). $CD8^+$ Treg cells have been subdivided into different populations. tTregs and pTregs $CD8^+CD25^+FoxP3^+$ that also express some molecules found in their $CD4^+$ counterparts, as CTLA-4 and GITR (Bienvenu et al. 2005). Their mechanisms of action include cell to cell contact, inhibition of CD40L on effector T cells, anergy and effector T lymphocytes conversion into Treg cells.

Naturally arising $CD8^+CD28^-$ Treg cells have been shown to play a role in EAE (Experimental Autoimmune Encephalomyelitis) resistance (Najafian et al. 2003). *In vitro*, they inhibit IFNγ production by $CD4^+$ T cells. Upon stimulation, these cells produce immunosuppressive cytokines but they are not required for *in vitro* suppression. On the contrary, they require cell to cell contact and APCs antigen presentation. But $CD8^+CD28^-$ cells are a heterogeneous population that also includes cytolytic cells (Pomié et al. 2008). Therefore, a better characterization of their phenotype is needed. $CD8^+CD122^+$ Treg cells either from thymic or peripheral origin, regulate effector responses by IL-10 secretion and by PD-1 (Programmed Death-1) expression. They inhibit T cell activation *in vitro* and are able to prevent EAE *in vivo* (Rifa'i et al. 2004).

$CD8^+CD11c^+$ regulatory T cells were first described as a cytotoxic population by Keizer et. al. (Keizer et al. 1987). *In vivo*, in a rheumatoid arthritis model it has been demonstrated that $CD8^+CD11c^+$ Tregs produce large quantities of IFNγ that induce IDO (indoleamine 2 3-dioxygenase) expression by DCs and monocytes, which then inhibit antigen-specific $CD4^+$ T effector responses (Seo et al. 2004). Therefore, $CD8^+CD11c^+$ Tregs mediate their effect by IFNγ secretion. They can develop from $CD8^+CD11c^-$ T lymphocytes by antigenic stimulation plus anti-4-1BB antibody (Vinay et al. 2009) $CD8\alpha\alpha^+$ thymic or peripheral Treg cells are TcR restricted by the invariant molecule Qa-1. They induce apoptosis of Th1 cells and regulate NKT cells. $CD8^+CD45RC^{low}$ Treg lymphocytes can have thymic or peripheral origin. They have been described in rat (Xystrakis, Dejean, et al. 2004; Guillonneau et al. 2007) and human (Ordonez L, et al. 2009). They express FoxP3 and CTLA-4 molecules and secrete Th2 cytokines. They display suppressive activity both, *in vitro* and *in vivo* (Xystrakis, Dejean, et al. 2004). Their mechanism of action includes IFNγ induced IDO expression by other cell types, as well as inhibitory cytokine secretion like fibrinogen-like protein 2 (FGL-2) (Li et al. 2010a).

Thymic or peripheral **$CD3^+CD4^-CD8^-$ T lymphocytes** (double negative, DNT) have been characterized as a regulatory population in mice (Zhang et al. 2000) and humans (Fischer et al. 2005). They produce high quantities of IFNγ and TNFα. DNT cells use a variety of mechanisms to mediate suppression, as direct killing of T cells in an antigen-specific manner via Fas/FasL, downregulation of costimulatory molecules by DCs, induction of DCs apoptosis, acquisition of antigens from DC membrane. The latest mechanism is called trogocytosis, where DNT cells incorporate membrane

fragments with cell surface molecules expressed by the DC that will allow them to eliminate $CD4^+$ or $CD8^+$ T lymphocytes with the same antigenic specificity by apoptosis (Ford McIntyre et al. 2008).

1.2.2 B lymphocytes

B lymphocytes develop in the bone marrow. As T lymphocytes, B lymphocytes also undergo multiple selection steps in order to generate a repertoire of non-auto-reactive immature B lymphocytes. Each B lymphocyte expresses a unique B cell receptor (BcR), a surface receptor composed of 2 identical heavy chains (μ or δ) and 2 identical light chains (λ or κ), which derive by somatic rearrangements and point mutations from coding genes. This structure is known as immunoglobulin (Ig) and is the membrane form equivalent to soluble antibodies.

Several developmental steps lead CLP to pro-B cell (no expression of membrane receptor) which evolves into pre-B (expressing a pre-BcR). Until this step, clones selection is not dependent on the antigen. Then they develop into immature B lymphocytes, which are negatively selected. This step is antigen-dependent: pre-B cells expressing a pre-BcR displaying high affinity for autoantigens have to edit their receptor, rearrange genes coding for light chain. After rearrangements, the pre-BcR affinity of the immature B cells is assessed again. Depending on the strength affinity of self-antigen recognition, immature B cells continue their development to mature B lymphocytes or suffer deletion or anergy (Murphy et al. 2008).

a. Effector B lymphocytes

B lymphocytes with a specific non-autoreactive BcR migrate from bone marrow to the spleen, where they become mature naive B cells. Mature B cells recirculate to secondary lymphoid organs, where they find the appropriate microenvironment to facilitate antigen encounter. After antigen encounter, B cells can be activated through two different ways: either (i) they are activated only by the direct recognition of soluble antigens, which triggers a rapid response that do not require T cell cooperation or (ii) B cells get activated by a T cell dependent mechanism, which is case for the majority of B cells. The latest, migrate to the T-B zone border, where they internalize and process the captured antigen through their BcR and they present it onto the cell surface in a peptide/MHC class II complex, acting as an APC. These complexes are recognized by helper T cells which are specific for the same antigen. This recognition triggers effector T cell activation and cytokine secretion. Then B cells can follow two differentiation pathways: either they become plasma cells (PC) that will secrete the first wave of antibodies, either they participate in the germinal center (GC) reaction (Victora & Nussenzweig 2012). In the GC there is intense B cell proliferation. Clone's selection and BcR maturation affinity processes take place. Antigens are retained mainly by follicular dendritic cells (FDC), and B cells capture those antigens and present them to T follicular helper (Tfh) cells, that in turn secrete IL-21, a cytokine that guides isotype switching towards IgG1 (Ozaki et al. 2002). Clones with the highest affinity are selected to become memory B cells or long-lived PC.

b. Regulatory B lymphocytes

In the mid-70's, a suppressive role for B cells was suspected, but it was not until 1996 that regulatory B cells where shown to play a role in mouse EAE (Wolf et al. 1996). Since then, two main populations of regulatory B cells have been reported in mice.

The first ones to be described were T2 precursor B cell from the marginal zone. They display a CD19+ $CD21^{high}$ $CD23^{+}$ $CD24^{high}$ $CD93^{+}$ phenotype and produce IL-10. Those cells were able to suppress collagen-induced arthritis mouse model after adoptive transfer (Evans et al. 2007). Then, B10 cells were identified as being a rare B cell subset, predominantly found in spleen, expressing $CD1d^{high}$ $CD5^{+}$ molecules and being characterized by its unique capacity to produce IL-10 in response to specific activation signals (Yanaba et al. 2008). They have been shown to inhibit T cell dependent inflammation in a mouse model (Bouaziz et al. 2008) and they can differentiate into plasmablasts secreting antigen-specific antibodies (Maseda et al. 2012). A major role for CD40 stimulation and IL-21 in the activation of IL-10 secretion by Bregs has been described (Yoshizaki et al. 2012).

More recently, it has been shown that B cell stimulation through BcR in combination with IL-21 triggers granzyme B production without perforin secretion. These cells could play a role in the regulation of autoimmune responses (Hagn et al. 2012).

Bregs can suppress different T effector pathways by multiple mechanisms, like inhibition of Th1 and Th17 differentiation, induction of Treg cells, or direct inhibitory effect on antigenic presentation function of DCs (Chesneau et al. 2013).

1.2.3 Innate lymphoid cells

Although some cells classified into the ILC family have long been discovered, the relationship between them and their common origin has only recently been elucidated. ILC belong to the innate immune system but derive from a lymphoid precursor. The main characteristics are the absence of rearranged receptors, the lack of myeloid markers and their lymphoid morphology. ILC have been classified into 3 groups, depending on their cytokinic profile and TF expression: ILC1 are IFNγ producers and include NK cells (explained later); ILC2 express GATA-3 TF and produce IL-5 and IL-13 and ILC3 express RORγτ and produce IL-17 and IL-22 (Spits et al. 2013). They seem to have important roles in protective immunity, against intracellular or extracellular pathogens and virus. Their dysfunction has been shown to be the cause of multiple inflammatory and autoimmune disorders, most of them affecting mucosa (airways and gastrointestinal tract) (Spits & Di Santo 2011).

1.2.4 NK cells

Natural Killer (NK) cells belong to the group 1 of the ILC family. They are part of the innate immune system and provide a rapid response against viral infections and transformed cells. They develop in the bone marrow from CLP, and they emerge to the periphery with full functional

competence. NK cell precursors give rise to immature NK cells that begin the NK cell education via self MHC class I molecules. Mature NK cells leave the bone marrow and populate peripheral lymphoid organs (Huntington et al. 2007).

Regulation of NK cells activity is possible thanks to the expression of activating and inhibitory receptors at their surface. MHC class I molecules are the ligands of KIR (Killer cell Ig-like Receptor), the main inhibitory receptor. Following the "missing self hypothesis" (Lanier 2005), when cells are infected by an intracellular pathogen, they downregulate the expression of MHC class I molecules at their surface. The balance between activator and inhibitor signals is then broken, NK are activated and have the full capacity to kill the target cell.

NK cells can also regulate the migration and activation state of other cells from the innate or adaptive immune system. By IFNγ secretion, NK cells can activate macrophages and DCs to produce proinflammatory cytokines and enhance their antigen presentation capacity (Degli-Esposti & Smyth 2005). NK cells also directly interact with T cells to promote Th1 proinflammatory deviation (Martín-Fontecha et al. 2004).

1.2.5 Natural Killer T cells

NKT cells are a population of mature lymphocytes coexpressing NK receptors and a TcR complex. There are two main subsets, depending if their selection is dependent or independent on the non-classical class I molecule, CD1d. Those cells which are dependent on CD1d selection display a semi-invariant TcR, composed of $V_{\alpha}14$-$J_{\alpha}18$ rearrangement that preferentially associates to a limited variety of V_{β} chains (Gapin et al. 2001). They are either $CD4^{+}$ or DN cells, displaying a memory or activated phenotype. *In vitro* and *in vivo* studies have shown that these cells are able to produce large amounts of IL-4. They do not lyse target cells as NK cells do, but they can redirect lysis of Fc receptor-bearing target cells (Macdonald 1995).

2 MYELOID CELLS

2.1 Ontogeny and development of myeloid cells

In mice, embryonic hematopoiesis takes place in two phases. Firstly, myelo-erythroid development takes place in the yolk-sac (called **primitive hematopoiesis**). After that, HSC are generated in the aorta-gonads-mesonephros (AGM) axis (called **definitive hematopoiesis**). In the mid-embryogenesis period, progenitor cells derived from both phases of hematopoiesis give rise to the fetal liver. Fetal liver becomes then the major hematopoietic organ and the main source of circulating monocytes during embryogenesis. After birth, fetal liver hematopoiesis is replaced by bone marrow hematopoiesis, which becomes the main hematopoietic organ in adult mice.

In the late 1960s, the work of R Van Furth and ZA Cohn allowed to classify highly phagocytic cells and their precursors in one system, which was called **Mononuclear Phagocyte System (MPS)**. Although initially it only included monocytes and macrophages, at the end of 60's all myeloid immune cells other than polymorphonuclear granulocytes where already included in the classification (van Furth & Cohn 1968). In the early 1970's, following their discovery by Steinman and Cohn, DCs were also included in this system (Steinman & Cohn 1973).

Myelopoiesis is the process of formation and development of myeloid cells, which takes place in bone marrow in adults. The last accepted classification of the MPS includes every differentiation stage since the first precursor cell to terminally differentiated cells. **Hematopoietic Stem Cells (HSC)** give rise to the two main immune cell lineages, **Common Lymphoid Progenitors** (Kondo et al. 1997) and **Common Myeloid Progenitors (CMP)** (Akashi et al. 2000). CMP proliferate and differentiate into **Granulocyte Macrophage Precursors (GMP)** which develop into terminally differentiated **granulocytes**, or **Macrophage-Dendritic cell Progenitor (MDP)**. MDP have lost the potential to develop into granulocytes and are committed to the mononuclear phagocyte lineage; therefore, these cells can only give rise to monocytes and DC restricted precursors (Fogg et al. 2006).

MDP give rise to the recently discovered precursor, **common Monocyte Precursor (cMoP)** and also to **Common DC Precursors (CDP)**. cMoPs differ from MDP only by the lack of Flt3 expression on their surface and give rise to **monocytes** (Hettinger et al. 2013). From cMoP, monocytes are the most terminally differentiated cells, being continuously released into the blood stream and recirculating in blood under steady state conditions. On the contrary, under inflammatory conditions, a subset of monocytes can transmigrate inside tissues, and give rise to other cell types, such as inflammatory macrophages and inflammatory DCs (explained in detail below). CDPs give rise to **plasmacytoid DC (pDCs)** that circulate in the blood and enter lymphoid tissues, or to **pre-conventional DC (pre-cDC)**, that migrate through the blood to home to lymphoid and non-

lymphoid tissues, differentiating into **conventional DC (cDC)**, either **CD8$^+$** or **CD11b$^+$ cDC** and into **CD103$^+$** or **CD11b$^+$ cDC** respectively.

For years, tissue resident **macrophages** were thought to derive exclusively from circulating blood monocytes. However, it has recently been shown that most tissue-resident adult macrophages derive from precursors during embryonic development (Hettinger et al. 2013), and are self-renewed *in situ*, independently of adult hematopoiesis (Hashimoto, et al. 2013). Langerhan cells (LC) derive also from embryonic precursors and self-renew independently of the pool of macrophages (Merad et al. 2002). Therefore, although macrophages and Langerhan cells are part of the MPS, they do not share the same developmental pathway than the rest of myeloid cells.

Each differentiation step during myelopoiesis involves cell fate decisions that restrict the potential to give rise to other cell types. These steps are tightly regulated by transcription factors, cytokines and intracellular signaling molecules. In fact, all along myelopoiesis, there is a balance between various TFs that define the developmental pathway of precursors, leading to different final fates, depending on their relative expression. Most of our knowledge about the importance of gene expression during myelopoiesis has only been possible thanks to the development of tools to abolish gene expression *in vivo*, like knock-out (KO) animal models, or to overexpress one specific gene by its insertion in a specific targeted locus, knock-in (KI) animal models.

One TF that plays an important role in early myeloid commitment is **PU.1** from the Ets family (Nerlov & Graf 1998; Anderson et al. 2000; Guerriero et al. 2014). PU.1 has been shown to play important roles since HSC stage, as its constitutive expression is needed for maintenance of the HSC pool in the bone marrow (Iwasaki et al. 2005). Concerning the myeloid lineage development, PU.1 is required for the generation of CMP and it is also critical to commit cells down the monocytic developmental pathway by antagonizing with C/EBPα, which, on the contrary, is known to promote granulocytic development (Dakic et al. 2005; Dahl et al. 2003; Reddy et al. 2002). Other TFs and intracellular signaling pathways are also important to drive monocytic development, like ICSBP/IRF-8 (Tamura et al. 2000), KLF4 (Feinberg et al. 2007) or MafB and c-Maf (Sieweke et al. 1996; Bakri et al. 2005; Hegde et al. 1999).

A key cytokine that regulates DC commitment in hematopoiesis is **Flt3L**. Its receptor, Flt3 (also known as CD135 and Flk2) is present since HSC stage, until cDC final differentiation, whereas it is lost in progenitors that are not committed to DC lineage (Merad et al. 2013). The two main subtypes of DCs, pDCs and cDCs are strongly reduced in Flt3L $^{-/-}$ mice (McKenna et al. 2000; Karsunky et al. 2003), which also display reduced numbers of MDPs and CDP precursors.

Another important growth factor in the differentiation, proliferation and survival of blood monocytes and macrophages is **M-CSF** (also known as Csf-1). Mice carrying the *op/op* recessive mutation, which affects the production of functional M-CSF (Yoshida et al. 1990; Wiktor-Jedrzejczak

et al. 1990), suffer a deficiency in mature macrophages and also in osteoclasts, which causes low bone remodeling capacity and osteoporosis (Wiktor-Jedrzejczak et al. 1982). M-CSF receptor (M-CSFR, Csf-1R or CD115) is expressed on monocytes, macrophages and DC and their precursors (Sasmono et al. 2003). IL-34, an alternative more recently discovered M-CSFR ligand has been shown to play an important role in myeloid development, as M-CSF-deficient mice have milder phenotype than M-CSFR-deficient mice (Lin et al. 2008; Wei et al. 2010). PU.1 TF activates M-CSFR gene transcription, as myeloid progenitors deficient in PU.1 do not express M-CSFR (Reddy et al. 1994).

GM-CSF (also known as Csf-2) is a growth factor that controls the differentiation of the myeloid lineage. It binds specifically to GM-CSFR (also known as Csf-2R), composed of two chains (α and β). This receptor is expressed on GMP, MDP, CDP and cDCs (Merad et al. 2013). Although it is critical for promoting DCs differentiation, it has been shown that DC development in lymphoid organs is not impaired in Csf-2$^{-/-}$ mice, even though they display reduced numbers of non-lymphoid tissue DCs (Greter et al. 2012).

MPS nomenclature has recently been unified. The new terminology is based on a two-level system: the first level is the classification on the basis of their origin and the second level depends on cells function, location and/or phenotype (Guilliams et al. 2014). Therefore, taking into consideration the new nomenclature, the actual proposed model of the MPS is summarized in **Figure 3**.

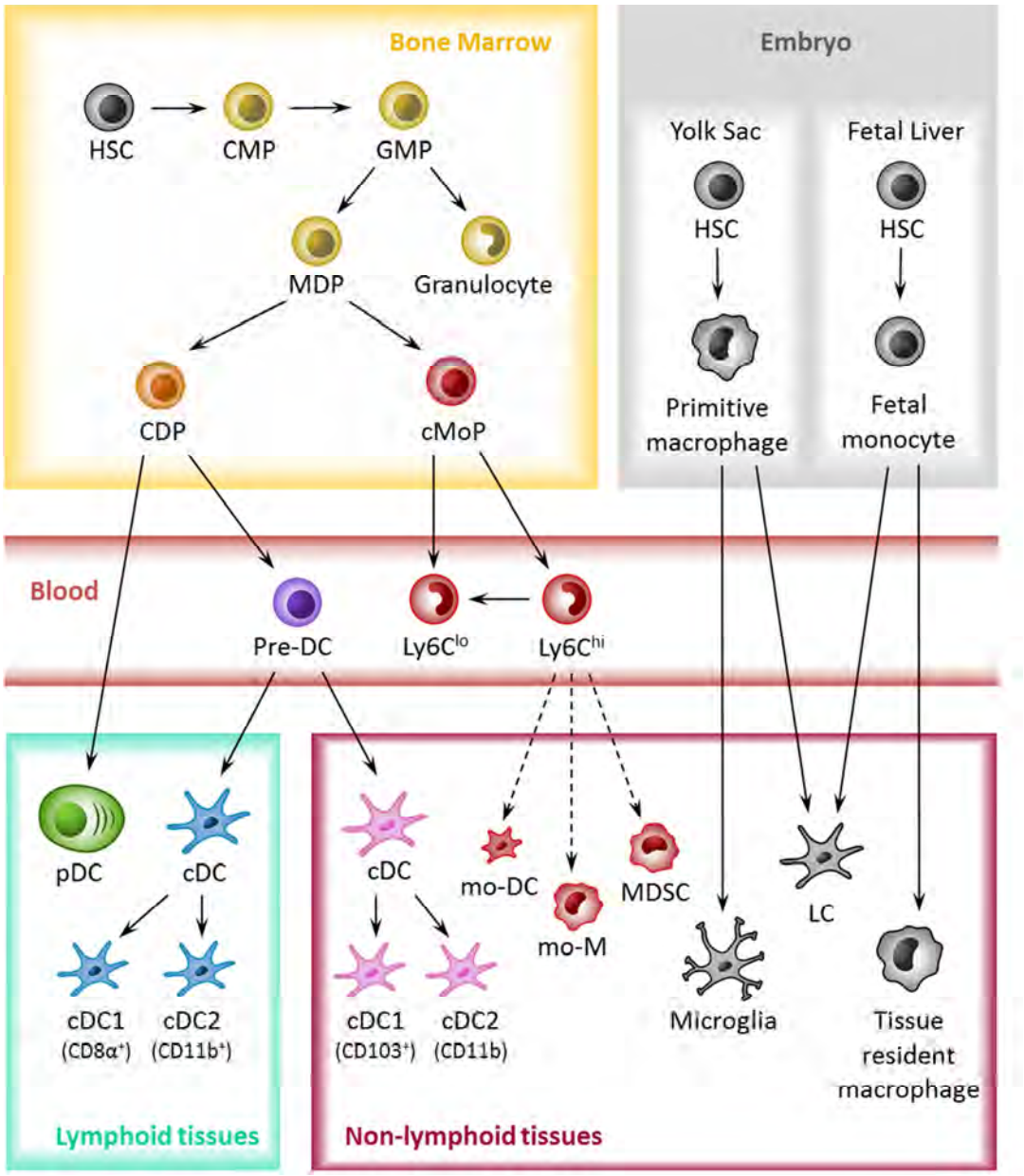

Figure 3. Myeloid cell development. HSC present in the bone marrow give rise to myeloid intermediates that leave the bone marrow to enter the blood. CDP differentiate into pDC and into pre-cDC, that circulate in blood and enter either to lymphoid tissues, where they give rise to CD8α$^+$ and CD11b$^+$ cDCs or to non-lymphoid tissues, where they give rise to CD103$^+$ or to CD11b+ cDC. cMoP give rise to two monocyte populations, Ly6C^{lo} and Ly6C^{hi}, that circulate in blood under steady state conditions. Under inflammatory conditions, in non-lymphoid tissues, Ly6C^{hi} monocytes develop into mo-DC, mo-M or MDSC. Tissue resident macrophages derive mostly from HSC present in the main hematopoietic sites of the embryo, either the yolk sac or the fetal liver. HSC, Hematopoietic Stem Cell, CMP, Common Myeloid Progenitor, GMP, Granulocyte Macrophage Precursor, MDP, Macrophage Dendritic cell Precursors, CDP, Common Dendritic cell Progenitor, cMoP, common Monocyte Progenitor, Pre-DC, pre-Dendritic Cell, pDC, plasmocytoid Dendritic Cell, cDC, conventional Dendritic Cell, mo-DC, monocyte-derived Dendritic Cell, mo-M, monocyte-derived Macrophage, MDSC, Myeloid-Derived Suppressive cell, LC, Langerhans Cell

2.2 Cell types

2.2.1 Monocytes

Monocytes represent around 5% of leukocytes in mouse blood. Originally, they were thought to be the precursors of most tissue-resident macrophages and inflammatory DCs. Nowadays monocytes are recognized as being an independent cellular system of effector cells, and not merely precursor cells (Ginhoux & Jung 2014).

Monocytes arise from myeloid precursors in both fetal liver and bone marrow during embryonic and adult hematopoiesis but under inflammatory conditions, they have also been shown to arise from the spleen (Hashimoto, Chow, Noizat, Teo, Beasley, Leboeuf, Christian D Becker, et al. 2013; Swirski et al. 2009). cMoP has been recently defined as the immediate precursor of monocytes (Hettinger et al. 2013).Their development and survival completely depend on the cytokine M-CSF, as mice defective in M-CSF or its receptor exhibit a profound monocytopenia (Wiktor-Jedrzejczak & Gordon 1996).

Among their functions, the most important one involves their great scavenger capacity, which allows them to remove apoptotic cells during homeostatic processes, development and also after inflammation. Thanks to their vast expression of scavenger receptors, they are also able to recognize toxic compounds, lipids and microorganisms. This recognition leads to their activation. Once stimulated, they produce large quantities of effector molecules involved in the defense of the organism. Insights in monocytes' development and function are extensively addressed in Auffray et al. (Auffray et al. 2009).

In mice, two different subsets of monocytes can be distinguished, based on functional and phenotypical differences:

The main subset of mouse monocytes is called **inflammatory monocytes**, which can be characterized as Ly6C^{hi} CX3CR1^{lo} CCR2^{hi}. Undifferentiated Ly6C^{hi} are not only found in blood but also in several tissues in steady state, including spleen, lymph nodes, skin and lungs (Jakubzick et al. 2013). The function of Ly6C^{hi} monocytes in blood under steady-state remains poorly defined. It has been suggested that it might be related to their high phagocytic capacity and their ability to access tissues and organs (Ginhoux & Jung 2014). During inflammation, these cells migrate from the bone marrow and are recruited to sites of inflammation or tissue remodeling in response to CCR2 ligands (CCL2 and CCL7). They extravasate to tissues and can give rise to other effector cells, as monocyte-derived macrophages and monocyte-derived DCs.

The second subset is found in resting and in inflamed tissues and display longer half-life than inflammatory monocytes. Phenotypically, these cells are characterized by Ly6C^{lo} CX3CR1^{hi} and CCR2^{lo} expression. In steady-state, they remain within blood vessels and migrate along vascular

endothelium. Their main function is to survey endothelial integrity, which is the reason why they are called **patrolling monocytes.** Extravasation is rare in the absence of inflammation, but under inflammatory conditions these cells are rapidly recruited to sites of infection, where they produce inflammatory mediators and chemokines involved in the recruitment of other effector cell types (granulocytes, inflammatory monocytes, NK cells and T cells). At this early stage of inflammation, Ly6C^{lo} monocytes are the only source of TNFα in the inflamed tissue, a central cytokine in mediating innate immune responses (Auffray et al. 2007). But this inflammatory response is only transient, as some hours later inflammatory monocytes are the ones in charge of the production of pro-inflammatory cytokines.

Accumulating evidence suggest that blood monocyte subsets represent stages in a developmental sequence, suggesting that Ly6C^{hi} monocytes differentiate into Ly6C^{lo} monocytes in circulation (Liu et al. 2009).

Following the early inflammatory response, patrolling monocyte initiate a M2-like macrophage differentiation program, displaying an alternative activated phenotype which take part in tissue remodeling after inflammation. On the contrary, inflammatory monocytes initiate a M1 type inflammatory response or differentiate into inflammatory DC, perpetuating the inflammatory microenvironment (**Figure 4**). Therefore, in inflamed tissues and during tissue remodeling monocytes can be macrophage precursors.

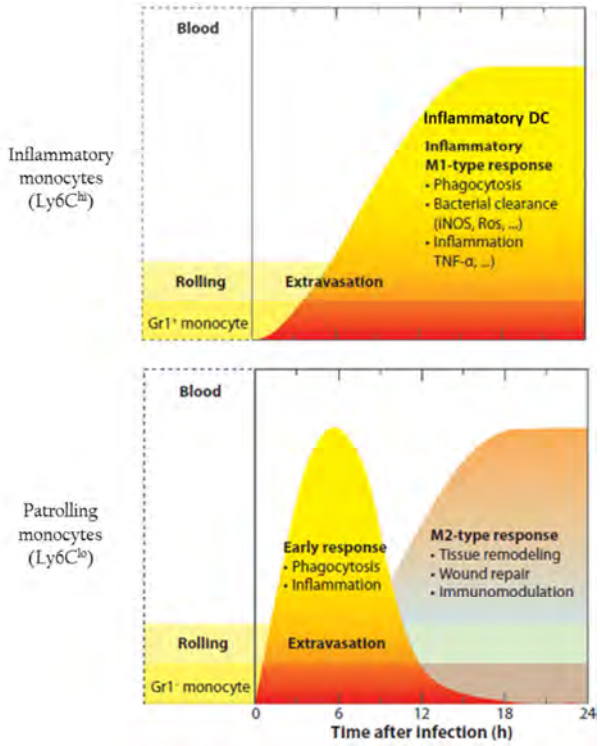

Figure 4. Differentiation fate of the two monocytes subsets in inflammatory conditions. Adapted from (Auffray et al. 2009).

Even if in steady-state conditions monocytes are not macrophages precursors, some exceptions exist. Inflammatory monocytes give rise to macrophages in tissues that are exposed to microbiota, like gut (where most of the specialized macrophage populations arise from Ly6C^{hi} monocytes) (Varol et al. 2009) and skin (where monocytes give rise to a proportion of dermal macrophage population) (Tamoutounour et al. 2013).

In humans, three populations of blood monocytes have been characterized. They are defined by the expression of two surface markers, CD14 and CD16: (i) CD14^{+}CD16^{-}, which are equivalent to Ly6C^{hi} in mouse, represents 80-90% of blood monocytes, express CCR2^{hi} CX3CR1^{lo} and produce IL-10, (ii) CD14^{+}CD16^{+}, that express CD64 and CD32 FcR, have phagocytic activity and produce TNFα and IL-1 in response to LPS and (iii) CD14^{dim}CD16^{+}, which are poorly phagocytic cells and do not produce TNFα nor IL-1 (Auffray et al. 2009).

2.2.2 Dendritic cells

DCs were discovered in 1973 by R Steinman and Z Cohn. DCs were firstly described as a cellular population that displayed a characteristic morphology with prolongations (later called dendrites) identified for the first time in mice lymphoid organs (Steinman & Cohn 1973). Nowadays, there is no doubt that DC are key players of immune responses, playing double faced roles, as they are able to induce primary immune responses, and on the other hand they are also able to regulate immune responses by tolerance induction.

DCs are a rare population, representing around 0.3 and 1-2% of total leukocytes in blood and lymphoid organs, respectively. In spite of their relatively low percentage, they are widespread around the body, not only present in lymphoid tissues but also in non-lymphoid tissues, particularly in mucosa membranes (skin, gut and lungs). This strategic location allows them to take part in host protection against invading organisms. Taking into consideration their wide localization, it was soon proposed that different subtypes of DCs might exist, with specialized functions depending on the tissue they reside.

In steady-state conditions, DCs function as sentinels of the immune system. When they first arrive to a peripheral tissue from the bone marrow, they are called immature DCs (iDC), as they have not encountered an antigen yet. They express a spectrum of membrane receptors that let them continuously take up local antigens and process them, in order to survey for a possible invasion. Under inflammatory conditions or after an infection, iDC can recognize PAMPs or DAMPs by through PRRs expressed at their surface. Binding of these receptors triggers a process of DC activation, which transitorily increases DC phagocytic capacity. DCs go through a maturation process, which involves cytoskeleton reorganization and expression of chemokine receptors, like CCR7, which will allow DCs to migrate to the T zone of draining lymph nodes. It is in secondary

lymphoid organs where mature DCs present processed antigens to naive T cells, triggering an antigen-specific adaptive immune response.

Therefore, DCs are considered the link between innate and adaptive immune responses, as they are able to activate cells from the innate immune system, such as macrophages, NK cells and eosinophils which participate in the elimination and clearance of pathogens from the site of infection and at the same tame they can activate naive T cells which will, in turn, differentiate and activate other cell types from the adaptive system such as B cells.

a. DC subsets and function

The existence of DCs subsets was only accepted in the mid-90's, when some experiments performed in mouse lymph nodes showed that only some DC expressed the CD8 surface marker whereas others did not (Shortman & Heath 2010). Later, those findings were extended to non-lymphoid tissue DCs. In humans, DCs can also be differentiated into subtypes, by differential expression of various surface markers.

Whereas in steady-state DCs can be divided into two major groups, cDC and pDC, under inflammatory conditions another important subset differentiates from blood monocytes, called inflammatory DCs. The subsets and homology between mouse and human DC populations in steady-state are represented in **Figure 5**.

Conventional DC (cDC)

Conventional DCs (cDCs) include lymphoid tissue DCs and non-lymphoid tissue DCs, which are localized in different tissues and express different surface markers but still share their origin and specialization. Recently, nomenclature of cDCs has been unified and DCs are assembled depending on the TF profile that regulates their development. Lymphoid tissue $CD8\alpha^+$ and non-lymphoid tissue $CD103^+CD11b^-$cDCs are grouped under the name of classical type 1 DCs (cDC1s), and their development depends on BATF3 TF. Lymphoid tissue and non-lymphoid tissue $CD11b^+$ and $CD103^+$ DCs are brought together under the name of classical type 2 DCs (cDC2s), and their development depends on IRF4 TF (Guilliams et al. 2014).

Lymphoid tissue DC

Lymphoid tissue–resident cDCs differentiate in and spend their whole life into lymphoid tissues. They represent the majority of the DC population in spleen and thymus, and only half of that in lymph nodes. In the steady-state, they are phenotypically immature cells, and become activated when they are faced to danger signals.

Their analogs in humans are CD1c$^+$ and CD141$^+$ DC (BDCA-1 and BDCA-3 DC respectively), which resemble blood DCs, and were found in spleen and tonsils. It has been suggested that they could be migratory DCs, as they have also been found in the dermis.

In mouse, cDCs comprise two main subsets, classified depending on their surface markers expression, into CD8$^+$ cDC or CD8$^-$CD11b$^+$ cDC.

CD8$^+$ DCs represent 20–40% of spleen and lymph node cDCs. They express the molecule CD8α, no or low levels of the integrin CD11b, and high levels of Flt3, a cytokine essential for their differentiation and proliferation.

Their main function is to sense pathogens and tissue damage, which is possible thanks to their strategic anatomical localization. In the spleen, they are located in the marginal zone (Reis e Sousa et al. 1997), where they filter blood antigens. In lymph nodes, they are located in the subcapsular sinus, which is the site of entry of afferent lymphatic vessels that drain non-lymphoid tissues (Qiu et al. 2009; Idoyaga et al. 2009). After antigen capture, CD8$^+$ cDCs migrate to the T cell zone of SLO where they present blood or tissue antigens to T lymphocytes.

Regarding their antigen presenting capacity, CD8$^+$ cDCs are very efficient stimulators of CD8$^+$ T cells and, to less extent, of CD4$^+$ T cells (Shortman & Heath 2010; Dudziak et al. 2007). This difference is mostly due to their preferential expression of molecules related to the MHC class I pathway of antigen presentation. A part from the classical MHC class I presentation pathway, they can also capture exogenous antigens and cross-present them to CD8$^+$ T cells. Antigen presentation pathways will be discussed in detail in Part II Section 2.1 of the introduction.

CD8$^+$ cDCs contribute to the induction and maintenance of central and peripheral tolerance. In the thymus, they play a key role in central tolerance by their participation in negative selection process of developing thymocytes and the induction of regulatory T cells. In the periphery, they are thought to participate in deletional tolerance of self-reactive T cells and the induction of antigen-specific Tregs (Merad et al. 2013).

The **CD8$^-$CD11b$^+$ cDC** subset lacks the marker CD8 and predominates among the lymphoid resident cDC population in all organs except the thymus. They also depend on Flt3L for their proliferation.

CD11b$^+$ cDCs express different PRRs than those present in CD8$^+$ cDCs, which allow them to recognize and get activated by different PAMPs.

On the contrary to CD8$^+$ DCs, the main role of CD11b$^+$ cDCs in T cell priming is to activate CD4$^+$ T cells, as they express higher levels of MHC class II compared with CD8$^+$ cDC. Similar to CD8$^+$ cDCs, CD11b$^+$ cDCs also contribute to the maintenance of central and peripheral tolerance by

inducing clonal deletion of autoreactive clones or differentiation of antigen specific T reg cells (Bonasio et al. 2006; Proietto et al. 2008).

Nonlymphoid tissue DC

cDCs represent 1–5% of tissue cells, depending on the organ. Non-lymphoid tissue DCs constantly migrate through afferent lymphatic vessels to T cell zones of tissue draining lymph nodes after antigen capture, already in a mature state. This is in contrast to lymph node-resident cDCs, which get to lymph nodes from blood precursors in an immature state. In response to inflammation, migration to lymph nodes increases (Jakubzick et al. 2008). These cDCs are called tissue-migratory DCs.

In humans, two populations of DC can be distinguished in dermis and lungs, one that displays $CD1a^+CD14^-$ phenotype and the other one which is $CD1a^-CD14^+$. Human epidermis contains Langerhans cells, expressing high levels of CD1a.

In mice, they consist of two major subsets: **$CD103^+CD11b^-$cDCs** and **$CD11b^+$ cDCs** , which are the analogs of the $CD8^+$ and $CD11b^+$ cDC subsets in lymphoid organs respectively.

Nonlymphoid tissue DCs are mainly found at the first barrier of host's protection, like skin and mucosa, where they encounter and capture invading organisms and initiate an adaptive immune response.

In the **skin**, myeloid immune cells are organized and located into specific sites. In the epidermis layer, a population of Langerhans Cells (LC) is found. LCs share characteristics with DCs and macrophages, and will be discussed in detail later on this chapter. In the dermis, there are two DC subpopulations: $CD103^+CD11b^-$Langerin$^+$ DC, which are very efficient at antigen cross-presentation to $CD8^+$ T cells and $CD103^-CD11b^{hi}$Langerhin$^+$ DC, which efficiently present antigens to $CD4^+$ T cells.

In the **gut**, an enriched population of $CD103^+$ DC that co-express CD8 marker and display low expression of MHC class II molecules has been described in Peyer's Patches. In lamina propria, CD103 and CX3CR1 expression help to distinguish between the two DC subpopulations that express different levels of CD11b: $CD11b^{hi}CD103^-CX3CR1^+$ and $CD11b^+CD103^+CX3CR1^-$.

Plasmacytoid DC (pDC)

Plasmacytoid DCs (pDC) develop in the bone marrow directly from CDP. They are mostly present in peripheral lymphoid tissues although they are also found in inflamed tissues. They play a main role in the anti-viral response, as they strongly express TLR (Toll-like Receptor) 7 and TRL9, which recognize viral ssDNA and ssRNA. After viral antigen recognition, they secrete high levels of type-I-IFN (Liu 2005). pDC development depends on the TF E2-2, a member of the E protein family

(Cisse et al. 2008). They play a crucial role in oral and mucosal tolerance to inhaled or ingested antigens, and also in the induction of intrathymic Treg cell development (Matta et al. 2010).

In humans, pDCs do not express CD11c marker but they express BDCA-2 and BDCA-4 (CD303 and CD304/Neuropilin-1) (Dzionek et al. 2000).

In mice, the pDC phenotype is described as $Ly6C^{+}B220^{+}CD11c^{low}CD4^{-}CD8\alpha^{-}CD11b^{-}CD137^{+}$. pDCs produce IL-12 and IFNα (Asselin-Paturel et al. 2001).

Inflammatory DC (infDC)

Inflammatory DCs (infDCs) refer to a DC population with a reinforcement function, which is absent from steady-state tissues and lymphoid organs (León et al. 2007). This population differentiates from circulating monocytes in response to inflammatory stimuli and disappears when the infection is resolved.

They are characterized by the expression of Ly6C, CD11b, MHC-II, and intermediate CD11c levels, although their phenotype depends on the nature of the stimuli that induces them and the microenvironment at the inflammatory focus (León et al. 2007).

infDCs arise from $Ly6C^{hi}$ monocytes, which are recruited to the site of inflammation thanks to their expression of CCR2 chemokine receptor. It has been reported that, in situations of stress, early hematopoietic precursors can differentiate directly into DCs, not following the normal differentiation pathway of myelopoiesis (Takizawa et al. 2012). infDC have been found in lymph nodes, draining sites of infection. This migration seems to be dependent on CCR7 (Segura & Amigorena 2013).

DCs which are differentiated in culture with GM-CSF from bone marrow precursors resemble infDCs (Xu et al. 2007). Therefore, GM-CSF was thought to play an important role in infDC differentiation (Shortman & Naik 2007). Later, it was shown that the absence of GM-CSFR does not impair the accumulation of infDCs in spleen after LPS injection or during infection (Greter et al. 2012).

infDCs present antigens to $CD4^{+}$ T cells. The type of T cell response that they polarize depends on the inflammatory environment and the type of infection, as both, Th1 and Th2 polarizations have been described to be induced by infDC (Segura & Amigorena 2013). They can also stimulate $CD8^{+}$ T cells through cross-presentation.

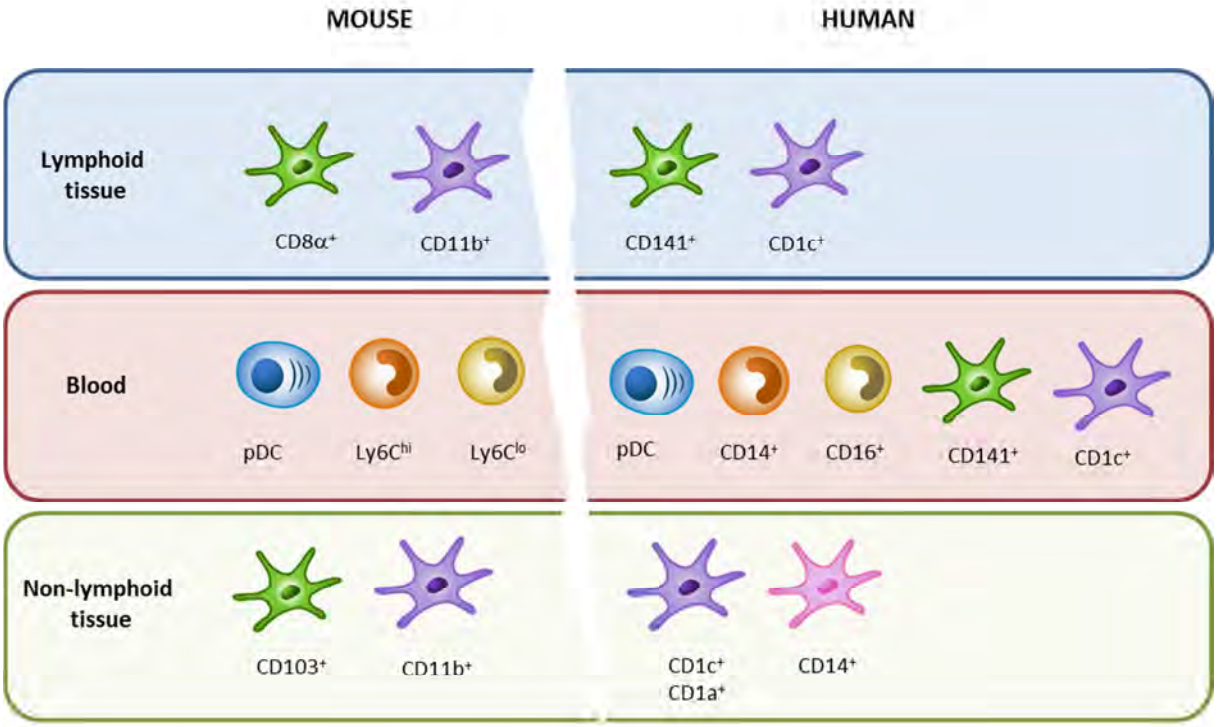

Figure 5. DC subsets in mouse and human. Most subsets have an homolog in mice and humans, which share origin and functionallity

2.2.3 Macrophages

Macrophages were discovered in the late 60's and were described as tissue-resident cells, not able to migrate to secondary lymphoid organs and inefficient at presenting antigens to naive T lymphocytes. On the contrary, they are tissue-resident myeloid cells specialized in the maintenance of tissue homeostasis and integrity at steady state. After an inflammatory response takes place they are in charge of tissue damage repair, as they display high phagocytic and degradative capacity.

Macrophage development depends on the growth factor M-CSF and its receptor, M-CSFR. The majority of tissue-resident macrophages are generated during embryonic hematopoiesis and not during myelopoiesis in bone marrow, as previously thought. Macrophages are able to proliferate locally in steady-state and in response to tissue injuries, being able to self-renew and preserve the tissue macrophage population; therefore, in those situations, monocytes would not be the major source of macrophages (Epelman et al. 2014). Adult microglia has been found to derive from primitive macrophages (Ginhoux et al. 2010). Other tissue resident macrophage populations are constantly replenished by Ly6C^{hi} monocytes, as intestinal macrophages, healthy skin or splenic marginal zone macrophages (Ginhoux & Jung 2014). Therefore, under certain conditions, monocytes can participate to the generation of macrophages' pool.

There is a deal concerning macrophages classification and nomenclature. Terminology used in the field is confusing, as each research group defines different nomenclatures, complicating the comparison of results between different studies. In 2014, a group of experts in the field of macrophages met and proposed a new nomenclature for *in vitro* generated macrophages, taking into consideration macrophage's source, activators used to "polarize" macrophage differentiation and surface markers as well as gene transcription profiles (Murray et al. 2014). In this manuscript, macrophages have been classified depending on their origin and the tissue where they reside. Different activation profiles are also explained.

a. Tissue-resident macrophage subsets

Macrophages display high degree of heterogeneity depending on the tissue where they are found. This heterogeneity reflects the multiple functions they carry on. Therefore, their classification depending on surface markers becomes a difficult task.

In each tissue, macrophages can be differentiated into two main subsets, depending on their precursor origin, which leads to different phenotypes: HSC-derived monocytes give rise to CD11b^{hi}F4/80^{int} macrophages and yolk-sac macrophages express CD11b^{int} F4/80^{hi}. An exception is the gastrointestinal tract, where all resident macrophages derive from blood monocytes (Bain et al. 2013).

Under steady-state conditions, macrophages are called differently, depending on the tissue they reside (Davies, Jenkins, et al. 2013). They express different surface markers and display tissue-specific functions (**Figure 6**):

- **Osteoclasts** are macrophages found in bone. Their main function consists in bone remodeling.
- **Alveolar macrophages**, found in lungs, are key cells in clearing surfactant.
- **Central nervous system macrophages**, which include various subsets with different origins, like microglia, perivascular macrophages, meningeal macrophages and choroid-plexus macrophages.
- **Kupffer cells**, which are specialized hepatic macrophages, playing a key role in erythrocyte's clearance and iron recycling.
- **Adipose tissue macrophages**
- In secondary lymphoid organs, there are different populations in spleen and lymph nodes. **Splenic macrophages** (sM) are further classified in two subpopulations, depending on their location at the spleen: marginal zone macrophages (mz-sM) (found adjacent to the marginal sinus, where blood circulates) and metallophilic macrophages (m-sM) (adjacent to white pulp, where they sample particles contained in the blood, playing an important role during infections). In lymph nodes, they are called

subcapsular macrophages, as they are located at subcapsular sinus, and it is the region where lymphatic fluid arrives, between capsule and cortex region.

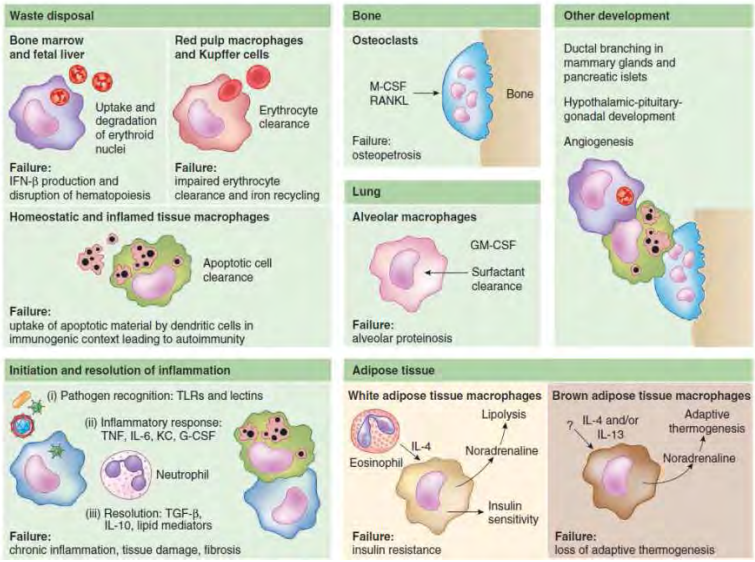

Figure 6. Tissue-resident macrophage subsets. Figure from (Davies, Jenkins, et al. 2013)

During inflammation, another type of macrophage arises, the so called **inflammatory-monocyte-derived macrophages**. In mouse, they originate from circulating inflammatory monocytes ($Ly6C^{hi}$). Inflammatory monocytes are recruited to the site of inflammation, where they differentiate into inflammatory macrophages and get activated by microenvironmental signals, leading either to (i) a pro-inflammatory phenotype (with increased microbicidal activity and pro-inflammatory cytokine secretion) or to (ii) an anti-inflammatory state (where they participate in tissue repair and secrete anti-inflammatory cytokines) (Liddiard et al. 2011).

b. Macrophage polarization

The main function of macrophages as cells from the innate immunity is to protect from microbial invasion. To do so, they are equipped with a variety of surface receptors that sense and recognize a broad range of microbial components which are not normally found in healthy tissues. Sensing of microbial components leads to macrophage activation and functional specialization, polarizing macrophages towards a phenotype which will determine the outcome of the response. Therefore, there is an adaptive component in the way phagocytes recognize pathogens, that makes it possible to establish a more accurate response depending on the stimulus, thus shaping a polarized response (Murray & Wynn 2011). Different polarization states are represented in **Figure 7**.

Classically activated macrophages (M1)

The first signal that macrophages need to become classically activated is the pro-inflammatory cytokine IFNγ. The major source of IFNγ production is activated $CD4^+$ Th1 cells and, to a lesser extent, Tc1 cells and NK cells. The second signal is TNF itself or a TNF inducer, like a TLR ligand. Therefore, Th1 lymphocytes and microbial products (such as LPS) can drive the polarization of macrophages into classically activated macrophages, also called **M1 macrophages** (Mosser & Edwards 2008).

Macrophages encounter pathogens at sites of tissue inflammation. Once classically activated, macrophages display an enhanced ability to kill and degrade intracellular microorganisms. This is possible due to their increased expression of iNOS (inducible Nitric Oxide Synthase) enzyme, which increases production of reactive nitrogen intermediates (RNI) and reactive oxygen species (ROS). Their phagocytic capacity is not increased compared to resting cells and they express low levels of mannose receptor and FcγRII. On the contrary, they increase the expression of MHC class II molecules, therefore becoming more efficient at antigen presentation.

They release large amounts of pro-inflammatory cytokines, importantly IL-12, IL-23 and TNF, and low levels of anti-inflammatory cytokines, like IL-10. Moreover, they secrete cytokines and chemokines that recruit and polarize T lymphocytes towards a Th1 phenotype, therefore amplifying and perpetuating a type 1 response.

Alternatively activated macrophages (M2)

Type 2 cellular responses are essential for the control of extracellular parasites (helminthes, protozoa and fungi), but can also have negative impact, contributing to allergy and infection's complications. Activated $CD4^+$ T helper 2 cells (Th2) produce IL-4 and IL-13 cytokines, which drive polarization to **M2 macrophages**.

M2 macrophages are characterized by high expression of scavenger, mannose and galactose receptors. They display high phagocytic activity, although they are not very efficient killers of intracellular bacteria since they cannot produce enough levels of NO due to the induction of the enzyme Arginase-1. They also upregulate MHC class II molecules, although they are inefficient at antigen presentation; instead, they display suppressive activity, inhibiting T cell proliferation.

M2 macrophages secrete high levels of the anti-inflammatory cytokine IL-10, and low levels of IL-12. Therefore, alternatively activated macrophages are considered as a regulatory cell type, being involved in tissue remodeling after injury.

M2 macrophage is a generic name that includes all alternative activated cells (i.e. activation pathways different from the classical activation pathway), which share some functional characteristics, such as low production of IL-12, and the involvement in type 2 responses,

immunoregulation and tissue remodeling. Even though, there exists a more accurate classification of M2 macrophages (Mantovani et al. 2004):

- M2a: they stand for the polarization induced by IL-4 and IL-13. They express high levels of Arginase-1 enzyme. They are involved in the perpetuation of a Th2 inflammatory response and are the main players during allergy.
- M2b: they are induced by exposure to immunocomplexes (IC) and TLR agonists or IL-1R. M2b macrophages produce high levels of inflammatory cytokines (IL-1, TNF and IL-6) but keep the M2 macrophage characteristics of high IL-10 and low IL-12 secretion, taking part in immune regulation.
- M2c: they are induced by IL-10 and glucocorticoid hormones and produce IL-10 and TGFβ, being implicated in tissue remodeling after injury.

	M1	M2a	M2b	M2c
Polarization	Classical	Alternative	Type II	Deactivated
Activation	IFNγ + LPS or TNF	IL-4 and IL-16	IC + TCR / IL-1R ligands	IL-10
Response	Th1 (inflammation, intracellular pathogen killing)	Th2 (inflammation, allergy, parasite killing)	Immunoregulation	Tissue repair

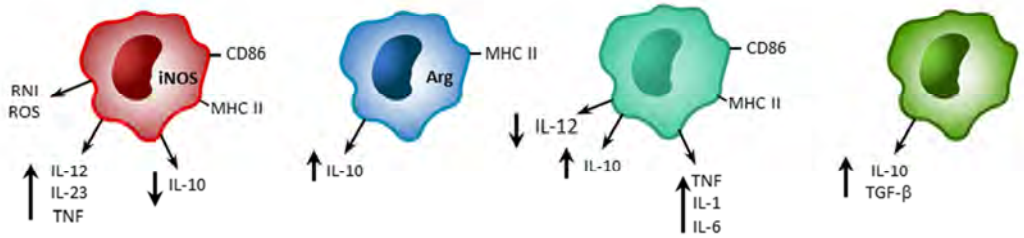

Figure 7. Macropahge polarization states. Adapted from (Mantovani et al., 2004)

Even if for practical reasons we try to classify macrophages depending on the stimulus that leads to their differentiation, macrophages should be considered as a plastic cell population, a continuum spectrum of polarization states, which influence their function, ranking from inflammation to tissue repair (Mosser & Edwards 2008).

c. Macrophages functions

Tissue macrophages express PRR which are classified according to their cellular location or the type of molecules they recognize: TLR, NLR (NOD-like receptors), lectins and scavenger receptors. Their expression depend on macrophage location and varies with the microenvironment to which macrophages are exposed.

After initial recognition of microbial or danger signal, tissue-resident macrophages get activated and produce inflammatory cytokines and chemokines which attract inflammatory leukocytes, like neutrophils and inflammatory monocytes. Monocytes differentiate into inflammatory macrophages, which arise as the main cell type in lesions during inflammation and disappear once the danger is overcome (Murray & Wynn 2011). After injury, many resident macrophages remain throughout inflammation. Although they are considered non-migratory cells, it has been suggested that some tissue-resident macrophages can migrate to draining lymph nodes in response to tissue injury at low frequency (Hashimoto et al. 2011).

Once inflammation is over, tissue-resident macrophages repopulate tissues due to enhanced proliferation and self-renewal capacity in response to growth factors, mainly M-CSF (Davies, Rosas, et al. 2013). In this context, there is no evidence of monocyte contribution to the macrophage pool, suggesting that both macrophage subsets play their roles at different moments during inflammation. At the resolution phase, macrophages clear apoptotic and damaged cells ensuring the restoration of tissue homeostasis (Gordon & Taylor 2005; Lucas et al. 2010).

2.2.4 Langerhans cells

Langerhans cells (LC) refer to the myeloid population that reside in the epidermal layer of the skin, both in steady-state an under inflammatory conditions. They account for 3-5% of all nucleated cells in the epidermis and are disposed in a network through their extended dendrites.

Phenotypically, they express myeloid surface markers, like CD11b and F4/80, low levels of MHC class II molecules and intermediate levels of CD11c. Their hallmark is the high expression of the C-type lectin langerin (CD207), although its expression is not confined to LC, as langerin$^+$ dermal DCs have identified (Merad et al. 2008). They constitutively secrete the anti-inflammatory cytokine IL-10.

They have a unique ontogeny, as they arise exclusively from embryonic macrophage precursors in the steady state. LCs are recruited to the epidermis layer of the skin prior to birth (Hoeffel et al. 2012), where they self-renew *in situ* independently from bone marrow precursors (Merad et al. 2002). On the contrary, yolk sac contribution to adult LC is minimal (Hoeffel et al. 2012). Local hematopoietic cell precursors could also contribute to LC homeostasis depending on physiological needs. However, during inflammation, circulating monocytes have been shown to replenish epidermal LC population (Ginhoux et al. 2006).

LC are totally absent in Csf-1R KO mice, but develop normally in Flt3 o FLt3L KO mice (Ginhoux et al. 2006). Interestingly, LC can develop in mice deficient for M-CSF (Witmer-Pack et al. 1993), suggesting that it is IL-34, the other known ligand for M-CSFR, the one responsible for LC

development (Y. Wang et al. 2012). Furthermore, IL-34 is produced at high levels by keratinocytes in the epidermis, whereas M-CSF levels are undetectable (Y. Wang et al. 2012). TGFβ is required for LC differentiation. It is expressed by keratinocytes and LC, and can act in an autocrine manner (Borkowski et al. 1996; Kaplan et al. 2007).

Great controversies exist regarding the classification of LCs as a subtype of DCs or of macrophages. As already mentioned, LCs derive from embryonic macrophages, depend on M-CSFR for their development, have poor migratory capacity to lymph nodes and have a similar gene profiling to macrophages. However, following activation LCs increase their migratory capacity and their gene profiling acquires hallmarks of DC signature.

LC function *in vivo* depends on the microenvironment where antigen encounter takes place. Some authors suggest that LCs are unable to prime T cell responses (Allan et al. 2003) as shown by experiments were LC did not induce $CD8^+$ T cell responses against Herpes Simplex virus-1. Those findings are in contrast with what had long been described in the literature. In a contact hypersensitivity mouse model, LCs have been shown to display redundant functions with dermal CD103+ DC (Kaplan et al. 2008).

2.2.5 Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) consist of a heterogeneous population of myeloid progenitor cells and immature myeloid cells (macrophages, dendritic cells and granulocytes in their immature form). These cells were described around the 1980's as being a natural suppressor population present in tumor-bearing mice which co-expressed CD11b and Gr1 (Ly6C and Ly6G antigens) on their surface (Strober 1984; Maier T, et al. 1989).

Nowadays, we know that MDSCs are not only generated intratumorally, but also under other chronic stress-causing agents or inflammatory stimuli. Polymicrobial sepsis, viral infection, sterile inflammation or organ transplantation induce a dramatic increase in MDSC recruitment to lymphoid organs (spleen, lymph nodes and bone marrow) or to the liver (Gabrilovich & Nagaraj 2009).

In healthy individuals, immature myeloid cells are constantly being generated in the bone marrow, and follow a normal developmental pathway, leading to DCs, macrophages and granulocytes; however, in cancer or under sustained inflammatory stimuli, there is discontinuation of the normal differentiation pathway, leading to excessive bone marrow myelopoiesis and the accumulation of immature myeloid precursors in the periphery, which constitute the MDSC subset (Gabrilovich et al. 2012).

Two phenotypically and functionally distinct MDSC subsets have been identified in mouse (Youn et al. 2008):

- **Polymorphonuclear (or granulocytic) MDSCs**, which are $CD11b^+$ $Ly6G^+$ $Ly6C^{+/-}$. They correspond to the main population of MDSC in mice but the less immunosuppressive one. Their mechanism of action is based on the suppression of antigen specific $CD8^+$ T cells by ROS production. Morphologically, they resemble neutrophils but their functions are very different, as neutrophils are not immunosuppressive and display high phagocytic capacity; moreover, polymorphonuclear MDSC express higher levels of certain enzymes (Aginase-1 and myeloperoxidase) and increased ROS production compared to neutrophils.

- **Monocytic MDSC**, which display a $CD11b^+Ly6G^-Ly6G^{hi}$ phenotype. They induce strong suppression of $CD8^+$ T cells through the expression of multiple enzymes and the generation of RNS. They could be mistaken for inflammatory monocytes, as they share phenotype and morphology but, unlike inflammatory monocytes, monocytic MDSCs express simultaneously high level of two enzymes, Arginase-1 and iNOS, which confers them highly suppressive capacity towards $CD8^+$ cells.

Both subtypes express MHC class I but not MHC class II molecules. As MDSC is an heterogeneous population composed of macrophages and DC progenitors, when these cells are cultured in the presence of specific cytokines and growth factors (IL-4 or TNFα plus GM-CSF) they can differentiate into mature macrophages and DCs, and increase their expression of MHC class II molecules (Bronte et al. 2000). Moreover, it has been shown that the transfer of MDSC into tumor-free mice results in the development of mature macrophages and DCs but instead, its transfer into tumor-bearing mice results in the generation of suppressive macrophages (Narita et al. 2009). The generation of suppressive DC from MDSCs has not yet been demonstrated.

MDSC display several mechanisms of T cell suppression. They can be classified into two types: those that avoid T cell proliferation and survival and those who influence the polarization or phenotypes of other cell types present at the target site (tumor, infection or graft).

When MDSCs interact with activated T cells, they generate oxidative stress by the production of ROS and RNS, peroxynitrite and hydrogen peroxide. This is possible thanks to the combined activity of NADPH oxidase, Arginase-1 and iNOS enzymes expressed by MDSC (Dilek et al. 2012). Hemeoxygenase-1 (HO-1) is involved in the response to oxidative stress and also associated with MDSC suppressive activity (De Wilde et al. 2009). HO-1 inhibition prevents MDSCs from secreting IL-10, one of the cytokines implicated in their immunosuppressive properties.

MDSC can also prevent T cell proliferation by T cell deprivation from nutrients, specifically modulating the availability of amino acids on the microenvironment. This is possible as they consume

L-arginine (through Arginase-1 expression) and L-cysteine (by converting cysteine from the medium into L-cysteine but not releasing it to the medium, as other antigen presenting cells acting as "feeder cells" do (Srivastava et al. 2010)) from the medium.

Another mechanism for preventing T cell activation is interfering with lymphocyte recirculation to secondary lymphoid organs. MDSC express ADAM17, a metallopeptidase that cleaves the integrin L-selectin (CD62L), involved in naive T cell migration to lymph nodes (Hanson et al. 2009).

Finally, MDSC can also have an indirect suppressive effect by promoting the development of other regulatory cell types or the deviation of the immune response towards an immunoregulatory phenotype. Through IL-10 secretion, MDSC can expand antigen-specific natural Tregs or induce $CD4^+$ Tregs from naive $CD4^+$ T cells (Huang et al. 2006). Some other factors like TGFβ or IFNγ (Huang et al. 2006) may be involved, as well as cell-to-cell contact (Pan et al. 2010). IL-10 secretion by MDSC can also modify myeloid cell subsets towards an anti-inflammatory phenotype. They promote macrophage polarization towards an M2 phenotype, decreasing IL-12 production (Sinha et al. 2007) and they inhibit TLR-induced IL-12 production by DCs (Hu et al. 2011).

Cells from the monocyte phagocyte system share common functions: inducing and regulating the immune response against pathogens. Even if they share their objective, a well-established developmental pathway exists, tightly regulated by cytokines, growth factors and transcription factors, which define each cell type differentiation. Terminally differentiated cells are plastic cells which can modify their phenotype and function depending on the surrounding environment. The tight collaboration between cell types results in the coordination of the immune response against invading organisms and leads to a good resolution of tissue inflammation, allowing the return to tissue homeostasis.

PART II. TRANSPLANTATION

1 TRANSPLANTATION HISTORY AND CURRENT LIMITATIONS

The term "transplantation" stands for the transfer of cells, tissues or organs from a donor to a recipient with the aim of restoring functions in the body. This process can concern vascularized tissues or entire organs, where there is a need of chirurgical intervention to link blood vessels, or non-vascularized tissues or cells.

Organ transplantation can be classified depending on graft origin; therefore, we can refer to autotransplant (transplant of tissues to the same person), isotransplant (transplant of organs or tissues from a genetically identical donor), allotransplant (transplant of organs or tissues from a genetically non-identical individual) and xenotransplant (transplant of organs or tissues from an individual of a different species).

The first well documented transplant dates from the 2nd century BC, where the indian surgeon Sushurta performed autograft skin transplantation for nose reconstitution. Centuries later, in 1883 T Kocher performed the first thyroid transplantation, which would be the model for organ transplantation as we know it nowadays. However, contemporary surgical techniques did not allow performing viable organ transplantation due to the incapacity to reestablish blood circulation after surgery.

It was not until the 20th century that entire organ transplantation could be efficiently performed, when A Carrel and C Guthrie developed a surgical technique to suture arteries and veins to avoid death by bleed.

But then another key problem was identified: the discovery of transplant immunity and rejection. In the late 1940s, P Medawar and co-workers dedicated strong efforts to improve the understanding of the immune system: in 1951 they identified immune responses and suggested the benefit of the use of immunosuppressive drugs in organ transplantation. This represents the first step towards the concept of tolerance.

The first successful kidney transplant was performed by J Murray and JH Harrison in 1954, but it was only possible because it was performed between identical twins; transplants between non-identical individuals suffered early acute rejection and graft failure.

At that moment, the first immunosuppressive drugs were starting to develop (cortisone and azathioprine) but it was not until the discovery of cyclosporine in 1970 that transplant surgery found a sufficiently powerful immunosuppressive drug.

During the last 20 years, major advances in organ transplantation have been performed: improvement in chirurgical technics, better organ conservation after recovery, improvement of life's quality, etc. Therefore, nowadays organ transplantation is the optimal treatment of choice for many patients with end-stage organ failure. Even though, new concerns still limiting transplantation practice, like organ scarcity, graft rejection prognosis or non-efficient long term treatment to avoid rejection.

Each year, thousands of transplants are performed all around the world. Even though, thousands more patients still registered on waiting list, which can represent until 7 times the number of performed grafts. As shown in **Table 1**, data from the US in 2011 evidence the lack of available organs.

Organ	Transplants (total number)		Patients on waiting list (end year)	Annual death rates (%)
Kidney	16812	5769 (Live donor)	88489	1,43
		11043 (Cadaveric donor)		3,99
Pancreas	137		529	4,84
Liver	6370	247 (Live donor)	16077	7,91
		6123 (Cadaveric donor)		12,37
Intestine	129		272	19,35
Heart	2326		3139	9,18
Lungs	1831		1685	12,37

Table 1. Organ transplant data for the United States in 2011. Data were obtained from SRTR (Scientific Registry of Transplant Recipients) website (www.srtr.org)

Even more, due to the relative short half-life of transplanted organs, some patients need to receive multiple transplants during their life, which contributes to the decrease of available organs. That is the main reason why other sources of organs are being considered, as interspecies (xenotransplantation) or composite tissue transplantation. Half-life of kidney transplant in the US, considering the origin of the organ, is shown in **Table 2**.

Donor	Transplants	Graft survival (time after transplantation, %)			
		3 months	1 year	5 years	10 years
Living	5769	98,4	96,7	84	61,9
Deceased	11043	96	92,2	72	45

Table 2. Kidney graft survival at different time points after transplantation. Data were obtained from SRTR (Scientific Registry of Transplant Recipients) website (www.srtr.org)

The major problem in organ transplantation, graft rejection, is due to the recognition of the graft as non-self by host's immune system. This recognition leads to an immune response which destroys transplanted organ or tissue. In the case of vascularized organ transplantation, three different types of rejection can be differentiated, depending on their kinetics and the mechanisms involved in the response.

The quickest mechanism of rejection, **hyperacute rejection**, takes place between minutes to hours after transplantation. It is due to the presence of pre-existent circulating antibodies directed mainly against donor HLA (Human Leukocyte Antigen) molecules (Halloran et al. 1990; Halloran et al. 1992). Those antibodies recognize and bind antigens at the surface of graft endothelial cells, triggering mechanisms that damage graft vessels and secreting damage mediators that allow cellular infiltration, mainly innate immune cells at first. Nowadays, this kind of rejection is rare due to the existence of tests performed before transplantation, as cross-match test, where HLA compatibility between donor and recipient is ensured.

The months following transplantation, **acute rejection** can occur. It can be mediated by cellular or humoral mechanisms. Donor or recipient APCs activate alloreactive T lymphocytes, which infiltrate the graft and destroy it (cellular mechanisms) (Lechler & Batchelor 1982). The *de novo* generation of alloantibodies by activated alloreactive B lymphocytes can also take place. Alloantibodies bind donor antigens at the graft, triggering graft destruction (humoral mechanisms) (Hippen et al. 2005). Prevention of acute rejection is now ensured in more than 85% of cases due to the use of immunosuppressive agents (Nankivell & Kuypers 2011). Anyway, toxicity by long-term immunosuppressive treatment can as well lead to graft lost in the long term (Bennett 1996).

Even though, the main problem which has not been solved yet is long-term allograft dysfunction or **chronic rejection**. It is a slow, progressive and irreversible graft destruction, characterized by an increase of intima's layer of graft vessel's thickness which ends up in arteriosclerosis leading to tissue ischemia, responsible of graft necrosis. Chronic rejection is due to persistent antigen-specific cellular and humoral immune responses against the graft, which enhances

the traffic and recruitment of inflammatory mediators to the graft through the activation of the endothelium, and the secretion of damage signals and free radicals which favor vessel's muscle's proliferation. Nowadays, there is no efficient treatment to deal with chronic rejection.

Current immunosuppressive treatments have proven to be effective in prevention of acute rejection in vascularized organ transplantation. In the case of non-vascularized tissue transplantation, such as skin transplantation, this strategy has little or no effect (Benichou et al. 2011). There is increasing evidence suggesting that those different outcomes could involve differences in alloantigen encounter due to the lack of vascularization of skin grafts at the time of their placement. One possibility is that trafficking of donor skin DCs via lymphatic vessels could increase the immunogenicity of the graft and prevent regulatory mechanisms which are classically associated to antigen delivery via blood vessels (Benichou et al. 2011).

2 IMMUNE RESPONSE IN TRANSPLANTATION

The immune system is taught to be able to differentiate self and non-self-molecules, and initiate an immune response since peptides or antigens recognized are non-self. Therefore, in the case of allotransplantation, where graft cells are not genetically identical to recipient cells, allograft is recognized as non-self and the immune system set up an immune response against it, called alloresponse.

Innate immune response plays a key role in the first place, being responsible of the inflammatory reaction that causes tissue damage (LaRosa et al. 2007). Factors like donor brain death, transplantation surgery or ischemia-reperfusion are at the origin of the generation of a pro-inflammatory environment and free radicals that activate innate immune cells, which preserve the inflammatory environment and recruit cells from the adaptive immune system to the graft.

Cells from the innate immune system, like macrophages and other phagocytic cells, express PRR that sense danger signals. This recognition triggers the activation of inflammatory gene transcription (IL-1, IL-6, TNF, IFN type I and chemokines), contributing to the local inflammatory environment. Innate immune cells like macrophages will also directly participate in the phase of rejection by antibody binding; NK cells and neutrophils can as well be attracted to the transplant and be activated, mediating tissue damage (van der Touw & Bromberg 2010). The permeability of endothelial cells from graft vessels change and there is release of factors that attract host inflammatory leukocytes to the graft. Activation of the innate immune response post-transplant is non-specific, and occurs independently of genetical differences between donor and recipient (Wood & Goto 2012).

Adaptive immune response takes place once innate immune response is established. This response is antigen-specific, therefore mediated by cells that express antigen specific receptors. Effector immune responses are divided in humoral or cellular responses. Preformed host alloantibodies will rapidly react against donor molecules, triggering graft destruction (Wood 1994). In the absence of preformed antibodies, the first step in adaptive immune response against a transplant is T lymphocyte recognition of alloantigens (Kim et al. 2008). Donor passenger leukocytes present at the graft are able to migrate to recipient's secondary lymphoid organs, where they encounter allospecific naive T lymphocytes, therefore starting an adaptive immune response. Depending on the nature of cells that recognize alloantigens, the triggered response can be effector or regulatory. Regulatory immune responses are possible thanks to cellular intermediates which avoid effector responses to take place. Adaptive immune response will be explained in detail in the following paragraphs.

2.1 Alloantigen recognition and effector immune response

In the context of alloresponse, target antigens are those which differ between donor and recipient. Hence, the most polymorphic antigens arise as the main targets. Those include ABO system antigens, MHC molecules (major histocompatibility molecules) and miH (minor histocompatibility antigens). Nowadays, there are pre-clinical procedures in order to match the maximum of antigens between donor and recipient. For instance, ABO antigens, localized at the surface of endothelial grafts cells, can be recognized by pre-existent recipient antibodies and trigger rapid graft destruction (Dausset J 1966). Even though, they do not represent a problem anymore as grafts are selected for ABO compatible donor-recipient.

Due to their highly genetic polymorphism and to their involvement in APC/T cell interaction, MHC molecules (HLA in human and H-2 in mice) represent the main target in allorecognition. MHC class I molecules are present in the surface of almost every nucleated cell type whereas MHC class II molecules are only expressed by professional APCs. Mechanisms of antigen presentation will be explained later in this Chapter. There are also MHC invariable molecules, MICA and MICB, found at the surface of endothelial cells. Their mismatch is associated with highest risk of rejection (Zou et al. 2007; Sumitran-Holgersson 2008).

Even if donor and recipient shared the same polymorphism regarding MHC molecules, there would still be other polymorphic non-MHC molecules encoded throughout the genome, not necessarily expressed by cells of the immune system, which would trigger an immune response. Those other molecules are called minor histocompatibility antigens (Dierselhuis & Goulmy 2009). Important miH antigens are the ones encoded by the Y chromosome, called male antigens. It has been demonstrated that there is increased risk of rejection when recipient is a female and donor is a male

(Gratwohl et al. 2008; Pabón et al. 2011). Male minor antigens will be explained in detail later, as it is the basis of the transplantation model we have chosen for our studies. Importantly, all codifying single nucleotide polymorphisms (SNPs) are miH.

2.1.1 T cell mediated response

a. T cell activation

For T lymphocytes to be fully activated, APC must provide them two signals. The first one is antigen presentation, which is the only one where antigenic specificity is considered. The second one, co-stimulation, reinforces the contact and modulates the strength of the first signal. As a result of the two mentioned signals, T lymphocytes secrete cytokines, mainly IL-2, which will contribute to the activation. Sometimes, cytokine secretion is considered as a 3rd signal. Each signal will be detailed in the following paragraphs. A schema representing the three signals is shown in **Figure 9**.

a. Antigen presentation to T cells

Antigen presentation pathways

The TcR complex, expressed at the surface of T lymphocytes, consists on the antigen specific TcR, composed of two chains, α and ß (for the majority of T lymphocytes), which recognizes a wide range of antigens through hypervariable regions called CDR (Complementarity Determining Regions) but lack a functional signaling intracellular domain. TcR is therefore associated with additional membrane molecules, the most important one being the CD3 complex, which mediate downstream signaling to T cells through their intracellular domain (Murphy et al. 2008).

T lymphocytes are not able to recognize soluble antigens through their TcR; instead, antigens have to be presented at the surface of a cell, being part of a self-MHC/peptide complex.

CD8$^+$ T lymphocytes recognize peptides complexed with **MHC class I** molecules, which are expressed at the surface of almost every cell type of the body. Those peptides come out from endogenous proteins present at the cytosol or from intracellular microbes or viruses infecting cells (cytosolic pathway). They are processed by the proteasome and enter the endoplasmic reticulum via the transporter protein TAP (Transporter associated with Antigen Processing), where they are loaded into MHC class I molecules and transported to the cell membrane. It is an important pathway as it allows every nucleated cell of the organism to present its cytosolic content to CD8$^+$ T lymphocytes. Therefore, if the cell is infected or if it expresses damaged or missfolded own proteins, those antigens will be presented at the cell membrane, triggering a cytotoxic response against the cell and destroying it.

In the case of $CD4^+$ T lymphocytes, they specifically recognize peptides complexed with **MHC class II** molecules. As APCs are the only cells to express MHC class II molecules, they are the only ones to present antigens to $CD4^+$ T lymphocytes. APCs endocyte or phagocyte exogenous antigens, either microbial components or dead cells, which are processed at endocytic vesicles and will be assembled to pre-formed MHC class II molecules in endosomes. Those vesicles reach cell surface and antigens are presented to $CD4^+$ T cells. As antigens do not reach the cytosol, this pathway is also called vacuolar pathway.

There exists a third mechanism of antigen presentation, discovered in the late 1970's, called **cross-presentation** (Bevan 1976). It happens when exogenous antigens enter into the cell by endocytosis and are presented in the context of MHC class I molecules, thus being recognized by $CD8^+$ T lymphocytes. Two cross-presentation pathways have been described: (i) the cytosolic pathway, where antigens reach the cytoplasm and are degraded by the proteasome, and (ii) the vacuolar pathway, where antigens stay in the phagosome, which fusions with lysosomes and antigens are degraded by lysosomal proteases. In both cases, antigens are loaded in MHC class I molecules. Even if all cells express MHC class I molecules, cross-presentation can only be performed by endocytic cells, mainly APCs, specially some DC subsets as $CD8^+$ DCs. First insights in cross-presentation were obtained in the mid 1990's (Heath et al. 2004), but intracellular processes involved in the conversion of exogenous antigens into MHC class I/peptide complexes are still poorly understood.

Alloantigen presentation in transplantation

Alloreactive T lymphocytes represent 3 to 5% of lymphocyte pool when there is low degree of incompatibility between donor and recipient. It can rise to 20% of the pool of lymphocytes when there is fully MHC mismatch (Suchin et al. 2001).

After transplantation, naive recipient alloreactive T lymphocytes get activated by the recognition of donor peptides presented by professional APCs. Normally, activation leads to the polarization of naive T lymphocytes towards effector lymphocytes, which initiate an immune response against the graft. The case of organ transplantation is the only one where T cells can be primed by three distinct pathways. Those three pathways are shown in **Figure 8**.

In the case of vascularized grafts, donor APC (mainly DCs) get activated in the graft, secrete pro-inflammatory molecules and overexpress costimulatory molecules and MHC molecules loaded with donor peptides. They acquire the capacity to migrate to draining lymph nodes, where they encounter both, $CD4^+$ and $CD8^+$ naive alloreactive T lymphocytes from recipient that will recognize the complex donor MHC/donor peptide and get activated. This process is called **direct presentation**. Normally, T lymphocytes should not recognize peptides loaded in non-self MHC molecules, but this

mechanism can be explained by the capacity of T lymphocytes to recognize MHC molecules by molecular mimics. This response is very quick and intense. Its importance in acute rejection phase was confirmed by the fact that depletion of donor leukocytes avoided graft rejection whereas the transfer of donor APC reestablished rejection (Talmage et al. 1976). As donor leukocytes have a limited half-life, the importance of direct presentation in allograft rejection decrease over time (Benichou et al. 1999).

The pro-inflammatory microenvironment created by surgery injuries or the graft itself recruits recipient's innate immune cells that infiltrate the graft (Penfield, Wang, et al. 1999). This takes place even when syngeneic grafts are performed (Penfield, Dawidson, et al. 1999). This rapid infiltration by recipient DC and inflammatory monocytes has recently been shown by intravital microscopy in a mouse skin graft model (Celli et al. 2011). Recipient's DCs capture donor antigens which derive from dead donor cells and process them, get activated and migrate to graft draining lymph nodes. In lymph nodes, donor antigens will be presented to alloreactive T lymphocytes in self-MHC molecules, process known as **indirect presentation** (Lechler & Batchelor 1982). This presentation is associated with chronic rejection but as well to acute rejection (Benichou et al. 1999; Shlomchik et al. 1999; Liu et al. 1996), and is the most important presentation pathway concerning the response to minor antigens (Fangmann et al. 1992).

A third mechanism of antigen presentation was described by Robert Lechler's team, the **semi-direct presentation**. It consists in the capacity of recipient DCs to capture entire intact MHC/peptide complexes expressed at the surface of donor DCs or endothelial cells. Those recipient DCs are then able to directly activate alloantigen-specific T cells (Herrera et al. 2004). Therefore, the same recipient DC can stimulate T lymphocytes in a direct and indirect way at the same time. The role of semi-direct presentation in transplantation has not yet been deciphered.

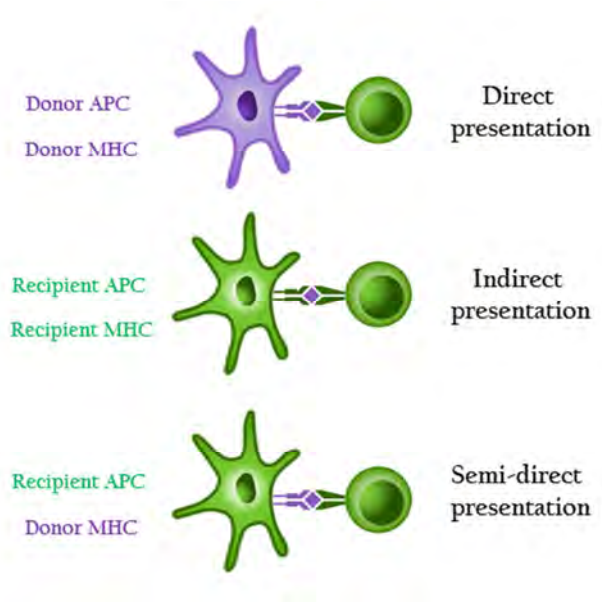

Figure 8. Alloantigen presentation in transplantation. Donor derived peptides are presented to recipient alloreactive T lymphocytes following three different pathways, direct, indirect or semi-direct presentation.

b. Costimulatory signals

In order to optimally activate an antigen-specific T lymphocyte that recognizes a specific MHC/peptide complex in the APC's surface, the APC has to provide a second positive signal, called co-stimulatory signal. If the APC is immature (i.e. lack or low expression of co-stimulatory molecules at the surface), T lymphocyte's activation is incomplete, which leads to T cells apoptosis or anergy (Gimmi et al. 1993).

Costimulation is mediated by multiple types of molecules. Co-stimulatory molecules are the ones which amplify the TcR signal, inducing an independent signal (Bjorndahl et al. 1989). Other molecules also play important roles, like integrins and accessory molecules that allow and stabilize the contact between the T cell and the APC during the formation of the immunological synapse. There are different families of co-stimulation molecules. The most important ones at the APC's surface are B7 family members, CD80 and CD86. Their ligands are CD28 family molecules (CD28 and CTLA-4) found at the surface of the T lymphocyte. Ligation of CD28 to CD80/86 transmits a positive signal of T cell survival and activation; on the contrary, ligation of CTLA-4 to CD80/86 transmits an inhibitory signal to the T cell. CD28 is constitutively expressed at the T cell membrane, whereas CTLA-4 is only expressed after T cell activation, and displays higher affinity to APCs B7 ligands. The balance between those two molecules at the T cell surface allows the regulation of T cell activation, avoiding excessive responses in inflammation or autoimmunity (Wood & Goto 2012).

Considering the important role costimulatory molecules play in immune responses, they are targeted in several therapeutic strategies concerning different diseases, with the goal of either,

enhance their activity (to boost immune system) or block their ligation (to get immune tolerance or a state of unresponsiveness).

c. Cytokine signal

The first two signals activate calcineurin signaling transduction pathways, MAPK and NF-κβ. Those pathways allow the expression of TF that trigger the transcription of many activation molecules at the T lymphocyte cell surface, as CD154 and CD25, or the secretion of cytokines, as IL-2.

CD25 is the α chain of the high affinity IL-2 receptor. Once expressed at the T cell surface, IL-2 can strongly bind IL-2R and transmit positive signals to T lymphocytes. IL-2 (and to less extent, IL-15) activate then mTOR (mammalian Target of Rapamycin) pathway via PI3K (Phosphatidyl Inositol 3 Kinase) to provide the third signal, inducing T lymphocyte's proliferation (Halloran 2004; Wood & Goto 2012).

Similarly to costimulatory molecules, in transplantation, inhibitors of IL-2 or IL-15 downstream pathways are gaining therapeutic interest.

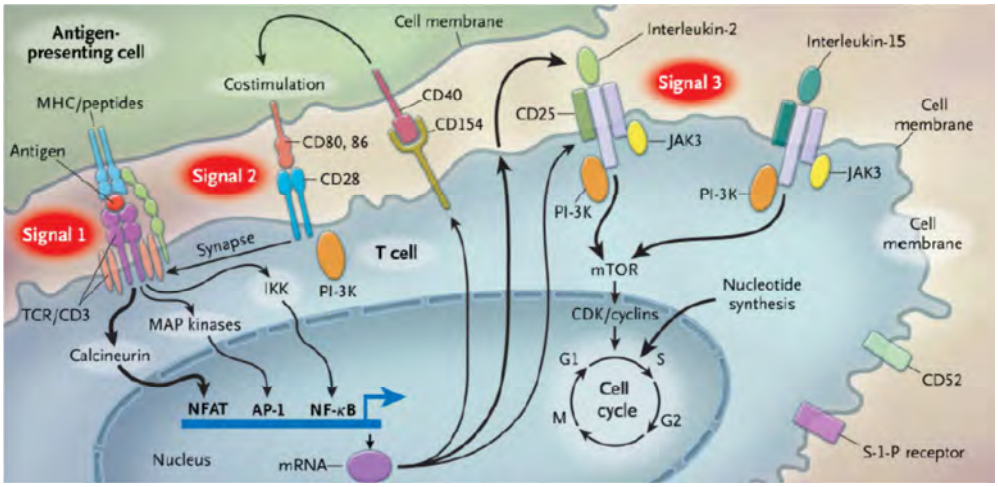

Figure 9. Schema representing the three signals needed for T lymphocyte activation. From (Halloran 2004)

b. T cell polarization

Once T lymphocytes get activated, depending on the cytokines present in the microenvironment and other signals they receive, T lymphocytes polarize towards different phenotypes, expressing different molecular profile in terms of genes and TFs that will influence their function and cytokine secretion profile.

Both, $CD8^+$ and $CD4^+$ T lymphocytes can differentiate into effector cells, which mediate graft rejection in transplantation, or into regulatory cells that will inhibit effector alloresponses towards

the graft. A summary of the T cell polarization and their roles in transplantation is shown in **Table 3**. of Regulatory cells will be detailed in the Part II Section 2.2.

CD8 polarization

In organ transplantation, indirect alloresponse can be mediated by $CD8^+$ T lymphocytes which recognize donor-derived peptides presented by self-MHC class I molecules on self-APCs, process known as cross-priming. This was first demonstrated by Matzinger et. al. in a skin transplantation model (Matzinger P et al. 1977). Semi-direct recognition lead to the concept of "three-cell model", where the $CD8^+$ cell is fully activated by both, its contact with the APC's MHC class I/peptide complex and the $CD4^+$ T cell that is being stimulated by the same APC through peptides presented in MHC class II molecules (Ridge et al. 1998).

Once activated, $CD8^+$ T cells differentiate into effector cytotoxic lymphocytes. CTLs migrate to the graft where they recognize their target cells by the expression of allogeneic MHC class I molecules and they release cytotoxic granules (containing cytotoxic molecules such as perforine/granzyme B) which initiate an apoptosis program. CTLs upregulate FasL, which binds Fas on the target cell, inducing apoptosis by caspase activation.

CTLs are classified into Tc1, Tc2 and the more recently described Tc17 subtype. Tc1 cells are generated when exposed to IL-12, cytokine mainly produced by DCs (Filatenkov et al. 2005). They secrete IFNγ and TNFα. After activation, they acquire effector cytotoxic functions, being involved in graft rejection. Cytotoxic Tc2 cells differentiate in the presence of IL-4, and secrete IL-4, IL-5, IL-6, IL-10 and IL-13 (Sad et al. 1995). In transplantation, Tc2 cells display opposite roles, depending on the context. They have been shown to inhibit GVHD (Graft Versus Host Disease) (Fowler & Gress 1998; Erdmann et al. 2004), but also to take part in graft rejection by eosinophil's recruitment (Delfs et al. 2001). A recently described population, Tc17, is differentiated in the presence of TGFβ, IL-23 and IL-6. They express RORγτ TF. They have been shown to induce acute cardiac graft rejection, as they take part in the recruitment of neutrophils to the graft (Burrell et al. 2008).

$CD8^+$ T cell activation does not always lead to effector cytotoxic populations. If alloantigens are cross-presented to $CD8^+$ T cells by immature DC, which lacks co-stimulation molecules, $CD8^+$ T lymphocytes do not get activated but die from apoptosis. This process is called **cross-tolerance** (Lutz & Kurts 2009), and plays an important role in mediating tolerance to the allograft.

CD4 polarization

It is well established that $CD4^+$ T cells activated via direct allorecognition and differentiated towards a Th1 profile provide help for the differentiation and expansion of anti-donor $CD8^+$ CTLs.

Naive alloreactive $CD4^+$ T cells can be activated by direct, indirect and semi direct pathway. Naive $CD4^+$ T cells are activated by DCs which present MHC class II/allopeptide complexes at their surface. They can polarize towards different T helper (Th) phenotypes.

In a proinflammatory environment, DAMPs stimulate APCs through TLR signaling. DAMPs trigger IL-12 secretion, which polarizes naive $CD4^+$ T lymphocytes towards a Th1 phenotype. Th1 cells are characterized by the secretion of IFNγ, which activates other cell types, like NK cells. They can mediate acute and chronic rejection (Obata et al. 2005). In the absence of a Th1 response, Th17 lymphocytes can mediate acute graft rejection (Yuan et al. 2008) through IL-17 production, which is proinflammatory *in vivo* and stimulates neutrophil migration towards the inflammatory site (Chadha et al. 2011). It has been described that Th2 cells also can initiate rejection (Barbara et al. 2000), and are mainly associated with chronic rejection (Illigens et al. 2009). IL-17 blockade allows a prolongation of graft survival in a rat heart transplantation model (Li et al. 2006). Th2 cells attract eosinophils to the graft and inhibit the differentiation pathway towards Th1 profile. In some models, accumulation of Th2 cells is been associated with tolerance profile (Amarnath et al. 2011).

Th9 cells secrete IL-9 and are able to recruit mast cells. They also produce IL-10 and some IL-21, but their role is unclear (Askar 2014). Tfh cells, found in lymph nodes, are specialized in helping B cells in germinal center reactions, including B cell proliferation, maturation affinity, switch recombination and differentiation into plasma and memory B cells. Therefore, effective humoral immunity depends on the support of B cell responses by Tfh cells (Breitfeld et al. 2000). They play a critical role in transplant alloimmunity and allosensitization (Askar 2014).

Table 3. Non exaustive summary of T lymphocyte polarization and involvement in the immune response in organ transplantation

Type T cell	Cell polarization	Mechanism/evidence for involvement in transplantation	Phase of transplantation	Model/ Species	Reference
$CD4^+$	Th1	Increase in mRNA IFNg expression	Acute rejection		Dallman et al., 1991; O'Connell et al., 1993
		IL-2 secretion mediated proliferation of alloreactive effector $CD8^+$ T cells			
		Macrophage activation			
		B cells activation and antibody production			
		Allograft damage by cytotoxicity through Fas/FasL interaction			
	Th2	Th1 inhibiton through IL-4 and IL-10 secretion	Delay acute rejection		Waaga et al. 2001
		Abrogation of $CD8^+$ CTL responses	Delay rejection	Skin allograft	
		Abrogation of $CD8^+$ CTL responses	Chronic rejection	Heart allograft	Illigens et al., 2009
		Increased IL-10 in allograft biopsies	Chronic rejection	Human renal	
		Eosinophil activation through IL-4 production	Rejection		Goldman et al. 2001; Braun et al. 2000; Surquin et al. 2005
		IL-4 production	Rejection		
	Th17	Recruitment of neutrophils and macrophages to the graft through IL-17 secretion, inflammation	Acute rejection		Laan et al. 1999, Agorogiannis et al., 2012
		Promotion of ectopic germinal centers through IL-21 secretion, local humoral response	Chronic rejection		Deteix et al. 2010
	Tfh	Humoral immunity, B cell activation	Chronic rejection		
$CD8^+$	Tc1	Cytotoxicity (granzime/perforine)			
		Apoptosis mediated through Fas/FasL			
	Tc2	Eosinophil recruitment to the graft	Rejection		
	Tc17	Neutrophils recruitment to the graft through IL-17 secretion, inflammation	Acute rejection	Heart allograft	
		Tissue lessions		Human GVHD	

2.1.2 B cell mediated response

B cells can take part in allograft rejection through different mechanisms. In their final stage of differentiation, B cells develop into antibody producing cells. They can also act as APCs, as they express MHC class II and costimulatory molecules. As APCs, B cells interact with T cells TCR, and T cells differentiate into helper cells that will, in turn, activate B cells and influence B cell differentiation into antibody producing cells or plasma cells. B cells have also been shown to promote alloreactive T cell differentiation into memory T cells (Ng et al. 2010), which deliver faster and stronger response towards the allograft.

The role of B cells in transplant rejection is still controverted, as B cell transcripts have been found in rejecting allografts (Sarwal et al. 2003) but operationally tolerant patients (i.e. patients who are spontaneously tolerant in the absence of immunosuppressor treatment) have been shown to display a B cell signature of genes (Newell et al. 2010; Sagoo et al. 2010; Pallier et al. 2010; Silva et al. 2012), therefore suggesting a role for B cells in transplantation tolerance.

a. Antibodies

Preformed antibodies against donor antigens are at the origin of hyperacute graft rejection, whereas antibodies generated post-graft, mainly directed against donor HLA molecules, are involved in acute and chronic graft rejection (Terasaki & Cai 2008). The role of preformed alloantibodies was demonstrated by a study where a strong correlation between the presence of anti-donor HLA class I specific antibodies and rejection was found (Halloran et al. 1990; Halloran et al. 1992). Anti-class I antibodies that persist after transplantation injure the endothelium of the microvasculature, leading to a rapid deterioration in graft function.

Macrophages and NK cells can bind the Fc portion of alloantibodies through FcR expressed at their membrane therefore recognizing graft molecules through the antigen specific portion of alloantibodies and triggering lysis of target cells through the secretion of cytotoxic mediators like ROS, NO or pro-inflammatory cytokines. This mechanism is called antibody dependent cellular cytotoxicity (ADCC).

b. Complement

The complement is a group of proteins found in the plasma with proteolytic activity. The complement cascade can be activated by 3 different pathways: classical, alternative or lectins pathway. Once activated, they cleave downstream proteins which will end up causing cell injury by the formation of a membrane attack complex (MAC) within the target cell membrane. One of the target cells are endothelial cells, therefore damaging endothelial vessels. Endothelial cells get activated and secrete proinflammatory cytokines and chemokines, which attract other pro-inflammatory cell types that infiltrate the graft.

Complement molecules are also target of alloantibodies. C4d complement deposits complexed with donor specific antibodies in patient's biopsies are associated with greater risk of acute humoral rejection (Nickeleit et al. 2002; Kato et al. 2003).

2.2 Tolerance induction in transplantation

Tolerance is the state of non-responsiveness in the presence of a particular antigen. Functional tolerance encompasses two processes. The first one consists in the elimination of autoreactive lymphocytes in primary lymphoid organs during lymphocytic maturation, called central tolerance. The second one, called peripheral tolerance, consists in the suppression of autoreactive lymphocytes that have escaped central tolerance and got to secondary lymphoid organs. Both mechanisms are ensured by different cell types at different anatomical locations.

The concept of tolerance in transplantation refers to the specific elimination of alloreactive lymphocytes that could damage the graft without interfering with the rest of the immune system functionality.

One of the early mechanisms used in transplantation to induce specific tolerance was the infusion of donor bone marrow into recipient. In animal models, this technique was able to prolong skin pancreatic islet allograft survival and to induce specific tolerance (Panijayanond P et al. 1974) and it correlated with the presence of donor class II mRNA in the recipient's thymus (Hale et al. 2002), meaning that central tolerance could be induced.

Those findings were translated into the clinics under guidance of Dr. Thomas Starzl and expanded by Ciancio et al. Transplant patients were injected donor bone marrow along with the graft. Bone marrow injections were safely tolerated, with no sign of GVHD. Unfortunately, patients experienced acute rejection episodes following similar kinetics than control patients (Ciancio et al. 2004).

2.2.1 Central tolerance

The thymus is the key player of experimental induction of transplant tolerance, as it is where self/non-self distinction is originated. In animal models, tolerance can be induced in a class I mismatched model of kidney transplant after a short cyclosporine treatment (Gianello et al. 1995). If recipients are thymectomized 21 days prior to kidney transplantation, tolerance induction is abolished (Okumi et al. 2008). Transplant tolerance is also induced by direct thymic inoculation of dominant allopeptides (Chowdhury et al. 1996; Oluwole SF et al. 1999). Those experiments evidence the role of the thymus and central tolerance in tolerance induction in organ transplantation.

Even though, induced regulatory mechanisms appear to be enough for the maintenance of transplant tolerance once established. Experiments using a miniature swine kidney transplant model show that if thymus is ablated 21 days after transplantation, tolerance persists. This suggests that thymic function is not required for the maintenance of tolerance (Vagefi et al. 2004).

Some populations of regulatory T lymphocytes arise from thymic selection and are expanded in periphery. Although thymic $CD4^+CD25^+FoxP3^+$ T reg cells are an important lineage that controls immune homeostasis, the ability to induce peripheral Tregs may be more important in the regulation of immune response in the case of organ transplantation (Kang et al. 2007). Naturally occurring regulatory $CD8^+CD122^+PD\text{-}1^+$ Treg population has been shown to inhibit skin graft rejection through IL-10 secretion and inhibition of IFNγ secretion by effector $CD4^+$ and $CD8^+$ T cells (Rifa'i et al. 2004).

Myeloid cells also play a key role in central tolerance induction. As already mentioned, cDC take part in thymic negative selection of T lymphocytes (Matzinger & Guerder 1989). pDC have also been found to be implicated in central tolerance induction, playing a role in the thymic selection of Treg cells that can preferentially secrete IL-10 in response to self-antigens in periphery (Matta et al. 2010).

2.2.2 Peripheral tolerance

When auto-reactive T lymphocytes escape to positive and negative selection in thymus, there are other peripheral mechanisms to avoid their activation. Those mechanisms are classified in T-cell intrinsic (direct action on effector T cells) and T-cell extrinsic (involvement of other regulatory cell types) mechanisms.

a. T-cell intrinsic mechanisms of tolerance

Several mechanisms concerning T cell modification or their capacity to respond to antigenic presentation are involved in tolerance induction (represented in **Figure 10**).

The simplest mechanism is **antigenic ignorance**. It takes place when an antigen is expressed or present in an anatomical location which is not accessible to immune system's cells; therefore, T lymphocytes are functional but they cannot set up an immune response against those antigens. Another reason for antigen ignorance is when antigens are expressed at very low levels and do not reach the threshold to trigger an immune response (Miller JF et al. 1993).

Active mechanisms of tolerance induction concern anergy, phenotype skewing and clonal deletion. **Anergy**, implies the functional inactivation of T lymphocytes, which is characterized by the absence of proliferation in front of a new stimulation (Jenkins & Schwartz 1987; Schwartz 1990). It is due to either, the lack of co-stimulation when a T lymphocyte encounters a specific MHC/peptide

complex on an APC surface or to the expression by the T lymphocyte of the inhibitory costimulatory molecule CTLA-4 expressed after T lymphocytes activation (Krummel & Allison 1995).

Even when T lymphocytes become fully activated, tolerance can still be maintained. In that situation, there is a shift of the nature of the response from a pathogenic effect, which involves tissue damage, towards a tolerogenic profile. The most common situation is to skew from a proinflammatory cytokinic profile (typical Th1 polarization) towards anti-inflammatory cytokine secretion (Th2 profile). This mechanism is called **phenotype skewing.** Transfer of Th2 lymphocytes have been associated with autoimmunity protection compared to transfer of Th1 lymphocytes (Bradley et al. 1999). In transplantation, tolerance is usually associated with a Th2 response, as transitory high levels of IL-4 in patient's serum before transplantation is correlated with a good prognostic of graft survival (Karczewski et al. 2008).

Another way to induce tolerance is by elimination of antigen-specific T cells by **clonal deletion**. It takes place because of repetitive stimulation of the T lymphocyte. In transplantation, clonal deletion occurs due to the constant presence of alloantigens. T cells are eliminated by apoptosis through Fas/FasL interaction, by a mechanism called AICD (Activation Induced Cell Death).

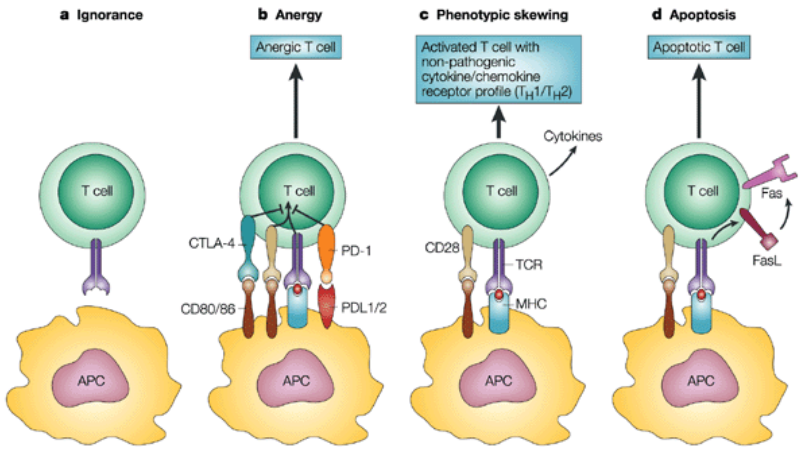

Figure 10. Mechanisms of peripheral tolerance induction. From (Walker & Abbas 2002)

b. T-cell extrinsic mechanisms of tolerance

T-cell extrinsic mechanisms involve the induction or regulatory cell types, which suppress T lymphocytes activation. Those regulatory cell types can derive from lymphoid or myeloid lineages.

Regulatory lymphocytes

Regulatory T lymphocytes

Peripherally induced Treg lymphocytes have been shown to be essential in the induction and maintenance of transplantation tolerance. Thanks to the use of animal models, their main role in the sets of autoimmunity and transplantation has been elucidated. It has been widely demonstrated that when Treg cells are depleted, auto or alloreactive T lymphocytes are overactivated. This overactivation ends up destroying self-organs or transplanted ones (Kang et al. 2007; Jiang et al. 2006; Wood & Sakaguchi 2003).

In transplantation, the balance between Treg and T effector lymphocytes will decide the outcome of the graft, either rejection or tolerance. Treg cells naturally present in the recipient are not enough to inhibit T cell activation. Therefore, an expansion of tTregs or induction of pTregs is needed, not only for tolerance induction but also for its maintenance.

The first evidence for $CD4^+$ T-cell-mediated immunoregulation in transplantation came from a heart transplant model in rats, where after cyclosporine treatment, a suppressive population of $CD4^+CD25^+$ T cells was induced. Induced $CD4^+$ Treg cells were able to transfer tolerance to newly grafted recipients (Hall et al. 1984; Hall 1985). However, their existence has long been. In 1995, Sakaguchi et al. demonstrated in mice that CD25 molecule was expressed by $CD4^+$ T cells that were able to suppress autoimmune diseases, whereas the $CD4^+CD25^-$ population was not (Sakaguchi et al. 1995). Since then, other $CD4^+$ regulatory cell types have been described.

The best characterized Treg cells are $CD4^+CD25^+FoxP3^+$ Treg cells. They are involved in tolerance induction and maintenance by direct action on effector cells (either by cell contact mechanisms or by secretion of inhibitory mediators, like IL-10 or TGFβ) or by regulatory APCs' induction. In clinical setting, high numbers of $CD4^+CD25^+FoxP3^+$ Treg cells have been found in blood of operational tolerant hepatic transplanted patients (Martínez-Llordella et al. 2007).

The suppressive population of Tr1 cells was induced *in vivo* in a mouse model of islet transplantation by the administration of IL-10 and rapamycin, inducing stable long-term tolerance. This Tr1 induced cell subset was able to transfer tolerance to newly grafted mice (Battaglia, Stabilini, et al. 2006). Using two different models of transplantation, Graca et al. and Sawitzki et al. showed that Tr1-like cells were enriched within tolerated grafts (Graca et al. 2002; Sawitzki et al. 2001).

Our group described another regulatory population, $CD4^+CD45RC^{low}$ Treg cells, which were increased in the graft of tolerant animals after DST (Donor-Specific Transfusion) in a fully mismatched rat heart transplant model. Induction of $CD4^+CD45RC^{low}$ population correlated with a decrease of $CD4^+CD45RC^{high}$ effector population (Josien R et al. 1995).

Although less studied, the role of $CD8^+$ Treg cells in the control of immune responses towards autoimmunity or transplantation is well established. Multiple populations of $CD8^+$ regulatory T cells have been identified, displaying different phenotypes and varied mechanisms of action.

In transplantation, $CD8^+FoxP3^+$ Treg cells have been found responsible of tolerance induction in a rat cardiac allograft model. Their mechanism of action involves upregulation of inhibitory receptors in DCs and graft endothelial cells (Liu et al. 2004).

$CD8^+CD28^{neg}$ cells are associated with the prevention of acute rejection in transplantation. In a mouse skin graft transplant model, they can be induced by continuous exposure of recipients to donor peptides, which induced a significative prolongation of graft survival (Sireci et al. 2009). They are as well induced after DST in a rat liver transplant model. $CD8^+CD28^{neg}$ T cell transfer avoids acute rejection but does not avoid chronic rejection (Liu et al. 2007). In humans, their role is controversial, as they have been associated to a protective profile but also to transplant rejection. In one study, lower numbers of $CD8^+CD28^{neg}$ cells have been found in the circulation of kidney transplant patients in acute rejection phase compared to stable patients (Karczewski et al. 2010). On the contrary, another study suggests that $CD8^+CD28^{neg}$ cells are the ones that mediate graft rejection, as they display a cytotoxic profile (they produce granzyme A and perforin) in kidney transplant patients in chronic rejection (Baeten et al. 2006).

Similarly, IL-10–secreting $CD8^+$ regulatory T cells could be generated *in vitro* by stimulation of naive $CD8^+$ T cells with allogeneic pDC. These $CD8^+$ Treg cells suppressed allospecific $CD8^+$ T cells proliferation through IL-10 secretion (Gilliet & Liu 2002).

$CD8^+CD45RC^{low}$ regulatory population exists naturally but can also be induced *in vivo* in a rat cardiac allograft model after blockade of CD40/CD40L costimulatory pathway by treatment of recipients with an adenovirus coding for CD40-Ig (Guillonneau et al. 2007). $CD8^+CD45RC^{low}$ population is responsible for the long term allograft survival, as their transfer to newly grafted recipients induce tolerance. This tolerance is donor specific as third party transplants are rejected. Their mechanism of action is through the molecule IDO and the cytokine IFNγ, as blockade of either of them prevents allograft survival prolongation (Guillonneau et al. 2007; Li et al. 2010b).

$CD8^+CD11c^+$ regulatory T cells were first described as a regulatory population induced in a collagen type II-induced arthritis (Seo et al. 2004). Recently, our group found an increase of donor-specific $CD8^+CD11c^+$ Treg cells in a mouse model of skin transplant where allograft survival prolongation was obtained by the combination of autologous tolerogenic DC plus αCD3 mAb treatment (Segovia et al. 2014). $CD8^+$ cells isolated from draining lymph nodes of tolerant mice were able to transfer tolerance into newly grafted mice without additional treatment.

A regulatory population of donor-specific T cells with phenotype $CD3^+CD4^-CD8^-$ (DNT) are induced by donor lymphocyte transfusion before transplantation. Those regulatory cells have been

shown to kill alloreactive $CD8^+$ T lymphocytes by Fas/FasL mediated mechanism of apoptosis and are able to inhibit the proliferation of $CD4^+$ T lymphocytes although the mechanism remains unknown (Zhang et al. 2000; Young et al. 2002). There are multiple transplantation settings where regulatory DNT cells have been involved, like GVHD (Young et al. 2003) xenotransplantation (Chen et al. 2003; Chen et al. 2005) and cardiac graft survival (Lee et al. 2005). Our group found out that DNT regulatory cells accumulated in the spleen of operationally tolerant rats in a model of heart allograft. In that model, tolerogenic DCs induced IFNγ expression in DNT cells. *In vivo* blockade of IFNγ resulted in allograft rejection (Hill et al. 2011).

The group of Strober demonstrated that **NKT cells** were able to prevent GVHD after allogeneic bone marrow transplantation (Pillai et al. 2007). On the contrary, in a model of allograft islet transplantation, there is evidence that NKT cells contribute to the early graft rejection through the activation of $Gr1^+CD11b^+$ effector cells by IFNγ synthesis (Toyofuku et al. 2006; Yasunami et al. 2005).

Regulatory B lymphocytes

The first evidence for a role of Breg cells in transplant tolerance came from a pancreatic islet allograft model, where B cell injection in conjunction with an anti-CD40L blocking antibody prolonged allograft survival (Parker et al. 1995). This benefic effect was confirmed in a fully mismatched mouse cardiac transplant model (Niimi et al. 1998) and in a rat kidney transplant model (Yan et al. 2002). Breg cells have been found to be responsible of tolerance induction in a model where CD45RB molecule was blocked by an antibody. The mechanism of tolerance induction was through prevention of T cell-B cell interaction (Deng et al. 2007) or blocking ICAM-1 interaction (Huang et al. 2008). Surprisingly, in that tolerance model, IL-10 expression by Breg cells was shown to inhibit B cell-mediated tolerance induction (Zhao et al. 2010). On the contrary, DM Rothstein's team identified TIM-1 (T cell Ig domain and mucin domain-1) expressing Breg cells which produce high levels of IL-10, responsible of mouse islet allograft rejection inhibition (Ding et al. 2011).

In a study performed in our laboratory using a fully mismatched rat cardiac allograft model, administration of LF15-0195, an inhibitor of NF-κβ, induces long-term cardiac allograft tolerance. The authors showed that B cells were accumulated in tolerated allografts, and those B cells displayed an inhibited phenotype, where switch from IgM to IgG was inhibited (Le Texier et al. 2011).

Human Bregs have extensively been studied in autoimmune diseases but some evidences point out that they could play an important role in tolerance induction or maintenance in operational tolerant patients. Studies performed in our laboratory by S Brouard's team showed that chronic rejection patients display lower absolute number of B cells (Louis et al. 2006). They also found that operational tolerant patients displayed a B cell transcriptional signature in peripheral blood (Brouard

et al. 2007) and that absolute numbers and B cell frequency in blood was increased (Pallier et al. 2010). B cell compartment of tolerant patients lack plasma cells and B cells display increased sensibility to apoptosis (Chesneau et al. 2014). Activated B cells from tolerant patients secrete more IL-10 than from healthy volunteers and stable patients (Chesneau et al. 2014). Other studies including larger numbers of patients evidenced overexpression of B cell genes in tolerant patients (Newell et al. 2010; Sagoo et al. 2010).

Regulatory myeloid cells

DCs play an important role in the induction and maintenance of peripheral tolerance. Their role as APCs can lead to T cell priming but also to tolerance induction to self-antigens expressed by non-lymphoid tissues in the absence of danger signals (Steinman & Nussenzweig 2002) or low costimulatory molecules, leading to T cell anergy or Treg generation. The first evidence for a role of DCs in tolerance induction in transplantation came from a rat heart transplant model where tolerance was induced by donor specific transfusion. Depletion of passenger leukocytes, mainly constituted by DCs, abrogated tolerance induction (Josien et al. 1998). *In vivo* targeting of DCs with apoptotic donor leukocytes has been shown to promote allograft survival in a mouse model of transplantation (Morelli AE 2005) which again confirms the crucial role of DCs in tolerance induction and its importance in organ transplantation.

Tregs can also induce a tolerogenic state in DCs. For instance, it has been shown that $CD4^+CD25^+$ Tregs are able to upregulate IDO expression by DCs, which is responsible for immune responses suppression (Mellor & Munn 2004).

Macrophages can be driven to a suppressor phenotype *in vivo* by treatment with M-CSF or IL-10 cytokines. Those induced macrophages inhibit T helper polarization whilst inducing regulatory T cells. Importantly, classical proinflammatory factors present at the graft after transplantation, as IFNγ, can also induce suppressor functions in macrophages by upregulating the expression of regulatory molecules like IDO, PD-L1 (Programmed Death-Ligand 1) and FasL (Wood et al. 2012).

MDSC also play a role in tolerance induction in transplantation. An expansion of MDSC was first reported in a rat model of kidney allograft tolerance (Dugast et al. 2008). De Wilde et. al. showed that endotoxin induced MDSC are able to significantly prolong skin allograft survival through IL-10 production enhanced by HO-1 expression (De Wilde et al. 2009). In humans, it has been recently shown that MDSC naturally accumulate in renal transplant patients and are able to suppress T cell activation and expand $CD4^+$ Treg cells *in vitro* (Luan et al. 2013). The authors found a correlation between the accumulation of MDSC after transplantation and an increase in Treg population *in vivo* (Luan et al. 2013).

PART III. THERAPIES IN TRANSPLANTATION

One of the major aims in organ transplantation field is to induce a state of tolerance or immunological ignorance towards the graft. Nowadays, acute graft rejection is well controlled thanks to the use of IS drugs, but chronic rejection remains the most challenging problem. Even more, the use of IS drugs has non-specific effects, therefore generating a state of global immunosuppression which can lead to undesired secondary effects and diseases. Later on, mAbs appeared as a good strategy as IS substitutes, as effects are more specific, due to the fact that they target a specific antigen. Both therapies induce a state of immunosuppression which is not donor-specific.

To overcome that problem, cell therapy has arisen as the best strategy. By *in vitro* or *in vivo* inducing regulatory cells specific towards donor antigens, the global state of immunosuppression is avoided while tolerance to the graft remains.

In this section, the three mentioned treatments which have been widely used in organ transplantation will be described.

1 IMMUNOSUPPRESSIVE DRUGS

Immunosuppressive drugs (IS) are a class of drugs that reduce the strength of the body's immune system. IS therapy after transplant is essential, as it efficiently decreases the host's immune response towards the new organ or tissue in order to avoid graft rejection.

Lymphocytes are the main players in the perpetuation of the immune response that leads to rejection. Therefore, most of the currently used IS agents target lymphocytes, by depleting them or blocking their signaling pathways, proliferation capacity or traffic to secondary lymphoid organs.

The drawback of IS drugs is that they display several non-desired effects, which can lead to secondary complications. The most important side-effect is the induction of a state of immunodeficiency, making patients more susceptible to infections and cancer development. Another important aspect of IS drugs is the toxicity which affects non-immune organs (mainly kidney), as targeted molecules can play a role in other non-immune cells.

IS drugs can be classified in two big groups: corticosteroids and small-molecule drugs. Some of the most commonly used IS drugs in organ transplantation will be described in the following paragraphs.

Corticosteroids

Corticosteroids are chemical compounds, analogs of natural hormones, which are involved in a wide range of physiological processes. They act by binding intracellular glucocorticoid receptors in

target cells, downregulating the transcription of several genes as pro-inflammatory cytokines. Their actions result in a decrease of the inflammatory response. Corticosteroids have been shown to impair monocyte/macrophage function and decrease the numbers of circulating $CD4^+$ T cells (Taylor et al. 2005).

Corticosteroids are the first line of treatment to prevent acute allograft rejection. The most commonly used are **Prednisolone** and **Prednisone**.

Small-molecule drugs

Most of small-drug molecules are derived from microbial products. They target evolutionary conserved proteins. A detailed description of their mechanism of action, clinical application and toxicity has been reviewed by P Halloran (Halloran 2004).

- **Antimetabolites:** Azathioprine
  It was the first drug to be used in organ transplantation, around 60's. Once metabolized, the resulting compounds are incorporated into replicating DNA, interfering with cell replication. They also block purine synthesis pathway, therefore interfering with lymphocytes proliferation. It also interferes with CD28 costimulation. The main side effect is dose-related bone marrow depletion.
- **Calcineurin inhibitors (CNIs)**: Cyclosporine, Tacrolimus (FK506)
  Cyclosporine was the pillar of immunosuppression through the 1980's. CNIs inhibit T cell activation and proliferation by blocking phosphorylation and nuclear translocation of NFAT (Nuclear Factor of Activated T cells), avoiding calcium signaling normally triggered by MHC/TCR interaction. The result is the blockade of transcription of cytokines such as IL-2. CNIs are associated with a range of side effects, including nephrotoxicity (Shihab 1996). These effects are reversible with a discontinuation of CNI therapy. Tacrolimus and cyclosporine provide good immunosuppression and give equivalent graft and patient survival although graft survival in patients receiving tacrolimus seems to be improved (Mayer et al. 1997).
- **Inhibitors of nucleotide synthesis:** Mycophenolate mofetil (MMF), Leflunomide
  MMF is the inhibitor of an enzyme expressed by activated lymphocytes, which is involved in the synthesis of guanosine nucleotides. Therefore, MMF blocks DNA synthesis and lymphocyte division. It has been shown to improve long-term renal graft function and graft survival in 3-4 years.
- **Target-of-rapamicyn (mTOR) inhibitors**: Sirolimus, Everolimus, Rapamycin
  They act by inhibiting mTOR enzyme activity, a molecule found downstream the signaling pathway of receptors that provide T lymphocyte signal 3. Therefore, mTOR

inhibitors prevent cytokines from activating cell cycle. Their side effects include metabolic, hematological and dermatological troubles.

The increasing number of potential combinations of available agents has led to a variety of immunosuppressive protocols used by different transplant centers. It is important to establish the best way to combine immunosuppressive therapy to adapt to individual patient's needs. The priority would be to select immunosuppressive regimens that minimize side-effects. The use of IS agents that share similar side-effects should be avoided.

2 MONOCLONAL ANTIBODIES

The first biologic agents to be used in clinical practice were polyclonal anti-thymocyte globulins (ATG). They were first described by Mechnikov in 1899 and used in transplantation since 1960. They are obtained from sera of rabbit (rATG) or horses (eATG) which have been immunized with human thymocytes. Therefore, the final product contains an heterogeneous mixture of polyclonal antibodies which target a variety of human molecules found at thymocytes' membrane. Its efficacy and tolerability in patients have made of it the most widely used induction agent in the United States. Some undesired symptoms related to cytokine release syndrome must be experienced.

In 1975 George Kohler and Cesar Milstein produced the first monoclonal antibody (Köhler & Milstein 1975). This technological advance opened a new window of possibilities to treat a wide range of immunological disorders. In the last two decades therapy using mAbs has greatly developed, with over 600 mAbs entering clinical studies and a total of 28 approved by the European Union and the American Food and Drug Administration (FDA) (Page et al. 2012).

The advantage of mAbs over ATG relies on their single specificity, which allows a more accurate immunosuppression. mAbs target surface epitopes of immune cells, triggering various mechanisms. Some of them will destroy target cells by fixing complement; others by modulating or shedding surface molecules, or by blocking their binding domain, rendering cells unresponsive to stimulation.

In allotransplantation, mAbs have been widely used as induction immunosuppression, mostly depleting mAbs. Induction immunosuppression consists on an intense prophylactic therapy used at the time of transplantation in order to promote graft acceptance by preventing early acute rejection (Kirk 2006). Some of the most relevant mAbs which have been used in clinics will be described in the following paragraphs, with specific emphasis on those which have been applied to organ transplantation. At the end of the chapter, **Table 4** summarizes experimental studies were the use of mAb therapy was able to induce immunological tolerance or significantly prolong graft survival in transplantation.

Anti-CD3

In 1979, P Kung and G Goldstein produced the first mouse mAb specific against human CD3 molecule, a mouse IgG2a, named **OKT3 (Muromab)** (Kung et al. 1979). It was also the first mAb to enter clinical practice in transplantation field, in the early 1980s, even before knowing its molecular target. OKT3 displayed similar properties as ATG.

Thanks to its strong immunosuppressive potency, its use was approved worldwide by 1984 in association with other conventional immunosuppressors. But at mid-1990s, its use was discontinued because of its severe side effects. At the same moment, other mAbs without acute side effects became available.

The most important undesired effect is called "flu-like" syndrome, which comprises high fever, chills, headache and gastrointestinal symptoms. Those effects are due to the transient activation of T cells when the antibody links the TCR/CD3 complex on the surface of circulating human T cells. This activation leads to the release of pro-inflammatory cytokines (IL-6, TNFα, IFNγ, GM-CSF, IL-2…) immediately after the first injection.

Despite this drawback, efforts to study anti-CD3 mAbs biology kept on. In late 1980s, JA Bluestone's team generated a hamster mAb which recognized the murine CD3 complex, 145-2C11 (Leo et al. 1987) and established a murine model of skin transplantation to study the *in vivo* effects of anti-CD3 mAb therapy (R Hirsch et al. 1988). They realized that αCD3 mAb immunosuppressive effect was independent of T cell depletion induction, but rather due to what they thought was T cell sequestration. They demonstrated that the reason why αCD3 mAb was a potent T cell activator *in vitro* and *in vivo* was due to the cross-linking of the mAb through its Fc portion. Therefore, they generated an αCD3-F(ab')2 fragment by pepsin digestion, which induced modulation of the CD3/TCR complex without transducing activation signals (Raphael Hirsch et al. 1988). They showed that Fab'2 conserved the immunosuppressive activity of the whole mAb *in vivo* while displaying reduced activating properties (Hirsch et al. 1990; Hirsch R et al. 1991). As F(ab) fragments have a much shorter half-life than a whole mAb, chimeric hamster 145-2C11-F(ab')2 region/mouse Fcγ portion (IgG3) where engineered. This chimeric mAb did not induce T lymphocyte activation, but promoted internalization of CD3/TCR complex, depleted T cells from blood circulation and prolonged skin graft survival (Alegre et al. 1995).

At that time, *in vivo* experiments using a model of cardiac allograft in rats demonstrated that a non-mitogenic mAb against the CD3 molecule (G4.18 mouse IgG3) was able to induce long-term donor-specific tolerance, as a second graft from the same genetically background was not rejected whereas a third party graft was (Nicolls et al. 1993).

Therefore, αCD3 mAbs regained interest when experimental evidence showed that they possess the unique ability to promote immunological tolerance (antigen-specific unresponsiveness) instead of a state of long-term generalized immunosuppression. Another important point was technical advances which made possible the generation of humanized non-mitogenic CD3 mAb (Chatenoud 2003). Humanized αCD3 mAbs have been produced by replacing mouse Fc portion of the Ab by a non-FcR-binding human IgG1 immunoglobulin chain (Riechmann et al. 1988). Two genetically engineered CD3-specific mAb have been used in phase I and II clinical trials:

- **Telizumab** (or huOKT3γ1(Ala-Ala)) is a humanized non-mitogenic OKT3 variant, where hypervariable regions of OKT3 (mouse IgG2a) have been transferred to a CH2 mutated human IgG1. It retains its *in vivo* immunosuppressive capacity and has proven effective to treat acute renal graft rejection (Woodle et al. 1999) as well as autoimmune diabetes.
- **Otelixizumab** (or ChAglyCD3) is a non-mitogenic version of rat IgG1 YTH12.5 Ab. Hypervariable regions of the Ab have been transferred to a human IgG1 lacking glycosylation sites in CH2 domain (Bolt et al. 1993), being unable to bind FcR. After *in vivo* administration, T cell proliferation and cytokine secretion are highly decreased. Its efficacy has been proven in a phase I clinical trial in kidney transplant patients (Friend et al. 1999).

Anti-CD3 mechanisms of tolerance induction have been widely studied (Chatenoud & Bluestone 2007; You et al. 2008). Immediately after injection, αCD3 mAb binds the CD3 complex at the cell surface, resulting in antigenic modulation through CD3 internalization or shedding, apoptosis induction of activated T cells by Fas/FasL interaction or in anergy induction. Therefore, there is a dramatic decrease in circulating T cell numbers. Once αCD3 mAb treatment is discontinued, CD3/TCR complex is newly expressed at T cell surface, and T cell pool returns to normal numbers shortly after (Chatenoud et al. 1982). The long-term effects of αCD3 mAb treatment rely on the induction of Treg cells, which suppresses $CD4^+$ effector T cells by TGFβ production (Belghith et al. 2003; You et al. 2007), presumably involving DC modifications that maintain the an anti-inflammatory environment. Recent studies have shown that the increase in Treg cells numbers is due to a preferential elimination of effector T cells by αCD3 mAb treatment rather than by Treg cells, the balance being an increase in the ratio Treg : T effector cells (Penaranda et al. 2011; You et al. 2012).

Anti CD25

CD25 is the α-chain of the trimeric high affinity IL-2 receptor expressed on T cells. When IL-2 binds to its receptor, it brings about a clonal expansion and activation of T lymphocytes. In the context of transplantation, targeting of IL-2Rα inhibits IL-2 mediated T cell proliferation and activation, thus inducing immunosuppression.

In the late 1980s, mAbs against IL-2R where shown to prevent and reverse acute heart allograft rejection and delay skin rejection in mouse (Kirkman et al. 1985; Granstein et al. 1986). They also proved to be safe and efficient in preventing kidney allograft graft rejection in primate (Reed et al. 1989). In 1987, Soulillou et. al. demonstrated that the administration of a blocking rat mAb IgG2a directed against IL-2Rα chain (33B3.1) in combination with prednisolone was able to prevent early acute kidney graft rejection in humans. It was clinically well tolerated and displayed less secondary effects as it did not induce a severe lymphopenia (Soulillou et al. 1987).

Those findings lead to the development of two modified anti-CD25 mAb which are still used as induction therapy in renal transplantation: **Basiliximab**, which is a recombinant chimeric mouse/human IgG1 mAb and **Daclizumab**, a humanized mAb. Those mAbs do not deplete T cells in humans. Even though, there Treg cell numbers are decreased, it does not seem to be clinically relevant. Their efficacy is comparable to that obtained with ATG.

Anti CD52

CD52 is a molecule expressed at the surface of T and B lymphocytes and to a lesser extent, on NK cells, monocytes, macrophages and granulocytes. The mAb which targets CD52 in humans, **Alemtuzumab** (Campath-1H), is a humanized IgG1. It induces massive lymphopenia by complement activation and ADCC. In parallel, Treg cell numbers remain constant.

It was introduced as induction therapy in renal transplantation at the end of the 1990s in combination with low dose of immunosuppressive drugs. It has been shown to effectively prevent early T cell mediated rejection.

Co-stimulation blockade:

- **CTLA-4-Ig** and its commercially available fusion proteins, **belatacept** and **abatacept**, target CD80/86, therefore blocking their interaction with CD28 and CTLA-4. By costimulation blockade, they promote T-cell tolerance. Belatacept, shows higher binding affinity and is currently approved for clinical use in renal transplantation (Vincenti et al. 2005; Vincenti et al. 2010).
- **Anti-CD28:** Antagonist antibodies targeting CD28 emerged as an alternative to the use of CD80/86 antagonists. It presents some advantages regarding CTLA-4-Ig, as they block CD28-mediated co-stimulatory signaling without impeding the co-inhibitory signals

delivered by CTLA-4 and PD-L1. Therefore, selective CD28 antagonists might show improved graft prolongation. The monovalent form of the mAb displays better outcomes because of multimerization prevention and the associated activation of PI3K. Divalent Abs induce cell proliferation and cytokine release in Fc-independent manner (Mary et al. 2013). Selective blockade of CD28 attenuates acute and chronic rejection of murine cardiac allografts (T. Zhang et al. 2011) and promotes Treg cell induction in organ transplantation (Poirier et al. 2010). An anti-CD28 monovalent F(ab)' has proven to be safe and efficient in humanized mice of GVHD (Poirier et al. 2012).

Anti CD40/CD40L

CD40 is a costimulatory molecule present at the surface of antigen presenting cells (APCs). CD40 binding to CD40L (CD40 ligand or CD154) at the T cell surface triggers APCs activation which in turn activates T lymphocytes.

Treatment with anti-CD154 results in potent immunosuppression in non-human primates, but carried some complications that stopped their further development. As an alternative, blockade of CD40 with a human anti-CD40 mAb have been shown to prolong renal and hepatic allograft survival in non-human primates (Oura et al. 2012).

Anti CD20

The negative impact of alloantibodies on acute rejection and long term allograft function has already been discussed. Even more, B cells are responsible of T cell mediated rejection, by their antigen presenting capacity.

The CD20 antigen is found at the surface of B lymphocytes, since the developmental state of pre-B cell to plasmablast. **Rituximab**, a mouse/human chimeric IgG1 mAb which targets CD20, induces B cell depletion through different mechanisms, such as complement-dependent cytotoxicity, ADCC and apoptosis induction. It was approved by the FDA as therapy to treat B-cell lymphomas in 1997. A pilot study was performed on renal transplant patients presenting acute humoral rejection, where rituximab treatment was able to improve renal function (Faguer et al. 2007).

Nowadays, monoclonal antibody therapy covers multiple fields, including cancer, infectious diseases, autoimmune diseases and transplantation. Only advances towards humanized antibodies made possible the reduction of important side effects of mAb therapy. The potential of some mAbs to expand immunoregulatory cells is important to induce tolerance in the setting of transplantation.

Table 4. Non-exhaustive list of target molecules of mAbs studied in animal models of transplantation

Target	mAb name	Isotype	Mechanism of action	Animal model/effect	References
TCRαβ	H57-597	hamster IgG	Reduction of antigen-specific T cells	Long-term fully mismatched heart allograft survival in mice	Miyahara et al., 2012
CD3	G4.18	mouse IgG3	Activated T cell depletion Antigenic modulation of TCR/CD3 complex	Long-term specific tolerance to fully mismatched heart allografts in rat	Nicolls et al., 1993
CD25	M7/20	rat IgM	Activated T cell depletion	Indefinite graft survival in fully mismatched heart mouse transplantation	Kirkman et al., 1985
CD28	sc28AT	Fusion protein	Block CD28/B7 co-stimulation	Block acute rejection of kidney transplantation in primates	Poirier et al., 2010
CD40	4D11	Fully human	Block co-stimulation CD40/CD154	Prolongation of kidney transplantation in primates	Imai et al., 2007
CD54 (ICAM-1)	1A29	mouse IgG1	Block leukocyte adhesion to endothelium	Tolerance induction of liver transplantation in rat (plus anti-CD25 and CSA treatment)	Gassel et al., 2000
CD80/86	CTLA4-Ig	Fusion protein	Block CD28/B7 co-stimulation	Indefinite graft survival in fully mismatched heart mouse transplantation	Pearson et al., 1994

3 CELL THERAPY IN ORGAN TRANSPLANTATION

Pharmacological or biological agents have not succeeded to achieve long-term immunological acceptance of transplants. In this context, cell therapy arises as a promising approach to struggle against late graft failure. Cell therapy consists in the use of *in vitro* derived cell types in order to induce donor specific tolerance while keeping the recipient immunocompetent.

3.1 Lymphoid cell therapy

Since their discovery, $CD4^+CD25^+FoxP3^+$ Tregs have been widely studied, aiming at their use as cell therapy. Treg cells characterization in humans took place in 2001 (Dieckmann et al. 2001) and the same year, their *in vitro* polyclonal expansion was reported (Levings et al. 2001).

Proof-of-concept of human Treg cell therapy for organ transplantation comes from various animal models. Transfer of Treg cells has proven to be effective to control immune responses in transplantation (Wood et al. 2012). *Ex vivo* expanded Treg cells can prevent transplant arteriosclerosis (Nadig et al. 2010) and skin allograft rejection in humanized mouse models (Issa et al. 2010), and efficiently protect from GVHD (Ermann et al. 2005).

Injection of both, donor (Di Ianni et al. 2011) and recipient (Trzonkowski et al. 2009) derived expanded Treg cells have successfully reduced the incidence of GVHD in humans. An alternative approach is to expand or enhance Treg cell function *in vivo*. This has successfully been performed in mice by injection of low-doses of IL-2 and rapamycin against acute GVHD (Shin et al. 2011). In humans, low-dose of IL-2 therapy was associated with Treg cell expansion and successful treatment of patients with chronic GVHD (Koreth et al. 2011).

Other challenges concern the cell source for Treg cell expansion. Thymus-derived Treg cells, as well as Treg cells isolated from cord blood and expanded *in vitro* have proven to be safe and efficient in preventing GVHD (Trzonkowski et al. 2009; Brunstein et al. 2011).

One major concern about the clinical use of Treg cells for organ transplantation is the lack of specific markers that clearly discriminates between Treg and effector T cells. Therefore, contaminating effector populations may exist in the *ex vivo* cultures of Treg cells. To overcome that problem, some groups add immunosuppressive agents (such as rapamycin) to the culture medium during expansion (Battaglia et al. 2005; Tresoldi et al. 2011). This strategy has increased the purity of Treg cells. Besides, a combination of Vitamine D3 (VitD3) with dexamethasone differentiates naive T cells into IL-10 producing regulatory T cells in mouse and human (Barrat et al. 2002).

Tr1 regulatory $CD4^+$ T cells have also been tested in multiple pre-clinical models of transplantation. Tr1 cell therapy leads to tolerance induction in two distinct models of mouse islet transplant. The authors showed that antigen-specific Tr1 cells induced better transplant tolerance

than polyclonally expanded Tr1 cells, by triggering of IL-10 secretion (Gagliani et al. 2010). Between 2000 and 2009, a clinical trial where high-risk of hematologic malignancies patients received T cell depleted haploidentical hematopoietic stem cell transplantation was performed. Patients were treated with donor T lymphocytes pre-treated with IL-10, which contained Tr1 cells. This clinical assay not only proved the safety and feasibility of Tr1 cell therapy (Battaglia, Gregori, et al. 2006) but also its efficacy in immune reconstitution of four long-term survival patients (Bacchetta et al. 2014).

Currently, there is no data available concerning the use of $CD8^+$ Treg cells in human clinical trials in transplantation. In our laboratory, the group of Dr. I Anegón is carrying on pre-clinical studies in humanized mice, in order to determine the efficacy of human $CD8^+CD45RC^{lo}$ Treg cells, a regulatory population identified in a rat model of CD40Ig-induced tolerance to heart transplant (Guillonneau et al. 2007).

Table 4 displays a summary of Treg cell clinical trials performed until 2012. Only one clinical trial included solid organ transplantation.

Group leader (location)	Number of patients	Type of transplant	Type of T cells transferred	Dose of T cells transferred	T_{Reg} cell *in vitro*-expansion protocol	Clinical outcome
Trzonkowski (Poland)	2: one with chronic GVHD (patient 1); one with acute GVHD (patient 2)	Bone marrow transplant (patient 1) or peripheral blood stem cell transplant (patient 2) from HLA-matched sibling donor	$CD4^+CD25^+$ $CD127^-$ donor T_{Reg} cells	1×10^5 cells per kg (patient 1) or 3×10^6 cells per kg (patient 2)	High concentration of IL-2 (1000 U ml^{-1}) and beads coated with CD3- and CD28-specific antibodies in a 1 to 2 ratio	Withdrawal of mycophenolate mofetil and a reduction in the dose of prednisone (patient 1); temporary clinical improvement (patient 2)
Blazar (Minnesota)	23	Double-unit umbilical cord blood from unrelated donors	$CD4^+CD25^+$ third-party cord blood T_{Reg} cells	1×10^4 to 3×10^6 per kg (day +1)	CD3- and CD28-specific antibody-coated beads and IL-2 for 18 days; 200-fold T_{Reg} cell population expansion achieved	The incidence of grade II–IV acute GVHD was reduced in the T_{Reg} cell treatment group
Martelli (Perugia)	28	HLA-haploidentical HSCT	$CD4^+CD25^+$ donor T_{Reg} cells	2×10^6 per kg (day −4)	No manipulation; freshly isolated cells were used	The incidence of acute and chronic GVHD was extremely low
Edinger (Regensburg)	9	HSCT in patients with a high risk of leukaemia relapse	T_{Reg} cells (>50% $FOXP3^+$), together with effector T cells to promote GVL effects	5×10^6 per kg	No manipulation; freshly isolated cells were used	Safe and feasible after the withdrawal of GVHD prophylaxis
Roncarolo (Milan)	16	HLA-haploidentical HSCT	Donor T_R1 cells	1×10^5 $CD3^+$ cells per kg (day +30) (with the exception of two patients who received 3×10^5 $CD3^+$ cells per kg)	Allostimulation in the presence of IL-10 for 10 days	Safe and feasible
Okumura (Japan)	13	Kidney	Recipient anergic cells	Anergic cells (day +12)	PBMCs from the recipient were co-cultured with irradiated (30 Gy) donor cells in the presence of CD80- and CD86-specific monoclonal antibodies for 2 weeks	Permitted the reduction of immunosuppression

FOXP3, forkhead box P3; GVHD, graft-versus-host disease; GVL, graft-versus-leukaemia; HSCT, haematopoietic stem cell transplantation; IL, interleukin; PBMC, peripheral blood mononuclear cell; T_R1, T regulatory type 1; T_{Reg}, regulatory T.

Table 5. Treg cell therapy in clinical trials in transplantation. From (Wood et al. 2012)

3.2 Myeloid cell therapy

The use of innate immune cells, like myeloid cells, as products for cell therapy is gaining interest. Myeloid cells play a role in the induction of immune responses but also in the induction of tolerance. Different strategies using myeloid cells have been tested and developed in animal models with the aim of translating results into clinical trials. Myeloid cells can be targeted *in vivo*, using immunosuppressive agents or mAb or can be generated *in vitro* from myeloid precursors. There are three main types of regulatory myeloid cells which have been well studied: tolDC, Mreg and MDSC.

In the next chapter, the different myeloid cell types candidates for cell therapy will be described, focusing on the different ways to obtain them *in vitro*, their *in vivo* efficacy and the advances in the understanding of their mechanisms of action.

3.2.1 Tolerogenic DCs (tolDCs)

Tolerogenic DCs are immature or semi-mature DCs which express classical DC markers (CD11c, CD11b) but display low constitutive expression of MHC molecules and lower expression of costimulatory molecules (CD80, CD86) compared to inhibitory molecules (PD-1). They maintain the capacity to acquire and present antigens to T cells but the lack of costimulation promotes T cell anergy or death. Moreover, they are resistant to maturation after exposure to maturation stimuli and induce low T cell proliferation *in vitro*.

a. *In vitro* generation of tolDC

Animal tolDC are derived from bone marrow precursors. The most common protocol to differentiate them uses GM-CSF and IL-4 cytokines. However, Lutz et. al. showed that DCs derived *in vitro* in the presence of low doses of GM-CSF and in the absence of IL-4 shared classical tolDC characteristics, as they displayed an immature phenotype, high endocytic capacity and did not induce allogeneic T cell proliferation (Lutz et al. 2000).

Typically, human tolDC are derived from peripheral blood monocytes, purified by elutriation or positive selection of CD14 cells and cultured in the presence of GM-CSF and IL-4, but it is also possible to differentiate them only with low doses of GM-CSF (Moreau, Varey, Bériou, et al. 2012).

Different pharmacological agents can be added to tolDC culture, in order to promote their tolerogenic phenotype and functions. The most commonly used are VitD3, rapamycin and dexamethasone. In human DC culture, the addition of the active form of VitD3 to the culture inhibits DC maturation (Penna & Adorini 2000). Rapamycin treatment has been shown to impair antigen uptake by human DC (Monti et al. 2003). Dexamethasone maintains the immature state of tolDC and induces IL-10 producing human MoDC (Xia et al. 2005). In mice, recipient-derived DC cultured in the

presence of rapamycin and pulsed with donor antigens lead to indefinite allograft acceptance and through increasing numbers of $CD4^+$ Treg cells (Turnquist et al. 2010). *In vivo*, these drugs also induce a tolerogenic profile on DCs. Treatment of graft recipients directly with VitD3 and MMF have been shown to induce tolerance to mouse pancreatic islet allografts presumably by downregulating costimulatory molecules on the surface of DCs and macrophages *in vivo*, which induces increased numbers of $CD4^+$ Tregs (Gregori et al. 2001).

A comparison of the three immunomodulatory agents in the generation of human clinical grade tolDCs has been performed by FE Borràs group (Naranjo-Gómez et al. 2011). The authors found relevant differences depending on the agent used. Only dexamethasone- and VitD3-cultured DCs displayed reduced expression of costimulatory markers and were able to secrete IL-10. Rapamycin treated DC displayed higher expression of costimulatory markers but, on the other hand, were the only ones to induce $CD4^+$ Tregs. Even though, none of them induced allogeneic T cell proliferation nor IFNγ production by T cells (Naranjo-Gómez et al. 2011).

An alternative approach for *in vitro* DCs manipulation is the addition of anti-inflammatory agents to the culture medium, like IL-10. Addition of IL-10 at the end of the regulates the expression of costimulatory molecules (Buelens et al. 1995) and leads to autocrine IL-10 secretion, which maintains them in an immature state (Corinti et al. 2001). DCs treated with IL-10 inhibit allogeneic T cell responses (Buelens et al. 1995) and induce $CD4^+$ and $CD8^+$ T cell anergy in an antigen-specific way (Steinbrink et al. 2002). Bone-marrow derived DCs generated *in vitro* with a combination of GM-CSF, IL-10, TGFβ and pulsed with LPS prevented lethal GVHD after bone marrow transplantation in sublethally irradiated mice (Sato et al. 2003).

Another protocol set up by the group of MG Roncarolo consists in the addition of IL-10 since the beginning of the culture. This strategy leads to the development of tolDC called DC-10 (Gregori et al. 2010). They exert their regulatory function by induction of antigen-specific IL-10 producing Tr1 cells. Equivalent DC-10 were found *in vivo* in humans (Gregori et al. 2010)

Our group generates human tolDC using low-dose GM-CSF alone (Moreau, Varey, Bouchet-Delbos, et al. 2012; Moreau, Varey, Bériou, et al. 2012), based on the efficacy of this methodology *in vivo* in multiple animal transplant models (Pêche et al. 2005; Bériou et al. 2005; Hill et al. 2011; Moreau et al. 2014). As previously described., human tolDC generated using low-dose GM-CSF display tolerogenic properties *in vitro* (Chitta et al. 2008). These cells express a tolerogenic phenotype, induce very low stimulation of allogeneic T cells, are semi-resistant to maturation after LPS and IFNγ stimulation and secreting IL-10 but not IL-12 stimulation (Moreau, Varey, Bouchet-Delbos, et al. 2012).

Other protocols involve the *in vitro* maturation of tolerogenic DC previously to administration. This process, called alternative activation, aims at increasing DCs migratory and antigen presentation capacity.Inflammatory cytokines such as TNFα, IFNγ and IL-1 or other cytokinic cocktails can be used, including molecules derived from bacterial products or stimulation through CD40 ligation (Cella et al. 1996; Cella et al. 1997).

b. Mechanisms of action of tolDC

Strong efforts have been made in understanding the mechanisms underlying tolDC immunosuppressive activity. As previously mentioned, it does not exist a unique molecule that renders DCs to a tolerogenic state. Therefore, depending on the factors used for the *in vitro* differentiation of tolDCs, suppressor mechanisms may also be different. In the literature, it has been described that tolDC express some immunomodulatory molecules, like HO-1, PD-L1/2, ILT3/4 and IDO or anti-inflammatory cytokines as IL-10 or TGFβ. The effect of those molecules as well as their importance in tolerance induction in transplantation has been reviewed by Morelli and Thomson (Morelli & Thomson 2007).

In our group, studies using rat autologous tolDCs showed that the enzyme iNOS is involved in the hypoproliferative properties of tolDC, as iNOS inhibition in DC by L-NMMA (NG-monomethyl-L-arginine) reestablishes T cell proliferation (Pêche et al. 2005). That observation is in agreement with other studies showing the importance of iNOS for T cell proliferation in rat and mouse (Powell et al. 2003; Bonham et al. 1996; Aiello et al. 2000).

Another important molecule is HO-1. We found that HO-1 blocked maturation of DCs in rats and humans and inhibited proinflammatory and allogeneic immune responses while preserving IL-10 production (Chauveau et al. 2005). We confirmed its expression on macaque tolDCs. *In vitro* inhibition of HO-1 in tolDC with SnPP (tin (Sn) protoporphryin-IX), was responsible for the loss of tolDC suppressive capacity (Moreau et al. 2009). Furthermore, *in vivo* inhibition of HO-1 by SnPP injections in cardiac allograft recipients, leads to the abrogation of allograft survival induced by tolDCs (Moreau et al. 2009)

EBI3 (Epstein-Barr virus Induced gene 3), a molecule that forms the interleukin IL-35 in conjunction with p35, was also found to be expressed by tolDC. EBI3 expression contributes to allograft survival, as its inhibition with a blocking antibody *in vivo* avoids the protective effect of tolDCs in a rat cardiac allograft model (Hill et al. 2011).

Some mechanisms imply direct effect of tolDCs on target cells. Indeed, it has been shown that tolDCs are able to induce T cell anergy, both *in vitro* and *in vivo*. Anergy is induced because tolDC lack costimulation molecules (Schwartz 1997) or by PD-L1 expression at the tolDC surface, which interacts with PD-1 at the T cell surface. TolDC can also eliminate reactive T cells by the induction of

clonal deletion, leading to apoptosis by AIDC (Activation Induced Cell Death), mainly through Fas-FasL interaction (Lu et al. 1997). IDO expression by tolDCs prevents proliferation of allogeneic T cells by degrading the essential amino acid tryptophan (Terness et al. 2002).

An important long-lasting mechanism of tolerance induction is tolDC expansion of thymic-derived natural $CD4^+$ Treg cells or induction of peripheral $CD4^+$ Tregs from naive $CD4^+$ T cells (Fujita et al. 2007). Again, multiple molecules can play a role in Treg generation/expansion. IDO expression by DC has been shown to induce Treg cells *in vitro* (Hill et al. 2007). DC-10 regulatory DCs have been shown to induce Tr1 cells by IL-10 secretion (Wakkach et al. 2003; Gregori et al. 2010) thanks to the ILT-4/HLA-G pathway, which impairs DC maturation. Little is known about the induction of $CD8^+$ Treg cells by tolDC treatment. In a mouse model of skin transplantation, our group has recently shown that mouse treated with tolDC and low dose of αCD3 mAb prolongs graft survival, which is associated with an increase of a Treg cell subtype expressing $CD8^+CD11c^+$ markers (Segovia et al. 2014). Those mechanisms are represented in **Figure 11**.

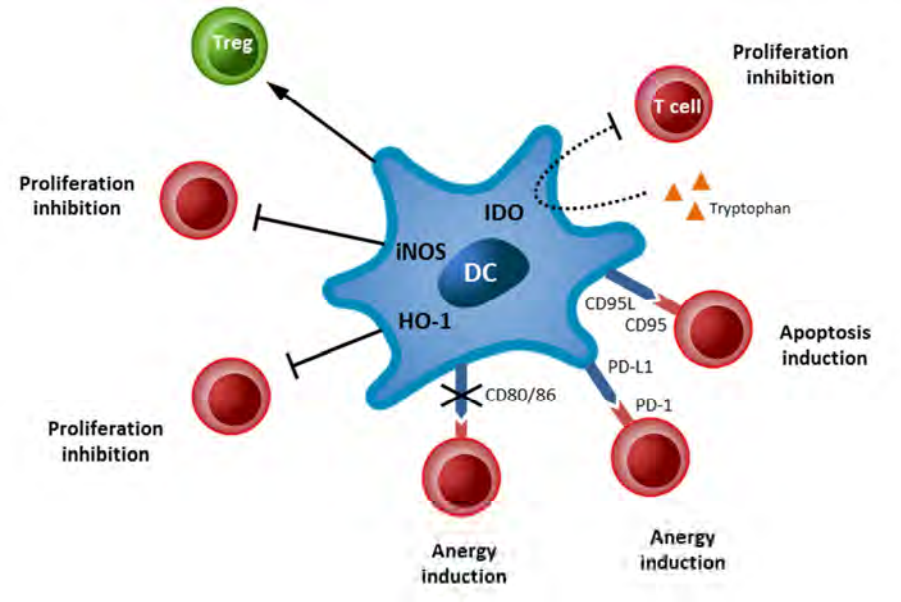

Figure 11. Mechanisms of action of tolDC.

The distribution of tolDC after injection depends on the type of DC generated, the administration route and the transplant model used. They are usually found in draining lymphoid organs, either lymph nodes or spleen. After intravenous injection of recipient derived DC in rat cardiac transplant model, DC migrate to the spleen (Pêche et al. 2005), whereas in a mouse skin transplant model they first migrate to the graft and then to graft draining lymph nodes (Segovia et al. 2014). Donor derived Rapa-differentiated DCs migrate to lymph nodes in a model of hematopoietic cell transplantation following intramuscular injection (Taner et al. 2005; Reichardt et al. 2008).

Expression of CCR7 by DCs directs them to secondary lymphoid organs, where they may exert their regulatory function. It has been shown that transfection of tolDC with an IL-10 homolog abolishes tolDC homing to secondary lymphoid organs (Garrod et al. 2006).

c. *In vivo* efficacy of tolDC in animal models and human clinical trials

TolDC injection has proven to be safe and efficient in a wide variety of animal models, with or without the combination of an immunosuppressive treatment. **Table 5** shows the capacity of inducing indefinite allograft survival with different types of *in vitro* differentiated, donor- or recipient-derived tolDC in rodent models of heart transplantation.

A. Promotion of indefinite heart graft survival by donor dendritic cells (DC).

DC Source	Species	DC treatment	Additional treatment	Route of injection	MST
MoDC	Rat	Granulocyte/macrophage -colony-stimulating factor (GM-CSF)		i.v.	>160 days
BMDC	Mouse	GM-CSF+GFβ	Anti-CD40L mAb	i.v.	>100 days (40%)
BMDC	Mouse	Low GM-CSF		i.v.	>100 days
BMDC	Mouse	NF-κB + rAd CTLA4Ig		i.v.	>100 days (40%)
BMDC	Rat	GM-CSF + IL-4	ALS	i.v.	>200 days† (50%)
BMDC	Mouse	Low GM-CSF	Anti-CD54 mAb + CTLA4Ig	i.v.	>100 days*

*Secondary challenge: cardiac allograft recipients were tested with skin grafts 30 days after heart transplantation, regardless of rejection. All 3rd party grafts were rejected; approximately 50% of donor skin grafts were accepted in anti-CD54 + CTLA4Ig-treated recipients.
†Secondary challenge post-100 days: 2nd donor heart accepted; 3rd party hearts rejected.
ALS: antilymphocyte serum; BMDC: bone marrow-derived dendritic cells; i.v.: intravenous; MoDC: monocyte-derived DC; MST: mean survival time; 'blank': none.

B. Promotion of indefinite heart graft survival by recipient DCs.

DC Source	Species	DC treatment	Additional treatment	Route of injection	MST
BMDC	Rat	Donor MHC I peptide (RT1.Au)	ALS	i.t.	>150 days*
BMDC	Rat	Donor MHC I peptide (RT1.Au)	ALS	i.v.	>200 days*
BMDC	Mouse	RAPA + donor cell lysate		i.v. (×3)	>100 days
BMDC	Rat	GM-CSF + IL-4	LF 15–0195†	i.v.	>100 days
BMDC	Mouse	GM-CSF + IL-4	NFκB ODN + donor-derived cell lysate	i.v.	>100 days (33%)
BMDC	Rat	Low GM-CSF + IL-4		i.v.	>100 days (20%)

*Secondary challenge post-100 days: 2nd donor heart accepted; 3rd party rejected.
†Deoxyspergaulin derivative.
ALS: antilymphocyte serum; BMDC: bone marrow-derived dendritic cells; i.t.: intra-thymic; i.v.: intravenous; ODN: oligodeoxyribonucleotides; RAPA: rapamycin; 'blank': none.

Table 6. TolDC therapy in animal models of heart allograft transplantation. A. Donor-derived tolDC therapy. B. Recipient-derived tolDC therapy. From (McCurry et al. 2006)

Our group has developed several animal models of transplantation (heart, kidney and skin) where safety and efficacy of non-pulsed autologous tolDC (ATDCs) have been tested. In rat heart transplant model, ATDCs alone were able to prolong allograft survival, and tolerance was achieved when ATDCs were injected with suboptimal immunosuppression using LF15-0195 (a deoxyspergualin analog which blocks NF-κβ signaling pathway) (Pêche et al. 2005; Bériou et al. 2005). Suppression mechanisms were found to be donor-specific, as third part transplants were rejected. ATDCs treatment has also been tested in two mouse transplantation models. In a minor antigen mismatch skin transplant model, ATDCs alone were not able to prolong allograft survival, but a significant prolongation was achieved when ATDC injection was combined with anti-CD3 mAb immunosuppression (Segovia et al. 2014). Similarly, in a pancreatic islet fully mismatch transplantation model, the combination of ATDC with anti-CD3 mAb treatment promotes permanent graft acceptance (Baas et al, 2014). Safety of IL-10-treated ATDC pulsed with a transgene has as well been tested in non-human primates, and no toxicity was detected after injection (Moreau et al. 2014).

Concerning human trials administering tolDC, in 2001, Dhodapkar et al. carried on a pilot study with healthy volunteers to evaluate the safety of autologous tolDCs injected through different routes of administration (Dhodapkar et al. 2001). Autologous tolDCs (loaded with MP Influenza peptide) were well tolerated and decreased effector T cell functions while increasing regulatory T cells frequency specific for Influenza virus (Dhodapkar et al. 2001).

Although no clinical trials using tolDCs in transplantation context have been reported until date, safety of autologous regulatory DC administration has been demonstrated in multiple clinical trials in the field of autoimmunity. The first human clinical trial using autologous regulatory DCs was performed by Giannoukakis et al. in type 1 diabetic patients. In that trial, DCs were targeted *in vitro* to down-regulate costimulatory molecules (Giannoukakis et al. 2011). Autologous DC injection was safe and the cells were well tolerated. There are currently two ongoing clinical trials in rheumatoid arthritis patients. The first one is a phase I study where tolDC are generated in the presence of a NF-κβ signaling inhibitor and loaded with citrullinated self-antigens (Thomas et al. 2011). The second clinical trial in development differentiates MoDC in presence of dexamethasone and VitD3 and activates them with monophosphoryl lipid A. *In vitro*, those cells are able to present antigens in the absence of costimulatory signals, therefore displaying hypoproliferative T cell capacity. Those DCs secrete high levels of IL-10 and TGFβ and low levels of IL-12, IL-23 and TNFα (Stoop et al. 2011). None of those clinical trials have shown the efficacy of tolDC therapy yet.

3.2.2 Regulatory macrophages (Mregs)

Macrophages are very plastic cell types, which can be easily driven to a polarization state or another. Therefore, it seems as an ideal cell type to modify *in vitro*, in order to obtain the desired characteristics and functions for its use in cell therapy. Nowadays, most of the work developed in the field of transplantation implying Mreg cell therapy, either in mouse or human, comes from findings of JA Hutchinson group. We will briefly describe what is known.

a. *In vitro* generation of Mregs

Mouse Mregs described by JA Hutchinson's team are derived from bone marrow $Ly6C^{+}CD11b^{+}$ sorted monocytes and cultured for 7 days in the presence of low dose M-CSF(5 ng/ml) and fetal calf serum (FCS) (10%) plus human AB serum (10%). They are pulsed with 25 ng/ml of IFNγ for the last 24h of culture. Those macrophages display typical macrophage markers (CD11b, F4/80, CD68, CD11a and CD14), intermediate levels of MHC class II molecules and CD80, but display other markers which distinguishes them from tissue macrophages (CD209, MARCO, Dectin-2) therefore representing a novel stage of macrophage polarization (Riquelme et al. 2013).

Human Mregs are differentiated in the same conditions as mouse Mregs (5 ng/ml M-CSF and only 10% of serum AB). They display high levels of HLA-DR and CD86 molecules, whereas there is low or none expression of CD14, CD16, CD80, CD163 and CD282 (J. a Hutchinson et al. 2011a).

b. Mechanisms of action of Mregs

In vitro, mouse Mregs are able to inhibit mitogen-driven T cell proliferation and preferentially eliminate allogeneic than syngeneic T cells in co-culture. Their mechanism of suppression relies on iNOS expression, as *in vitro* proliferation was restored when iNOS is inhibited by addition of L-NMMA to the allogeneic co-culture. *In vivo*, Mregs are able to significantly prolong cardiac graft survival in fully mismatched mice transplant model. iNOS plays an important role in tolerance induction *in vivo* as Mregs derived from iNOS-deficient mouse are not able to prolong allograft survival (Riquelme et al. 2013).

Human and murine Mregs injected intravenously first migrate to lungs and then the liver, the spleen, and the bone marrow. Interestingly, they have not been found in lymph nodes (J. a Hutchinson et al. 2011a). Their chemokine receptor expression profile remains unknown.

Mechanisms of action of *in vitro* generated Mregs as well as other mechanisms that have been described for alternatively activated macrophages to regulate immune responses are represented in **Figure 12**.

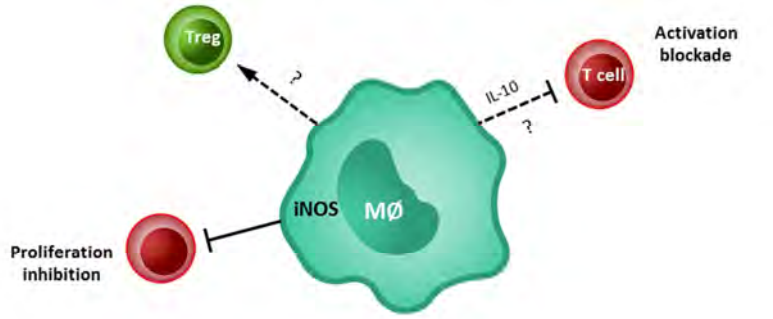

Figure 12. Mreg mechanisms of action. Dashed lines indicate suggested mechanisms, which take place by alternatively activated macrophages but have not been found in *in vitro* generated Mreg

c. *In vivo* efficacy of Mregs in animal models and human clinical trials

In mice, a single injection of 5 millions Mregs 8 or 35 days before the transplantation prolongs heart allograft survival (Riquelme et al. 2013). The authors showed that only donor Mregs were effective, as mice treated with recipient derived Mregs rejected the graft with the same kinetics than untreated grafted mice. Mreg injection in combination with rapamycin treatment improved their effect (Riquelme et al. 2013).

The first clue for the use of macrophages as therapy in human transplantation came from the evidence that myeloid cells which naturally differentiated from early myeloid precursors could induce indefinite allograft acceptance (Fändrich et al. 2002). F Fandrich's laboratory made strong efforts to obtain TAIC (Transplant Acceptance-Inducing Cells), which is a crude preparation mixture of differently activated macrophages, proving its efficacy in several animal models of transplantation. In 2003, human trials using TAIC-I (phase I) confirmed the feasibility and tolerability of TAIC administration to renal transplant patients (Hutchinson, Riquelme, et al. 2008). In 2005, TAIC-II (phase II) trial aimed at assessing the immunological effects of TAIC preparations in kidney transplant patients. Treatment with TAIC was successfully minimized to low-dose tacrolimus monotherapy in four out of five patients and rejection did not occur in 2 out of five patients (Hutchinson, Brem-Exner, et al. 2008).

Even if the TAICs contain populations of regulatory macrophages, such a heterogeneous population cannot be used as a medical product. Therefore, JA Hutchinson's laboratory focused in the generation of purer and uniformer Mreg preparations. Clinical grade Mregs have recently been injected into two renal transplant patients (J. a Hutchinson et al. 2011a) in combination with low-dose tacrolimus monotherapy without induction therapy. Both patients display excellent graft function 3 years later.

3.2.3 Myeloid-derived suppressor cells (MDSC)

MDSC were firstly characterized as inhibitors of anti-tumor immune responses, therefore allowing cancer progression. MDSC are not present in steady-state but only after inflammation. In mouse, MDSC are well defined by $CD11b^+$ and $Gr\text{-}1^+$ markers, although they constitute a heterogeneous cell population. A variety of suppressor mechanisms have been identified.

a. *In vitro* generation of MDSC

Several protocols exist for *in vitro* MDSC differentiation. Rossner et al. performed the first study of *in vitro* MDSC generation (Rössner et al. 2005). They transiently obtained MDSC in DC cultures from BM cells, either in presence of low GM-CSF for 8-10 days or after 3-4 days under high GM-CSF culture conditions. Therefore, they were firstly considered as DC myeloid precursors which were $CD11c^-Ly6C^+$. Those cells displayed high *in vitro* suppressive capacity in allogeneic mixed leukocyte reaction. Other groups focused on MDSC generation from mouse stem cells (Zhou et al. 2010). In mouse, G-CSF has also been used instead of or in combination with GM-CSF also generating *bona-fide* MDSC (Highfill et al. 2010).

To efficiently expand MDSC *in vitro*, other factors were added to the culture medium. IL-6 appeared as a complement cytokine which, in addition to GM-CSF, was shown to generate large numbers of MDSC from mouse and human bone marrow cells in a controlled manner, thus being suitable for their therapeutic use (Marigo et al. 2010). Those cells showed higher suppressive capacity of T cell responses *in vitro* and *in vivo* when compared to MDSCs generated only with GM-CSF and were able to induce tolerance in a mouse model of islet transplantation (Marigo et al. 2010). Exogenous IL-13 has also been described as being an optimal complement for MDSC generation. MDSCs generated with GM-CSF and IL-13 could prevent GVHD more efficiently than those generated only in presence of GM-CSF (Highfill et al. 2010).

In humans, addition of other factors to MDSC's culture has proven to be helpful in the maintenance of MDSC's suppressive capacity. Obermajer et al. have successfully generated human MDSCs in presence of GM-CSF+IL-4+PGE2 (Prostaglandin E2) (Obermajer & Kalinski 2012). PGE2 had been described as a proinflammatory molecule which suppresses the differentiation of human monocytes into DCs (Kaliński et al. 1997). In their model, PGE2 is necessary and sufficient to redirect the differentiation of human DC into MDSC (Obermajer et al. 2011).

b. Mechanisms of action of MDSC

As for macrophages, the wide variety of protocols to *in vitro* differentiate MDSC leads to cells which mediate their suppressive functions through different molecular mechanisms.

In *in vivo* models of tumor and autoimmune diseases MDSC have been shown to inhibit T cell proliferation. Although their capacity to inhibit $CD8^+$ antigen specific responses has been demonstrated in tumors (Movahedi et al. 2008), there is no data which supports the existence of an antigen specific mechanism of suppression for $CD4^+$ T cells (Gabrilovich et al. 2012).

Several molecules have been associated with MDSC's immunosuppressive potential. *In vitro* MDSC's suppressive mechanisms were shown to require cell to cell contact for NO suppressive mediator synthesis, but they were independent of TNF and TGFβ (Rössner et al. 2005). In rats, MDSC infiltrating tolerated allografts inhibited proliferation of effector T cells and induced a contact-dependent apoptosis in an iNOS-dependent manner (A.-S. Dugast et al. 2008). In mice, transfer of *in vitro* generated MDSC prevented GVHD via IL-10 and iNOS (Zhou et al. 2010). IL-13 addition to MDSC culture upregulates Arginase-1 enzyme. Arginase-1 mediates MDSC's suppressive effect by inhibiting T cell proliferation due to the depletion of L-arginine from the microenvironment (Highfill et al. 2010). The enzyme HO-1 has also been shown to suppress alloreactivity (De Wilde et al. 2009). The induction of C/EBPβ transcription factor was found to be important for MDSC activity in allograft transplantation (Marigo et al. 2010). Lastly, MDSC are able to induce regulatory $CD4^+$ T lymphocytes (Zhou et al. 2010). A schema representing the known mechanisms is shown in **Figure 13**.

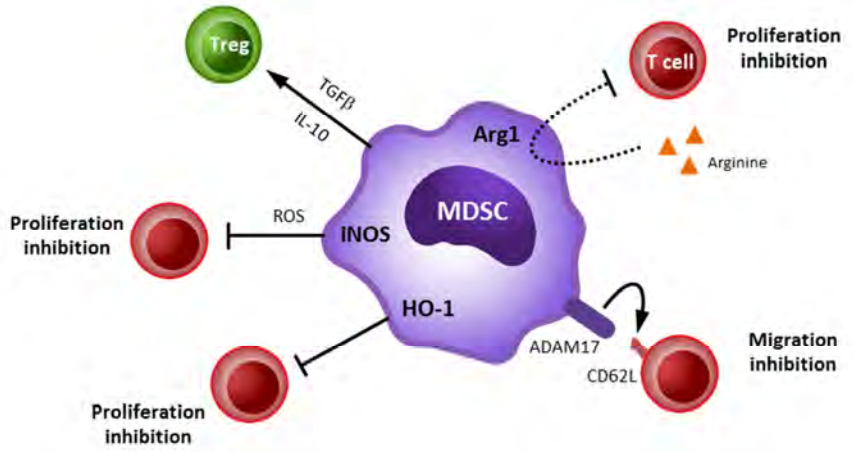

Figure 13. Mechanisms of action of MDSC.

MDSC express chemokine receptors, like CCR2 and CX3CR1, that attracts them towards sites of inflammation (Movahedi et al. 2008). Furthermore, CCR7 and CD62L expression homes them to secondary lymphoid organs (Highfill et al. 2010).

c. *In vivo* efficacy of MDSC in animal models and human clinical trials

MDSC have been found to be naturally increased in tumor-bearing mouse models (Strober 1984) and to accumulate in kidney allograft in a rat renal transplantation model where allografts were tolerated (A.-S. Dugast et al. 2008).

A protective role of MDSC has been well documented in literature in several autoimmune and transplantation animal models (Cripps & Gorham 2011; Feinberg et al. 2007). MDSC have been shown to be able to prolong minor antigen and fully mismatched allogeneic transplant in mouse skin transplant models (Zhang et al. 2008; De Wilde et al. 2009). MDSC derived from *in vitro* bone marrow culture have successfully been tested in islet allograft models, where MDSC were efficient at inducing long-term graft survival (Marigo et al. 2010; Chou et al. 2012) and to prevent GVHD (Highfill et al. 2010).

After transplantation, MDSC are believed to migrate from the bone marrow to the allograft (Garcia et al. 2010). *In vivo* expanded MDSC, transferred to a mouse skin graft recipient, migrated to the spleen (De Wilde et al. 2009). *In vitro* generated MDSC injected into a GVHD model were found in lymphoid tissues and sites of inflammation (Zhou et al. 2010; Highfill et al. 2010). In cancer, MDSC are located within tumors (Kusmartsev et al. 2005).

Importantly, MDSC have never been evaluated as cell therapy in humans yet. Therefore, evidence for *in vivo* efficacy of *in vitro* generated MDSC therapy is only available from animal models. The most important concern in the use of MDSC as cell therapy is the fact that they are a heterogeneous population of immature myeloid cells, not finally differentiated. There is evidence for MDSC potential to differentiate into DC or macrophages *in vivo* after injection (Narita et al. 2009).

3.3 Points to consider before performing a clinical trial

Some pilot clinical trials using immune cell therapy have been performed in the field of organ transplantation or other diseases with an alteration on the immune system component, such as autoimmune diseases or cancer. Mesenchymal Stem Cells (MSC) have successfully been applied to the clinical setting in several phase I trials, proving safety, feasibility and efficacy in preventing acute cellular rejection and inducing long-term stable graft functions (Franquesa et al. 2013). But still there are multiple questions that remain open in order to optimize and standardize clinical protocols. In the following paragraphs these questions will be addressed, focusing on tolDC therapy which are the most widely studied.

Considering the *in vitro* generation of myeloid regulatory cells, one of the major concerns in the translation of animal model's advances into clinical practice is the source of precursor cells. Whereas

in rodents myeloid regulatory cells are derived from bone marrow precursors, in humans, the main source of cells is monocytes form peripheral blood. In bone marrow, different developmental stages of myeloid precursors are found, whereas blood monocytes are terminally differentiated cells. In both cases, the *in vitro* culture in the presence of the adequate growth factors can divert or reprogram the differentiation process towards the desired final product.

In order to compare the importance of the precursors origin in the generation of regulatory myeloid cells, our group and others have generated in parallel tolDC from blood monocytes and from bone marrow cells in non-human primate models (Ashton-Chess & Blancho 2005; Moreau et al. 2008). Each origin lead to different tolDC populations, as bone marrow derived DC displayed a more heterogeneous phenotype and induced more expansion of natural Tregs than monocyte derived DC (MoDC) (Moreau et al. 2008). Even if tolDC display different characteristics depending on the precursor cell-type, both terminally differentiated tolDCs are able to efficiently inhibit T cell responses and do not upregulate typical DC maturation markers.

Another important aspect is whether myeloid precursors derive from donor or recipient. The most widely used strategy is to derive myeloid cells from donor precursors (allogeneic cells). This approach has the advantage of tolerizing recipients towards donor antigens before the transplant. Although it seems a good strategy, the group of A.E. Morelli nicely demonstrated that donor -derived DC are killed by host NK cells shortly after their injection. Therefore, their efficacy seems to be due to reprocessing of donor antigens which are presented by endogenous DCs (Yu et al. 2006; Divito et al. 2010). Therefore, the advantage of donor-derived DC compared to DST (where recipients become sensitive to donor antigens by blood infusion) is that the injected product is purer. A different strategy which has gained importance during the last years is to use recipient derived tolerogenic myeloid cells loaded with donor antigens. Once injected, those cells will present donor-derived antigens in a tolerogenic way due to the lack of costimulatory molecules. This strategy brings about other concerns, i.e. the procedure to load donor antigens into myeloid cells.

Our group carried out an original approach which consists on the differentiation of recipient-derived tolDC (ATDCs) unpulsed with donor antigens (Bériou et al. 2005; Moreau et al. 2009; Hill et al. 2011; Segovia et al. 2014). This strategy displays multiple advantages, as there is no risk of sensitization towards the donor, avoiding immune system's priming and immune response against the graft at the moment of the transplantation. Moreover, recipient-derived cells are not eliminated by recipient's immune system, so there is no risk of cellular rejection.

Use of autologous regulatory myeloid cells has some important advantages in clinical application. In France, 90% of performed transplants are performed from cadaveric donors. Autologous cell therapy is compatible with deceased donor transplantation, as there is no need to

know the donor before the transplantation. Cellular products can be prepared in advance and preserved frozen until the time of transplantation.

The route of administration of the cell product is a critical step that dictates the function of injected cells. Normally, subcutaneous or intradermal via is considered immunogenic or will not have any effect, whereas intravenous via is tolerogenic (Schuler et al. 2003). That difference can be explained by the context where antigens are recognized by recipient's immune system, as skin and dermis are entry sites for pathogens and cells injected in that context will be recognized as exogenous, triggering an immune response towards them.

Although cell therapy is a promising strategy to decrease immunosuppressive treatments in organ transplantation, low doses of immunosuppressive drugs will continue to be administered to patients, due to their great efficacy in controlling acute rejection phases. Therefore, clinical protocols using cell therapy must be designed taking into consideration commonly used immunosuppressive agents and their effect on injected cells. One of the drawbacks of using different cell products in cell therapy is that IS protocols cannot be generalized, as each agent will affect each cell type in a different way, the objective being to maintain tolerogenic and regulatory properties of each individual cell type. Some studies have been performed considering the influence of IS on DC function *in vivo* (Hackstein & Thomson 2004), whereas studies of their influence on Mreg and MDSC are still limited. Co-administration of immunosuppressive drugs must be carefully considered, as some drugs have the potential to enhance the immunorregulatory functions of transferred cells whereas others will inhibit their regulatory pathways.

Safety of cell therapy using myeloid derived cells has been evaluated in phase I clinical trials in human renal transplantation and type-1 diabetes with Mregs (J. a Hutchinson et al. 2011b) and tolDC (Giannoukakis et al. 2011) respectively. Until date, no adverse effects have been reported. Those studies are summarized in **Table 6**.

One important aspect when translating *in vitro* generation of tolDC into clinics is to validate their generation from patients' blood precursors, as they are normally under immunosuppressive treatments or dialysis at the time of transplantation and beginning of cell therapy. Using the same protocols as for the generation of tolDC from healthy volunteers , the generation of tolDC with the same characteristics has proven to be successful in the case of patients with rheumatoid arthritis (Harry et al. 2010), relapsing-remitting multiple sclerosis (Raïch-Regué, Grau-López, et al. 2012) and patients with renal graft failure awaiting for a transplant (unpublished data from our group).

Other important points to consider are the time of administration (prior, peri or post transplantation), the number of injections and the number of cells administered. Those points have been recently reviewed by Moreau et. al. (Moreau, Varey, Bériou, et al. 2012; Moreau, Varey, Bouchet-Delbos, et al. 2012).

RMC	Source	Study name	Clinical	Protocol	Outcome
Immature DC	Autologous blood monocytes cultured in GM-CSF and IL-4 pulsed with Ag	N/A	N/A	2×10^6 s.c.	Ag-specific inhibition of $CD8^+$ effector T cell function and generation of $CD8^+$ Treg
Immature or tolDC	Autologous unmanipulated DC (n=3) or DC treated with antisense oligonucleotides for CD40, CD80, and CD86 (n=7)	N/A	Type-1 diabetes (Phase I safety study)	10^7 cells intradermally every 2 weeks for four doses	10/10 no adverse events; significant increase in peripheral $B220^+CD11c^-$ B-cell frequency
Mreg (TAIC)	Donor splenic mononuclear cells cultured in M-CSF and stimulated with IFN-γ	TAIC-I	Deceased-donor renal transplantation	$1.0–7.52\times10^6$ cells/kg by central venous infusion d5 (patient receiving 0.55×10^6 cells/kg excluded); tacrolimus, sirolimus, and GC triple therapy for first month then weaned to tacrolimus monotherapy with progressive tacrolimus weaning	8/10 weaned from steroids; 6/10 weaned to low-dose tacrolimus monotherapy
Mreg (TAIC)	Donor PBMC cultured in M-CSF and stimulated with IFN-γ then cocultured with recipient PBMC before infusion of all cells	TAIC-II	Living-donor renal transplantation	$1.74–10.39\times10^7$ cocultured cells/kg by central venous infusion d5; ATG (d0, d1, and d2), prednisolone and tacrolimus started at 8–12 ng/mL trough levels and weaned to 5–8 ng/mL; prednisolone stopped by 10 weeks if graft function stable	5/5 no adverse events; 3/5 on low-dose tacrolimus monotherapy; 1/5 withdrawn from all immunosuppression for 8 months
Mreg (TAIC)	Donor PBMC cultured in M-CSF and stimulated with IFN-γ then cocultured with recipient PBMC before infusion of all cells	Case study (TAIC-II)	Presensitized living-related renal transplantation	6.9×10^7 cocultured cells/kg by central venous infusion d-17; ATG (d0, d1, and d2), prednisolone and tacrolimus started at 8–12 ng/mL trough levels and weaned to 4–8 ng/mL at week 35	No acute rejection episodes up to 27 months; donor-specific hyporesponsiveness and loss of donor-specific Ab
Mreg	Donor PBMC cultured in M-CSF and stimulated with IFN-γ	N/A	Living-donor renal transplantation	2 mg/kg/day azathioprine beginning 3 days before central venous infusion of Mreg (7.1 or 8×10^6 cells/kg) until 8 week post-Tx; Mreg given 6–7 days before Tx; tacrolimus and prednisolone begun day of Tx and prednisolone weaned by 10 weeks resulting in tacrolimus monotherapy (4–8 ng/mL trough)	n=2 patients maintained on low-dose tacrolimus monotherapy with excellent graft function and no rejection episodes >3 year post-Tx

N/A, not applicable; Tx, transplant.

Table 7. Regulatory myeloid cell clinical trials in humans in autoimmunity and transplantation. From (Rosborough et al. 2014)

3.4 The ONE Study: a comparative phase I/II clinical trial

Human clinical trials using regulatory cell types that have been performed during the last years include only TAIC cells and Mregs. Influence of different immunosuppressive regimens has only been tested in animal models. Combination of all IS drugs currently used in clinical transplantation has not been considered. Therefore it becomes difficult to evaluate and compare the efficiency of each regulatory cell type applied and to foresee the effects of those cells when injected in conjunction with a minimal immunosuppressive treatment.

The ONE Study is a phase I/II cell therapy clinical trial involving research teams from 5 different countries where six promising regulatory *in vitro* derived cell types will be evaluated. The main objectives of The ONE Study are:

- The *in vitro* generation of distinct populations of regulatory immune cells
- The comparison of the immunosuppressive activities of those regulatory cells
- The test of each type of regulatory population in renal transplant recipients under the same immunosuppressive protocol

Cells that will be generated *in vitro* and compared in The ONE Study are listed in **Table 7**.

Patients will receive only one dose of regulatory cells (either before or after transplant), and patients will be treated with decreasing doses of Prednisolone during 14 weeks, MMF during 48 weeks and Tacrolimus. The reference group will follow a classical immunosuppressive protocol, which includes two doses of induction with Basiliximab, 14 weeks of Prednisolone and MMF and Tacrolimus during the whole life of the graft.

In the first place, this phase I/II clinical trial will focus on the safety of the use of purified regulatory cells in conjunction with a decrease in immunosuppression doses and in the duration of the treatment. Then, efficacy of each cell type will be evaluated. Patients will be followed regularly for rejection or tolerance biomarkers.

This clinical trial, where our group participates, will bring new insights in the biology of those cells *in vivo* in the context of transplantation and will be a proof of concept of cell therapy as the new era of donor specific tolerance induction.

Table 8. Regulatory cells that will be tested in the clinical trial The ONE Study

Cell type	Origin	Sorting	Expansion	Center	Country
Treg	Recipient PBMC	Enrichment CD25 beads Flow cytomeytry sorting $CD4^+CD25^+CD127^{neg}$	*In vitro*	University Hospital Regensburg	Germany
		Flow cytomeytry sorting $CD4^+CD25^+CD127^{neg}$ Co-culture with donor B lymphocytes (UCSF) or PBMC (MGH)	*In vitro* αCD3/28 beads	University of California, San Francisco Massachussets General Hospital	USA
		$CD8^+$ depletion	*In vitro* αCD3/28 beads	University of Oxford/King's College London (frozen cells)	UK
		Enrichment CD25 beads	Rapamycin and IL-2 culture	Charite Hospital Berlin (fresh cells)	Germany
Tr1	Recipient PBMC	Enrichment CD4 beads	Co-culture with donor DC-10	Fondazione Centro San Raffaele, Milan	Italy
Mreg	Donor PBMC	Enrichment CD14 beads	Culture with M-CSF + IFNγ	University Hospital Regensburg	Germany
TolDC	Recipient PBMC	Enrichment CD14 beads	Culture with GM-CSF	Nantes University Hospital	France

PART IV. RESEARCH PROJECT

As already discussed in the introduction, myeloid cell therapy arises as a good strategy to induce donor-specific tolerance towards an allograft. Our team has been working for several years in the generation of autologous tolDC that mediate allograft survival in several animal models of transplantation. Previous studies performed by our group have shown that unpulsed autologous tolDC therapy is safe and efficient in combination with suboptimal doses of immunosuppressor agents. Those results were obtained in a fully mismatched rat cardiac transplant model, where tolDC were injected in conjunction with the immunosuppressor LF 15-0195 (Bériou et al. 2005), in a mouse minor antigen skin transplant model by injection of tolDC plus αCD3 mAb (Segovia et al. 2014) and in a fully-mismatched mouse islet transplant model (Baas et al, 2014).

Even more, our group has studied the *in vitro* and *in vivo* mechanisms responsible of tolDC regulatory functions. The enzyme HO-1 was found to inhibit DC maturation while conserving IL-10 expression in rat (Chauveau et al. 2005) and to be involved in the *in vivo* regulatory effect of tolDC (Moreau et al. 2009). Upregulation of the cytokine EBI3 by tolDC was found to be responsible of the induction of a DNT regulatory cells in rat cardiac transplant model which mediated their immunorregulatory functions through IFNγ secretion (Hill et al. 2011). The tolerance induction in rat heart transplant was donor-specific (Bériou et al. 2005). We recently published that the mechanism of tolerance induction in the mouse skin transplant model is mediated by tolDC cross-presentation male antigens, which induces donor-specific $CD8^+CD11c^+$ regulatory T cells (Segovia et al. 2014). The work developed by our group is summarized in **Table 8**.

Table 9. Summary of MC Cuturi's group using ATDC therapy in animal models of transplantation

Species	Model		Immunosuppression/ treatment	Cells injection (days)	Cell dose	Effect and mechanism	Reference
Rat	Heart transplantation	Fully mismatch	non	-1	$7x10^6$	prolongation of allograft survival iNOS expression by ATDC	Peche et al. 2005
Rat	Heart transplantation	Fully mismatch	non	-1	$3x10^6/7x10^6/15x10^6$	no modification of the effect of different doses	Beriou et al. 2005
				-1/4	$7x10^6$	no better effect than -1	
				-1/6/13	$7x10^6$	no better effect than -1	
				0	$7x10^6$	same effect than -1	
			rapamycin	-1	$7x10^6$	inhibition of ATDC effect	
			s/o LF 15-0195	-1	$7x10^6$	donor specific allograft tolerance	
Rat	Heart transplantation	Fully mismatch	s/o LF 15-0195	-1	$7x10^6$	HO-1 is required for ATDC effect	Moreau et al. 2009
Rat	Heart transplantation	Fully mismatch	s/o LF 15-0195	-1	$7x10^6$	EBI3 expression by ATDC is required DNT cells induction and IFNγ is required	Hill et al. 2011
Mouse	Skin transplantation	Minor antigen	αCD3 mAb	-1	10^6	prolongation of allograft survival cross-presentationby ATDC is required	Segovia et al. 2014
Mouse	Islet transplantation	Fully mismatch	αCD3 mAb	-1	10^6	prolongation of allograft survival role of CD4 Treg	Baas et al. *in press*

1 OBJECTIVES OF THE PRESENT STUDY

Myeloid cells displaying regulatory properties have been described in a variety of animal models of transplantation, autoimmunity or cancer (detailed in Part III Section 2.2 of the introduction). Immature DCs and macrophages are present in tissues under steady-state conditions, where they play an important role in tissue homeostasis and self-tolerance. Under inflammatory conditions, suppressive DCs and macrophages are responsible of limiting the inflammatory process. In this situation, MDSCs are attracted to the inflammatory focus, limiting T cell activation (Riquelme et al. 2012).

These three types of naturally-arising regulatory myeloid cell (RMC) types are the ones that are currently being developed for *ex-vivo* cell therapy, particularly in organ transplantation. Our knowledge about the mechanisms of action of RMCs has long been elucidated but surprisingly, progress towards clinical applications has been limited.

In order to use cell products as a therapeutical agent, several questions should be assessed. One important question is whether *ex vivo* differentiated RMC arise from donor or recipient precursors. Another question is which immunosuppressive regimen should be applied, which may mostly depend on the cell type injected and their mechanism of action.

Therefore, the hypothesis of this work is: **"The three RMC types exhert their actions by different mechanisms, which can determine different therapeutic capacities in a transplantation context or potentiate different therapeutic outcomes depending on the immunological context of the model they where are administered".**

To adress this issue, the main aim of this thesis has been to compare *in vitro* and *in vivo* the three regulatory myeloid cell types with more clinical potential, in order to determine different mechanisms of action *in vitro* and *in vivo* whilst minimizing other factors that could bias results.

TolDC, Mreg and MDSC have been generated *in vitro* from mouse bone marrow precursors and injected in autologous way one day before the transplant. Even more, cells were injected in the absence of additional immunosuppressive agents, in order to evaluate their natural potential when injected into an immunocompetent host. To our knowledge, this is the first study where a direct comparison of the three RMC types is performed.

Another important issue we addressed was the comparison of the therapeutical potential of MDSC in two different mouse models, transplantation and autoimmunity. MDSC are controverted to be used in cell therapy, as they constitute a heterogeneous population composed of immature myeloid cells. Therefore, in this project, we wanted to test MDSC capacity regulate immune responses *in vivo* and to elicit their *in vivo* mechanisms of action in a model of autoimmunity and transplantation.

2 TRANSPLANT MODEL OF CHOICE

In order to assess the therapeutic potential of *in vitro* derived regulatory myeloid cells and to understand the mechanisms underlying their effect, we have used a mouse skin transplant model. In that model, 1 cm^2 of C57BL/6 male tail skin is grafted onto the back of C57BL/6 female recipients. In that system, MHC class I and class II molecules are identical between donor and recipient. The immune response against the graft is therefore due to the allogeneic recognition of minor histocompatibility (mH) antigens. In that setting, the main mH targets are male antigens (the HY system).

In human transplantation, even when major histocompatibility complex molecules are matched between donor and recipient, it is not sufficient for long-term graft survival in the absence of immunosuppressors. The first evidence of the existence of other histocompatibility loci in humans came from the fact that rejection still happened when skin transplantation was performed between identical siblings. Subsequent studies realized that those mH antigens can be responsible for graft-versus-host and host-versus-graft diseases in bone marrow transplantation (Goulmy et al. 1996).

In mice, responsiveness to HY antigens varies enormously between strains. This difference relies on MHC haplotypes. Females of $H2^b$ haplotype strains (i.e. C57BL/6) are strong responders which can reject syngeneic male skin grafts. In contrast, females of most $H2^d$ strains do not respond against HY derived peptides and some $H2^k$ strains can only respond after previous immunization (Fierz et al. 1982). The strongest response for $H2^b$ haplotype rely on $CD8^+$ T cell responses restricted to locus D^b.

Human and mice mH have been elucidated and studied at a molecular level (Simpson et al. 2001; Simpson et al. 2002). $H2^b$ haplotype HY peptide epitopes restricted to MHC class I and class II molecules have been identified. The two main MHC class I peptides associated with the H2-D^b molecule are WMHHNMLDI, which originates from the *Uty* gene (Greenfield et al. 1996), and KCSRNRQYL, which originates from the *Smcy* gene. The peptide originated from *Uty* gene is the immunodominant one, as there are more clones isolated with specificity for *Uty* peptide than for *Smcy* peptide (Gavin et al. 1994) and *Uty* peptide affinity for D^b molecule is greater than *Smcy* peptide (Millrain et al. 2001).

E. Simpson's group has studied the importance of mH antigens in organ transplantation for many years. Those scientists found out that the administration of immature DCs pulsed with *Uty* peptides before male skin graft was performed induced a state of non-responsiveness against the graft. On the contrary, when immature DCs were pulsed with *Dby* peptide (the immunodominant MHC class II associated peptide), male skin grafts were rapidly rejected (James et al. 2002).

$CD4^+$ helper cells were found to be critical for the development of cytotoxic $CD8^+$ cells in the context of a response to mH and also to be required for $CD8^+$ antigen specific response that leads to graft rejection (VanderVegt & Johnson 1993).

3 SKIN IMMUNE SYSTEM

Transplantation of large patches of allogeneic skin is the best alternative to heal burn injuries. While vascularized organ early transplant rejection can be controlled by the use of immunosuppressive treatments, they have little or no effect in skin transplantation (Benichou et al. 2011). Skin grafts rapidly trigger a potent inflammatory immune response which leads to graft rejection.

In skin transplantation, skin DCs (normally referred to as graft passenger leukocytes) play a main role in the initiation of the adaptive immune response against the allograft. After transplantation, donor DCs (both, dermal DCs and LCs) migrate out of the graft through lymphatic vessels and reach recipient's draining lymph nodes, were they present donor antigens to naive alloreactive T lymphocytes. Activated lymphocytes migrate and infiltrate the graft, rapidly rejecting it.

Acute rejection of allogeneic skin transplants can be mediated by either $CD4^+$ or $CD8^+$ T cells activated through the direct allorecognition pathway. It is worth noting that humans, contrary to mice, display high frequencies of alloreactive memory T cells that recognize MHC molecules at the surface of graft endothelial and epithelial cells, therefore playing an important role in the perpetuation of direct allorecognition following the elimination of DCs or other APCs of donor origin (Bingaman & Farber 2004). Even though, direct allorecognition is sufficient but not necessary, as the indirect pathway on its own can also lead to rejection (Lee et al. 1994).

RESULTS

In order to evaluate the efficacy of cell therapy in transplantation and to study the *in vivo* mechanisms underlying their beneficial effect, our laboratory developed a minor antigen (male onto female) mouse skin transplant model, as previously explained in Part IV Section 2.

Our group has focused on the efficacy of autologous unpulsed tolDC in preventing allograft rejection in rat and primate transplantation (Pêche et al. 2005; Segovia et al. 2014). A protocol for the *in vitro* generation of tolDC from mouse bone marrow was established by our group (Segovia et al. 2011). Cell therapy using autologous tolDCs in conjunction with low dose of immunosuppressive treatment lead to prolongation of allograft survival in skin transplant (Segovia et al. 2014) and islet transplant (Baas, et al. 2014) models.

The first part of the work developed during this thesis consisted in the *in vitro* generation of MDSC as previously described (Marigo et al. 2010) and to test MDSC potential as cell therapy in two different animal models, autoimmune model of type 1 diabetes and in transplantation. Whereas MDSC were not able to prevent diabetes onset, they could prolong allograft survival. Those results and possible mechanism of action are explained in Article I.

The second part of the work developed during this thesis consisted in the comparison of the three RMC types which have more potential to be applied in the clinical setting of transplantation: tolDC, MDSC and regulatory macrophages (Mregs). To do so, we developed a protocol for the *in vitro* generation of regulatory macrophages, detailed in Article II. The three RMC types were compared for their *in vitro* capacity to inhibit T cell activation, and their *in vivo* efficacy to prolong allograft survival after injection of each RMC as an autologous cell therapy unpulsed with donor antigens and without additional immunosuppressor treatment. Those results are included in Article III.

ARTICLE I: THERAPEUTIC POTENTIAL OF MDSC IN AUTOIMMUNITY AND TRANSPLANTATION

1 INTRODUCTION TO ARTICLE I

Evidence for MDSC protective role comes from studies where *in vivo* induction or injection of *in vitro* generated MDSCs, which have shown to control a variety of clinical settings where the immune system is involved. *In vitro*, GM-CSF culture of bone marrow cells supplemented with IL-6 have been shown to generate MDSC with strongest immunosuppressive activity *in vitro* than MDSC generated in presence of GM-CSF or G-CSF alone (Marigo et al. 2010). The same authors showed that those *in vitro* generated MDSC were able to prolong mouse islet allograft survival *in vivo*.

The aim of this study was to assess MDSC suppressive capacity *in vitro* and *in vivo* in mouse models of autoimmunity and transplantation without additional combination treatment.

We were able to *in vitro* generate MDSC as previously described by Marigo et.al (Marigo et al. 2010). As expected, MDSC displayed suppressive capacity *in vitro*. To test the *in vivo* efficacy of the use of MDSC as cell therapy, MDSC were tested in two mice animal models. Whereas MDSC treatment was not able to prevent type 1 diabetes development, it was efficient to prolong skin graft survival. *In vivo* analysis of the mechanisms underlying graft survival prolongation evidenced the increased expression of activation molecules at the surface of T cells and myeloid cells of treated mice when compared to untreated grafted mice. This observation leads to the hypothesis that MDSC could be generating a window of systemic exhaustion of the immune system, allowing the graft to survive through a temporary ignorance mechanism.

Therefore, our results show that MDSC therapy can lead to opposite outcomes depending on the model. Whereas their systemic activation would be beneficial in the transplantation setting, it would be detrimental in a potent autoimmunity model. An important point is that MDSC is a heterogeneous population, and its potential to develop into immunogenic myeloid cells once injected has already been demonstrated (Schmidt et al. 2013). Strategies to maintain MDSC in an immature state after injection should be developed before using this cellular population in cell therapy.

2 ARTICLE I

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PLOS ONE

Evaluation of the Therapeutic Potential of Bone Marrow-Derived Myeloid Suppressor Cell (MDSC) Adoptive Transfer in Mouse Models of Autoimmunity and Allograft Rejection

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Abstract

Therapeutic use of immunoregulatory cells represents a promising approach for the treatment of uncontrolled immunity. During the last decade, myeloid-derived suppressor cells (MDSC) have emerged as novel key regulatory players in the context of tumor growth, inflammation, transplantation or autoimmunity. Recently, MDSC have been successfully generated in vitro from naive mouse bone marrow cells or healthy human PBMCs using minimal cytokine combinations. In this study, we aimed to evaluate the potential of adoptive transfer of such cells to control auto- and allo-immunity in the mouse. Culture of bone marrow cells with GM-CSF and IL-6 consistently yielded a majority of $CD11b^{+}Gr1^{hi/lo}$ cells exhibiting strong inhibition of $CD8^{+}$ T cell proliferation in vitro. However, adoptive transfer of these cells failed to alter antigen-specific $CD8^{+}$ T cell proliferation and cytotoxicity in vivo. Furthermore, MDSC could not prevent the development of autoimmunity in a stringent model of type 1 diabetes. Rather, loading the cells prior to injection with a pancreatic neo-antigen peptide accelerated the development of the disease. Contrastingly, in a model of skin transplantation, repeated injection of MDSC or single injection of LPS-activated MDSC resulted in a significant prolongation of allograft survival. The beneficial effect of MDSC infusions on skin graft survival was paradoxically not explained by a decrease of donor-specific T cell response but associated with a systemic over-activation of T cells and antigen presenting cells, prominently in the spleen. Taken together, our results indicate that in vitro generated MDSC bear therapeutic potential but will require additional in vitro factors or adjunct immunosuppressive treatments to achieve safe and more robust immunomodulation upon adoptive transfer.

Citation: Drujont L, Carretero-Iglesia L, Bouchet-Delbos L, Beriou G, Merieau E, et al. (2014) Evaluation of the Therapeutic Potential of Bone Marrow-Derived Myeloid Suppressor Cell (MDSC) Adoptive Transfer in Mouse Models of Autoimmunity and Allograft Rejection. PLoS ONE 9(6): e100013. doi:10.1371/journal.pone.0100013

Editor: Ryan M. Teague, Saint Louis University School of Medicine, United States of America

Received December 6, 2013; **Accepted** May 21, 2014; **Published** June 13, 2014

Funding: This work has been carried out thanks to the support of the Labex IGO project (n° ANR-11-LABX-0016-01) funded by the «Investissements d'Avenir» French Government program, managed by the French National Research Agency (ANR). This work was also supported by funds from IHU-Cesti (Investissement d'Avenir ANR-10-IBHU-005, région Pays de la Loire et Nantes Métropole). LD was supported by an Inserm-Région Pays de la Loire fellowship. CL was supported by an Inserm "young researcher" CDD. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Myeloid-derived suppressor cells (MDSC) comprise a heterogeneous population of myeloid cells at various stages of differentiation accumulating during pathological situations, such as tumor development or inflammation, and with the ability to suppress T-cell responses [1,2,3]. In mice, MDSC are broadly defined as $CD11b^{+}$ $Gr1^{+}$ cells and have been shown to exhibit a variety of suppressor mechanisms [4,5].

Growing evidence indicate a central role of MDSC in diverse models of autoimmune diseases [6] including type 1 diabetes [7,8], arthritis [9], colitis [10], alopecia areata [11], myocarditis [12] or experimental autoimmune encephalomyelitis (EAE) [13,14,15]. A protective role of MDSC has also been documented in the context of allogenic transplantation [4,16,17,18,19,20,21]. Interestingly, a recent report linked the accumulation of MDSC with $FoxP3^{+}$ regulatory T cells (Tregs) in kidney-transplanted patients [22].

Thus, similarly to Tregs [23], MDSC represent a novel regulatory cell type that could be manipulated to achieve immune tolerance in the context of autoimmunity or transplantation. Although injections of G-CSF [24], LPS [18] or IL-33 [25] have been shown to favor the generation of endogeneous MDSC in allograft recipient mice, a promising and clinically applicable approach would consist in the adoptive transfer of in vitro-generated MDSC. In this regard, the study by Rossner et al. initially paved the way towards MDSC generation from bone marrow (BM) cells using GM-CSF [26]. Alternatively, Zhou et al. demonstrated the development of MDSC from mouse stem cells

[27]. Other studies reported that BM cells co-cultured with hepatic stellate cells could lead to the production of MDSC effectively preventing murine islet allograft rejection [28] or colitis [29]. Generally, GM-CSF, in conjunction with tumor cells conditioned culture medium, appeared as a pivotal cytokine for the generation of MDSC [30,31]. IL-6 has subsequently been identified as a potent complement to GM-CSF for the generation of both mouse and human MDSC [32,33]. Importantly, Marigo et al. showed that mouse bone marrow-derived MDSC generated with GM-CSF and IL-6 exhibit a stronger immunosuppressive activity in vivo and could induce long-term survival of pancreatic islet allograft upon repeated adoptive transfer [32]. This latter study opened an avenue to the generation of these cells in great numbers and in a controlled manner for their use in cellular immunotherapy.

In the current study, we investigated and compared the suppressive potential of BM-derived MDSC generated in vitro with GM-CSF and IL-6, without combination treatment, in different mouse models of auto- and allo-immunity.

Results

Based on the method described by Marigo et al. [32], we cultured BM cells from naive mice with GM-CSF and IL-6 and examined their phenotype after 4 days. We routinely obtained > 90% of $CD11b^+$ cells that could be subdivided in $Gr1^{hi}$ and $Gr1^{low}$ cells (Figure 1A and B). $Gr1^{low}$ cells, which contain the majority of $CD11c^+$ cells (Figure 1C), were shown to exhibit the highest suppressive activity [32]. Attributing the term MDSC to immature myeloid cells requires the demonstration of an immunosuppressive function, at least in vitro. As shown in Figure 2A and B, BM cells cultured with GM-CSF and IL-6 efficiently prevented $CD8^+$ T cell proliferation in a dose-dependent manner, reaching >80% inhibition at a ratio of 2:1 (MDSC:T cells).

We then examined the suppressive potential of these MDSC in vivo. In order to best reproduce a T cell response triggered by a cellular antigen, we immunized mice with COS cells transfected with a plasmid encoding a non-secreted fusion protein linking the ovalbumin peptide SIINFEKL ($OVA_{257\ 264}$) to GFP. In this system, the OVA peptide is presented to $CD8^+$ T cells by recipient APCs on their MHC class I molecules through the processes of phagocytosis and antigen cross-presentation. The injection of $CD8^+$ T cells from TCR-transgenic OT-1 mice then allows the monitoring of an antigen-specific T cell reponse in vivo, as depicted in Figure 3A and C. Immunization with OVA-expressing COS cells resulted in a strong $CD8^+$ T cell proliferation while control COS cells did not. Concomitant adoptive transfer of MDSC and immunization did not prevent this proliferation (Figure 3B). We then hypothesized that, rather than significantly altering proliferation, MDSC could influence their differentiation into CTLs. However, as shown in Figure 3D, MDSC failed to impact antigen-specific T cell cytoxicity.

To assess the effect of MDSC adoptive transfer in a more physiological context, we made use of a model of type 1 diabetes [34,35] in which autoimmunity is induced by the injection of $CD8^+$ OT-1 T cells in conjunction with a polyclonal anti-OVA antibody (Ab) into RIP-mOVA transgenic mice (membrane OVA is expressed as a neo-antigen by the pancreatic beta cells under the rat insulin promoter). In our hands, and as previously established [34], virtually all mice become diabetic within 5 to 12 days. Single adoptive transfer of MDSC on the day of OT-1 and Ab injection did not prevent diabetes development (Figure 4A). We reasoned that MDSC might benefit from an inflammatory milieu to stably exert their suppressive function on T cells. However, neither two consecutive adoptive transfers of MDSC, 2 and 5 days after OT-1 and Ab injection (Figure 4B), nor a single injection at day 5 using twice as much cells (data not shown) significantly impinged on the progression of the disease. Finally, we tested whether the loading of MDSC with the antigenic peptide before injection could potentiate the suppression by promoting their interaction with the diabetogenic T cells. Strikingly, this approach seemed to rather exacerbate the development of the disease, since treated mice developed accelerated diabetes compared to control mice (Figure 4C).

Next, we tested whether adoptive transfer of MDSC could modulate a polyclonal response in the context of allograft rejection. As shown in Figure 5A, while male skin grafts transplanted onto female recipients were rejected within 19 to 28 days, two injections of syngenic (female) MDSC, the day before transplantation and at day 6 post-transplantation, were sufficient to prolong graft survival. A single injection of LPS-activated MDSC (LPS was added to the MDSC culture for the last 5 hours) on the day of transplantation similarly achieved a significant outcome (Figure 5B). However, this effect was markedly and reproducibly enhanced with five weekly consecutive injections, leading to graft survival up to 40 days (Figure 5C).

To understand the beneficial effect of MDSC adoptive transfers on skin graft survival, we investigated the immune cell composition directly in the graft as well as in the draining lymph nodes and in the spleen, two weeks post-transplantation (after three weekly injections of MDSC). Few or no injected MDSC were detected (using the congenic marker Ly5.1) suggesting that these cells are rapidly eliminated or preferentially home to a distinct location than the skin graft, the draining lymph nodes or the spleen. Surprisingly, we found that skin grafts from both untreated and MDSC-treated mice showed similar numbers of total infiltrated leucocytes (data not shown). In fact, the proportion of $CD4^+$ T cells was even increased in MDSC-treated mice (Figure 6A) whereas no difference was observed for $CD8^+$ T cells (Figure 6B). In addition, donor-specific $CD8^+$ T cells were found in similar numbers both in skin grafts (Figure 6C) and in the periphery (Figure 7A). The proportions of $CD19^+$ B cells, $CD3^-$ $NK1.1^+$ NK cells, $CD3^+$ $CD4^+$ or $CD3^+$ $CD8^+$ T cells were not altered by MDSC adoptive transfers (data not shown). $FoxP3^+$ cell numbers among $CD4^+$ T cells were increased in skin-grafted mice compared to naive mice but no significant differences were observed between untreated and MDSC-treated mice (Figure 7B). As expected, increased numbers of $CD25^+$ and $CD69^+$ T cells were detected mostly in the draining lymph nodes of skin graft recipients compared to naive mice (Figure 7C and D). However, MDSC adoptive transfers did not prevent this activation phenotype. On the contrary, we observed a dramatic increase of $CD25^+$ and $CD69^+$ T cell numbers in MDSC-treated mice, almost exclusively in the spleen (Figure 7C and D), a phenomenon that was also associated with increased numbers of MHC II^+ and $CD86^+$ cells (Figure 8A and B). Thus, MDSC adoptive transfers, rather than specifically suppressing the allogenic immune response, appears to induce a state of systemic activation that correlates with prolongation of skin graft survival.

Discussion

Compelling evidence from animal models suggest a great potential of MDSC adoptive transfer for preventing graft rejection or treating autoimmune disorders. For example, MDSC from tumor-bearing mice have been shown to prevent the onset of type 1 diabetes when co-transferred with diabetogenic $CD4^+$ T cells [7]. Similarly, MDSC purified from LPS-treated mice are capable

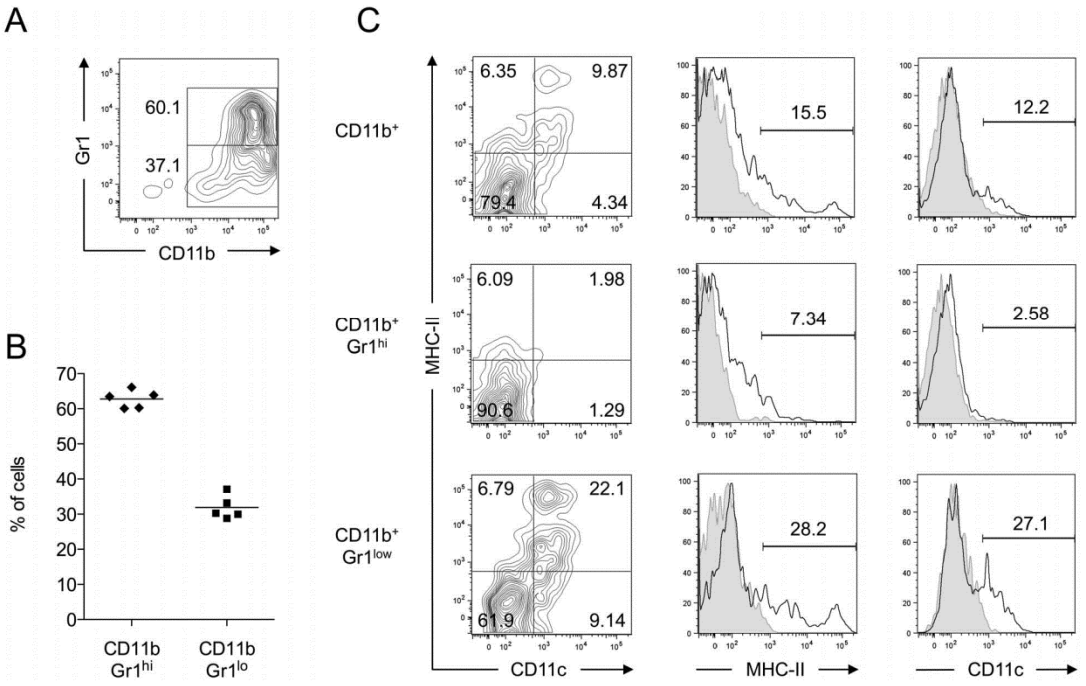

Figure 1. Phenotype of BM-derived MDSC. (A) BM cells from naive mice were cultured in the presence of GM-CSF and IL-6 for 4 days. Surface expression of CD11b and Gr1 was measured by flow cytometry. (B) Quantification of the relative proportions of CD11b$^+$ Gr1^{hi} and CD11b$^+$ Gr1^{low} populations in independent preparations. (C) Expression of CD11c and MHC II on total CD11b$^+$ cells or in Gr1^{hi} and Gr1^{low} populations. Gray areas represent fluorescence minus one (FMO) controls. Data show representative results from at least four independent experiments.
doi:10.1371/journal.pone.0100013.g001

of prolonging skin allograft survival [18]. These findings prompted us to embark on a study to assess their therapeutic potential in mouse models of autoimmunity and transplant rejection. A translational view implicates the development of a clinically acceptable method for the production of these cells. Marigo et al. provided convincing data highlighting the high suppressive activity of MDSC generated from BM cells using GM-CSF supplemented with IL-6, for the in vivo inhibition of T cell responses as well as the prevention of allogenic islet rejection [32].

While we succeeded in producing in vitro suppressive CD11b$^+$ cells using this approach, we did not observe any alteration of in vivo antigen-specific CD8$^+$ T cell responses or autoimmune diabetes development after adoptive transfer of these cells. It is important to note that this stringent in vivo experimental procedure involves a high number of strongly reactive monoclonal (OT-1 TCR transgenic) T cells. Furthermore, the expansion of diabetogenic CD8$^+$ T cells is not the result of homeostatic proliferation since RIP-mOVA mice are non-irradiated lymphosufficient hosts, but strictly depends on robust Fc receptor-mediated OVA cross-presentation by DCs [34].

Surprisingly, we found that loading MDSC with the neoantigen OVA peptide rather exacerbated than dampened the development of the disease. This observation was reproduced using in vivo cytotoxicity assay (data not shown). The culture of whole BM cells with GM-CSF and IL-6 results in an heterogeneous mixture of myeloid cells, a fraction of them likely bearing the potential to differentiate into highly immunogenic DCs. Additional factors, such as PGE_2 [36] or subset separation before injection, may help to maintain a suppressive homogeneity. The use of few markers expressed at the surface of MDSC obviously does not satisfy the requirement of a pure and stable suppressive population. In this regard, CD11b$^+$ Gr1$^+$ cells have also been described as immunostimulatory during tumor growth [37] or autoimmunity [38]. Taken together, these observations could raise doubts over the safety of BM-derived myeloid cell transfer, potentially detrimental in specific inflammatory situations.

The transplantation of male skin onto female recipients mounts a progressive expansion of low frequency polyclonal T cell clones leading to graft rejection. In this model, in contrast to type 1 diabetes, we found that multiple injections of MDSC significantly prolonged graft survival. It is tempting to speculate that a continuous treatment could result in long-term acceptance of the graft, as shown by Marigo et al. in pancreatic islet transplantation [32]. Of note, two injections of MDSC were not sufficient to prevent or delay rejection of complete mismatch skin grafts (Balb/c onto C57BL/6 mice, data not shown) pointing to the limit of these in vitro generated MDSC to impinge, by themselves, on a strong allogenic response, yet in the same manner as in vitro expanded Tregs, alone, failed to provide significant graft prolongation in a complete mismatch setting, in lymphosufficient mice [39].

These results also emphasize the need for identifying strategies to increase and preserve the suppressive ability of MDSC after transfer in order to reduce the frequency of injections. Indeed, MDSC have been shown to rapidly differentiate into mature myeloid cells in the absence of tumor-derived factors or sustained inflammation [40,41]. In this regard, Greifenberg et al. originally demonstrated that LPS + IFN-γ combination considerably

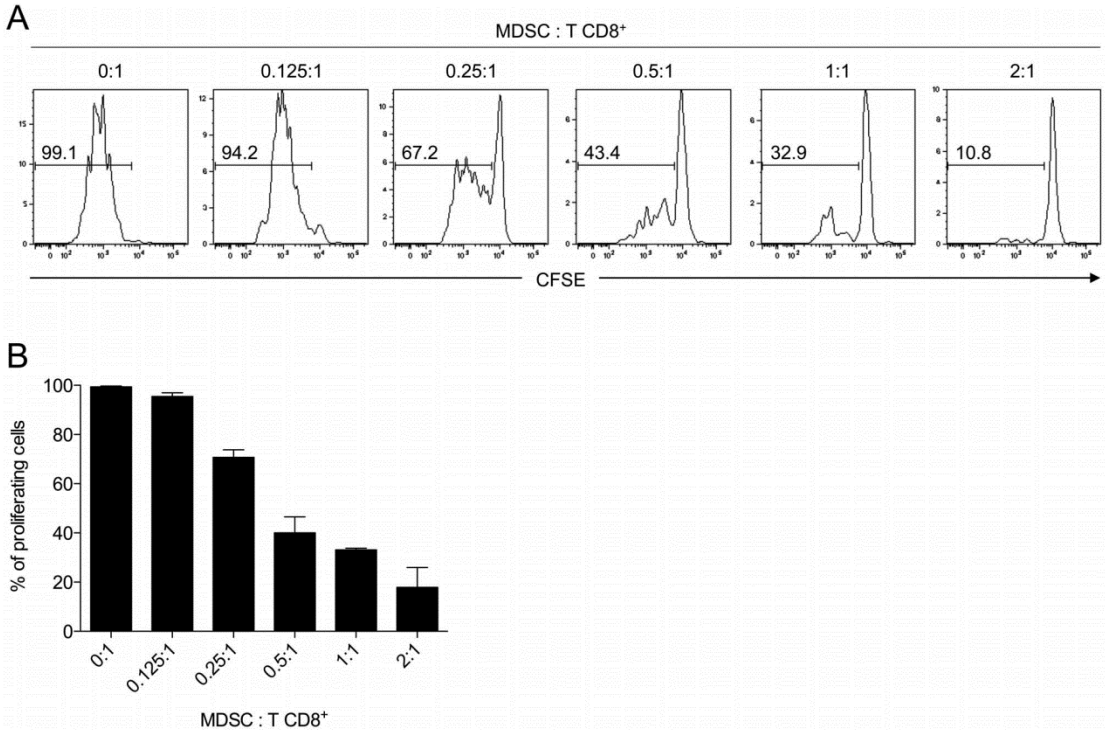

Figure 2. BM-derived MDSC efficiently inhibit T cell proliferation *in vitro*. $CD8^+$ T cells were purified from OT-1 transgenic mice and labeled with CFSE before anti-CD3/CD28 bead stimulation. MDSC generated in vitro with GM-CSF and IL-6 were added to T cells at different ratios. After three days of culture, the percentage of proliferating cells ($CFSE^{low}$) in $CD8^+$ cells was assessed by flow cytometry. Representative histograms of CFSE dilution (A) and quantification of triplicates for each condition are shown (B). Data are representative of three independent experiments.
doi:10.1371/journal.pone.0100013.g002

augmented the suppressive capacity of MDSC by impairing DC differentiation [42]. Similarly, Highfill et al. showed that addition of IL-13 in BM cells cultured with GM-CSF and G-CSF resulted in the production of potently suppressive MDSC that efficiently inhibited graft-versus-host disease [43]. Thus, amongst other strategies that have been reported to promote MDSC activation/expansion [5], our results support the relevance of this approach since a single injection of LPS-activated MDSC was sufficient to induce a significant prolongation of graft survival. It remains to be evaluated whether additional injections of these activated cells will reinforce this beneficial effect and if the addition of IFN-γ (or other cytokines) could further boost their suppressive function in vivo.

Mechanistically, we have found that the beneficial effect of MDSC infusions on skin graft survival was paradoxically not explained by a decrease of donor-specific T cell response but rather associated with an over-activation of T cells and antigen presenting cells. The fact that this observation was prominently made in the spleen suggests that MDSC transfers could create a window of systemic exhaustion in the immune system allowing the allogenic graft to survive, a phenomenon that would terminate immediately after cessation of the therapy, then excluding any mechanism of long term tolerance. Thus, while this effect is associated with delayed graft rejection in the setting of transplantation, it appears inefficient or rather detrimental during the developpement of a fast and potent autoimmune response. These differential outcomes stress the need to carefully evaluate MDSC adoptive transfer therapies, or any other approaches, by using carefully chosen models in relation with the clinical aim.

Interestingly, Treg therapy alone in lymphosufficient hosts, even in an antigen-specific fashion, similarly fails to induce a long-term protection from allograft rejection [39,44]. The combination of MDSC and Treg cell therapies could result in a synergistic effect. Indeed, numerous reports have shown that MDSC promote the development and homeostasis of Tregs over $CD4^+$ T effector cells [45], notably in the context of type 1 diabetes [7,8]. Moreover, MDSC can capture and present exogenous antigens to their MHC class II molecules which can be drastically upregulated upon IFN-γ stimulation [46]. Treg accumulation has also been attributed to monocytic suppressive cells [19]. Thus, in spite of a recent study that challenged this view concerning granulocytic MDSC [47], these results generally argue for a beneficial interplay between Tregs and MDSC that could be relevant in the context of cellular therapy. Athough similar levels of $FoxP3^+$ Treg were found in the draining lymph nodes or spleen of MDSC-treated mice, the detection of a potential beneficial effect of MDSC on these cells in the periphery will probably require the examination of the (donor) antigenic specificity. Moreover, it will be interesting to determine whether the increase of $CD4^+$ T cells that we observed in the skin grafts of MDSC-treated mice could reflect an influx of Treg that would be mostly specific for the donor antigens.

In summary, in the present study, we have compared the potential of in vitro generated MDSC adoptive transfer in relevant and distinct in vivo models of immune response. Our data

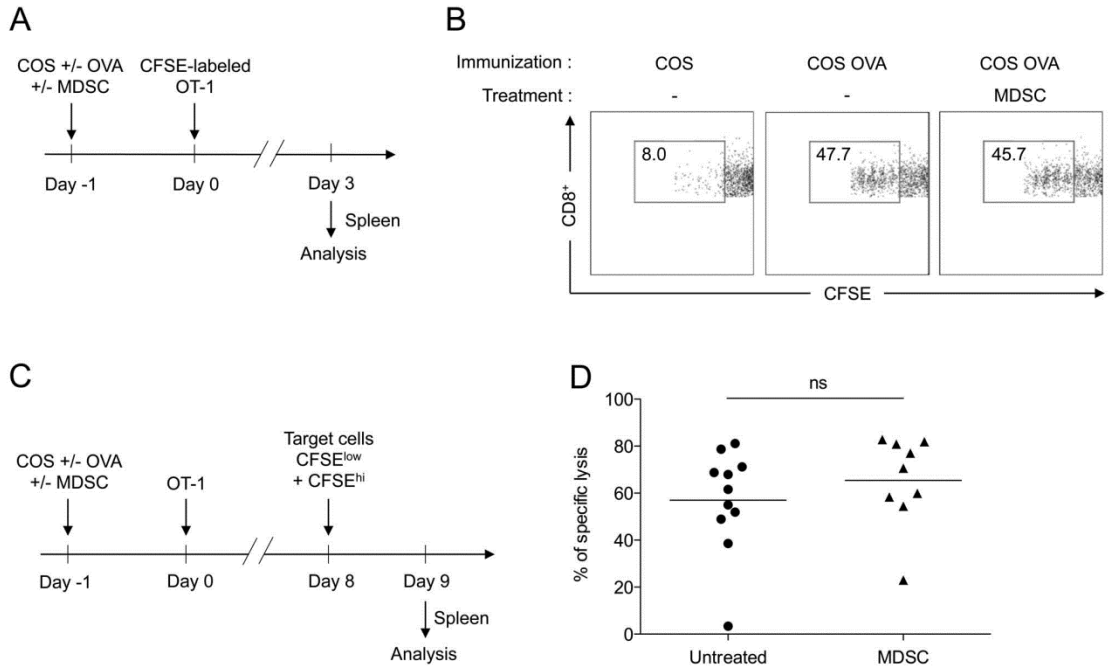

Figure 3. Adoptive transfer of BM-derived MDSC does not alter antigen-specific CD8$^+$ T cell responses. (A–B) In vivo proliferation assay: COS cells transfected with a plasmid coding for GFP fused to the $OVA_{257-264}$ peptide (COS OVA) or GFP alone (COS) were injected i.v. into mice with or without MDSC generated in vitro with GM-CSF and IL-6. Responder CD8$^+$ T cells purified from OT-1 TCR-transgenic mice were labeled with CFSE and injected i.v. the following day. After 3 days, spleens of recipient mice were harvested to assess CFSE dilution by flow cytometry. Data are representative of two experiments. (C–D) In vivo cytotoxicity assay: CD8$^+$ T cells purified from OT-1 TCR-transgenic mice (non labeled with CFSE) were injected in COS GFP/OVA-immunized mice as described above. After 8 days, CFSE-labeled CD45.1$^+$ target cells either loaded with $OVA_{257-264}$ (CFSE^{hi}) or control (CFSE^{low}) peptides were injected. Specific lysis was determined the next day by flow cytometry by measuring the relative proportion of each population in the spleen of MDSC-treated or untreated mice compared to non-immunized mice. Data show results from four independent experiments with 9 to 11 mice per group.
doi:10.1371/journal.pone.0100013.g003

highlight the need to refine the in vitro generation of homogeneous, stable and strongly suppressive myeloid cells before considering a therapeutic approach, most likely with combination treatments.

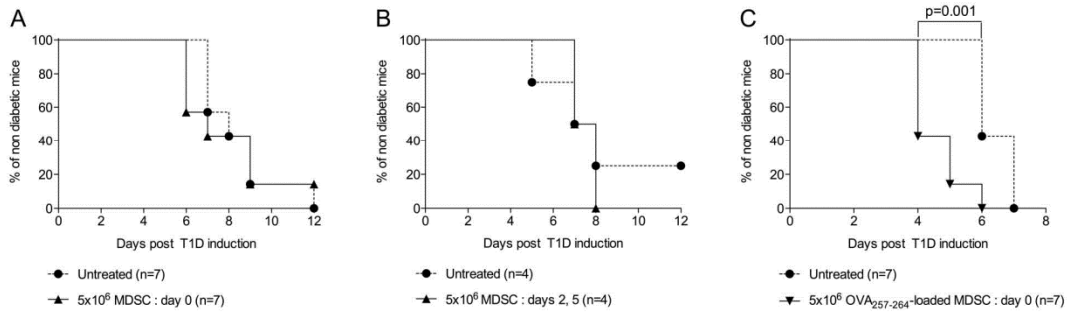

Figure 4. Adoptive transfer of BM-derived MDSC does not prevent the development of autoimmune diabetes. Type 1 diabetes was induced in RIP-mOVA mice by injecting (i.v.) naive CD8$^+$ T cells from OT-1 TCR-transgenic mice together with an anti-OVA polyclonal antibody (i.p.). Blood glycemia was monitored every day during at least 12 days. Indicated numbers of MDSC generated in vitro with GM-CSF and IL-6 were adoptively transferred at day 0 (A) or at days 2 and 5 (B). Alternatively, MDSC were loaded with the $OVA_{257-264}$ peptide before injection at day 0 (C). In each experiment, MDSC-treated mice were compared to a group of untreated mice.
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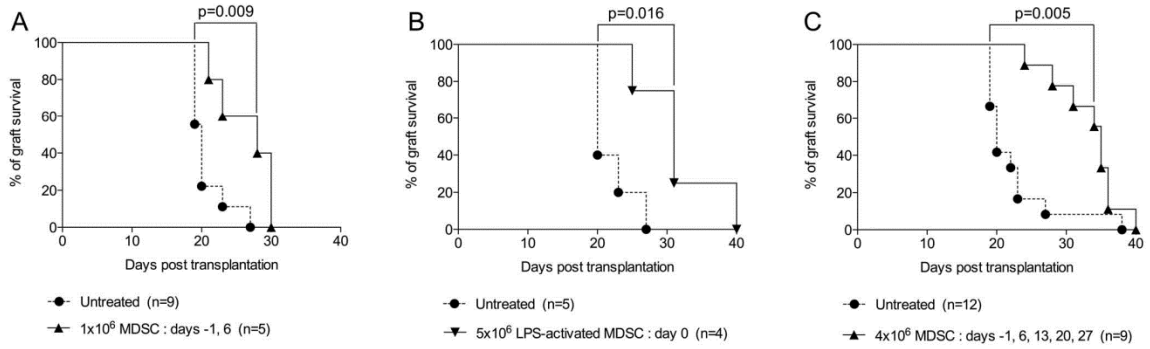

Figure 5. Adoptive transfer of BM-derived MDSC prolongs skin allograft survival. (A) Male skin grafts were transplanted onto females recipients treated or not at days −1 and 6 post-transplantation, with one million autologous (female) MDSC generated in vitro with GM-CSF and IL-6. (B) Alternatively, LPS was added in vitro for the last 5 hours of the MDSC culture and five million cells were injected at day 0. (C) Male skin grafts were transplanted onto females recipients treated or not at days −1, 6, 13, 20 and 27 post-transplantation, with four million autologous (female) MDSC generated in vitro with GM-CSF and IL-6. Graft survival was monitored every other day from day 7 post-transplantation.
doi:10.1371/journal.pone.0100013.g005

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the protocol approved by the Commitee on the Ethics of Animal Experiments of Pays de la Loire (Ref: CEEA.2012.211 and CEEA.2013.9).

Mice

C57BL/6 mice were purshased from Janvier (France). RIP-mOVA (C57BL/6-Tg(Ins2-TFRC/OVA)296Wehi/WehiJ) transgenic mice [48] were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). For this line, hemizigous mice were maintained in the laboratory by breeding transgenic mice, selected by PCR genotyping, with wild-type C57BL/6 mice. OT-1 TCR-transgenic mice (C57BL/6-Tg(TcraTcrb)1100Mjb/Crl) [49] and Ly5.1 congenic mice (B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyCrl) were purshased from Charles Rivers (France).

Reagents

Murine GM-CSF was from Peprotech (Neuilly-sur-Seine, France). IL-6 and LPS were from Sigma-Aldrich (Saint-Quentin Fallavier, France). CFDA-SE (CFSE) was from Molecular Probes (Montluçon, France). OVA (SIINFEKL) and Smcy (KCSRNRQYL) peptides were from PolyPeptide (Strasbourg, France). Anti mouse CD11b biotin (M1/70) (used with streptavidin APC or streptavidin APC-Cy7), CD11b APC-Cy7 (M1/70), CD11c PE-Cy7 (HL3), I-A^b FITC (AF6-120.1), Gr1 PE (Ly6C/G, RB6-8C5), CD45.1 APC (A20), CD45.2 APC-Cy7 (104), CD45.2 PerCP-Cy5.5 (104), CD19 APC (1D3), NK1.1 PE (PK136), CD3ε PerCP-Cy5.5 (145-2C11), CD3ε Pacific Blue (500A2), CD3ε FITC (145-2C11), CD4 PE-Cy7 (RM4-5), CD8α Pacific blue (53-6.7), CD8α APC-Cy7 (53-6.7), CD8α PerCP-Cy5.5 (53-6.7), FoxP3 Alexa Fluor647 (MF23), CD25 PE (704), CD69 FITC (H1.2F3), and CD86 FITC (B7.2, GL1) were from BD PharMingen (Le Pont de Claix, France). Male antigen UTY-specific CD8$^+$ T cells were detected using a PE labelled Pro5 MHC Pentamer (H-2D^b, WMHHNMDLI) (ProImmune Limited, Oxford, UK).

Generation of BM-derived MDSC

MDSC were generated as previously described [32]. Tibias and femurs from C57BL/6 mice were removed and BM was flushed. Red blood cells (RBCs) were lysed with ammonium chloride. To obtain BM-derived MDSC, 2.5×10^6 cells were plated into dishes with 100 mm diameter in 10 mL of complete medium, which consisted of 10% heat-inactivated fetal bovine serum (Lonza, Levallois, France), nonessential amino acids, 1 mM sodium

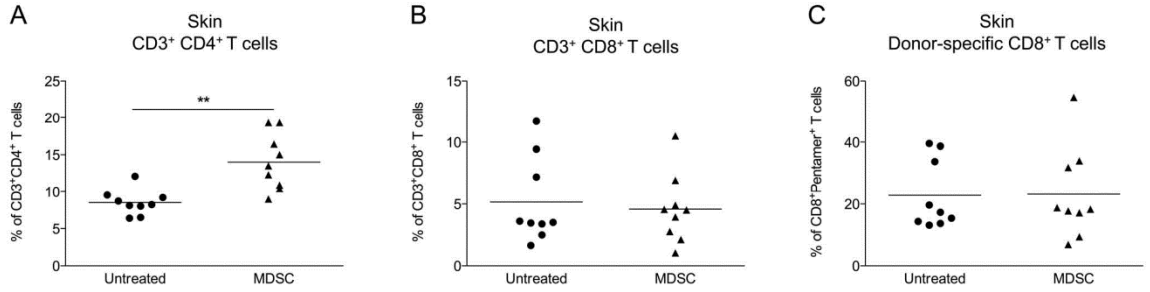

Figure 6. Adoptive transfer of BM-derived MDSC does not prevent lymphocyte infiltration in skin allografts. Male skin grafts were transplanted onto females recipients treated or not at days −1, 6 and 13 post-transplantation with four million autologous (female) MDSC generated in vitro with GM-CSF and IL-6. Skin grafts were harvested 14 days after transplantation and infiltrated leukocytes were analyzed by flow cytometry. Results are expressed in percentages of CD3$^+$ CD4$^+$ T cells (A), CD3$^+$ CD8$^+$ T cells (B) and donor-specific Pentamer$^+$ cells among CD8$^+$ T cells (C). Data show results from two independent experiments with 4 to 9 mice per group. **p<0.01.
doi:10.1371/journal.pone.0100013.g006

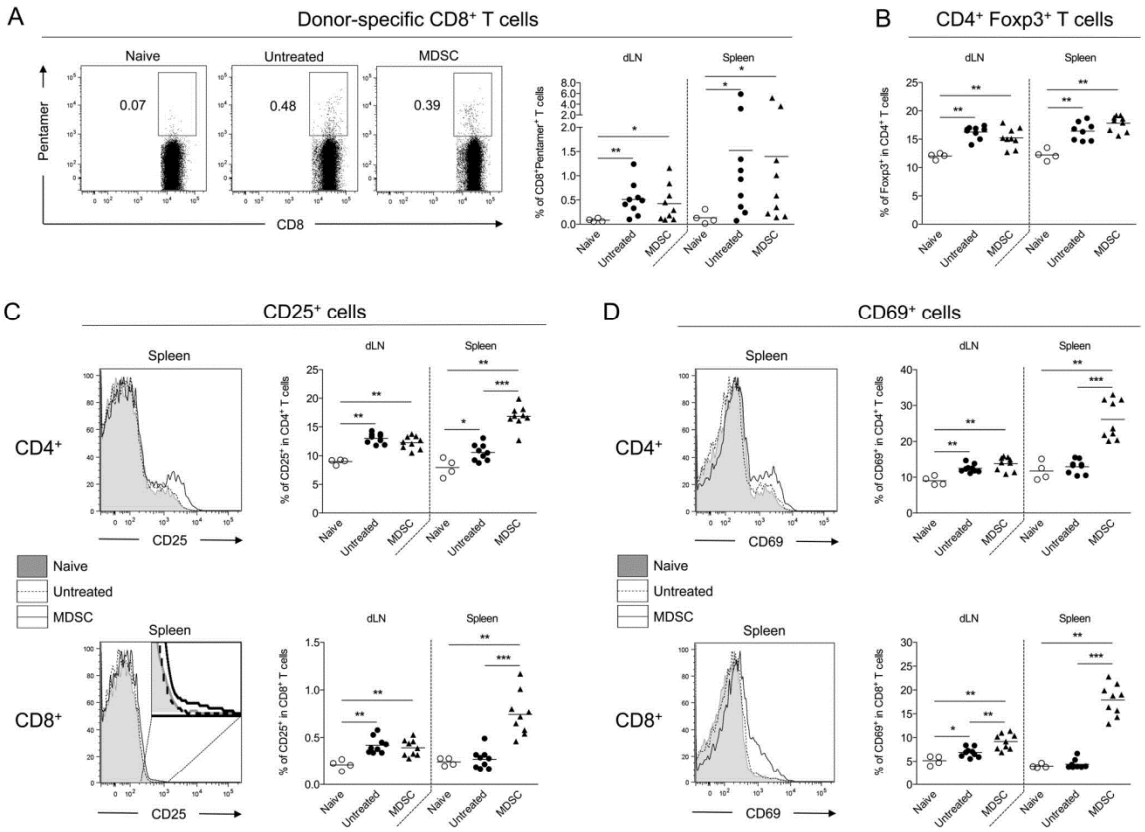

Figure 7. Adoptive transfer of BM-derived MDSC is associated with increased numbers of CD25$^+$ and CD69$^+$ cells, mainly in the spleen. Male skin grafts were transplanted onto females recipients treated or not at days −1, 6 and 13 post-transplantation with four million autologous (female) MDSC generated in vitro with GM-CSF and IL-6. Draining lymph nodes and spleen were harvested from skin-grafted mice 14 days after transplantation or from naive mice for flow cytometry analysis. (A) Representative staining and quantification of donor-specific Pentamer$^+$ CD8$^+$ T cells in naive or skin-grafted mice. (B) Quantification of FoxP3$^+$ cells among CD3$^+$ CD4$^+$ T cells. (C, D) Representative stainings and quantifications of CD25$^+$ (C) and CD69$^+$ (D) among CD3$^+$ CD4$^+$ or CD3$^+$ CD8$^+$ T cells. Data show results from two independent experiments with 4 to 9 mice per group. *p<0.05, **p<0.01, ***p<0.001.
doi:10.1371/journal.pone.0100013.g007

pyruvate, 10 mM HEPES (all from Gibco, Saint Aubin, France), 2 mM glutamine and 50 µM β-mercaptoethanol (both from Sigma-Aldrich) in DMEM base (Gibco). Medium was supplemented with GM-CSF (40 ng/ml) and IL-6 (40 ng/ml) cytokines. Cells were maintained at 37°C in 5% CO_2-humidified atmosphere. After 4 days, cells were washed twice before flow cytometry analysis, in vitro culture or in vivo injection. In some experiments, LPS was added (1 µg/mL final) for the last 5 hours of the culture.

In vitro Proliferation Assay

Responder CD8$^+$ T cells were purified (CD8a$^+$ T cell Isolation Kit II, Miltenyi Biotec, Paris, France) from spleens of naive C57BL/6 mice, labeled with CFSE and plated at the concentration of 2×10^4 cells/mL in 96-well round bottom plate in 200 µL final of complete medium. Anti-CD3/28 microbeads (Life Technologies, Saint Aubin, France) were used at a 1:1 ratio and increased numbers of BM-derived MDSC were added. After 3 days, CFSE dilution in CD8$^+$ T cells was analyzed by flow cytometry.

Immunization with OVA-expressing COS Cells

COS cells were transfected (Lipofectamine Transfection Reagent, Life Technologies) with plasmids (pCI-neo backbone, Promega, Charbonnières-les-Bains, France) coding for GFP alone or GFP fused to $OVA_{257\ 264}$ sequence (SIINFEKL peptide) at N-terminal. After 48 hours, COS cells expressing GFP alone (control COS) or $OVA_{257\ 264}$-GFP fusion protein (COS OVA) were trypsinized and washed in PBS before i.v. injection (1×10^5 cells). Transfection efficiency routinely reached 40–50% of GFP$^+$ cells.

In vivo Proliferation Assay

Experimental scheme is depicted in Figure 3A. C57BL/6 mice were immunized with control COS or COS OVA cells and co-injected (i.v.) or not with 6.5×10^6 BM-derived MDSC. The next day, 5×10^6 CD8$^+$ T cells purified (CD8a$^+$ T cell Isolation Kit II, Miltenyi Biotec) from pooled spleens and lymph nodes of OT-1 TCR-transgenic mice were labeled with CFSE and injected (i.v.). After 3 days, spleens were harvested and CFSE dilution in injected CD8$^+$ T cells was analyzed by flow cytometry.

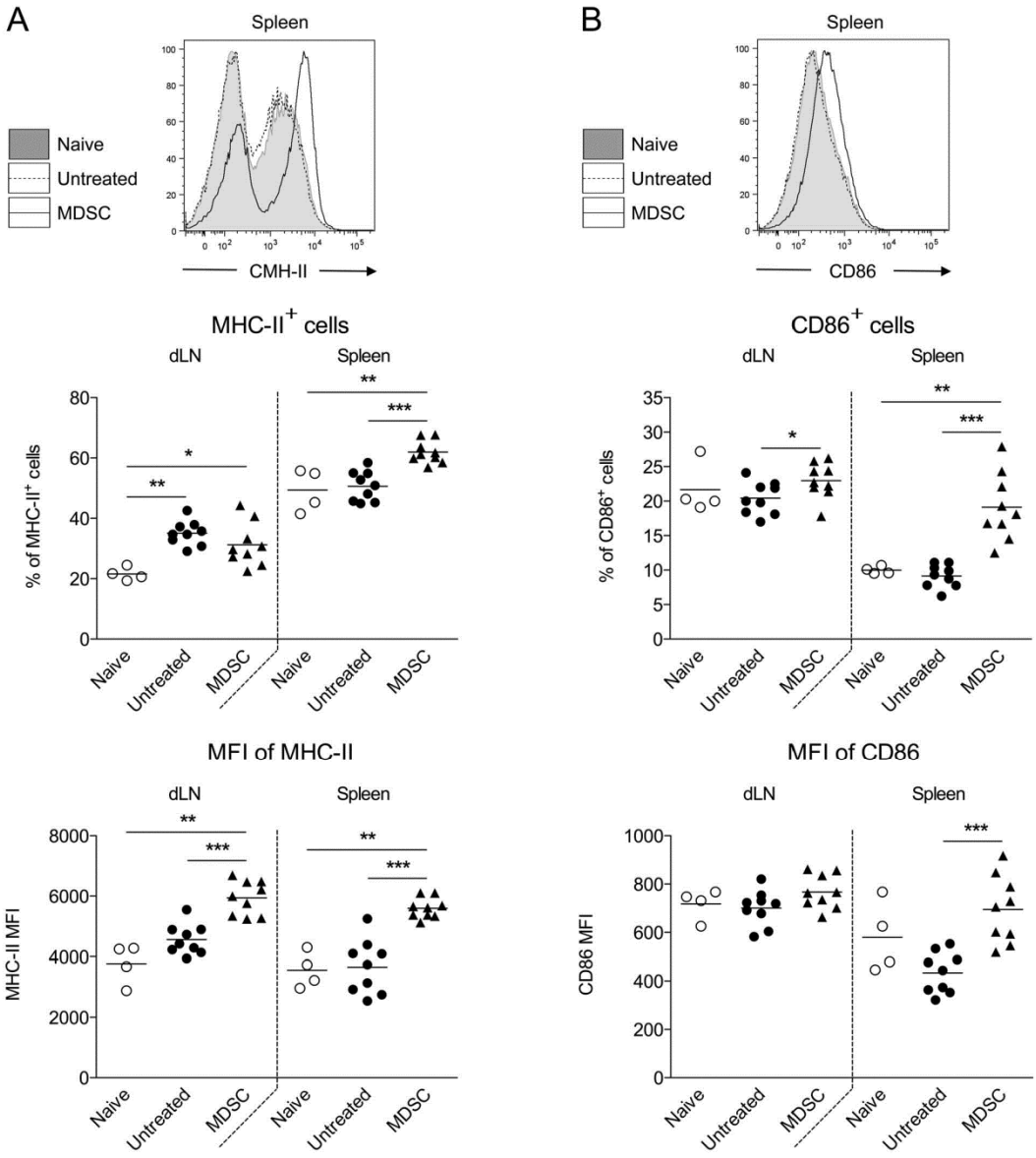

Figure 8. Adoptive transfer of BM-derived MDSC is associated with increased numbers of MHC II$^+$ and CD86$^+$ cells, mainly in the spleen. Male skin grafts were transplanted onto females recipients treated or not at days −1, 6 and 13 post-transplantation with four million autologous (female) MDSC generated in vitro with GM-CSF and IL-6. Draining lymph nodes and spleen were harvested from skin-grafted mice 14 days after transplantation or from naive mice for flow cytometry analysis. Representative stainings and quantifications of MHC II$^+$ (A) and CD86$^+$ (B) cells in naive or skin-grafted mice. Data show results from two independent experiments with 4 to 9 mice per group. *p<0.05, **p<0.01, ***p<0.001. doi:10.1371/journal.pone.0100013.g008

In vivo Cytotoxicity Assay

Experimental scheme is depicted in Figure 3C. C57BL/6 mice were immunized with control COS or COS OVA cells and co-injected (i.v.) or not with 5×10^6 BM-derived MDSC. The next day, 0.25×10^6 CD8$^+$ T cells purified (CD8a$^+$ T cell Isolation Kit II, Miltenyi Biotec) from pooled spleens and lymph nodes of OT-1 TCR-transgenic mice were injected (i.v.). After 8 days, spleens cells from Ly5.1 mice (CD45.1$^+$ cells) were labeled with 4 µM or 0.2 µM of CFSE to obtain CFSE^{hi} and CFSE^{lo} populations respectively loaded with control Smcy and $OVA_{257\ 264}$ peptides and were injected (i.v.) at a 1:1 ratio (1.6×10^6 cells for each population). The next day, spleens were harvested to measure the relative proportions of each population within CD45.1$^+$ cells by flow cytometry. Specific lysis was determined by calculating the percentage of decrease of the CFSE^{hi} population in immunized mice compared to non-immunized mice.

Induction of Autoimmune Diabetes

Diabetes was induced in RIP-mOVA mice as previously described [34]. Briefly, 6 to 8 week-old RIP-mOVA mice were injected intravenously with 5×10^6 $CD8^+$ T cells purified ($CD8a^+$ T cell Isolation Kit II, Miltenyi Biotec) from pooled spleens and lymph nodes of OT-1 TCR-transgenic mice together with intraperitoneal administration of 1 mg anti-OVA IgG. Anti-OVA serum was obtained from ovalbumin (OVA)-hyperimmunized rabbits (Covalab, Villeurbanne, France) and IgG were purified by protein A affinity chromatography. Endotoxin-free OVA protein was from Profos (Regensberg, Germany). Blood glucose levels were measured with a StatStrip Xpress Glucose/Ketone Meter monitoring system (Nova Biomedical, Les Ulis, France). Mice were considered diabetic after two consecutive measurements >250 mg/dL.

Skin Transplantation

Mice were anesthetized with a mixture of 5% xylazine (Rompun) and 18% ketamine in PBS (170 µL) injected intraperitoneally (8.5 mg/kg of xylazine and 76.5 mg/kg of ketamine per mouse). Square skin grafts (1 cm^2) were prepared from the tail of male wild-type C57BL/6 donors and transplanted on the shaved left flank of C57BL/6 female recipients. The grafts were fixed to the graft bed with 10–12 interrupted sutures and were covered with protective tape. The first inspection was carried out seven days later and graft survival was monitored every other day. Rejection was defined as complete sloughing or a dry scab.

Analysis of Cellular Populations in Skin Graft Recipients

Fourteen days after skin transplant, mice were sacrificed and draining lymph nodes, spleen and skin graft were harvested. Skin grafts were processed using collagenase D (Sigma-Aldrich) for 45 min at 37°C. Cells were fluorescently labeled and cellular populations were analyzed by flow cytometry.

Statistical Analysis

Statistical analyses were performed with Graphpad Prism 5.0 (La Jolla, CA, USA) using the Mann-Whitney test. Survival rates were compared using the Log-rank (Mantel-cox) test. Statistical significance was defined as $p<0.05$.

Author Contributions

Conceived and designed the experiments: LD LCI GB MH YD MCC CL. Performed the experiments: LD LCI LBD GB EM CL. Analyzed the data: LD LCI GB MCC CL. Wrote the paper: LD YD CL.

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ARTICLE II: *IN VITRO* GENERATION OF MOUSE REGULATORY MACROPHAGES

1 INTRODUCTION TO ARTICLE II

Previous work developed by our demonstrated the ability of autologous tolDC therapy in inducing a prolongation of allograft survival in rat transplant model (Pêche et al. 2005; Bériou et al. 2005). The work performed by Segovia et al. allowed the generation of autologous tolDC from mouse bone marrow precursors, which were also able to prolong allograft survival (Segovia et al. 2011; Segovia et al. 2014). Other regulatory myeloid cells have recently proven to be effective to prolong allograft survival in animal models. Macrophages display important plastic characteristics that make them a suitable cell type for *in vitro* modification to be used as cell therapy. Riquelme et al. described a protocol to generate murine regulatory macrophages, by culturing sorted bone marrow precursors with low doses of M-CSF and stimulating them with IFNγ the last 24h of culture (Riquelme et al. 2013). Importantly, those Mregs efficiently prolong allograft survival when generated from donor precursors, but were unable to prolong allograft survival when derived from autologous bone marrow precursors.

The aim of this part of the thesis was to develop a protocol for the *in vitro* generation of regulatory macrophages (Mreg) from mouse bone marrow precursors which could prolong allograft survival when used autologous cell therapy. Different doses of recombinant mouse M-CSF were used, as well as different culture days, medium, serum and plastic support. The best conditions in terms of yield and phenotype were obtained after 15 days of culture with 0.2 ng/ml of rmM-CSF. In this article, the detailed method to generate Mregs from mouse bone marrow precursors is explained. Also some assays to determine Mreg function are described. Mreg are hypostimulatory *in vitro* and also keep the capacity to efficiently endocyte and degrade antigens and bacteria.

2 ARTICLE II

Generation and characterization of mice regulatory macrophages

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Key words: regulatory macrophages (Mreg), cell therapy, *in vitro* differentiation, bacterial killing assay (BKA), suppression

Abstract

In the last years, cell therapy has become a promising approach to therapeutically manipulate immune responses in autoimmunity, cancer and transplantation. Several types of lymphoid and myeloid cells origin have been generated *in vitro* and tested in animal models. Their efficacy to decrease pharmacological treatment has successfully been established. Macrophages play an important role in physiological and pathological processes. They represent an interesting cell population due to their high plasticity *in vivo* and *in vitro*. Here, we describe a protocol to differentiate murine regulatory macrophages *in vitro* from bone marrow precursors. We also describe several methods to assess macrophage classical functions, as their bacterial killing capacity and antigen endocytosis and degradation. Importantly, regulatory macrophages also display suppressive characteristics, which are addressed by the study of their hypostimulatory T lymphocyte capacity and polyclonal T lymphocyte activation suppression.

Introduction

Pharmacological and biological compounds are widely used to treat immunological disorders that lead to an excessive response of the immune system. Even though, those agents unselectively target important cellular and molecular pathways, leading to undesired side-effects. Therefore, cell therapy arises as a good strategy to modulate immune responses by induction of specific peripheral tolerance[1].

Macrophages constitute a highly heterogeneous cell subset, comprising cells derived from different embryonic and adult precursors. Macrophages are involved in both, physiological and pathological processes[2]. In steady-state, macrophages are responsible of maintaining homeostasis in a variety of peripheral tissues (dermis, bone, lungs, spleen, adipose tissue…) by clearance of apoptotic cells, development and metabolic regulation. They also play fundamental roles as immune sentinels. After pathogen encounter, macrophages get activated and are responsible of the initiation of an inflammatory microenvironment which triggers the recruitment of other immune cell types, which perpetuate inflammation. Meanwhile, macrophages are also involved in the resolution of inflammatory processes, through phagocytosis of cellular debris and tissue reconstitution[3].

Those antagonistic functions displayed by macrophages in vivo give a hint of the plasticity of this population. Therefore, *in vitro* differentiation of macrophages from precursor cells or *ex vivo* modification of isolated macrophages can be a good strategy to polarize macrophages towards a regulatory profile for their use in clinical practise.

Regulatory macrophages (Mreg) have already been generated in mouse[4] and humans[5]. In the setting of transplantation, those Mreg have successfully been able to prolong cardiac graft survival in a fully-mismatched mouse transplant model. A clinical trial including two patients has also shown their capacity to maintain graft functionality with only tacrolimus monotherapy after Mreg administration[6].

In this Chapter, we describe a methodology to generate *in vitro* another subset of Mreg using only low doses of M-CSF. Mreg conserve the functional characteristics of classical macrophages while being able to suppress polyclonal T lymphocytes activation and to be hypostimulatory in in vitro allogeneic co-cultures.

4

Materials

General materials:

- Complete medium: 1x DMEM medium (Gibco) supplemented with 10% FCS (Lonza), 0,05 mM β-ME (Sigma-Aldrich), 2mM L-glutamine, 1mM sodium pyruvate, 10mM HEPES, 1x MEM NEAA, 100 U/ml penicillin, 0,1 mg/ml streptomycin (all from Gibco)
- Sterile PBS 1x (Phosphate Saline Buffer, pH 7,2-7,4)
- PFE: sterile PBS 1x supplemented with 2% FCS and 2 mM EDTA
- Vacuum-driven filtration system, 0.22 µm (Millipore)
- 15- and 50-ml centrifuge tubes (Greiner bio-one)
- 24-well flat bottom plates and 96-well round bottom plates (BD Falcon)

Material for in vitro Mregs generation

- Complete medium
- Mouse recombinant M-CSF (mrM-CSF) (Peprotech)
- 6- to 8-week-old C57BL/6 mice (Janvier, France)
- 2.5 ml syringe and 26 G needle
- RBCL (Red Blood Cell Lysis) solution: 0.15 M NH_4Cl, 0.01 M $KHCO_3$ and 100 µM Na_2EDTA, filtered and adjusted to pH 7.4
- 100 µm nylon filters (BD Falcon)
- Non cultured-treated 10-cm Petri dishes (VWR)

Material for bacterial killing assay

- Antibiotic-free complete medium: complete medium without penicillin and streptomycin
- FCS (Lonza)
- *E. coli* bacteria (Agilent Technologies)

- Liquid LB médium (Sigma-Aldrich)

- LB-agar plates (Sigma-Aldrich)

- Gentamicin (Sigma-Aldrich)

- 0.5% Sodium Deoxycolate (Sigma-Aldrich)

Material for endocytosis and antigen degradation

- OVA-Alexa Fluor647 (Invitrogen)

- DQ-OVA (Invitrogen)

- PFA 2% (Paraformaldehyde 2%)

Material for allogeneic coculture and suppression test

- 6- to 8-week-old C57BL/6 or Balb/C mice (Janvier, France)

- 100 µm nylon filters (BD Falcon)

- CD90.2 or Pan T Isolation Kit II, mouse (Miltenyi Biotech)

- AutoMACS separator or columns and magnets (Miltenyi Biotech)

- CFDA-SE (CarboxyFluorescein DiAcetate Succinimidyl Ester) (Invitrogen)

- DynaBeads mouse T-Activator CD3/28 (Gibco): anti-CD3 and anti-CD28-coated microbeads

- BD™IMagnet (BD Biosciences Pharmigen)

Methods

Differentiation of Mregs from bone marrow precursors

In order to generate Mreg *in vitro*, several culture conditions were tested: harvesting time points, serum amounts and lots, type of plastic recipient for cell culture and mrM-CSF concentrations. Macrophages that displayed the desired phenotype and function were obtained after 15 days of culture in complete medium supplemented with 10% of FCS and cultured in untreated Petri dishes with 0.2 ng/ml of mrM-CSF. Cell yields obtained using the different conditions are shown in **Table 1**. The following protocol considers the chosen culture conditions:

1. After sacrifice, remove tibias and femurs from 6- to 8-weeks old C57BL/6 mice (Note 1) and flush bone marrow using a 2.5 ml syringe and a 26 G needle.
2. Recovered cells into a 50 ml tube and centrifuge them for 10 min at 500x g. Discard supernatant and resuspended cell pellet in 5 ml of RBCL solution for 5 min in order to eliminate erythrocytes.
3. Add 45 ml of PFE to stop the lysis and centrifuge 10 min at 500x g. Discard supernatant.
4. Repeat step 3.
5. Resuspend cell pellet in 10 ml of PFE and filter cells using a 100 µm-filter.
6. Determine cell number and resuspend cells into pre-warmed complete medium to a final concentration of 10^6 cells/ml. Add 0.2 ng/ml of mrM-CSF.
7. Dispatch 10 ml of the suspension into each Petri dish. Incubate cells at 37°C and 5% CO_2.
8. At day 3 of the culture, add 10 ml of pre-heated complete medium supplemented with 0.2 ng/ml of mrM-CSF to each Petri dish.

9. At day 7, replace 10 ml of medium from each plate. Centrifuge cell suspension, discard supernatant and resuspend pellets in the same volume to the initial of pre-heated complete medium supplemented with 0.2 ng/ml of M-CSF.
10. At day 15, discard medium containing non-adherent cells. Add 10 ml of cold PFE are to each Petri dish. Adherent cells are harvested by pipetting up and down. Mregs are collected into 50 ml tubes and centrifuged. Supernatant is discarded and pellets are resuspended in PFE and pooled into a final volume of 10 ml of PFE.
11. Phenotypical characterization of recovered macrophages can be performed by flow cytometry. Typically, virtually all recovered Mregs are CD11b/F4/80 positive, whereas half of the population expresses CD11c and MHC class II markers and Gr1 marker is absent (**Figure 1**).

Study of macrophages function

Bacterial Killing Assay

In this assay, we evaluate the capacity of macrophages to kill internalized bacteria. The readout is the quantification of live bacteria at different times after phagocytosis. The following protocol has been modified from Sokolovska et.al. [7]

1. Culture and harvest macrophages as previously described. Wash cells with PFE. Count cell numbers and resuspend cell pellet in antibiotic-free complete medium to a concentration of 10^6 cells/ml. Dispose 1 ml per well in a 24-well flat-bottom culture plate. Each well represents a condition or a time point. It is recommended to perform three replicates per point. Allow macrophages to adhere to the bottom of the culture plate for 1h at 37°C
2. Prepare live *E. coli* (Note 2):
    a. Day -2: Spread *E. coli* into LB-agar plates and incubate ON at 37°C

b. Day -1: Grow one separate colony into liquid LB ON at 37°C

c. Day 0:

i. Make serial dilutions of bacteria into pre-warmed liquid LB

ii. Incubate different dilutions at 37°C for 1-2h

iii. Determine the optical density (OD) of the suspension at 600 nm (Note 3) and calculate bacterial concentration (x) as follows:

$$x = \frac{measured\ O.D.\ x\ 5x10^8\ CFU/ml}{1\ O.D.}\ \text{(CFU/ml)}$$

iv. Resuspend bacteria into antibiotic-free complete medium to a concentration of 10^7 bacteria/ml (Note 4)

3. Infect macrophages by adding 10µl of the bacterial preparation to each well and centrifuge the plate 4 min at 500x g.
4. To allow macrophages phagocyte *E. coli*, incubate plates at 37°C 5% CO_2 for 30 min.
5. Wash wells twice with warm PBS. Add 500 µl of complete medium supplemented with XX µg/ml of gentamicin. Incubate for 1h at 37°C. This step allows killing of remaining non-phagocyted bacteria.
6. Wash wells twice with warm PBS. Add 500 µl of complete medium with 5% FCS containing X µg/ml of gentamicin. This is time point 0 (Note 5). Incubate for the desired time points.
7. Harvest macrophages by washing wells twice with warm PBS and scraping.
8. To assess intracellular bacteria, centrifuge cell suspension, discard supernatant and lyse macrophages by adding 100 µl of 0.5% Sodium Deoxycolate (Note 6). Pipet up and down and vortex vigorously to release intracellular bacteria (Note 7).
9. Make serial dilutions of the lysate and plate bacteria onto LB-plates. Incubate ON at 37°C and count CFU. Results can be expressed as remaining "alive bacteria per

initial macrophage number" (Note 8). Titration of CFU counts can be performed in order to choose the best MOI to perform further (**Figure 2**).

Endocytosis and antigen degradation

To perform this protocol, two ovalbumin (OVA) modified proteins are used. OVA protein conjugated with Alexa Fluor 647 fluorochrom (OVA-AF647) is used to evaluate endocytosis. AF647 fluorochrom displays invariable fluorescence despite differences in environmental factors. To evaluate antigen degradation DQ-OVA is used. DQ-OVA is a self-quenched conjugate that only emits fluorescence upon proteolytic digestion. DQ-OVA fluorescence excitation and emission are close to fluorescein (FITC) but contrary to FITC labelled proteins, DQ-OVA is labelled with a photostable dye in a pH range of 3 to 9, which makes it suitable for phagosomal antigen degradation studies.

1. Culture and harvest macrophages as previously described. Wash cells with PFE. Count cell numbers and resuspend cell pellet in complete medium to a concentration of 10^6 cells/ml.
2. Place 1 ml of the cell suspension into as many tubes as conditions to test. At least two 15 ml tubes are needed, one for the sample and one for the negative control.
3. Place one tube containing cells at 37°C and another on ice (negative control) for at least 30 min.
4. Add both fluorescent OVA proteins into macrophage cell suspension at a final concentration of 1 µg/ml each.
5. Incubate tubes for the desired time points either at 37°C or on ice.
6. Resuspend each tube and recover 100 µl of each cell suspension. Stop endocytosis and degradation by placing them rapidly into ice and wash with cold PFE. From this point on, cells must be kept on ice (Note 9).

7. Cells can directly be analysed by flow cytometry or fixed with PFA 2% for 20 min, then washed with PFE and stored at 4°C (Note 10).
8. Determine the percentage and the mean fluorescence intensity in the APC channel (for endocytosis) and FITC channel (for antigen degradation). **Figure 3** shows an example of the endocytic and degradative capacity of Mreg at different time points. Whereas Mregs continuously endocyte OVA particles, degradation rises a maximal kinetics at 60min.

Assessment of Mregs immunoregulatory properties

Allogeneic coculture

In order to assess the ability of Mreg to induce hypoproliferation of allogeneic T lymphocytes, co-cultures between complete mismatched cells are performed. Proliferation of responder cells from Balb/C mice are used as the readout of the assay.

1. Prepare macrophages dilutions:
    a. Culture and harvest macrophages as previously described. Wash cells with PFE. Count cell numbers and resuspend the pellet in complete medium to a concentration of $5x10^6$ cells/ml.
    b. Perform serial dilutions. Ratios can range from 1:2 to 1:128 (Mregs:T lymphocytes).
2. Prepare allogeneic T lymphocytes from spleen or lymph nodes (LN) from Balb/C mice:
    a. Crush spleen or LN on a 100 µm nylon strainer with a syringe.
    b. Rinse the strainer with PFE and recover cells in a 50 ml tube. Centrifuge the cell suspension.

c. Discard supernatant and resuspend cellular pellet in 5 ml of RBCL solution for 5 min in order to eliminate erythrocytes.

d. Add 45 ml of PFE to stop the reaction and centrifuge 10 min at 500x g. Discard supernatant.

e. Repeat step d.

f. Resuspend cell pellet in 10 ml of PFE and filter them using a 100µm-filter.

g. Determine cell number and proceed to magnetic separation of T lymphocytes, following the manufacturer's instructions.

h. After purification, label T lymphocytes with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE). Wash to remove the excess of dye.

i. Determine lymphocyte numbers and resuspend them in complete medium at a density of 10^6 cells/ml.

3. Pour the following into round-bottom 96-well plates (Note 11)

   - 100 µl of Mreg suspension (of each dilution) or 100 µl of complete medium as a basal T lymphocyte proliferation control.

   - 100 µl of Balb/C CFDA-SE labelled T lymphocytes

4. Culture plates at 37°C and 5% of CO_2 for 4 days

5. Assess T lymphocyte's proliferation by CFDA-SE dilution using flow cytometry. An example of the hypostimulatory capacity of Mregs is shown in **Figure 4.**

Suppression test

In this protocol, T lymphocytes are polyclonally stimulated with αCD3/αCD28-coated microbeads (Note 12). Syngeneic Mregs are added to the culture to test their suppressive capacity.

1. Culture and harvest macrophages as previously described. Wash cells with PFE. Count cell numbers and resuspend the pellet in complete medium to a concentration of 10^6 cells/ml.
2. Prepare syngeneic T lymphocytes from C57BL/6 mice as already explained in "Allogeneic coculture" (step 2). Resuspend CFDA-SE labelled T lymphocytes to concentration of 10^6 cells/ml.
3. Prepare polyclonal stimulation: wash αCD3/αCD28 coated microbeads with PBS using the BD™IMagnet and resuspend them in complete medium to a final concentration of $2x10^5$ beads/ml.
4. Pour the following into round-bottom 96-well plates:
   - 100 µl of C57BL/6 CFDA-SE labelled T lymphocytes
   - 50 µl of αCD3/αCD28 coated-beads
5. Incubate for 30 min at 37°C and 5% of CO_2. (Note 13)
6. Add 50 µl of Mreg suspension or 50 µl of complete medium as a proliferation control.
7. Culture plates at 37°C and 5% of CO_2 for 4 days.
8. Assess T lymphocyte's proliferation by CFDA-SE dilution using flow cytometry. Mreg are able to efficiently suppress polyclonal T lymphocyte proliferation (**Figure 5**).

Notes

1. BM derived Mreg can be generated from any mouse strain. However, functional differences may be observed depending on the mouse strain they derive from [8].
2. Other bacterial strains can be used. In that case, it is important to adapt reagents used for bacterial growth and spread to the bacterial strain used.
3. Bacteria should be used when they are at the exponential phase of the culture, obtained when OD_{600nm} = 0.4-0.6
4. The initial bacterial concentration depends on the MOI chosen for the experiments. It is recommended to try different MOIs before starting experiments.
5. Phagocytic capacity of macrophages can also be analysed by counting internalized bacteria at time point 0, expressing values as "number of CFU/initial bacteria numbers".
6. Alternatively, macrophages can be lysed by resuspension in 0.2% Triton-X 100.
7. At that point, cell lysates can be conserved at 4°C for some days.
8. In some cases, it may be important to count living macrophages after harvesting (Step 8). Values can then be expressed as "number of CFU/10^3 live macrophages".
9. The same tube is used for each time point. When needed, take 100 µl of the cellular dilution for endocytosis/antigen degradation analysis.
10. Cells can be stained for a viability dye before flow cytometry analysis. It must be essential when treating cells with new drugs which may affect cell viability.
11. It is recommended to perform triplicates of each ratio.
12. Alternatively, αCD3-coated plates and soluble αCD28 antibody stimulation can be used.

13. This step is to facilitate the recognition between antibodies and their target surface molecules before addition of Mregs. If coated plates and soluble antibodies are used, this step can be skipped.

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Initial concentration (cells/ml)	Volume (ml/dish)	rmM-CSF (ng/ml)	Culture time (days)	
			7	15
10^6	10	0.2	0.3	2.3
		0.4	0.4	2.7
		1	1.2	2
2×10^6	10	0.2	0.8	2
		0.4	0.8	2.8
		1	0.8	2.9

Table 1. Yield of adherent cells recovery under different culture conditions. Two initial cell concentrations (10^6 or $2x10^6$ cells/ml) were cultured for the indicated times (7 or 15 days) in presence of different doses of rmM-CSF (from 0.2 to 1 ng/ml). Numbers show the yield of recovery of adherent cells ($x10^6$). Around 95% of adherent cells displayed $CD11b^+F4/80^+$ phenotype

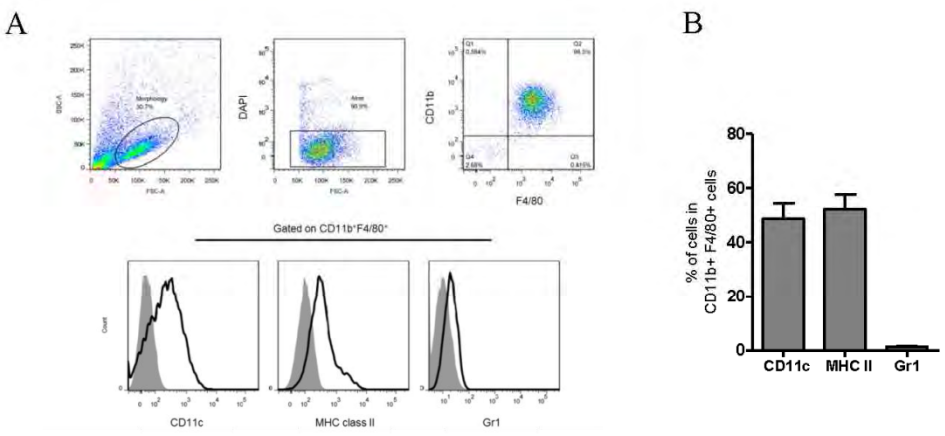

Figure 1. Mreg phenotype at day 15. Adherent cells were harvested at the end of the differentiation period. Phenotype was assessed by flow cytometry. A) Representative plots of Mreg phenotype. B) Mreg surface markers expression in $CD11b^+$ $F4/80^+$ cells (mean 93,6 ± 2,1). Data are represented as mean ± SEM of 4 independent experiments.

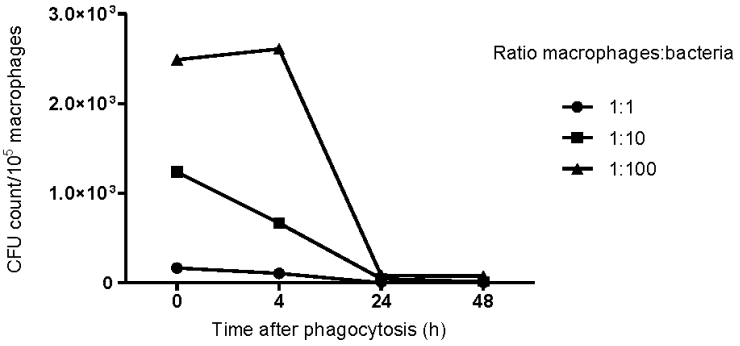

Figure 2. Macrophages bacterial killing kinetics. Evaluation of *E. coli* killing capacity was assessed as previously described. A representative experiment evaluating different time points and different MOIs (ratio 1:1 (circles); 1:10 (squares), 1:100 (triangles)) is shown.

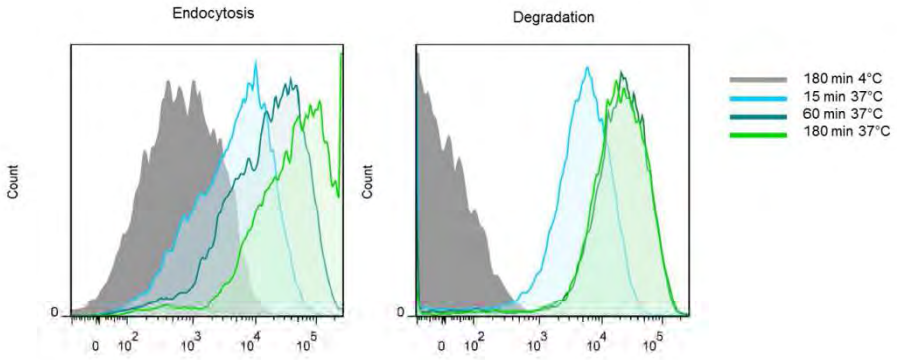

Figure 3. Endocytosis and antigen degradation kinetics. Evaluation of endocytosis and antigen degradation were assessed as previously described. A representative histogram displaying different time points is shown. 4°C control is shown filled in grey.

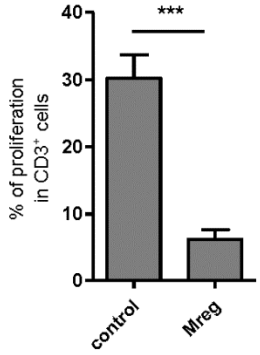

Figure 4. Mregs display hypostimulatory capacity in allogeneic co-cultures. M reg capacity to stimulate allogeneic T lymphocytes was evaluated in direct 4d co-cultures as previously described. The figure shows a 1:8 ratio (myeloid cell:T lymphocyte). Control cells were generated by culturing bone marrow precursors with 40 ng/ml of GM-CSF for 8 days. Data are represented as mean ± SEM of 3 independent experiments. Statistical analyses were performed using Mann-Whitney test, two-tailed, ***$p<0.0001$.

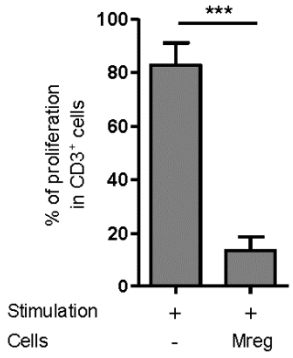

Figure 5. Mregs suppress polyclonal T cell activation. M reg capacity to stimulate suppress aCD3/aCD28-stimulated T lymphocytes proliferation was evaluated as previously described (Section XX). The figure shows a 1:2:1/5 ratio (myeloid cell:T lymphocyte:microbeads). Data are represented as mean ± SEM of 3 independent experiments. Statistical analyses were performed using Mann-Whitney test, two-tailed, ***$p<0.0001$.

ARTICLE III: COMPARATIVE STUDY OF *IN VITRO* GENERATED TOLDC, MREG AND MDSC

1 INTRODUCTION TO ARTICLE III

Cell therapy is a promising approach to induce donor-specific tolerance, but the mechanisms of action of those cells have to be further studied.

This study consisted in the comparison of three types of regulatory myeloid cells. First, we were interested in deciphering if the mechanisms of allogeneic T cell suppression were different between the three RMC types. Then, we wanted to compare the *in vivo* efficacy of each RMC type when injected in an autologous way without combination treatment.

First, we have focused on testing the capacity of RMC to stimulate allogeneic T cells and whether they were able to induce T cell activation or T cell death when co-cultured with allogeneic T cells. Then, we have analyzed RMC capacity to modify T cells in a permanent way, by anergy induction, or if their action only lasts while they are present in co-cultures.

In vivo, we have determined that all RMC are able to induce prolongation of allograft survival. Differences in efficiency may depend on the *in vivo* mechanism each of those RMC type induce, and deserves deeper studies concerning RMC homing *in vivo* and phenotype modification after exposure to a pro-inflammatory graft environment.

2 ARTICLE III

Comparative study of the immunoregulatory capacity of *in vitro* generated TolDC, Mreg and MDSC

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ABSTRACT

Regulatory myeloid cell therapy is a promising strategy to deal with immunological disorders like autoimmune diseases and organ transplantation. Nowadays, several types of regulatory myeloid cells are being developed for clinical use, the best studied ones being tolerogenic dendritic cells, regulatory macrophages and myeloid-derived suppressor cells. Those cells are generated using different protocols and cytokines but their phenotype often overlap, raising the question about their real differences. In this study, we aimed at generating all three types of regulatory myeloid cell from the same mouse strain and to compare their *in vitro* properties and evaluate their potential in a model of skin transplantation. This study shows that the three cell types present some common but also some specific markers. They also display differences regarding their APC functions or their effect on T cells. Lastly, all the cell type are able to prolong graft survival. Using the same mouse strain and the same assays, this study highlights for the first time a comparison of TolDC, Mreg and MDSC about their phenotype, their functions and their effect on transplantation survival.

INTRODUCTION

Although current therapies in organ transplantation are efficient in the short-term prevention of allograft rejection, long-term graft failure remains an unresolved problem [1]. Even more, current treatments can lead to drug-related side-effects or to secondary complications due to the induction of recipient's general immunosuppression. In that context, cell therapy arises as a good strategy, aiming at the induction of permanent donor-specific tolerance and reduction of immunosuppressor doses [2]. Safety and efficiency of multiple regulatory cell types differentiated *in vitro* or expanded *ex vivo* have been tested in animal models of transplantation [3].

Regulatory myeloid cells (RMC), as tolerogenic DC (TolDC), regulatory macrophages (Mreg) and myeloid-derived suppressor cells (MDSC) are gaining interest as therapeutic agents, due to their unique capacity to modulate effector T cell activity by directly targeting activated T lymphocytes or by regulatory T cell induction [4], [5]. In animal models, RMC therapy has been shown to be effective in graft survival prolongation [6]–[9] and to treat autoimmune disorders [10]–[12]. Promising results have been obtained by clinical trials evaluating safety and efficacy of RMC therapy in transplantation [13] and autoimmunity [14]. *Ex vivo* human RMC generated for cell therapy are derived from blood monocytes, under different *in vitro* conditions [15]. Since RMC share the same precursor, display similar phenotype and immunosuppressive function, the question that arises is: are they different cellular populations?

Here, we generated and characterized three myeloid suppressive populations with cell therapy potential: TolDC, Mreg, and MDSC. We compare their characteristics *in vitro* and *in vivo* in a murine model of skin transplantation. We could demonstrate that, although they share some phenotypic and functional similarities, they constitute different cell subsets. In

fact TolDC are $CD11c^{+}$ $CD11b^{+}$ $F4/80^{-/+}$ $CD169^{-}$ $Gr1^{-}$, Mreg are $CD11c^{+}$ $CD11b^{+}$ $F4/80^{+}$ $CD169^{+}$ $Gr1^{-}$ and MDSC are $CD11c^{-}$ $CD11b^{+}$ $Gr1^{+}$. Whereas TolDCs and Mregs are able to efficiently endocyte and degrade antigens, MDSC are not. *In vitro* experiments show that TolDCs induce T cell hyporesponsiveness, whereas MDSC induce T cell apoptosis. *In vivo*, autologous injections of all RMC were able to induce prolongation of graft survival but at different doses suggesting different mechanisms of action. Our results show that TolDC, Mreg and MDSC are different and that we should tested them in other settings in order to optimize cell therapy approaches.

MATERIALS AND METHODS

Mice and Ethics Statement

6 to 8 weeks old C57BL/6 and Balb/C mice were purchased from Janvier (France). This study was carried out in strict accordance with the protocol approved by the Committee on the Ethics of Animal Experiments of Pays de la Loire (Ref CEEA.2013.9).

Cell preparations

Bone marrow precursors for the generation of all RMC populations were obtained from tibias and femurs of C57BL/6 female mice. TolDC were generated as previously described [16]. Bone marrow precursors were cultured at a density of $0.5x10^6$ cells/ml for 8 days in 100mm untreated Petri dishes with 10ml of complete RPMI 1640 medium (100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 mM HEPES, 2 mM glutamine, 1mM sodium pyruvate, 1x MEM NEAA, all from Gibco), 0.05 mM 50µM β-ME (Sigma-Aldrich) and 10% FCS (HyClone) and in the presence of low doses of GM-CSF (0.4 ng/ml) (from COS supernatant). Mreg were generated by culture of bone marrow precursors at a density of 10^6 cells/ml for 15 days in 100mm untreated Petri dishes, each dish containing 10ml of complete DMEM medium (supplemented as for TolDC) and 10% FCS (Lonza) and in the presence of low dose M-CSF (0.2 ng/ml, Peprotech). For TolDC and Mreg, 10 ml of medium supplemented with cytokines were added at day 3 of culture and 10 ml of medium were replaced at day 6 and 7 of culture respectively. Harvesting of adherent RMC was performed by flush with cold PBS, 2% of FCS and 0.1mM of EDTA. MDSC were generated as previously described [17]. Bone marrow precursors were cultured at a $2.5x10^6$ cells/ml density for 4 days into 100mm culture-treated Petri dishes (Falcon BD) in 10 ml of complete DMEM medium and 10% of FCS (Lonza). Medium was supplemented with 40 ng/ml of GM-CSF (Peprotech) and 40 ng/ml of

IL-6 (Sigma-Aldrich). All, adherent and non-adherent MDSC were harvested as previously described.

Control cells were obtained by culturing bone marrow precursors at a density of $0.5x10^6$ cells/ml for 8 days in 100mm untreated Petri dishes with 10ml of complete RPMI 1640 medium and 10% FCS (HyClone) and in the presence of 40 ng/ml of GM-CSF (from COS supernatant) and were matured by addition of 1 µg/ml LPS (Sigma) the last 24h of culture.

Morphologic analysis and Hematoxylin Eosin staining

Morphological observations were performed on a Nikon Eclipse TS100 (Nikon) at x20 (NA0.4) optic magnification. Images were acquired with a Canon Power Shot G11digital camera (Canon).

One hundred µl of $3x10^5$ cellular suspensions were seeded onto Poly-L-lysine coated slides and incubated for 10min at 37°C. Hematoxylin-Eosin staining is performed by incubating slides 5 min into Meyer Hematoxylin solution (Sigma-Aldrich), washing in distillate water, 20 seconds of 1% eosin staining and rinsing twice in distillate water. Slides were observed on a Nikon Eclipse E600 microscope (Nikon) at x20 optic magnification. Images were acquired with a digital camera DXM 1200 (Nikon) using the software Nikon ACT-1.

Flow cytometry and antibodies

RMC phenotypic characterization was performed with the following mAbs: anti-CD11b (APC-Cy7 conjugated, BD Pharmingen), anti-CD11c (PE-Cy7 conjugated, BD Pharmingen), anti-MHC class II (I-Ab eFluor®450 conjugated, eBiosciences), anti-F4/80 (PE-Cy5 conjugated, eBiosciences), anti-CD169 (APC conjugated, eBiosciences), anti-Gr1 (PE

conjugated, BD Pharmingen), anti-CD86 (PE conjugated, BD Pharmingen), anti-CD80 (FITC conjugated, BD Pharmingen), anti-CD40 (APC conjugated, BD Pharmingen). Surface staining of T cells was performed with anti-CD3, anti-CD4, anti-CD8, anti-CD69, anti-CD25, anti-CD95 mAbs. Dead cells were excluded by DAPI positive staining or Viability Dye eFluor®506 staining (eBiosciences). FACS Canto II (BD Biosciences) was used to measure fluorescence and data were analyzed using FlowJo software, version 7.6.5 (Tree Star Inc.).

Maturation resistance

RMC were harvested and seeded at a density of 10^6 cells/ml in 48 well flat bottom plates (Falcon BD). RMC were stimulated with 1 µg/ml of LPS (Sigma-Aldrich) or left untreated for 48h. Cells were harvested and analyzed using flow cytometry.

Antigen internalization and degradation assay

Endocytosis and degradation were determined using Alexa Fluor 647-conjugated OVA (OVA-AF647) and DQ-OVA (both from Invitrogen). RCMs were incubated with 1 µg/ml of each OVA protein for different time points at 37°C and 5% of CO_2. Cells were washed with cold PBS and fixed with 2% PFA until flow cytometry analysis (FACS Canto II, BD). A control at 4°C was included for each time point.

***In vitro* allogeneic co-culture**

To test RMC stimulatory capacity, T cells were purified from Balb/C spleen using the PanT Kit (Miltenyi) and labeled with CFSE probe (Invitrogen). Cells resuspended in 1ml PBS were stained with 5 µM of CFSE, for 5 min at RT, then 2 volumes of FCS were added and cells were washed twice with PBS supplemented with10% of FCS. 10^5 T cells per well were seeded in 96-round bottom well plates (Falcon BD) and decreasing amounts of C57BL/6 derived RMC were added to T cell culture (from $5x10^4$ to $1.5x10^3$ cells). Each point was

performed in triplicates. Four days after, T cell proliferation was measured by CFSE dilution flow cytometry using a FACS Canto II (BD).

To test cytokine secretion, 10^6 T cells were co-cultured with varying amounts of RMC in 24-well plates for 4 days. Then, supernatants were recovered and IFNγ and IL-10 were measured by ELISA (BD OptEIA).

***In vitro* suppression assay**

T cells were purified from C57BL/6 spleen using the PanT Kit (Miltenyi) and CFSE-labeled (Invitrogen). 10^5 T cells per well were seeded in 96-well plates (Falcon BD) and polyclonally stimulated with $4x10^4$ anti-CD3/28 Dynabeads (Invitrogen). RMC were then added to the culture, and incubated at 37°C for 4 days. Proliferation was analyzed by CFSE dilution assessed by flow cytometry (FACS Canto II, BD).

T cell re-stimulation after co-culture

Magnetically purified, CFSE-labeled Balb/C T cells were co-cultured with each C57BL/6 derived RMC type for 3 days (ratio 2:1). Balb/C T cells were then re-isolated using mouse CD90.2 magnetic beads (Miltenyi) and re-stimulated with splenic irradiated (35 Grays) $CD11c^+$ DCs purified with $CD11c^+$ magnetic beads (Myltenyi) in a ratio 4:1 for 3 more days. At day 6, total proliferation was measured by CFSE dilution (FACS Canto II, BD) and IFNγ secretion was measured in culture supernatants by ELISA (BD OptEIA).

Skin transplantation and treatments

C57BL/6 male tail skin was grafted on female recipients as previously described [17], [18]. Recipients were either injected with 10^6 or $3x10^6$ of autologous non-pulsed RMCs intravenously the day before transplantation or left untreated. Seven days later, the plaster was

cut off and the graft survival was monitored on a daily basis. Rejection was defined as complete sloughing or a dry scab.

Statistical analysis

Statistical analyses were performed with Graphpad Prism 5.0 (La Jolla, CA, USA) using the Mann-Whitney test. Survival rates were compared using the Log-rank (Mantel-Cox) test. Statistical significance was defined as $p < 0.05$.

RESULTS

In vitro derived RMC display different phenotype

Regulatory myeloid cells were differentiated *in vitro* from bone marrow precursors from naive C57BL/6 mice under different culture conditions. As previously described, total bone marrow was cultured in the presence of low doses of GM-CSF (0.4 ng/ml) for 8 days to obtain TolDC [16], in the presence of low doses of M-CSF (0.2 ng/ml) for 15 days to obtain Mreg (Carretero-Iglesia et al. *submitted*) or in the presence of 40 ng/ml GM-CSF and 40 ng/ml IL-6 for 4 days to obtain MDSC [17], [19] (**Figure 1a**). Control myeloid cells were differentiated from bone marrow cells cultured for 8 days with 40 ng/ml of GM-CSF and matured with 1µg/ml of LPS during the last 24h of differentiation. Each RMC type displayed characteristic morphology. TolDC displayed round-shape morphology, prominent cytoplasm and small prolongations, and usually clustered together. Non-adherent cells were discarded, as only adherent cells displayed suppressive capacity [16]. Mreg displayed a central body, eccentric nuclei and some long prolongations, but clusters were not observed. A heterogeneous population of MDSC was obtained, composed of different cell sizes, adherent and non-adherent, some of them clustering together (**Figure 1b**). For comparison, morphology of control myeloid cells was also analyzed (**Suppl Fig 1a**). The yield of recovery was variable between cell types. The most little recovery was obtained from Mreg cultures, followed by TolDC and then MDSC (**Suppl Fig 2**).

Phenotypically, all RMC expressed CD11b myeloid cell marker and different levels of CD11c expression as well as other lineage specific surface markers. Therefore, TolDCs expressed $CD11b^{+}$ $CD11c^{+}$ MHC class II^{low}, Mregs displayed $CD11b^{+}$ $CD11c^{+}$ MHC class II^{low} and typical macrophage markers $F4/80^{hi}$ $CD169^{+}$ and MDSC were $CD11b^{+}$ $CD11c^{-}$ $CD86^{low}$ $F4/80^{-}$ and expressed the typical granulocytic and MDSC marker Gr1. All RMC

expressed low levels of the costimulatory marker CD86 (**Figure 1c**). For comparison, phenotype of control myeloid cells was analyzed. They expressed higher levels of MHC class II and the costimulatory molecule CD86 than RMC (**Suppl Fig 1b**). Other tested markers that were not expressed were CD8, CD4, CD103 and CD205 (data not shown).

RMC are functionally different cell types

Two important characteristics of RMC are their resistance to maturation stimuli as well as their capacity to endocyte antigens to present them to T lymphocytes. In order to elicit if RMC are resistant to maturation stimuli, RMC were exposed to LPS for 48h and upregulation of costimulatory molecules as well as MHC-II molecules was assessed by flow cytometry. As shown in **Figure 2a**, TolDCs and MDSCs are resistant to maturation. On the contrary, Mregs overexpress MHC-II and CD40 molecules after LPS stimulation.

Then, we evaluated RMC capacity to endocyte and degrade exogenous antigens. By using OVA beads conjugated with Alexa Fluor 647, we could determine that TolDCs and Mregs displayed high endocytic capacity, which increases over time. On the contrary, MDSCs were not able to endocyte antigens, even 24h after antigen exposure (**Figure 2b**). To evaluate the degradative capacity of RMC, they were incubated with DQ-OVA, a protein probe-labeled and quenched that emits fluorescence after degradation. Fluorescence was assessed at different time points. As shown in **Figure 2b**, TolDC and Mregs were able to efficiently degrade antigens, whereas MDSC were not, probably due to the lack of antigen uptake.

Mregs and TolDCs induce T cell hyporesponsiveness whereas MDSCs induce T cell apoptosis

In order to elicit the mechanisms of tolerance induction set up by each RMC type, we assessed the capacity of RMC to stimulate allogeneic T cell proliferation. We performed direct co-cultures of RMC:allogeneic T cells at different ratios. T cells were stained with

CFSE at day 0 and $CD4^+$ and $CD8^+$ T cell division was determined at day 4 by flow cytometry. TolDCs and Mregs did not stimulate $CD4^+$ nor $CD8^+$ T cell proliferation at any RMC:T cells ratio. MDSCs did not induce T cell proliferation at high ratios but were able to stimulate $CD4^+$ and $CD8^+$ T cell proliferation at lower ratios (**Figure 3a**). T cell activation was assessed after 4 days of co-culture (at 1:8 ratio) by surface staining of CD25 and CD69 molecules. The lack of proliferation observed in TolDC co-cultures was associated with a lack of T cell activation, whereas T cells co-cultured with Mreg or MDSC displayed an activated phenotype (**Figure 3b**).

We then wondered if RMC were able to polarize T cell response towards pro or anti-inflammatory phenotype. We measured IFNγ and IL-10 production in co-culture supernatants. IL-10 secretion was not detected in any condition (data not shown). On the contrary, low but some IFNγ production was detected in supernatants of MDSCs-T cells co-cultures whereas it was not detected in TolDCs or Mregs co-culture (ratio 1:8) (**Figure 3c**). Therefore, these results suggest that MDSC could polarize T cell responses at low ratios towards a Th1 proinflammatory profile *in vitro*, whereas TolDC and Mregs do not induce a Th1 T cell polarization.

Persistent TCR stimulation can promote elimination of activated T cell clones through activation-induced cell death (AIDC), which implies binding of Fas-FasL molecules at the T cell surface. Hence, we examined whether allogeneic co-culture of T cells with each RMC type was able to upregulate Fas expression by T cells. Both, MDSCs and Mregs induced Fas expression at the surface of $CD4^+$ and $CD8^+$ T cells whereas TolDC co-culture did not (**Fig 3d**). Moreover, in MDSCs co-culture, Fas upregulation was correlated with significantly increased T cell death (**Figure 3e**).

Taken together, these results demonstrate that RMC use different mechanisms to control T cell responses. TolDCs induce T cell hyporesponsiveness and low T cell activation in direct co-cultures, presumably due to the low expression of costimulatory molecules. Mregs do not induce allogeneic T cell proliferation but T cells display an over-activated profile, as determined by upregulation of activation molecules and Fas on their surface. On the other hand, MDSCs co-culture induces the upregulation of activation markers and Fas expression, leading to T cell death induction.

TolDCs, Mregs and MDSCs suppress polyclonal T cell proliferation

In order to evaluate the suppressive capacity of RMC, purified T lymphocytes were stained with CFSE and stimulated with αCD3/CD28 coated microbeads (ratio 1:1/5) in the presence of each RMC type (ratio 1:2) in syngeneic conditions. T cell proliferation was assessed after 4 days of co-culture by flow cytometry. All RMC were able to inhibit syngeneic polyclonal T cell proliferation, although TolDCs and Mregs inhibition was stronger than MDSCs for both, $CD4^+$ (mean of 9.68% ± 3.17 and 4.57% ± 0.8 versus 49.38% ± 11.76 of proliferation respectively) and $CD8^+$ T cells (mean of 12.87% ± 5.31 and 15.02% ± 5.81 versus 44.03% ± 4.7 of proliferation respectively) (**Figure 4**).

TolDC induce T cell unresponsiveness

To assess if co-culture of RMC with T cells induce a state of unresponsiveness in allogeneic T lymphocytes, Balb/C T lymphocytes where magnetically purified 3 days after co-culture with each type of C57BL/6 derived RMC (ratio 1:2) and re-stimulated for 3 more days by C57BL/6 splenic irradiated DCs in alloantigen-specific manner (ratio 4:1) (schematic representation of the experiment on **Figure 5a**). $CD4^+$ and $CD8^+$ T lymphocytes co-cultured with Mregs and MDSCs were able to proliferate in response to a second allogeneic stimulus whereas TolDCs-co-cultured T lymphocytes displayed lower proliferation (**Figure 5b**).

Equally, a decrease in IFNγ production was observed by T cells previously co-cultured with TolDCs when compared to other co-cultures (**Figure 5c**).

Adoptive transfer of autologous RMC prolong graft survival

To test the *in vivo* efficacy of each type of autologous RMC in transplantation, we used a mouse minor histocompatibility model in which male skin is grafted onto female recipients. RMCs were intravenously injected the day before transplantation. Recipients were fully immunocompetent and RMC injection was not associated to any immunosuppressor treatment. Injection of 10^6 autologous TolDC slightly prolonged graft survival (median 31 days vs 21 days untreated mice), whereas injection of $3x10^6$ autologous TolDC did not prolong graft survival when compared to untreated mice (median 25 days vs 21 days untreated mice) (**Figure 6a**). 10^6 recipient derived Mreg injection did not prolong allograft survival (median 23.5 days). On the contrary, when $3x10^6$ autologous Mreg were injected alone, prolonged graft survival was observed (median 27 days), even long term graft acceptance was achieved in 10% of recipients (**Figure 6b**). A single dose of autologous MDSC injection prolonged graft survival when 10^6 cells were injected (median 28 days), and their effect was even more important when cell dose was increased to $3x10^6$ cells (median 45 days) (**Figure 6c**).

DISCUSSION

A constant improvement of short-term prevention of allograft rejection has been shown in the organ transplantation field in the last years but long term graft failure remains an unresolved problem. Current treatments need the life use of immunosuppressors that do not prevent chronic rejection and lead to strong drug-related side effects, as increased risk of infection and cancer [20]. Even more, the use of these drugs fails to induce antigen-specific tolerance. In that context, cell therapy with regulatory cells arises as a good strategy, aiming at regulate the anti-donor alloresponse and allow the reduction of immunosuppressors administration. In this context the European consortium « The ONE Study » aim to develop and compare cell therapy protocols using different types of regulatory cells (TolDC, Mreg, CD4 Treg and CD4 Tr1 cells) in living donor kidney transplantation [5].

Recently different types of myeloid cells with regulatory function (RMC), as tolerogenic DC (TolDC), regulatory macrophages (Mreg) and myeloid-derived suppressor cells (MDSC) have been described and tested in preclinical models of transplantation and autoimmunity with promising results. Those cells are generated in human from circulating $CD14^+$ PBMC using different protocols and even if each population displays some specific markers, they share some phenotypic and functional properties [3], [21].

In this manuscript we aimed at generating and characterizing TolDC, Mreg and MDSC from the same animals and compare their phenotype and function in order to identify whether there is a better candidate for its use in cell therapy in organ transplantation. The three types of RMC were differentiated *in vitro* from mouse bone marrow precursors under different culture conditions. At the end of the culture they show different recovery yield and characteristics. TolDC displayed round-shape morphology, prominent cytoplasm and small prolongations, and usually clustered together. They are characterized by the high expression

of CD11c, CD11b, low expression of MHC class II and costimulatory antigens in the absence of CD169 and Gr1 markers. We have previously shown that the administration of autologous TolDC in combination to anti-CD3 treatment was able to prolong skin and islets allograft survival in mice ([22] and Baas et al. *in press*).

Mreg show eccentric nuclei and some long prolongations adhering to the plastic without clustering. They express high CD11b and CD11c, low MHC class II molecules and F4/80^{hi} CD169^{+} typical macrophage markers in the absence of Gr1. This phenotype is in accordance to *in vitro* generated Mreg previously described by Riquelme et al. The authors have shown that IFNγ stimulation at the end of Mreg culture brings macrophages to a novel state of activation, conferring them *in vitro* and *in vivo* regulatory capacity, as shown in a model of mouse cardiac transplant, where donor macrophages injected 8 days before the transplant were able to prolong allograft survival [7].

As expected obtained MDSC were a heterogeneous population composed of different cell sizes, a mixture of adherent and non-adherent cells. They display the typical phenotype, characterized by CD11b^{+}, Gr1^{+} and MHC class II^{-} cells, as we and others have previously described [17], [19]. MDSC generated with GM-CSF and complemented with IL-6 have been shown to display stronger immunosuppressive activity in mouse and human [19], [23]. Importantly, *in vivo*, multiple injections of GM-CSF plus IL-6 differentiated MDSC were able to induce long-term allograft survival in a pancreatic islet allograft mouse model [19].

We have also shown that those three RMC populations are functionally distinct *in vitro*, even if all three RMC populations are able to inhibit the proliferation of polyclonally activated T lymphocytes. TolDCs are resistant to maturation and display high endocytic capacity, a characteristic of immature DC which is not shared by control mature cells (data not shown). Mregs upregulate some maturation markers after proinflammatory stimulation but

still efficiently endocyte and degrade antigens. MDSC show resistance to maturation through LPS stimulation but they are not able to endocyte and therefore degrade antigens. Those different characteristics can lead to differences in the mechanisms for T cell response suppression.

Results obtained by direct co-culture of each RMC with allogeneic T lymphocytes demonstrate that RMC use different mechanisms to control T cell responses. As already shown by Lutz et al. [24], TolDCs induce T cell hyporesponsiveness and low T cell activation, presumably due to the low expression of costimulatory molecules (**Figure 2a**). Mregs do not induce allogeneic T cell proliferation but T cells display an activated profile, as determined by the upregulation of activation molecules CD25 and CD69 and Fas on their surface. Riquelme et al. [7] described that Mreg induce apoptosis of specific allogeneic T cells by the induction of iNOS. Even if we found an increase expression of Fas by activated T cells (**Figure 3d**) we do not confirm the increase in apoptosis. This can be due to the fact that our cells are not stimulated by IFNγ and they can have different mechanisms of regulation. On the other hand, MDSCs co-culture induces the upregulation of CD69 and Fas expression, leading to an increase of T cell death. IFNγ secretion by T cells was only detected in MDSC co-cultures, suggesting that, at given ratios, MDSC are able to polarize T cell responses towards a Th1 phenotype. All these results taken together suggest that RMC play their suppressive role following different *in vitro* mechanisms of T cell suppression. Whereas co-culture with TolDC induces a profound influence in the proliferation, activation, and re-activation capacity of T cells, Mreg induce a state of non-proliferative "altered" activation of T cells, which consequences remain to be determined. Activation and cell death induction, maybe because of over-activation, seems to be MDSC mechanism of action. It has already been described that two of the mechanisms MDSC use is T cell apoptosis induction [25] or T cells deprivation of nutrients [26].

After primary allogeneic co-culture of T cells with RMC, a secondary stimulation with mature immunogenic DC lead to T cell proliferation and IFNγ secretion of T lymphocytes co-cultured with Mreg and MDSC, but T lymphocytes co-cultured with TolDC did not respond to secondary stimuli. This result highlights the need of Mreg and MDSC to be present at the site of effector T cell activation in order to mediate the suppressive function, therefore implying either a cell-to-cell contact mechanism or a mechanism implying diffusion of mediators. On the contrary, TolDC co-culture seems to affect T cells in a way that persists, even in the absence of TolDC and even after a second challenge. One hypothesis of TolDC mechanism of action could be induction of T cell anergy by incomplete activation of T cells due to the deficient co-stimulation of TolDC [27].

In vivo, we have shown that injection of a single dose of all autologous non-pulsed RMC is able to significantly prolong allograft survival. Assessing the efficacy of cell therapy in the absence of combined immunosuppression is of the most importance, as immunosuppressor drugs can modify the activity of injected cells, therefore masking the contribution of cell therapy to graft survival prolongation. Even more, autologous and non-pulsed with donor-derived peptides cell therapy provide practical advantages compared to donor-derived RMC generation, as there is no need to know the donor before transplantation, there is less risk of sensitization due to contaminant cell products and injected cells are not recognized as non-self, therefore limiting the chances of destruction by the host's immune system [15].

Even though multiple mechanisms were considered in our *in vitro* tests, other mechanisms or the combination of multiple mechanisms could be taking place. Indeed, it will be interesting to identify whether the *in vitro* suppressive effect is mediated by soluble factors or cell-to-cell contact [8], [28]. Furthermore, the involvement of molecules and enzymes

which have already been described as mediating RMC mechanisms of tolerance must also be addressed [3], [6]. Finally, the real contribution of each of those mechanisms to the *in vivo* effect of each RMC will be investigated in the future.

Using the protocols previously described we have generated from the same animals three types of RMC that can be distinguished by their morphology, their phenotype and their *in vitro* effects. We show that all RMC used have an immunoregulatory effect on skin allograft survival. Nevertheless, we have recently shown that MDSC could have different effect depending on the model used [17]. Those results highlight the necessity to better characterize the effect of RMC in different models of autoimmunity and transplantation and then adjust the timing of injection and the doses, to optimize the choice of the type of cell for the induction of tolerance.

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FIGURE LEGENDS

Figure 1. Morphology and phenotype of RMC. (a) Schema representing the *in vitro* differentiation process of each RMC type derived from bone marrow precursors. **(b)** Morphology of RMC in culture and hematoxylin and eosin staining. **(c)** Phenotype of surface markers defining each RMC. Histograms are representative of 5 independent experiments.

Figure 2. RMC display functional differences. (a) Expression of maturation associated markers under normal conditions (medium) and after 48h of LPS stimulation (LPS) (n=3). **(b)** Representative histogram (left) and kinetics (right) of RMC antigen endocytosis and degradation. Data are representative of 3 independent experiments.

Figure 3. Suppression of T cell proliferation by RMC is mediated through different mechanisms. T lymphocytes were purified from naive Balb/C mice and labeled with CFSE before performing a 4 days allogeneic co-culture with C57BL/6 *in vitro* derived RMC **(a)** Proliferation of $CD3^+CD4^+$ or $CD3^+CD8^+$ T lymphocytes in allogeneic co-culture was assessed by CFSE dilution using flow cytometry (n=2) **(b)** Activation markers expression was assessed at day 4 by analyzing CD25 and CD69 staining in $CD3^+CD4^+$ or $CD3^+CD8^+$ T lymphocytes (ratio 1:8) (n=3-4) **(c)** IFNγ secretion was assessed in co-culture supernatants at day 4 by ELISA (ratio1:8) (n=4) **(d)** Fas receptor expression was assessed by flow cytometry in $CD3^+CD4^+$ or $CD3^+CD8^+$ T lymphocytes at day 4 (ratio 1:8) (n=4) **(e)** Dead cells were analyzed gating on $CD3^+CD4^+$ or $CD3^+CD8^+$ T lymphocytes and selecting the positive

population for DAPI staining (n=5). Statistics were performed using Mann-Whitney nonparametric test (*p<0.05, **p<0.001, ***p<0.0001).

Figure 4. All RMC suppress polyclonally stimulated T cells. anti-CD3/28 polyclonally activated C57BL/6 CFSE-labeled T lymphocytes were cultured alone or with syngeneic RMC for 4 days (ratio 2:1). **(a)** Representative histogram of $CD3^+CD4^+$ or $CD3^+CD8^+$ T lymphocyte proliferation. **(b)** Percentage of polyclonal proliferation after 4 days of co-culture with RMC measured by CFSE dilution (n=4). Statistics were performed using Mann-Whitney nonparametric test (*p <0.05, ***p<0.0001).

Figure 5. TolDC induce T cell unresponsiveness. Purified Balb/C T lymphocytes were labeled with CFSE and co-cultured for 3 days with each C57BL/6-derived RMC type or control cells (ratio 2:1). T lymphocytes were reisolated using magnetic beads and restimulated with irradiated C57BL/6 splenic mature DC for 3 more days (ratio 4:1). **(a)** Schema representing the experimental design. **(b)** Proliferation of $CD3^+CD4^+$ or $CD3^+CD8^+$ T lymphocytes measured by CFSE dilution at day 6 (n=3) **(c)** IFNγ secretion of the last 3 days of co-culture was assessed in supernatants at day 6 by ELISA (n=3)

Figure 6. A single dose of adoptively transferred RMC alone is able to prolong graft survival. Male skin grafts were transplanted onto female recipients. Recipients were treated with different doses of each of autologous RMC the day before transplantation (10^6 cells, black dashed line or $3x10^6$ cells, black line) or left untreated (grey line). **(a)** TolDC administration **(b)** Mreg administration **(c)** MDSC administration. Survival rates were

compared using the Log-rank (Mantel-Cox) test. Statistical significance was defined as *$p<0.05$, **$p<0.01$.

Figure S1. Morphology and phenotype of control cells. (a) Morphology of control cells in culture and hematoxylin and eosin staining. **(b)** Phenotype of surface markers defining control myeloid cells. Histograms are representative of 5 independent experiments.

Table S1. Yield of recovery of each cell type. Data are represented as cells recovered per plate (left) or per 10^6 initial cultured cells (right). Data ± SEM.

Figure 1

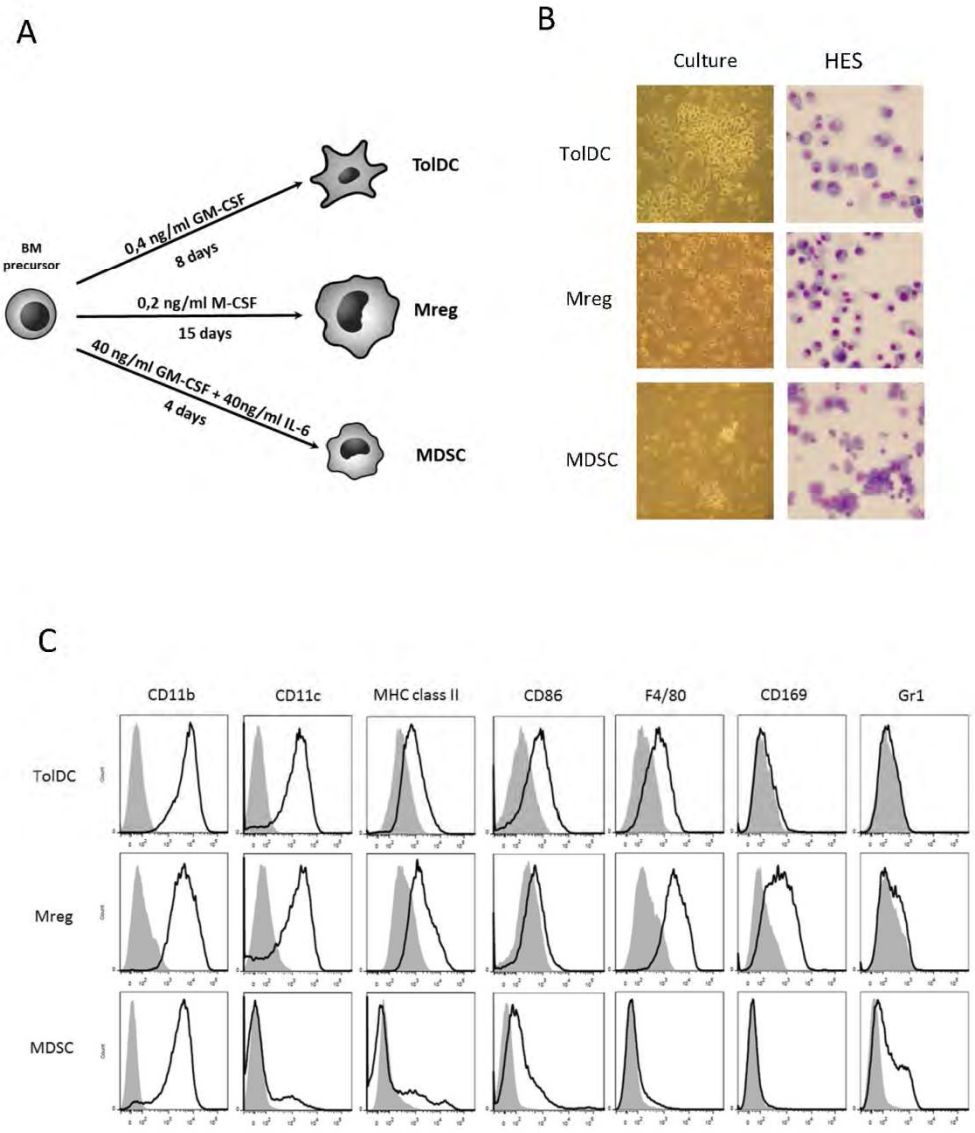

A
BM precursor
0,4 ng/ml GM-CSF
8 days
TolDC
0,2 ng/ml M-CSF
15 days
Mreg
40 ng/ml GM-CSF + 40ng/ml IL-6
4 days
MDSC
B
Culture
HES
TolDC
Mreg
MDSC
C
CD11b
CD11c
MHC class II
CD86
F4/80
CD169
Gr1
TolDC
Mreg
MDSC

Figure 2

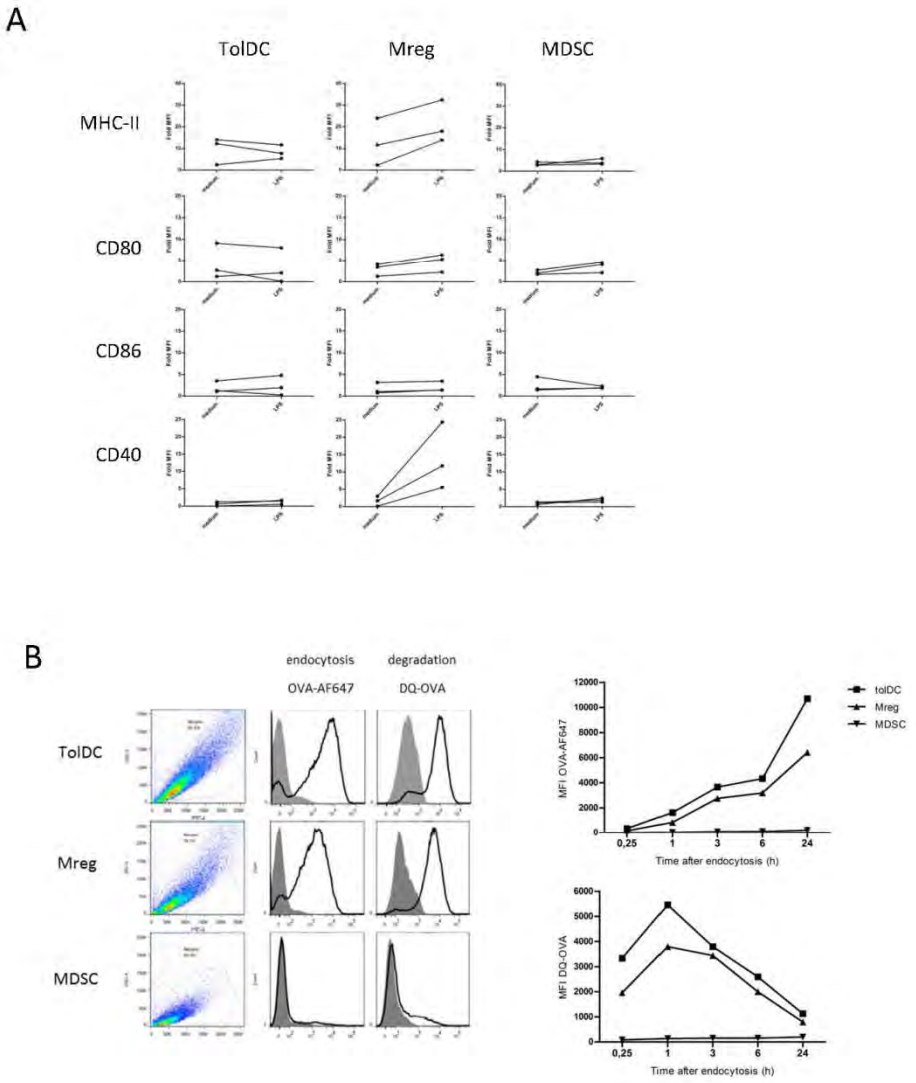

A
TolDC
Mreg
MDSC
MHC-II
CD80
CD86
CD40
B
endocytosis
OVA-AF647
degradation
DQ-OVA
TolDC
Mreg
MDSC
MFI OVA-AF647
Time after endocytosis (h)
tolDC
Mreg
MDSC
MFI DQ-OVA
Time after endocytosis (h)

Figure 3

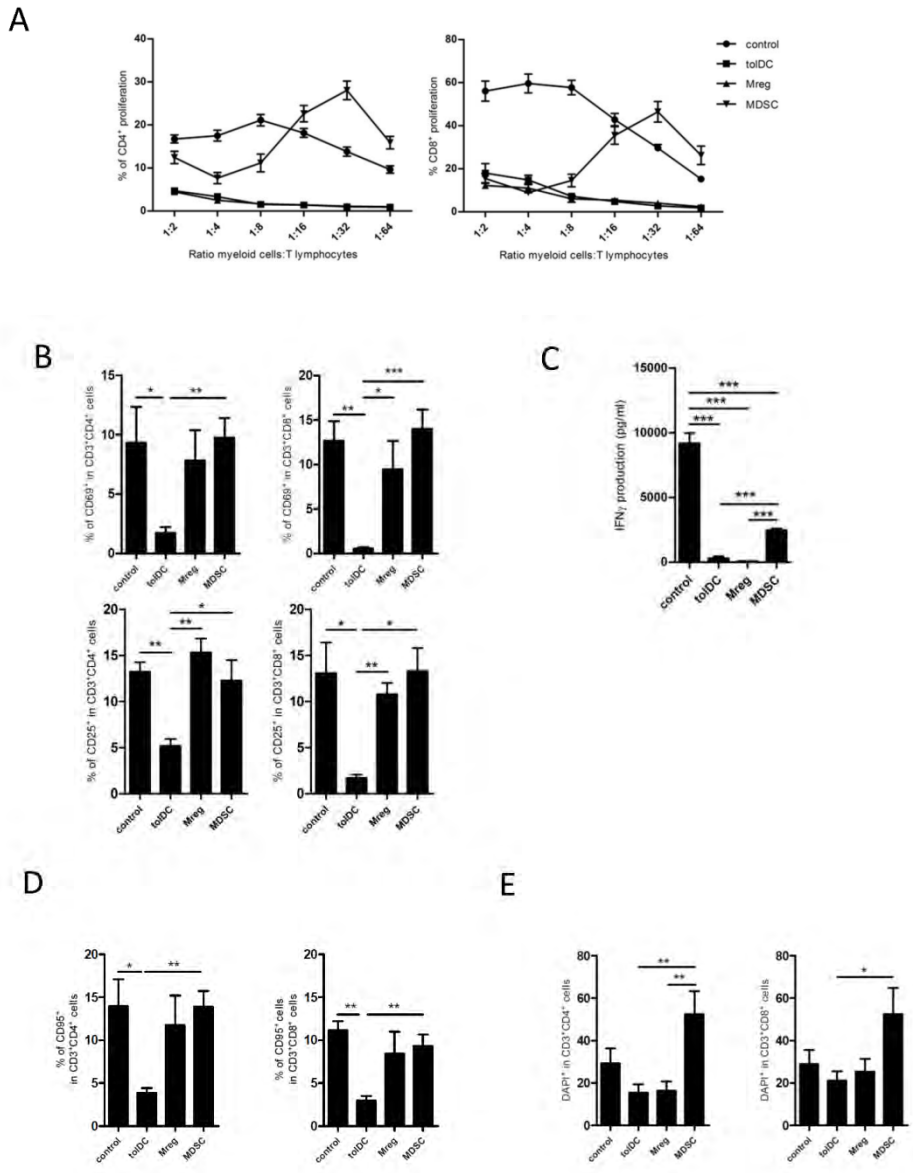

A
% of CD4+ proliferation
% CD8+ proliferation
Ratio myeloid cells:T lymphocytes
control
tolDC
Mreg
MDSC
B
% of CD69+ in CD3+CD4+ cells
% of CD69+ in CD3+CD8+ cells
% of CD25+ in CD3+CD4+ cells
% of CD25+ in CD3+CD8+ cells
C
IFNγ production (pg/ml)
D
% of CD95+ in CD3+CD4+ cells
% of CD95+ in CD3+CD8+ cells
E
DAPI+ in CD3+CD4+ cells
DAPI+ in CD3+CD8+ cells

Figure 4

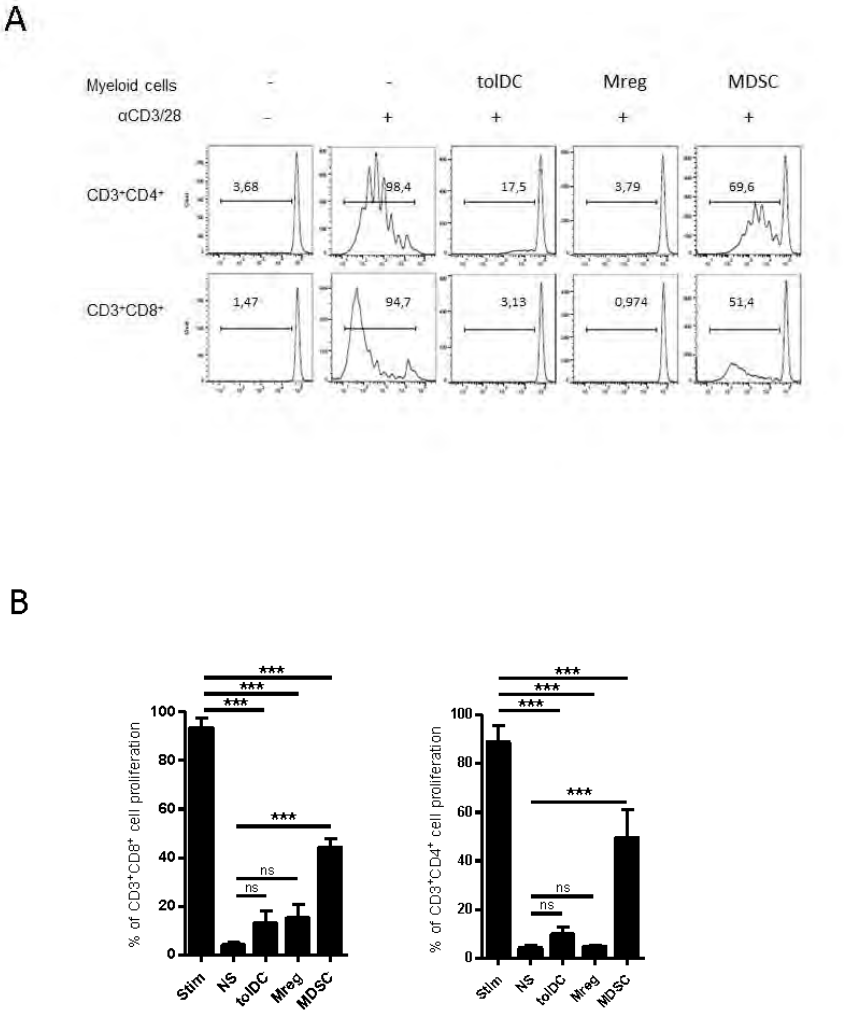

A
Myeloid cells
αCD3/28
tolDC
Mreg
MDSC
CD3+CD4+
3,68
98,4
17,5
3,79
69,6
CD3+CD8+
1,47
94,7
3,13
0,974
51,4
B
% of CD3+CD8+ cell proliferation
% of CD3+CD4+ cell proliferation
***
ns
Stim
NS
tolDC
Mreg
MDSC

Figure 5

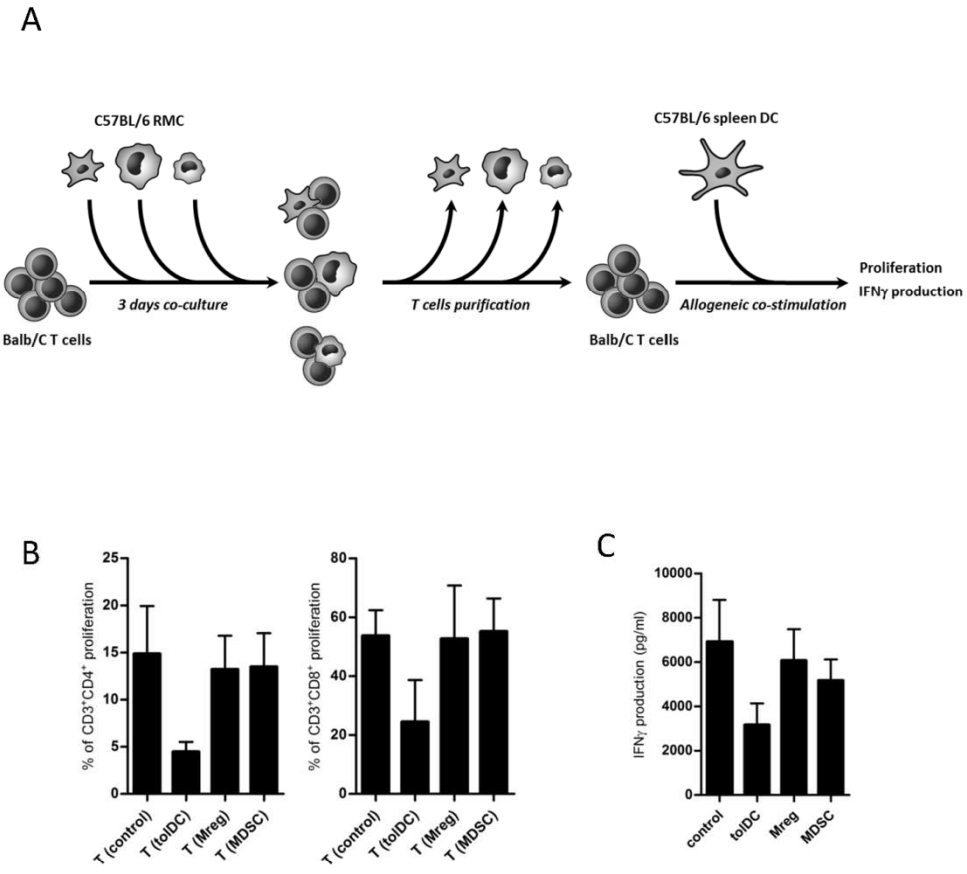
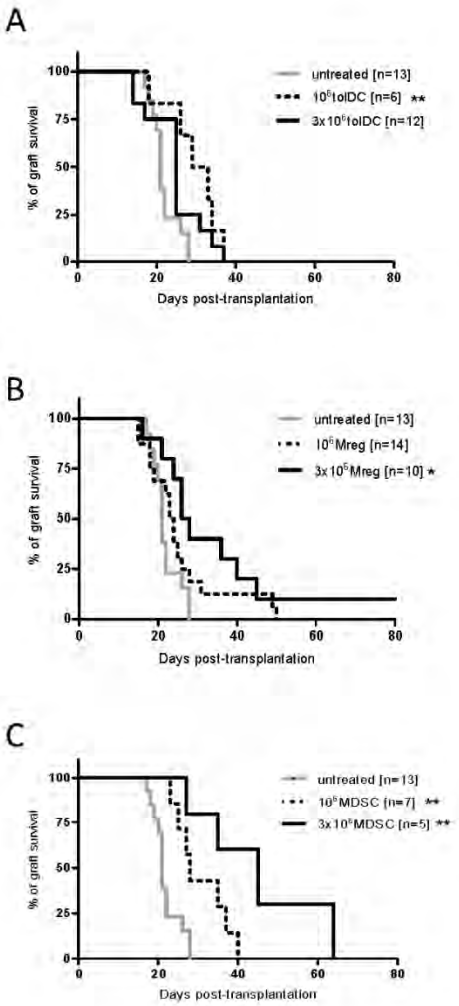
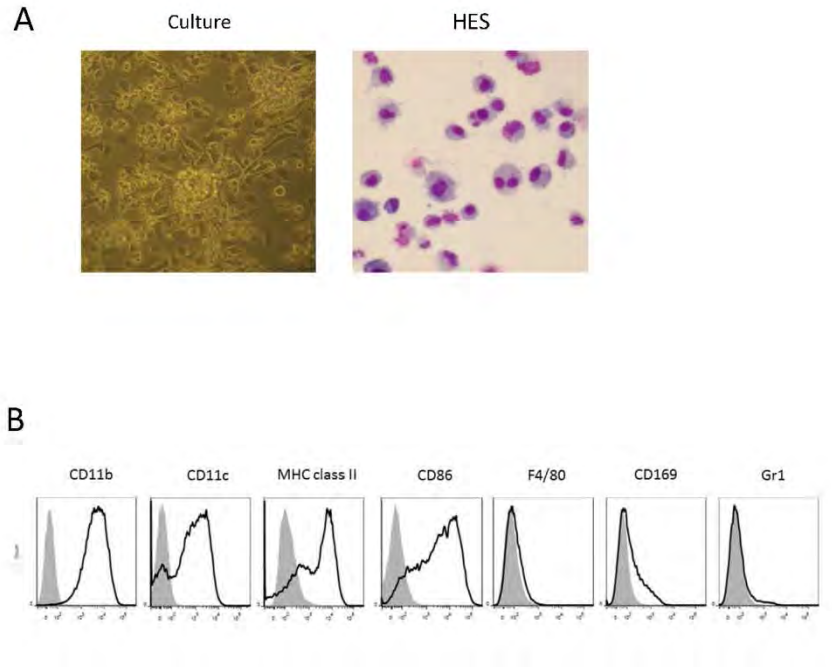

A
C57BL/6 RMC
C57BL/6 spleen DC
3 days co-culture
T cells purification
Allogeneic co-stimulation
Proliferation
IFNγ production
Balb/C T cells
Balb/C T cells
B
% of CD3+CD4+ proliferation
% of CD3+CD8+ proliferation
T (control)
T (tolDC)
T (Mreg)
T (MDSC)
C
IFNγ production (pg/ml)
control
tolDC
Mreg
MDSC

Figure 6


A
100
75
50
25
0
% of graft survival
0
20
40
60
80
Days post-transplantation
untreated [n=13]
10⁶tolDC [n=6] **
3x10⁶tolDC [n=12]
B
100
75
50
25
0
% of graft survival
0
20
40
60
80
Days post-transplantation
untreated [n=13]
10⁶Mreg [n=14]
3x10⁵Mreg [n=10] *
C
100
75
50
25
0
% of graft survival
0
20
40
60
80
Days post-transplantation
untreated [n=13]
10⁶MDSC [n=7] **
3x10⁵MDSC [n=5] **

Supplementary figure 1



Supplementary Figure 2

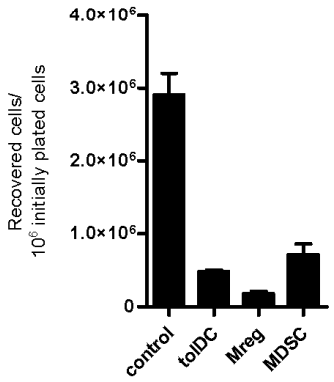


Table 10. Summary of phenotypic and functional characteristics of each RMC type

		TolDC	Mreg	MDSC
RMC characteristics	Morphology	Round-shape, prominent cytoplasm, small prolongations	Central body, eccentric nuclei, some prolongations	Heterogeneous
	Phenotype	$CD11b^{+}$ $CD11c^{+}$ MHC class II^{low}	$CD11b^{+}$ $F4/80^{hi}$ $CD169^{+}$ $CD11c^{+}$ MHC class II^{low}	$CD11b^{+}$ $Gr1^{+}$ $CD11c^{-}$ MHC class II^{-} $CD86^{low}$ $F4/80^{-}$
	Maturation resistance	yes	no	yes
	Endocytosis/degradation	yes	yes	no
T cells co-culture with RMC	Proliferation induction	no	no	at low ratios
	Activation markers	no	yes	yes
	IFNγ production	no	no	yes
	Fas expression	low	yes	yes
	Death induction	low	low	yes
	Suppression polyclonal stimulation	yes	yes	yes/low
T cells co-culture with RMC re-stimulation	Proliferation induction	low	yes	yes
	IFNγ production	low	yes	yes

DISCUSSION AND CONCLUSION

Organ transplantation is the best strategy to treat organ failure. From the very first transplant, what would become the most important problem of organ transplantation arose. Host immune system recognizes alloantigens present in the transplanted graft as non-self antigens, therefore initiating a strong immune response directed towards the graft which leads to rejection, rendering the organ non-functional.

A recent approach to induce graft survival in the long term has focused on cell therapy. Cell therapy consists in the *ex vivo* modification or culture of cell types under tolerogenic conditions, therefore generating a final cellular product that can be administered to patients in order to achieve a state of tolerance towards the graft. The advantage of cell therapy over classical immunosuppressive treatments relies on the fact that it does not consist on a passive mechanism of effector cell depletion, but represents an active mechanism, as suppressor cells can modify the graft environment by the secretion of anti-inflammatory molecules, or they can induce or expand regulatory cells, therefore inducing long-lasting treatments, and without side-effects. Moreover, cell therapy provides antigen-specific tolerance instead of a generalized host immunosuppression.

Autoimmunity is another disorder where cell therapy arises as a promising strategy. The aim of therapies in autoimmunity consists on limiting an immune response of the host against self-organs. Therefore cell therapy can be used to induce peripheral tolerance towards self antigens. Although both situations share the same objective, the way to address cell therapy is different, as in the case of autoimmunity cell therapy is applied once the disease is ongoing.

Regulatory Myeloid cells, as regulatory macrophages (Mreg) myeloid derived suppressive cells (MDSC) and tolerogenic dendritic cells (tolDC), appear to be good candidates to induce a regulation of the immune response. During my thesis I have generated and characterized RMC and I have compared their *in vitro* and *in vivo* effect in order to better understand their mechanisms of action and optimize their utilization for immunoregulation.

In previous studies, most of the *in vitro* differentiated tolDC are generated in presence of GM-CSF and IL-4. Lutz and collaborators demonstrated that DC obtained with GM-CSF alone displayed a more immature phenotype and were able to induce a prolongation of allograft survival on a mouse transplant model (Lutz et al. 2000). Following that discovery, our laboratory has focused on the generation and characterization of tolDC from mouse bone marrow cells (Segovia et al. 2014) and from human monocytes (Moreau, Varey, Bouchet-Delbos, et al. 2012) in the presence of low doses of GM-CSF. We have shown in mice that low GM-CSF tolDC cells display immature phenotype and are efficient to prolong allograft survival when they are injected in autologous way one day before the transplant in combination with suboptimal doses of immunosuppressor treatment (Segovia et al. 2014)(Baas et al., 2014)

Most of the work using Mreg as cell therapy in transplantation comes from the group of JA Hutchinson. They have focused on the regulatory population of macrophages obtained by culturing bone marrow or blood precursors 7 days with low dose of M-CSF and stimulated with IFNγ during the last 24h of the culture, both in mouse and humans respectively (J. A. Hutchinson et al. 2011; Riquelme et al. 2013). Donor Mreg treatment in a mouse fully mismatch cardiac transplant model efficiently prolongs allograft survival when Mreg are administered 8 days before the transplant (Riquelme et al. 2013). On the contrary, the authors found that recipient derived Mreg did not prolong allograft survival in this setting. Mreg has also been administered to two kidney transplant patients in a clinical trial, proving its feasibility and efficiency (J. a Hutchinson et al. 2011a).

There are multiple protocols to generate MDSC *in vitro*. MDSC obtained by culture of bone marrow precursors in the presence of GM-CSF and IL-6 for 4 days displayed the most suppressive function (Marigo et al. 2010). Multiple injections of those cells have been shown to significantly prolong islet transplant survival (Marigo et al. 2010).

In the first part of this study, I have compared the phenotype and the capacity to prolong allograft survival of the three *in vitro* generated regulatory myeloid cells, in order to evaluate the most promising cell type for therapy in transplantation. This is of the most importance, as to date, there are no studies were mechanisms and efficacy of regulatory myeloid cells are directly compared.

The second part has consisted in the comparison of the efficacy of cell therapy using MDSC in two completely different mouse models of autoimmunity and transplantation.

Part I: Comparison of RMC as transplantation therapy

During this study, I have compared the three types of RMC which are under consideration or already developed for clinical use as cell therapy. Therefore, this work has consisted on the *in vitro* generation of three types of RMC: tolDC, Mreg and MDSC. Their *in vitro* characteristics and potential to inhibit T cell activation have been tested and their *in vivo* capacity to prolong graft survival has been compared. To our knowledge, it is the first time that direct comparison is performed. The fact of manufacturing the three cell types at the same laboratory and by the same person allows a direct comparison of their characteristics and their efficacy.

1.1 *In vitro* RMC generation and characterization

I have generated RMC from bone marrow cells in different culture conditions. Our results show that tolDC cluster together and display typical DC markers, as they are MHC class II$^+$, CD11c$^+$, CD11b$^+$ and CD169$^-$ Gr1$^-$. Mreg cell culture evidence the presence of non-clustered cells, displaying a phenotype characteristic of macrophages, as they express MHC class II$^+$, CD11c$^+$, CD11b$^+$, F4/80$^+$,CD169$^+$ but are Gr1$^-$. MDSC were generated as previously described (Marigo et al. 2010). MDSC is a heterogeneous cell population, which we confirmed by microscopic observation. They displayed the typical phenotype CD11b$^+$, Gr1$^+$, CD11c$^-$, MHC class II$^-$.

In order to *in vitro*, characterize the generated RMC, I have evaluated two well-known characteristics of myeloid cells: their capacity to become fully activated after their encounter with pro-inflammatory stimuli and their capacity to endocyte and degrade antigens.

With a view to use i*n vitro* generated RMCs for cell therapy, it is essential to obtain cells which are resistant to maturation. We have shown that, after 48h exposure to LPS, tolDC and MDSC do not upregulate co-stimulatory molecules, as CD80, CD86 of CD40, and the expression of MHC class II remains constant. Exposure of Mreg to LPS did not induce the upregulation of B7 family co-stimulatory molecules CD80 and CD86. On the contrary, they increased the expression of CD40 and the upregulation of MHC class II molecules. LPS is an inducer of the second signal needed for macrophages polarization towards M1 phenotype. However, macrophages need a first signal delivered by IFNγ in order to trigger classical activation. Even more, it has been shown that macrophages activated with IFNγ display suppressive activity *in vitro* and *in vivo* (Riquelme et al. 2013).

Another important property of myeloid cells, especially APCs, is antigen endocytosis and degradation capacity. We have shown that tolDC and Mreg keep a high endocytic and degradative capacity which defines myeloid cells, whereas endocytosis and therefore degradation is not detectable by MDSC. MDSC have been shown to be able to induce antigen specific tolerance (Nagaraj et al. 2007). As we have demonstrated that they are not able to endocyte, a possible mechanism explaining

that effect may be trogocytosis i.e. a process of membrane exchange that can contain MHC/peptide complexes (Hudrisier et al. 2001).

The mentioned mechanisms are important in both extremes of immune responses. Immunogenic and tolerogenic capacities of APCs involve their ability to present antigenic peptides complexed with MHC molecules at the surface. In the first case, antigen presentation comes along with high expression of co-stimulatory molecules in order to fully activate T lymphocytes. In the second case, when antigen presentation is not associated with an over-expression of co-stimulatory molecules, antigen-specific T cells recognize the peptide/MHC complex but they cannot get fully activated, due to the lack of second signal, resulting in anergy or apoptosis, therefore leading to tolerance induction.

1.2. Mechanisms involved in RMC function

RMC can suppress immune responses through a variety of mechanisms, as already described in the introduction. Those mechanisms involve expression of immunomodulatory molecules or enzymes, which stabilize and maintain the tolerogenic phenotype of RMC and, in turn, induce the secretion of anti-inflammatory cytokines. Other active mechanisms have also been described, which affect effector T cells, either by direct death or apoptosis induction or by blocking their activation/proliferation capacity. Synergy between different mechanisms can also take place. In this study, we have assessed some of the possible mechanisms of tolerance induction. We have shown that *in vitro* mechanisms to induce effector T cell unresponsiveness vary between each RMC type.

First of all, I have observed that all three cell types were suppressive *in vitro*, as assessed by their ability to inhibit strong anti-CD3/anti-CD28 polyclonal T cell proliferation. As a perspective, in order to elicit the mechanisms underlying the suppressive function, it would be interesting to determine if anti-inflammatory cytokines are secreted to the supernatant of this assay and, if so, to block them using mAbs, to confirm their involvement. Another mechanism described to mediate suppression is cell-to-cell contact. Transwell assays would be a good strategy to elicit the contribution of this mechanism to RMC inhibition of polyclonal activation.

A classical feature of tolDC is the induction of T cell hyporesponsiveness. We have confirmed tolDC hypostimulatory capacity by performing allogeneic co-cultures. TolDC did not induce T cell proliferation either at high or at low ratios. Hyporesponsiveness induction was associated with the absence of IFNγ secretion by T cells. Neither $CD4^+$ nor $CD8^+$ T lymphocytes displayed CD69 or CD25 activation markers. We did not find upregulation of Fas by T lymphocytes, meaning that clonal deletion may not be the mechanism involved in tolDC suppressive function. T cell stimulation by

immunogenic DCs three days after T cell co-culture with tolDC did not restore proliferation or IFNγ secretion by T lymphocytes, suggesting that tolDC were responsible of T cell anergy induction.

Mregs were able to efficiently inhibit polyclonal T cell proliferation, and did not induce T cell proliferation or IFNγ production. However, T lymphocytes co-cultured with Mregs upregulated activation markers and Fas at the surface. It has already been shown that expression of activation markers do not always correlate with T cell proliferation (Gaus et al. 1994). Mechanisms involved in T cell hyporesponsiveness were not T cell death induction or anergy, as T cells primed by Mregs were able to proliferate in response to a second stimulus given by immunogenic DC, which enhanced IFNγ production. Riquelme et al. found that co-culture of T cells with immunogenic DC after 3 days of co-culture with Mreg did not enhance the production of IFNγ by allogeneic T lymphocytes (Riquelme et al. 2013), which means that two different mechanisms of tolerance induction may be occurring, which most probably depends on the modification of gene expression by the presence of IFNγ at the end of the culture. Therefore, other mechanisms, probably involving cytokine release or cell-to-cell contact may be involved in the *in vitro* T cell hypo-response induced by Mreg co-culture. Those results do not support the hypothesis of long term tolerance mechanism induction, but he hypothesis that Mreg need to be present in order to control T cell proliferation.

MDSC displayed low stimulatory capacity of both, $CD4^+$ and $CD8^+$ T lymphocytes when co-cultured at high ratios. On the contrary, when MDSC were co-cultured at low ratios, therefore less MDSC per T cell, they induced T lymphocyte proliferation. This result suggests that MDSC may need cell to cell contact, provided when they are cultured at high ratios, to mediate their suppressive function. Similarly to the results shown by Mreg co-cultures, T lymphocytes were able to upregulate activation markers after 4 days of co-culture, as well as Fas molecules. MDSC induced some IFNγ secretion in co-cultures, suggesting a possible polarization towards a Th1 profile. We could also observe a specific $CD4^+$ and $CD8^+$ deletion, suggesting that one possible mechanism of MDSC to induce tolerance could be induction of apoptosis of activated T lymphocytes. To confirm that mechanism, other experiments should be performed, like annexin or caspase staining.

A long-lasting mechanism of tolerance induction by regulatory myeloid cells is the expansion of regulatory T cells. In our experiments, we did not find a significant increase of $CD4^+FoxP3^+$ Treg cells in any co-culture. As a perspective, other regulatory T cell phenotypes should be further analyzed, as induction of Tr1 cells or $CD8^+CD11c^+$, which we found increased by tolDC treatment *in vivo* (Segovia et al. 2014).

Therefore, in this study, we found different possible mechanisms involved in the suppressive capacity of each RMC type. The contribution of other known mechanisms and inhibitory molecules or cytokines should be further addressed.

1.3. *In vivo* effect of RMC

The three types of RMC were tested in autologous condition in a mouse model of male onto female skin graft. Our results have shown that only one injection of autologous RMC the day before the transplant is enough to induce a prolongation of allograft survival.

MDSC were the most effective cell type to induce allograft survival, and its effect was dose-dependent, as injection of 3 x 10^6 cells could improve the prolongation of graft survival compared to 10^6 cells. Those results are in line with another study where multiple injections of MDSC significantly prolong allograft survival (Marigo et al. 2010). It would be important to test if multiple injections in our setting could improve the effect of MDSC in inducing allograft survival prolongation.

Mreg administration was effective only when 3 x 10^6 cells were injected. Other studies injecting Mreg for transplantation used 5 x 10^6 cells and Mregs derived from the donor (Riquelme et al., 2013), and the transplant model was fully mismatched. Higher doses will be tested in our model.

We had previously shown that tolDC injected alone did not improve graft survival compared to untreated mice in a model of mouse skin graft (Segovia et al. 2014) and neither in a model of islet transplant (Baas et al., 2014). Surprisingly, in our new experiments, we have found a small but significant improvement in graft survival. On the contrary, higher doses of tolDC could not improve the effect.

Part II. Comparison of MDSC cell therapy in two mouse models

Another part of this work has consisted on the comparison of the therapeutic potential of MDSC in two different models of mouse auto- and allo-immunity. The potential of MDSC to inhibit immune responses has widely been studied in tumor development. In that setting, the aim of therapeutical products is to boost immune responses to eliminate tumoral cells. Therefore, MDSC elimination by *in vivo* targeting is becoming of the most interest (Gabrilovich & Nagaraj 2009). On the contrary, in auto- and allo-immunity, the aim is to induce tolerance towards auto- or allo-antigens. In that context, it is essential to suppress host immune responses; therefore, MDSC innate potent suppressive capacity has been considered as a good strategy to achieve immunological tolerance in autoimmunity (Cripps & Gorham 2011) and allogeneic transplantation (Dilek et al. 2010).

In vitro MDSC generation with GM-CSF and IL-6 showed strong *in vitro* and *in vivo* inhibitory capacity in a mouse model of islet transplantation (Marigo et al. 2010). We generated MDSC following the same protocol. Phenotype of the obtained MDSC was in accordance to what had already been described, as cells expressed the markers CD11b and Gr-1.

MDSC were able to suppress polyclonal T cell proliferation *in vitro*. On the contrary, we could not observe a suppression of antigen specific $CD8^+$ T cell responses in an *in vivo* proliferation assay, where MDSC were adoptively transferred to OT-I mice immunized with OVA(Ovalbumin)-transfected COS cells. We studied the possibility that MDSC were affecting the function of $CD8^+$ T cells, differentiating them into cytotoxic cells, instead of suppressing their proliferation. However, we did not find an increase in the percentage of specific lysis of target cells loaded with OVA peptide in an *in vivo* cytotoxic assay of mice treated with MDSC compared to untreated mice.

In order to assess the therapeutic potential of MDSC in a physiological context without combination of IS treatment, we adoptively transferred MDSC in two different models where a beneficial role for MDSC subset had already been described: autoimmunity (Yin et al. 2010) and transplantation (Marigo et al. 2010).

Administration of MDSC in a mouse type-1 diabetes model did not prevent diabetes development, neither one nor multiple cellular injections. Surprisingly, loading of MDSC with the antigenic peptide accelerated the development of autoimmunity. Our results are in line with the observation made by Yi et al., where authors found an expansion of MDSC associated with the development of EAE (Yi et al. 2012). They found that MDSC efficiently expanded Th17 $CD4^+$ T cells that contribute to the pathogenesis of EAE. Targeting of MDSC *in vivo* significantly reduced the severity of the disease.

On the contrary, adoptive transfer of MDSC into a mouse minor histocompatibility skin graft model efficiently prolonged graft survival. As performed by Marigo et al., multiple injections of MDSC

were administered (Marigo et al. 2010). Two independent intravenous injections of 10^6 MDSC at the time of the transplant were enough to prolong allograft survival. Even more, 5 injections of 4 x 10^6 MDSC increased the beneficial effect. This suggests a dose-dependent effect of MDSC administration. The potential to induce long-term acceptance of the graft has already been demonstrated by a continuous treatment with MDSC (Marigo et al. 2010). Therefore, we can hypothesize that increasing the number of injections or the dose could lead to a better outcome of the graft in transplantation. We studied the *in vivo* mechanisms that lead to the prolongation of allograft survival. Interestingly, we did not find an increase in the $CD4^+FoxP3^+$ Treg cell population, as previously described (Adeegbe et al. 2011) nor an increase in $CD8^+$ donor-specific T cells. Instead, we found an over-activation of T and myeloid cells (mainly in the spleen) in MDSC treated mice compared to untreated grafted mice, suggesting that MDSC could create a window of systemic exhaustion, allowing the graft to survive as long as the treatment is carried on. Therefore, this mechanism would exclude any long-term tolerance induction mechanism.

Those different outcomes observed in the two animal models lead to two main conclusions. First of all, cell therapy must be carefully considered, depending on the clinical setting. In this study, we have shown that whereas MDSCs effect through over-activation of the immune system is beneficial in transplantation, it is detrimental concerning a clinical setting based on the development of a fast destructive autoimmune response. This observation brings about the difficulty of treating autoimmune disorders, as once the disease is detected, the proinflammatory environment is already existent; therefore, when cell therapy is applied, it is not in a preventive way, but in a curative way, which makes the treatment less effective. On the other hand, cell therapy can be applied before or around the time of transplantation, therefore being able to create an appropriate environment for injected cells to act. The second conclusion concerns the need to consider other protocols to generate MDSC that take into account the need to preserve the suppressive capacity and the immature state of MDSC once injected. MDSC have been shown to differentiate into mature DC *in vivo* in the absence of sustained inflammation (Sade-Feldman et al. 2013). Protocols stimulating MDSC culture (Greifenberg et al. 2009) prior to injection have shown to enhance MDSC suppressive capacity and to block their capacity to develop into DC. But whether cells cultured with pro-inflammatory signals represent a safe strategy for human cell therapy remains to be elucidated.

Part III. Important points to consider before applying cell therapy to transplantation

- Choice of donor or recipient precursors

Regarding tolDC cell therapy, most of the literature is based on the effect of injection of donor-derived tolDC or recipient derived cells pulsed with donor antigens (Moreau, Varey, Bouchet-Delbos, et al. 2012). The limited data available for the *in vitro* generation of Mreg and their application in the transplantation setting involve only the results obtained by the group of J. Hutchinson. In their experiments, they have also derived Mreg cells from donor sorted bone marrow precursors (Riquelme et al. 2013) and in humans, they use donor blood monocytes (J. A. Hutchinson et al. 2011). Injection of recipient derived Mregs a week before transplantation did not induce allograft survival prolongation. MDSC are derived *in vitro* from both, donor and recipient peripheral blood in humans and bone marrow in mice (Obermajer & Kalinski 2012). Even though, the majority of studies use *in vivo* induced MDSC transfer or simply the *in vivo* induction of MDSC in recipient animals, without *ex vivo* modification (Rosborough et al. 2014).

Although those strategies have been shown to be efficient at inducing allograft survival prolongation, we believe that recipient derived cell therapy may represent an advantage regarding multiple points considering safety and efficacy. The first important thing is that, by using autologous cell therapy, there is no risk of donor sensitization due to the presence of contaminant cells that could be contained in the final cell product. Another point is that there is less risk of elimination of the injected cells by host's immune system, as non-self recognition cannot occur. Indeed, in the case of tolDC therapy, it has been shown that donor-derived tolDC are rapidly eliminated by host NK cells, suggesting that their tolerogenic effect may be due to reprocessing of donor antigens by recipient DCs (Divito et al. 2010; Z. Wang et al. 2012). A third consideration involving tolDC therapy is the fact that in order to be able to migrate to lymphoid organs and present antigens to T cells, immature DC must be "alternatively activated", which involves their *in vitro* stimulation with LPS or other cytokinic cocktails before their injection (Anderson et al. 2008; Raïch-Regué, Naranjo-Gómez, et al. 2012). In contrast, autologous tolDC do not need to be activated in order to be efficient, therefore reducing the risk of maturation after injection, avoiding becoming immunogenic (Pêche et al. 2005; Segovia et al. 2014).

According to that, in this work we have injected unpulsed recipient-derived regulatory myeloid cells as cell therapy for transplant treatment. We have shown that all the three tested RMC types were able to prolong allograft survival in a mouse model of minor histocompatibility skin transplant when they were injected in an autologous way.

- Time of administration and doses

In transplantation, the time of cell administration is another important parameter. Normally, the first (or the only) cell injection is performed before the time of transplantation. Depending on the type of cell injected and if it derives from donor or recipient, some differences are reported.

There are some reports where authors inject alloantigen-pulsed autologous tolDC 7 days before transplantation (Garrovillo et al. 1999). In the case of unpulsed autologous tolDC, our group performed a study comparing the efficacy of different administration timings (Bériou et al. 2005). Allograft survival in a rat cardiac transplant model was not improved by the administration of repeated injections of tolDC (days -1 and 4 or -1, 6 and 13) nor by the injection of cells on the day of transplantation when compared with one single injection at day -1. Donor-derived Mreg injection in transplantation has been shown to be effective for fully mismatch allograft survival prolongation when administered 8 and 35 days before transplantation (Riquelme et al. 2013). The sources of MDSC treatment in transplantation are varied (*in vitro* generated or *in vivo* induction/transfer). Considering *in vitro* bone marrow derived MDSC, it is common to perform multiple cell injections. In a mouse islet transplant model cells have been injected on days 0, 7, 14 and 21 (Marigo et al. 2010). In a model of GVHD cells have been injected at day 0 (Highfill et al. 2010).

Therefore, considering that the only available data regarding autologous unpulsed cell injection efficacy did not show an improvement in allograft survival either by multiple injections or by different administration timing, we choose to administer the three types of RMC in an autologous way one day before the transplant.

Regarding cell doses to be administered, we must consider that it may vary depending on the animal model used. Autologous tolDC were administered to rats at 3, 7 and 15 million cells per animal, without displaying an improvement in allograft survival (Bériou et al. 2005). There is no data available concerning the dose-effect of Mreg injection in mice. Considering MDSC in islet allotransplantation, 10^7 cells were injected multiple times in recipient mice (Marigo et al. 2010). A dose-dependent improved outcome of MDSC injection (between 2 and 6 million) was observed in the setting of GVHD (Highfill et al. 2010).

In the present study, we compared several doses and administration times of MDSC in a mouse skin transplantation model. We show that two injections of 10^6 autologous MDSC, at days -1 and 6, where enough to prolong allograft survival. We observed that this effect was enhanced with 5 weekly consecutive injections and using a dose of 4 x 10^6 cells per injection. Therefore, we cannot conclude if the improved effect is due to the dose or to the increased frequency of injection.. We demonstrated that a single injection of 5 x 10^6 LPS-treated MDSC the day of the transplant was able to significantly prolong allograft survival, displaying a better outcome than twice injection of non-activated MDSC. The greater efficacy of stimulated MDSC in transplantation had already been

demonstrated and attributed to the fact that stimulation preserves MDSC's suppressive capacity (Greifenberg et al. 2009).

To compare the *in vivo* suppressive capacity of the three RMC types, we tried two different doses, either 10^6 or 3 x 10^6 cells. tolDC displayed better outcome when 10^6 cells were injected. On the contrary, injection of 3 x 10^6 Mreg cells was needed to prolong allograft survival. Finally, both doses, 10^6 and 3 x 10^6 MDSC injection significantly prolonged allograft survival, and in a dose-dependent manner. Interestingly, we could demonstrate that only one injection of MDSC was able to prolong allograft survival. This may represent an advantage in the clinical setting.

-Trafficking and migration

The ability of RMC to migrate to the adequate anatomical locations will determine the efficiency of the treatment. It has been described that expression of several surface molecules, in particular chemokine receptors, guides RMC towards secondary lymphoid organs, where antigen-specific T cells recognize MHC/peptide complexes, and RMC can exert their regulatory function. In other settings, RMC could migrate directly to the graft in order to pick up antigens or to mediate their regulatory function.

From experiments performed by our group, we could evidence that autologous tolDC injected in combination with anti-CD3 mAb treatment in a skin graft mouse model migrated first to the skin graft (detected at day 7 after transplant) and then to graft draining lymph nodes (at day 14), where they presumably present antigens captured in the graft to alloreactive lymphocytes in a tolerogenic way (Segovia et al. 2014). Our group also showed autologous tolDC migration to the spleen in a rat cardiac allograft model (Pêche et al. 2005). TolDC have been shown to express CCR7, which directs them to secondary lymphoid organs (Garrod et al. 2006).

In mouse, donor Mregs were detected one day after injection, displaying a wide distribution in non-lymphoid organs and the spleen, but were not detected neither in lymph nodes nor in bone marrow. One week after injection, very few cells were detected, and most of them localized in the lungs (Riquelme et al. 2013). Chemokine receptors expressed at Mreg surface are not known. So whether they migrate to secondary lymphoid organs, where they may exert their suppressive functions remains to be determined.

The chemokine receptor pattern of MDSC, CCR2 and CX3CR1, direct them to sites of inflammation (Movahedi et al. 2008; Huang et al. 2007). They also express the integrin CD62L and the chemokine receptor CCR7 that may direct them to secondary lymphoid organs (Highfill et al. 2010; Movahedi et al. 2008). To date, their preferential migration is still unknown. In GVHD, *in vitro* generated MDSC traffic to peripheral lymphoid tissues and sites of inflammation (Highfill et al. 2010). In the present study, we tracked injected MDSC in spleen, graft draining lymph nodes and skin graft

14 days after transplant and we were unable to find them. This suggests that MDSC could be rapidly cleared after injection or that they are located into other lymphoid or non-lymphoid tissues.

It would be important to track RMC location at different times after injection, in order to elicit their *in vivo* mechanisms of action: whether they need to traffic to the graft before migrating to secondary lymphoid organs, whether they exert their regulatory functions intragraft or in distal sites, etc. Another very important thing is to define the presence or absence of a range of chemokine receptors at the RMC surface before and after injection, to determine if the *in vivo* environment changes their phenotype, especially their pattern of integrins and chemokine receptor expression, which directs cells towards specific locations.

- Synergy with immunosuppressive drugs

Even if immunosuppressor drugs mainly target T and B lymphocytes response, they have also been shown to exert varied effects on host myeloid cells differentiation and function. At the present time, there is no proof that cell therapy alone can be applied in the clinical settings. Ongoing cell therapy clinical trials to assess the efficacy of cell therapy are associated with an immunosuppressive protocol using lower doses of immunosuppressive agents than the currently used. Therefore, as cells are still administered in conjunction with immunosuppressive agents, it is worth analyzing their effect on *ex vivo* generated regulatory myeloid cells in the context of cell therapy.

The influence of IS drugs on tolDC has been widely assessed. On the contrary, studies of their influence on Mregs and MDSC are still limited. The main problem of the *in vivo* injection of immature DC is their risk of maturation once injected. Immature DC could become immunogenic DC able to activate an immune response against a foreign antigen, instead of tolerizing it (Barratt-Boyes et al. 2000). The differentiation of tolDC *in vitro* in the presence of some of these IS drugs has been shown to generate cells that maintain a stable immature phenotype (Rosborough et al. 2014). But the most relevant studies are the ones that address how IS drugs target RMC after *in vivo* injection.

Therefore, we should carefully consider the association of IS drugs to RMC therapy if we want to combine both, and study their effect in phenotype and function modification *in vivo*.

To be able to control immune responses against tissues in diseases as autoimmunity and transplantation is of the most relevance. Current therapies used in organ transplantation allow controlling acute responses but are unable to generate long-term tolerance of the allograft. In that context, cell therapy using regulatory immune cells is a promising strategy to control alloresponses. Although multiple studies show the *in vivo* efficacy of cell therapy, their *in vitro* and *in vivo* mechanisms of action are still poorly studied.

During this thesis, we have compared the *in vitro* efficacy of three regulatory myeloid cells to control T lymphocyte response and their capacity to induce allograft survival *in vivo*. We have demonstrated that each population display different phenotypes and mechanisms of action *in vitro*, and that their capacity to induce graft survival prolongation is not the same.

We have also shown that cell therapy using MDSC can have to different outcomes depending on the model used. Whereas MDSC are unable to control *in vivo* the development of autoimmune type 1 diabetes in mouse, they are efficient at graft survival prolongation in a mouse skin graft model.

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ANNEXES

American Journal of Transplantation 2014; XX: 1–11
Wiley Periodicals Inc.

doi: 10.1111/ajt.12708

Autologous Dendritic Cells Prolong Allograft Survival Through *Tmem176b*-Dependent Antigen Cross-Presentation

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The administration of autologous (recipient-derived) tolerogenic dendritic cells (ATDCs) is under clinical evaluation. However, the molecular mechanisms by which these cells prolong graft survival in a donor-specific manner is unknown. Here, we tested mouse ATDCs for their therapeutic potential in a skin transplantation model. ATDC injection in combination with anti-CD3 treatment induced the accumulation of $CD8^+CD11c^+$ T cells and significantly prolonged allograft survival. TMEM176B is an intracellular protein expressed in ATDCs and initially identified in allograft tolerance. We show that *Tmem176b*$^{-/-}$ ATDCs completely failed to trigger both phenomena but recovered their effect when loaded with donor peptides before injection. These results strongly suggested that ATDCs require TMEM176B to cross-present antigens in a tolerogenic fashion. In agreement with this, *Tmem176b*$^{-/-}$ ATDCs specifically failed to cross-present male antigens or ovalbumin to $CD8^+$ T cells. Finally, we observed that a *Tmem176b*-dependent cation current controls phagosomal pH, a critical parameter in cross-presentation. Thus, ATDCs require TMEM176B to cross-present donor antigens to induce donor-specific $CD8^+CD11c^+$ T cells with regulatory properties and prolong graft survival.

Keywords: Autologous dendritic cells, cellular therapy, cross-presentation, ion channel

Abbreviations: ATDC, autologous tolerogenic dendritic cell; BMDCs, bone marrow dendritic cells; DCs, dendritic cells; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; PMA, phorbol myristate acetate; Treg, regulatory T cell; V-ATPase, vacuolar ATPase

Received 05 November 2013, revised 18 December 2013 and accepted for publication 07 January 2014

Introduction

Innovative therapeutic strategies are needed to diminish the harmful effects of immunosuppressive drugs used to treat patients with autoimmune diseases and transplant recipients. Cell therapy using dendritic cells (DCs) is a promising approach to decrease doses of immunosuppressives in these settings (1,2). Our group has previously characterized, phenotypically and functionally, rat and mouse autologous tolerogenic dendritic cells (ATDCs) (3–6). We showed that ATDC therapy, in association with suboptimal doses of immunosuppression, induces a prolongation of allograft survival in a donor-specific manner (3,4,7,8). It is important to note that, in these studies, ATDCs were not pulsed with donor antigens. This strategy is most clinically relevant because cells can be prepared in advance from patients placed on the transplant waiting list (9). We are currently developing a clinical protocol to inject ATDCs into kidney graft recipients in a phase I/II clinical trial (The ONE Study, 7th Frame Program, European Commission). Other ongoing clinical trials examine the potential of ATDCs in autoimmune patients (10,11).

In the current study, we wished to determine the molecular mechanisms by which mouse ATDCs can prolong graft survival in a donor-specific manner. We observed that ATDC injection generates donor-specific $CD8^+$ regulatory T cells (Tregs). We investigated the role of *Tmem176b*, a gene encoding for a transmembrane protein of unknown function, in which we previously identified as preferentially

expressed in immature DCs (12,13). We found that ATDCs required *Tmem176b* expression to exert their immunoregulatory function *in vivo*. We further show that TMEM176B is responsible for a phagosomal cation current that is needed to control phagosomal pH in DCs, a critical parameter in the cross-presentation pathway (14–20).

Materials and Methods

Mice

A *Tmem176b*$^{-/-}$ (KO) mouse was generated in the 129/SvJ strain and heterozygous mice were backcrossed for 10 generations onto the C57BL/6 background (Janvier, Saint Berthevin, France). WT C57BL/6 littermate controls were obtained in our animal facility. MataHari CD8$^+$ TCR transgenic and *β2-microglobulin*$^{-/-}$ (B6.129P2-B2m^{tm1Unc}/J) mice were kindly provided by Olivier Lantz. All animal experiments were performed under specific pathogen-free conditions in accordance with the European Union Guidelines. All animal studies were conducted according to the guidelines of the French Agriculture Ministry. The studies were approved by the Veterinary Departmental Services committee, La Chapelle-Sur-Erdre, Paris, France (no. E.44011, 75-1554), and all experiments were carried out in compliance with the ethical rules of the INSERM.

Bone Marrow DCs

Bone marrow DCs (BMDCs) were generated as previously described (6). For the sake of clarity, BMDCs are referred to as ATDCs along the text. Briefly, bone marrow precursors were cultured for 8 days in the presence of low doses of granulocyte macrophage colony-stimulating factor (0.4 ng/mL). By day 8, adherent cells were recovered and used for *in vitro* and *in vivo* experiments.

Skin transplantation and treatments

C57BL/6 male tail skin was grafted on female recipients as previously described (21). One million WT or *Tmem176b*$^{-/-}$ (KO) female ATDCs were injected intravenously (i.v.) the day before transplantation. One microgram anti-CD3 antibody (145-2C11, kindly provided by J. Bluestone) per mouse was injected intraperitoneally at days −1, +1, +3, +5 and +7 following skin transplantation. Graft survival was followed every other day.

Reagents and antibodies

Endotoxin-free OVA protein was from Profos (Regensberg, Germany). OVA (SIINFEKL), Smcy (KCSRNRQYL) and Uty (WMHHNMDLI) peptides were from Polypeptide (Strasbourg, France). Fluorescein isothiocyanate (FITC), PKH-26 and latex beads amine-modified polystyrene fluorescent red were from Sigma (St. Quentin Fallavier, France). Fluoprobes 647 was from Fluoprobes (Montluçon, France). DDAO-SE and OVA-Alexa 647 were from Molecular Probes (Montluçon, France). Anti-CD4 Pacific blue, anti-CD8 PECy7, anti-CD69 Biotin, anti-Vα2 FITC, CD19 APC and Annexin V APC were from BD (Le Pont-De-Claix, France). Anti-cathepsin S antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). H-2D^b WMHHNMDLI (Uty), H-2D^b KCSRNRQYL (Smcy), H-2K^b SIINFEKL (OVA), H-2K^b VNHRFTLV (*Trypanosoma Cruzi*, control) pentamers were from Proimmune (Oxford, UK). Pentamers stainings were performed according to the manufacturer's instructions (Pro5®Recombinant murine MHC Pentamer; ProImmune, Oxford, UK). The pentamers used in this study were H-2D^b WMHHNMDLI (Uty) and H-2D^b KCSRNRQYL (Smcy).

Adoptive cell transfer

CD8$^+$ T cells from graft-draining lymph nodes (axillary lymph node) were purified using a FACSAria (BD). Purified CD8$^+$ T cells (>95% purity) were injected i.v. (2×10^5 cells) the day before grafting male skin on female recipients that did not receive any additional treatment.

Antigen presentation assays

Five thousand WT or KO ATDCs were plated in triplicate in 96-well plates and incubated with indicated concentrations of soluble OVA, OVA-coated beads or SIINFEKL peptide for 45 min at 37°C. After extensive washing, cells were treated overnight with 0.25 μg/mL lipopolysaccharide, then washed a second time before adding 5×10^4 DDAO-labeled OT-1 CD8$^+$ or OT-2 CD4$^+$ T cells purified with the AutoMACS device (Miltenyi Biotec, Paris, France). Upon a 4-day culture, proliferation of T cells was analyzed by assessing DDAO dilution in CD8$^+$Vα2$^+$ (OT-1) or CD4$^+$Vα2$^+$ cells (OT-2). In assays based on direct presentation of OVA as an endogenous antigen, ATDCs were electroporated at 300 V, 150 μF (4-mm gap electroporation cuvette, Gene Pulser II apparatus; BioRad, Hercules, CA) with different doses of an *in vitro* synthesized mRNA (mMESSAGE mMACHINE Ultra Kit; Ambion, Austin, TX) coding for a protein fusioning the OVA peptides (for OT-1 and OT-2) and green fluorescent protein (GFP). In HY antigen experiments, 5×10^3 female WT or KO ATDCs were incubated with male *β2-microglobulin*$^{-/-}$ splenocytes at different ratios for 2 h in 96-well plates. After extensive washing, the splenocytes were eliminated while adherent ATDCs remained attached. Purified Uty-specific TCR transgenic CD8$^+$ MataHari T cells were then added (5×10^4 cells). After a 20 h culture, CD69 expression was assessed by flow cytometry on CD8$^+$ T cells.

Endocytosis and phagocytosis measurement by flow cytometry analysis

WT and KO ATDCs were pulsed with different doses of OVA-Alexa 647 for 15 min and then chased for 30 min at 37 or 4°C. To study phagocytosis, ATDCs were pulsed with fluorescent beads (Sigma) at different dilutions.

Measurement of phagosomal pH

Phagosomal pH was measured by flow cytometry analysis as previously described (14). Briefly, 3 μm polybeads amino were covalently coupled with FITC (pH sensitive) and FluoProbes 647 (pH insensitive) and used to pulse/chase cells at 37°C.

Electrophysiology and intra-oocyte pH measurements

Oocytes were surgically removed from MS222 (0.4%)-anesthetized *Xenopus laevis* female and dissociated under gentle agitation by a 2–3 h incubation in an OR2 solution (in mM NaCl 82; KCl, 2; $MgCl_2$, 1; HEPES, 5; pH 7.2) supplemented with collagenase 1A (1 mg/mg). Oocytes were then injected with 40 nL of *in vitro* synthesized *Tmem176b* mRNA at 1 μg/μL (mMESSAGE mMACHINE Ultra Kit). *Tmem176b* was fused to a signal peptide sequence (N-terminal) from pSecTag2B (Invitrogen, Carlsbad, CA) and to V5 + 6-His tags (C-terminal). The day after injection, the oocytes were placed in a pH 8 solution (in mM, NaCl, 100; KCl, 3; $MgCl_2$, 2; HEPES, 15; pH 8) that was changed daily. Two to three days later, currents were recorded in two-electrode voltage-clamp using a geneclamp500 amplifier (Axon Inst., Foster City, CA) interfaced to a personal computer using the Digidata 1200 interface and the pClamp software (ver 7.0; Axon Inst.). Prior to recording, oocytes were incubated in phorbol myristate acetate (PMA) at 100 nM in the pH 8 solution for 20–30 min. Currents were filtered at 100 Hz and digitized at 0.5 kHz before storage and further analysis. During recording, oocytes were continuously superfused with the pH 8 solution. On TMEM176B-expressing oocytes, induction of an inward current was obtained by switching to a pH 5 solution (in mM NaCl, 100; KCl, 3; $MgCl_2$, 2; MES, 15; pH 5).

Statistical analysis

Results were expressed as mean ± SD. Statistical significance was evaluated using a one-way analysis of variance test. $p < 0.05$ was considered significant. Kaplan–Meier tests were performed for survival analysis.

Results

Tolerogenic DCs prolong allograft survival in combination with anti-CD3 treatment

The main objective of this work was to determine the molecular mechanisms that allow ATDCs to prolong graft survival in a donor-specific manner. We used a mouse minor histocompatibility transplantation model in which male skin is grafted onto female recipients. We examined the effect of ATDC injection in recipient mice the day before transplantation. Injection of WT (female) ATDCs had no effect on graft survival as compared to untreated mice (Figure 1A). However, a synergistic effect was observed when female recipients were co-treated with WT ATDCs and peritransplant anti-CD3 antibody therapy. In fact, anti-CD3 antibody treatment alone prolonged skin graft survival, but its effect was significantly improved when associating ATDC injection the day before transplantation (Figure 1A). To understand the mechanism by which ATDC + anti-CD3 therapy prolongs allograft survival we carried out experiments aiming at tracking injected ATDCs *in vivo*. We analyzed the graft, the draining-lymph node and the spleen 7 and 15 days after transplantation. ATDCs were found in skin graft both at days 7 and 15 posttransplantation whereas they could be detected in the draining lymph node only at day 15 (Figure S1). ATDCs were not found in the spleen (data not shown). These observations strongly suggest that injected ATDCs uptake donor antigens in the graft and subsequently migrate to the draining lymph node.

Tolerogenic DCs induce regulatory CD8$^+$ T cells in a Tmem176b*-dependent manner*

We previously identified the gene *Tmem176b* (*Torid*) as overexpressed in tolerated allografts in the rat and preferentially associated with the immature state of DCs (12,13). We generated a *Tmem176b*-deficient mouse (Figure S2) that showed no obvious developmental abnormalities. Strikingly, when ATDCs were generated from *Tmem176$^{-/-}$* (KO) mice, prolongation of skin allograft survival was completely lost (Figure 1A). We then reasoned that injected ATDCs could induce or activate donor-specific Tregs through donor antigen presentation in the graft-draining lymph node. We first quantified the presence of CD4$^+$Foxp3$^+$ T cells. No significant differences were observed between the different treatment groups in the graft-draining lymph nodes (Figure S3). It was reported that male skin graft survival can be prolonged by tolerizing donor-specific CD8$^+$ T cells (22). We then studied different phenotypes associated to CD8$^+$ Tregs. Neither CD8$^+$Foxp3$^+$ (23) nor CD8$^+$CD28$^-$ (24) T cells were induced by ATDC + anti-CD3 treatment (Figure S4). However, CD8$^+$CD11c$^+$ T cells, a phenotype previously described in Tregs (25), were found to be significantly increased in the graft-draining lymph node of female recipients treated with WT ATDCs but not in KO ATDC-treated nor in untreated mice. This was true for the percentage and absolute numbers of CD8$^+$CD11c$^+$ T cells (Figure 1B). To functionally demonstrate that WT ATDCs + anti-CD3 treatment does indeed induce CD8$^+$ Tregs, we realized adoptive cell transfer experiments into otherwise untreated females receiving male skin grafts (Figure 1C). Transfer of CD8$^+$ T cells purified from the graft-draining lymph node of WT ATDC-treated mice significantly prolonged the graft survival compared to the ones purified from untreated rejecting mice or mice treated with anti-CD3 alone ($p < 0.05$). In contrast, CD8$^+$ T cells from animals treated with KO ATDCs as well as the ones from mice treated only with anti-CD3 failed to prolong allograft survival. TMEM176B expression by ATDCs is therefore required for the induction of regulatory CD8$^+$ T cells.

Male antigen cross-presentation by tolerogenic DCs is required for regulatory CD8$^+$ T cell induction and allograft survival prolongation

To further elucidate the mechanisms by which ATDCs prolong allograft survival and induce CD8$^+$CD11c$^+$ Tregs in a *Tmem176b*-dependent manner, we examined whether Tregs were donor-specific. We first analyzed the presence of donor-specific CD8$^+$ T cells in the graft-draining lymph node by using Uty-MHC class I and Smcy-MHC class I pentamers. Our results showed a significant increase of donor-specific CD8$^+$CD11c$^+$ T cells in WT ATDC-treated mice compared to untreated ones ($p < 0.05$) but not in *Tmem176b$^{-/-}$* ATDC-treated recipients (Figure 1D). The fact that CD8$^+$CD11c$^+$ cells were stained with MHC pentamers strongly suggests that those cells are T cells and not conventional DCs. We then quantified the presence of donor-specific CD8$^+$ T cells among total CD8$^+$ T cells in the draining lymph node of animals undergoing different treatments (Figure 1E). WT ATDCs + anti-CD3 treatment was associated with increased donor-specific CD8$^+$ T cells as compared to naïve, untreated and anti-CD3 treated. In contrast, recipients treated with KO ATDCs + anti-CD3 were unable to expand donor-specific CD8$^+$ T cells in the graft-draining lymph node.

We observed that WT and KO ATDCs both migrated to the graft-draining lymph node (Figure S1). Moreover, no phenotypic difference was observed between WT and KO ATDCs (Figure S5). We then hypothesized that *Tmem176b$^{-/-}$* ATDCs fail to process donor antigens through the cross-presentation pathway. We speculated that loading *Tmem176b$^{-/-}$* ATDCs with the already processed donor minimal peptides issued from Uty and Smcy proteins should rescue the generation of donor-specific CD8$^+$ T cells. Remarkably, when KO ATDCs were loaded with Uty or Smcy peptides before injection, the numbers of donor-specific CD8$^+$ T cells reached the levels observed in recipients treated with WT ATDCs (Figure 1E). Importantly, the injection of KO ATDCs loaded with Uty and Smcy peptides prolonged allograft survival in a similar manner to WT ATDCs (Figure 1F). The fact that the effect of *Tmem176b*-deficient ATDCs can be rescued by loading them with the minimal Uty and Smcy peptides strongly suggests that they fail to process and present donor antigens through the cross-presentation pathway.

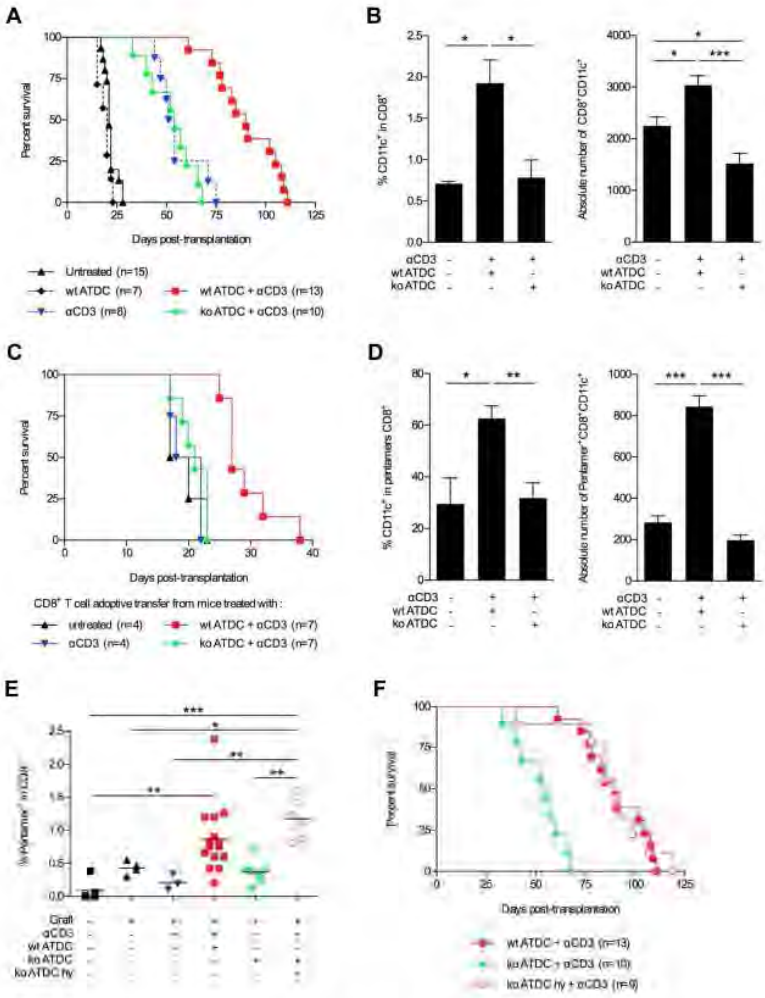

Figure 1: *Tmem176b*$^{-/-}$ (KO) ATDCs fail to prolong skin allograft survival in anti-CD3-treated mice. (A) Female C57BL/6 recipients were grafted with skin from male C57BL/6 donors. Groups of mice underwent the indicated treatments detailed in the Materials and Methods section. Graft survival was monitored every other day. (B) At day 15 post-transplantation, the presence of $CD8^+CD11c^+$ regulatory T cells was determined by flow cytometry in the graft-draining lymph node of mice left untreated, treated with WT ATDCs + anti-CD3 or *Tmem176b*$^{-/-}$ (KO) ATDCs + anti-CD3. n = 5 for each group. $CD8^+CD11c^+$ were defined as $CD11c^{int}$ to exclude $CD11c^{hi}$ conventional DCs. Relative (percentage) and absolute numbers are shown. $^*p < 0.05$. (C) Male skin-grafted female recipients were left untreated or injected with WT ATDCs + anti-CD3, *Tmem176b*$^{-/-}$ (KO) ATDCs + anti-CD3 or anti-CD3 alone. At day 30 post-transplantation, $CD8^+$ cells were purified from the graft-draining lymph node from the different groups and 2×10^5 cells were adoptively transferred into female recipients grafted with male skin, without any other treatment. Graft survival was monitored every other day. $^{***}p < 0.001$. (D) At day 15 post-transplantation, the relative (percentage) and absolute numbers of $CD11c^{int}$ cells among donor-specific $CD8^+$ T cells from the graft-draining lymph node were determined by flow cytometry using Uty pentamers in the indicated groups. n = 5 for each group. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. (E) At day 15 post-transplantation, graft-draining lymph nodes from mice undergoing the indicated treatments were recovered, and cell suspensions were stained to detect donor-specific $CD8^+$ T cells using Uty pentamers. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. (F) KO ATDCs were pulsed with a mix of Uty and Smcy peptides (15 μM) before injection and their effect on graft survival was compared to unpulsed WT or KO ATDCs in combination with anti-CD3 therapy. ATDCs, autologous tolerogenic dendritic cells; DCs, dendritic cells.

Taken together, our results show that *Tmem176b* expression is required by ATDCs to prolong allograft survival by inducing $CD8^+$ Tregs through a mechanism probably involving donor antigen cross-presentation.

Tmem176b *is required for efficient antigen cross-presentation by DCs*

We then directly assessed whether KO ATDCs failed to cross-present exogenous antigens *in vitro*. Cross-presentation of both soluble OVA and OVA-coated latex beads to OVA-specific OT-1 $CD8^+$ T cells was impaired in KO ATDCs compared to WT ATDCs (Figure 2A). However, KO and WT ATDCs were equally effective at stimulating the proliferation of OT-1 cells in the presence of the preprocessed, minimal peptide SIINFEKL (Figure 2A). Knock-down of *Tmem176b* expression in WT ATDCs using two different siRNAs (13) also resulted in reduced OVA cross-presentation (Figure 2B). Thus, TMEM176B is required for the cross-presentation of soluble and particulate OVA by ATDCs. When OVA was expressed endogenously after OVA mRNA electroporation of *Tmem176b*-sufficient or deficient ATDCs, the levels of presentation to OT-1 cells were similar, indicating that TMEM176B is not involved in the endogenous pathway of MHC class I-restricted antigen presentation (Figure 2C). We then determined whether TMEM176B could regulate the processing of other exogenous antigens. We reasoned that cross-presentation of male antigens from live cells may be a pertinent system to test in sight of our *in vivo* results described above. Female WT or KO ATDCs were incubated with live male *β2-microglobulin*$^{-/-}$ splenocytes and then co-cultured with MataHari transgenic $CD8^+$ T cells, which are specific for the male antigen, Uty. KO ATDCs failed to cross-present the cell-associated male antigens as efficiently as the control cells (Figure 2D, left panel). In contrast, when the ATDCs were loaded with the minimal Uty peptide, WT and KO ATDCs exhibited identical capacities to stimulate antigen-specific $CD8^+$ T cells (Figure 2D, right panel). Importantly, KO ATDCs were as efficient as WT ATDCs in stimulating the OVA-specific $CD4^+$ OT-2 T cells (Figure 2E). Similar results were obtained using WT and KO splenic DCs *ex vivo* (Figure S6). Therefore, the absence of TMEM176B impairs the cross-presentation of two different model antigens but spares both the presentation of these antigens to $CD4^+$ T cells and the endogenous presentation to $CD8^+$ T cells.

Taken together, our data indicate that *Tmem176b* is required to cross-present antigens to $CD8^+$ T cells, whereas it is dispensable for presentation to $CD4^+$ T cells through the MHC class II pathway.

TMEM176B is responsible for a cation conductance that regulates phagosomal pH in DCs

The observation that TMEM176B is involved in antigen processing in the cross-presentation pathway prompted us to interrogate the molecular mechanisms mediating this effect. Proteomic studies suggested a phagosomal localization for TMEM176B in macrophages (26,27). TMEM176B co-localized with soluble OVA in a population of scattered vesicular compartments (Figure S7A). In addition, TMEM176B expression was observed surrounding phagocytosed latex beads and in purified phagosomes (Figure S7B and data not shown), indicating that TMEM176B is indeed localized to the phagosomal membrane. To understand the mechanism by which TMEM176B could regulate antigen cross-presentation, we next investigated its function in endosomes and phagosomes. Internalization of soluble OVA (Figure 3A and B) and phagocytosis of fluorescent latex beads (Figure 3C and D) were not affected in KO ATDCs compared to WT counterparts. Similarly, internalization of antigenic material from live cells was not affected (Figure 3E and F). Thus, TMEM176B does not regulate antigen internalization.

We then analyzed phagosomal pH, one of the upstream mechanisms that controls antigen processing at the phagosomal lumen. Indeed, acidification of the phagosomal lumen in DCs has been linked to excessive antigen degradation (14). Moreover, neutralization of the phagosomal lumen pH using chloroquine has been shown to promote antigen export to the cytosol and thus cross-presentation (28). We measured the phagosomal pH in WT and KO ATDCs. Consistent with our previous work (14,15), WT DCs displayed a near-neutral pH even 2 h after phagocytosis (Figure 4A). However, in clear contrast, we observed that KO ATDCs displayed a striking, although transient, alkalinized phagosomal pH. In fact, the pH values were at least 1.5 pH units higher in KO ATDCs compared to WT ATDCs during the first hour following phagocytosis. DCs are known to actively alkalinize phagosomes. The alkalinized phagosomal pH in KO ATDCs is therefore compatible with deficient acidification mechanisms.

It has been suggested that TMEM176B could be involved in ion flux (29,30). In this regard, we and others have previously shown that *Tmem176b* presumably shares a common ancestral gene with the MS4A gene family that includes CD20, a B cell specific protein proposed to function as a store-operated calcium channel (12,31). We directly tested the hypothesis that TMEM176B could function as an ion channel. We expressed TMEM176B in *Xenopus* oocytes and recorded the electric activity under whole-cell patch clamp. Because TMEM176B is localized in intracellular compartments, surface expression was achieved by a 20–30 min pretreatment with PMA (data not shown) as described for other ion channels (32). This treatment resulted in the development of an inward current at a holding potential of −40 mV that was activated by acidification of the extracellular solution to pH 5 (Figure 4B). Further analysis revealed that TMEM176B forms a nonselective monovalent cation channel because the current was abolished by substitution of extracellular Na^+ with NMDG (*N*-methyl-D-glucamine) but not K^+ and was not affected by substitution of intracellular Cl^- with gluconate. This conductance was not blocked by Cd^{2+} (0.5 mM), La^{3+}

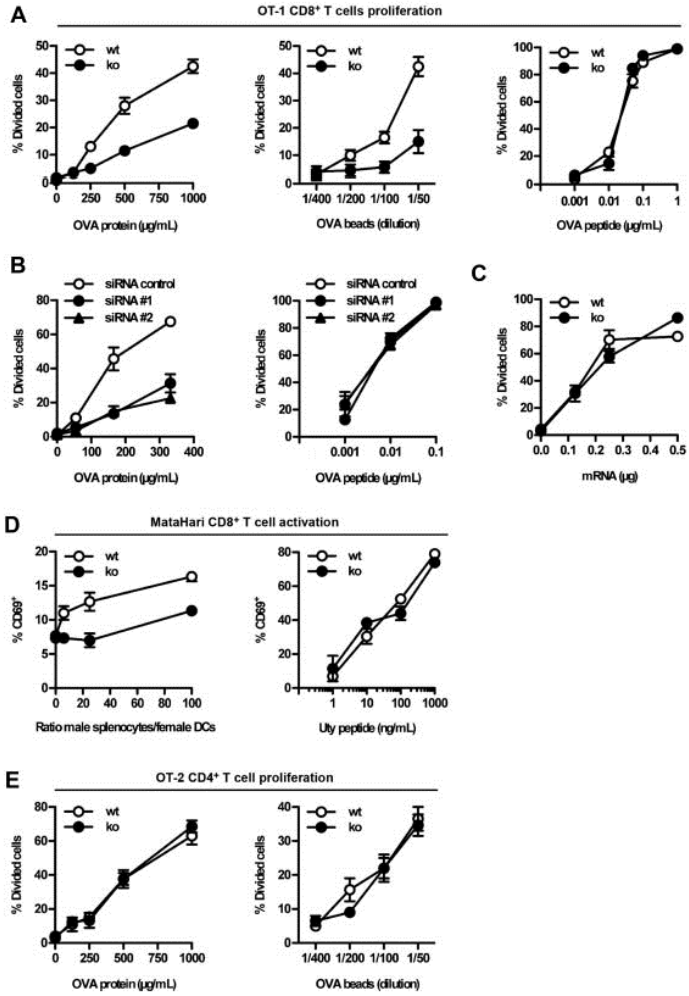

Figure 2: Defective *in vitro* antigen cross-presentation by *Tmem176b*$^{-/-}$ (KO) ATDCs. ATDCs from *Tmem176b*$^{-/-}$ (KO) mice and WT controls were examined *in vitro* to compare their capacities to present antigens to CD8$^+$ and CD4$^+$ T cells. (A) Soluble OVA (left panel), OVA-coated latex beads (center panel) and OVA-minimal peptide SIINFEKL (right panel) were used at different concentrations to treat WT (open symbols) and KO (full symbols) ATDCs. CD8$^+$ T cell proliferation was assessed as described in the Materials and Methods section. One experiment representative of 10 is shown for both the OVA protein and the minimal peptide. n = 5 for the OVA-coated beads. (B) Similar experiments were performed using WT ATDCs treated with control siRNA (open symbols) or two different anti *Tmem176b*-specific siRNAs (filled symbols). The OVA protein-treated ATDCs are depicted in the left panel whereas the right panel shows proliferation upon treatment with the SIINFEKL peptide (n = 3). (C) To study OVA presentation when expressed as an endogenous antigen, mRNA coding for the GFP fused to the SIINFEKL peptide was electroporated into WT (open symbols) and KO (filled symbols) ATDCs (n = 2). (D) Left panel: Male *β2-microglobulin*$^{-/-}$ splenocytes were incubated for 2 h at 37°C with different ratios of adherent female WT or KO ATDCs. The splenocytes were extensively washed, and MataHari CD8$^+$ T cells were then co-cultured with the ATDCs for 20 h. CD69 expression on CD8$^+$ T cells was determined by flow cytometry. Right panel: WT or KO ATDCs were treated for 45 min at 37°C with different doses of the minimal Uty peptide. Upon extensive washing, MataHari CD8$^+$ T cells were co-cultured with ATDCs for 20 h. CD69 expression on CD8$^+$ T cells was determined by flow cytometry (n = 3). (E) Similar experiments to those described in (A) were performed using OT-2 CD4$^+$ T cells (n = 3). ATDCs, autologous tolerogenic dendritic cells; GFP, green fluorescent protein; siRNA, small interfering RNA.

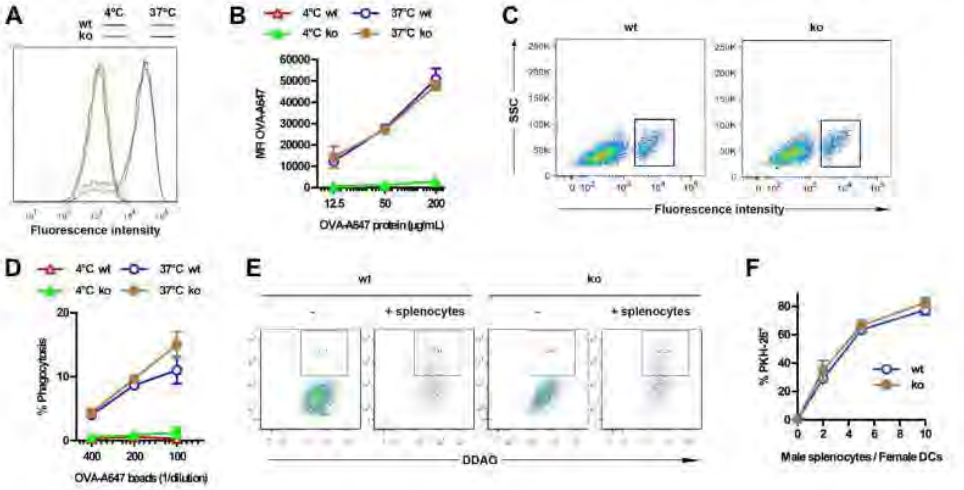

Figure 3: *Tmem176b* deficiency does not alter antigen internalization by ATDCs. (A,B) Soluble antigen endocytosis was compared between WT and *Tmem176b*$^{-/-}$ (KO) cells. ATDCs were pulsed for 15 min at 4°C or 37°C with OVA-Alexa 647. Sixty-minute chase was then performed before analyzing fluorescence intensity by flow cytometry. A representative histogram using 200 µg/mL OVA-Alexa 647 is shown (n = 3) in (A). Quantification ± SD is shown in (B). (C,D) Phagocytosis of particulate antigens was compared between WT and KO cells. ATDCs were pulsed for 15 min with fluorescent beads and then extensively washed and chased for 30 min. The percentage of ATDCs that internalized beads was quantified by flow cytometry. Representative dot plots of ATDCs treated with beads at 37°C are shown (n = 3) in (C). Quantification ± SD is shown in (D). (E,F) Male *β2-microglobulin*$^{-/-}$ splenocytes were labeled with PKH-26 and co-cultured at 37°C with DDAO-labeled WT or *Tmem176b*$^{-/-}$ ATDCs at different ratios. Upon a 2-h culture, the percentage of PKH-26^{+} cells among DDAO^{+} ATDCs was determined by flow cytometry. A representative experiment at a 2:1 ratio is shown in (E). Quantification ± SD is shown in (F). ATDCs, autologous tolerogenic dendritic cells.

(1 mM), Gd^{3+} (1 mM), nifedipine (10 µM), niflumic acid (100 mM) or amiloride (20 µM). Additionally, this conductance could not be activated directly by intracellular acidification because injection of methane sulfonic acid (40 nL, 150 mM) into the oocyte had no effect.

Our electrophysiological results define TMEM1768 as an acid sensitive, nonselective cation channel. Because the phagosomes from KO ATDCs showed excessive alkalinization, we questioned whether TMEM1768 might provide a phagosomal Na^{+} efflux conductance that permits greater proton pumping by the vacuolar ATPase (V ATPase) through charge compensation. Consistent with this hypothesis, removal of Na^{+} from the phagosomal lumen by incubation of cells in extracellular NMDG solution during phagocytosis led to the alkalinization of WT but not KO phagosomes (Figure 4C). In these experiments, the mean fluorescence intensity of FITC was not saturated at the pH observed in the KO phagosomes, ruling out a lack of alkalinization due to technical reasons. Furthermore, KO phagosomes had profoundly impaired phagosomal V ATPase activity, which was assessed by measuring luminal pH changes following bafilomycin treatment. Inhibition of the V ATPase increased the pH of WT but not KO ATDC phagosomes (Figure 4D). This result suggested that the cationic conductance mediated by TMEM1768 promotes V ATPase activity and phagosomal acidification in DCs.

Discussion

The injection of donor derived tolerogenic DCs before transplantation has been shown to prolong allograft survival (2,33). However, Divito et al (34) elegantly showed that endogenous DCs mediate this effect, presenting alloantigens on self MHCs. Injecting autologous DCs appears as a different approach and a clinically innovative and feasible strategy to control pathogenic immune responses. In humans, a pioneering study by Dhodapkar et al (35) demonstrated the feasibility and safety of injecting ATDCs in healthy volunteers. Injection of DCs was associated with antigen specific inhibition of effector T cell function and induction of antigen specific $CD8^{+}$ Tregs *in vivo* (35,36). We are currently developing a protocol to inject ATDCs into kidney graft recipients in a phase I/II clinical trial (The ONE Study, 7th Frame Program, European Commission). We have shown that human monocyte derived DCs do express TMEM1768. Cross presenting human DCs such as blood

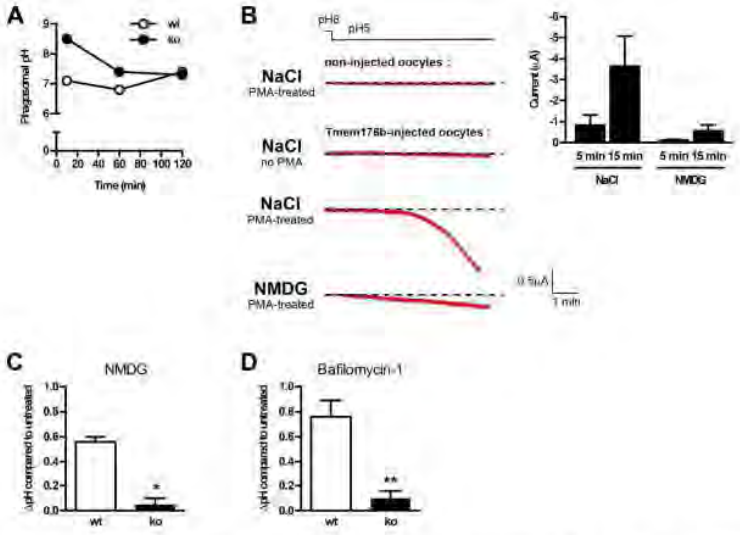

Figure 4: TMEM176B is responsible for a cation conductance that regulates phagosomal pH in ATDCs. (A) Phagosomal pH was measured in WT and *Tmem176b*$^{-/-}$ (KO) ATDCs at different time points using flow cytometry analysis as described in the Materials and Methods section (n = 5). (B) *Xenopus* oocytes were injected with *Tmem176b* mRNA, and currents were recorded in voltage-clamp 2–4 days later as detailed in the Materials and Methods section. Before recording, translocation of TMEM176B to the plasma membrane was induced by a 30-min treatment with PMA (100 nM). The left panel shows typical current recordings in extracellular buffers containing NaCl or NMDG. The right panel shows the means ± SD of the currents recorded in the presence of Na^+ or NMDG. The currents were quantified 5–15 min after holding the extracellular pH at 5 (n = 5–7). (C) Phagosomal pH was measured by flow cytometry in WT and KO ATDCs. Na^+ was substituted with NMDG in the extracellular buffer. The Na^+ buffer contained 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 3 mM glucose, and 10 mM HEPES, pH 7.4. In the NMDG buffer, 140 mM NMDG was substituted for Na^+. WT but not KO DCs exhibited a pH increase. (D) In phagosomal pH experiments, ATDCs were treated with the V-ATPase inhibitor, bafilomycin-1 (400 ng/mL). The phagosomal pH increased in WT but not in KO DCs. ATDCs, autologous tolerogenic dendritic cells; DCs, dendritic cells; PMA, phorbol myristate acetate; V-ATPase, vacuolar ATPase.

8DCA3$^+$ DCs and tonsil resident 8DCA1$^+$ DCs, 8DCA3$^+$ DCs and pDCs may express this cation channel (37).

Humanized anti CD3 antibodies are undergoing clinical evaluation in the field of autoimmunity with encouraging results (38). In mice, anti CD3 treatment induces apoptosis of activated T cells. Phagocytosis of apoptotic cells by macrophages and immature DCs triggers TGF β production, leading to induction of CD4$^+$Foxp3$^+$ Tregs (39). In type 1 diabetes patients undergoing anti CD3 treatment, increased CD8$^+$CD25$^+$FOXP3$^+$ Tregs were associated with clinical response (40). We have shown in the present study that CD8$^+$ cells purified from grafted mice treated only with anti CD3 antibodies were not able to prolong allograft survival when transferred into grafted mice. Thus, in agreement with the pentamer staining experiments, anti CD3 treatment alone is not able to trigger CD8$^+$ Tregs. Moreover, we studied whether ATDC injection alone could generate Tregs. We observed that ATDC injection alone failed to accumulate donor specific CD8$^+$ T cells (n = 3, data not shown). These results strongly suggest that ATDC and anti CD3 injections synergize to generate Tregs. We propose that T cell death might create a tolerogenic environment allowing ATDCs to generate Tregs in this model and prolong allograft survival.

Herein, we showed that injected ATDCs need to cross present donor antigens in a *Tmem176b* dependent manner to generate CD8$^+$ Tregs and prolong allograft survival in the mouse. These observations may explain the challenging observation that autologous (not donor derived) DCs prolong allograft survival in a donor specific manner (3). These observations may have practical consequences when setting up clinical protocols. For example, if ATDCs are to be used with calcineurin inhibitors, specific precautions should be taken since several of these drugs are known to interfere with antigen processing by DCs (41).

Although cross presentation triggers immunity, in some circumstances cross presentation can lead to immunological

tolerance. Abortive proliferation of $CD8^+$ T cells is one of the best studied mechanisms by which cross-presentation can regulate immune responses (42). A much less characterized mechanism is the possibility that cross-presentation may lead to generation of $CD8^+$ Tregs. We have previously suggested that rat plasmacytoid DCs may generate $CD8^+$ Tregs through cross-presentation in a transplantation model (43). Herein, we show novel evidence supporting this concept. We performed cell transfer experiments to determine whether the donor-specific $CD8^+$ T cells have regulatory properties. Due to the low percentage of donor-specific cells in the graft-draining lymph node and the total amount of cells recovered, we did not succeed in purifying sufficient numbers of donor-specific $CD8^+$ T cells or $CD8^+CD11c^+$ T cells to be used in adoptive transfer experiments. However, we believe that our results strongly support the interpretation that donor-specific $CD8^+CD11c^+$ T cells are indeed Tregs and not effectors. In fact, adoptive transfer of total $CD8^+$ cells from WT ATDC-treated recipients but not from untreated ones or from *Tmem176b*$^{-/-}$-treated ATDC mice significantly prolonged skin allograft survival. The mechanisms by which donor-specific $CD8^+CD11c^+$ Tregs impair graft rejection deserve a deep characterization, which is beyond the scope of this study. $CD8^+CD11c^+$ Tregs have been previously characterized in other models. Indeed, Seo et al (25) have shown that injection of anti-4-1BB (CD137) antibodies suppressed collagen-induced arthritis in mice by expanding $CD8^+CD11c^+$ Tregs.

We have recently shown that phagosomal pH is actively regulated in DCs and impacts antigen cross-presentation (14–16,18–20). In DCs, two multiprotein complexes tightly regulate the phagosomal pH. The NADPH-oxidase 2 promotes alkalinization, whereas the proton pump, V-ATPase, acidifies the phagosomal lumen (14–16,18–20). Proton pumping by V-ATPase generates a voltage across the membrane of the phagosome, which is intraluminally positive, antagonizing further inward transport of proton equivalents (44–47). Therefore, neutralizing counterion conductances are needed to prevent the generation of charge imbalances across the phagosomal membrane and to allow vesicular acidification. However, the molecular identities of these neutralizing counterion conductances remain unknown.

Intracellular ion metabolism has been linked with important biological processes in DCs such as migration (48) and inflammasome activation (49–51). To the best of our knowledge, this is the first time that intracellular ions are suggested to play a role in antigen processing by DCs. The acid-activated cation channel function described here for TMEM176B provides a mechanistic explanation for the transiently alkalinized phagosomal pH observed in KO DCs. This evidence is consistent with a role for TMEM176B as a cation channel responsible for the counterion conductances involved in regulating phagosomal pH. Notably, counterion conductances are postulated to regulate the rate, rather than the extent, at which vesicular organelles are acidified. Casey et al proposed that given sufficient time, even small conductances can cause V-ATPases to reach the maximal theoretical pH gradient (47). According to this suggestion, the presence of anionic counterionic conductances, such as chloride channel transporters present in KO phagosomes, may explain why the phagosomes were able to achieve normal pH values at late time points.

In conclusion, here we characterized cellular and molecular mechanisms required by a clinically relevant therapeutic approach. TMEM176B arises as a novel partner for tolerogenic DCs. Its function in controlling ionic homeostasis may be relevant to understand the role played by this protein in other cells.

Acknowledgments

This work was funded by Fondation Progreffe, Vaincre la Mucoviscidose and IMBIO. MH was supported by a Junior Basic Science grant from ESOT. We are grateful to Philippe Hulin from the IFR26 MicroPIcell imagery core facility Nantes, France, for excellent assistance with confocal microscopy, and to Claire Usal and Emmanuel Merieau for their great help in mouse experimentation. *Tmem176b*$^{-/-}$ (KO) mice were generated by "La Clinique de la souris," Strasbourg, France. Jeffrey Bluestone and Giovanna Lombardi critically read the manuscript.

Disclosure

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

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Supporting Information

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

Figure S1: Migration of ATDCs into skin graft and graft-draining lymph node. (A) Male skin-graft female recipients ($CD45.2^+$ cells) were treated with anti-CD3 with or without injection of ATDCs prepared from Ly5.1 mice. ATDCs ($CD45.1^+$) were tracked by flow cytometry in the graft-draining lymph node (dLN) and in the skin graft. (B) Frozen sections from the graft-dLN harvested 15 days after skin transplantation and analyzed through epifluorescence microscopy. ATDCs were labeled with PHK-26 (red) before injection for subsequent tracking in vivo. Tissues were counter-stained with DAPI (blue staining). No red staining was found in mice treated with anti-CD3 antibody whereas PKH^+ cells were observed in similar numbers in all WT or *Tmem176*$^{-/-}$ (KO) ATDC injected mice. Panels show representative results from the analysis of 6 mice per condition.

Figure S2: Generation of a *Tmem176b*$^{-/-}$ (KO) mouse. Genomic organization and targeting of a region including exon 1 and exon 2 (including the ATG start codon) of the *Tmem176b* gene. Homologous recombination was performed using ES cells derived from 129/SV mice (Institut Clinique de la Souris [ICS], Strasbourg). Heterozygous mice were backcrossed onto the C57BL/6 background 10 times and then intercrossed to generate +/+ (WT), +/− and −/− (KO) mice. Genotyping by PCR on genomic DNA using specific primers is shown.

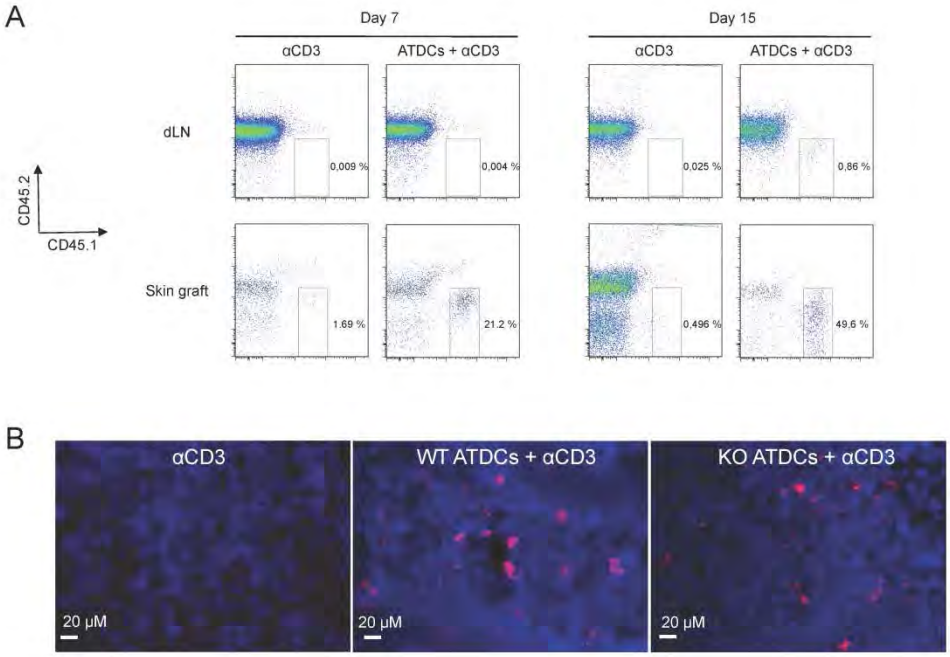
Figure S3: ATDCs + anti CD3 treatment is not associated with increased $Foxp3^+$ cells. Graft-draining lymph nodes were harvested 15 days after skin transplantation. Cells were labeled with anti CD19, CD8 and Foxp3 antibodies and donor-specific pentamers and analyzed by flow cytometry. $CD8^+CD19^-$ cells were gated and analyzed for Foxp3 expression in pentamers positive and negative populations. No significant differences were observed. Representative stainings are shown for each treatment (KO = *Tmem176b*$^{-/-}$).

Figure S4: ATDCs + anti CD3 treatment is not associated with increased $CD8^+$ Pentamer$^+$ $CD28^-$ cells. Graft-draining lymph nodes were harvested 15 days after skin transplantation. Cells were labeled with anti-CD19, and CD8 antibodies and donor-specific pentamers and analyzed by flow cytometry. $CD8^+CD19^-$Pentamer+ cells were gated and analyzed for CD28 expression. No significant differences were observed. Representative stainings are shown for each treatment (KO = *Tmem176b*$^{-/-}$).

Figure S5: Phenotypic characterization of *Tmem176b*$^{-/-}$ (KO) ATDCs. WT and *Tmem176b*$^{-/-}$ (KO) mouse ATDCs were analyzed for surface expression of the indicated markers by flow cytometry.

Figure S6: Splenic CD11chi *Tmem176b*$^{-/-}$ (KO) DCs specifically fail to cross-present OVA to $CD8^+$ T cells. WT or *Tmem176b*$^{-/-}$ (KO) splenic CD11chi DCs were incubated with OVA protein (A,C,E,G) or SIINFEKL class I peptide (B,D) or with the ISQAVHAAHAEINEAGR class II peptide (F,H) for 45 min at 37°C and at different doses. Cells were washed and then co-cultured for 72 h with OT-1 (A–D) or OT-2 T cells (E–H). The percentage of dividing cells (A, B, E, F) or $CD69^+$ cells (C, D, G, H) was determined by flow cytometry.

Figure S7: TMEM176B localizes at endosomal and phagosomal membranes of human DCs. A: Human monocyte-derived DCs (Mo-DCs) were generated as previously described (Hill et al 2007) and treated with 50 μg/mL OVA-Alexa 488 (Molecular Probes) for 15 min at 37°C and were then washed and chased for 15 min. Mo-DCs were then fixed with acetone and stained with anti human *Tmem176b* polyclonal antibody (Sigma). Confocal microscopy analysis is depicted, showing four different planes (0.4 μm) for one cell. Orthogonal analyses (X-Z and Y-Z) depicted at right show the co-localization of OVA-488 and *Tmem176b* staining. At least 50 cells were analyzed from each donor (n = 4). **B:** Mo-DCs were incubated with 1 μm latex beads for 15 min and were then extensively washed and chased for 30 or 120 min. Cells were then fixed and stained with anti-TMEM176B antibody. A representative cell of at least 50 analyzed for each donor is shown (n = 4).



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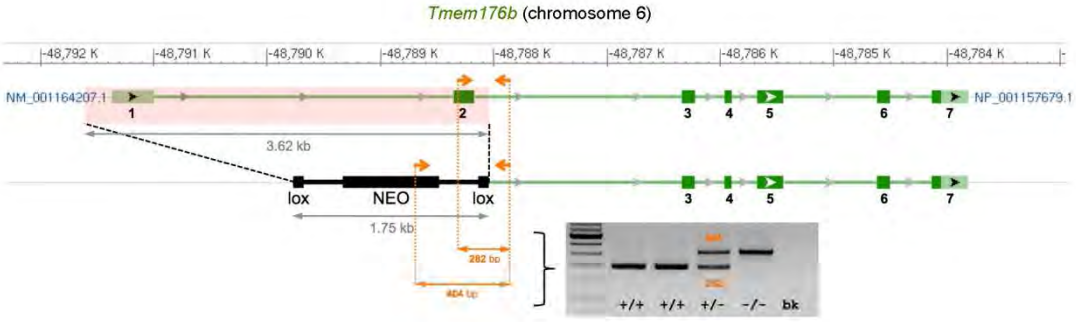

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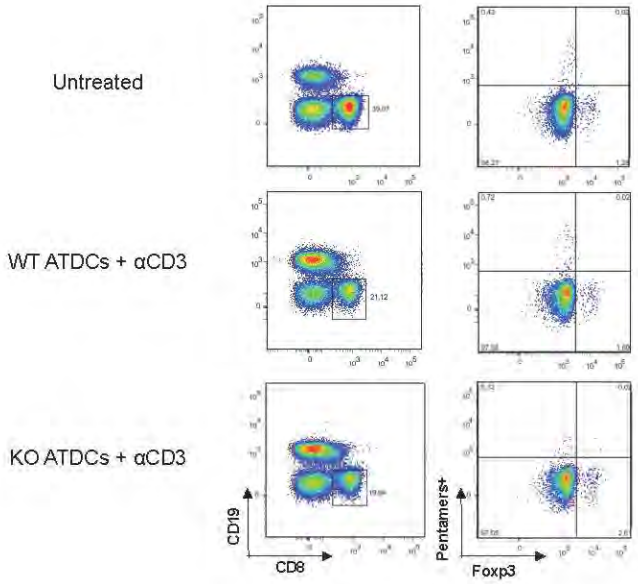

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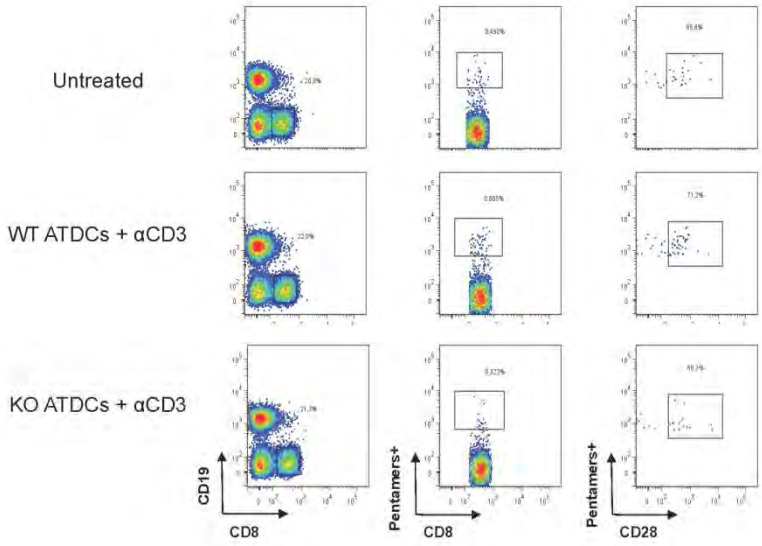

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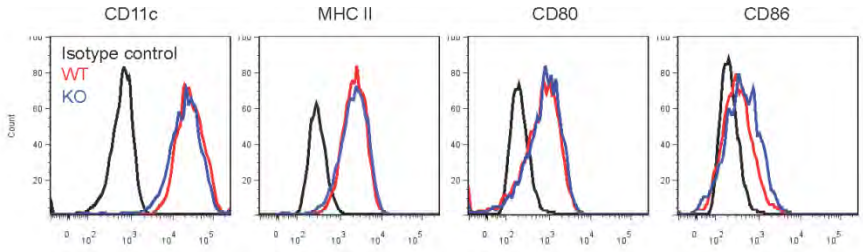

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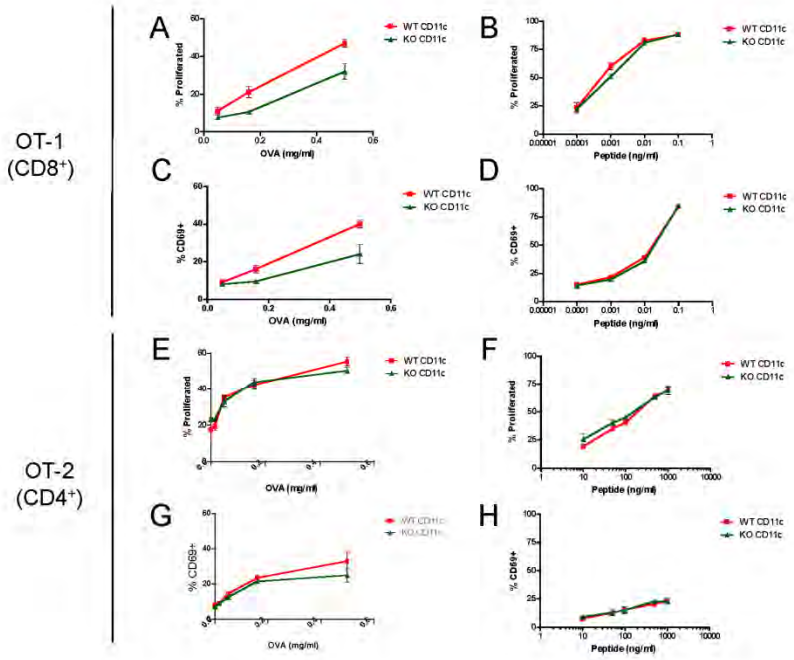

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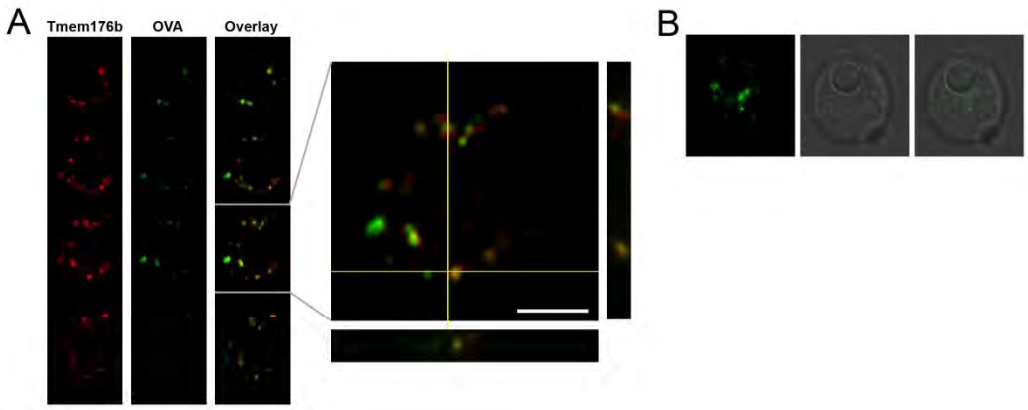

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COMMUNAUTÉ D'UNIVERSITÉS ET ÉTABLISSEMENTS

UNIVERSITÉ DE NANTES

Thèse de Doctorat

Laura CARRETERO-IGLESIA

Autologous regulatory myeloid cell therapy in transplantation

Thérapie cellulaire utilisant des cellules régulatrices myéloïdes autologues en transplantation

Abstract

The aim in organ transplantation is to induce specific long-term allograft tolerance. Current therapies control short-term allograft rejection but are inefficient against late graft failure. Moreover, they carry important side-effects, rendering patients vulnerable to other diseases. New therapies are nowadays being developed. The use of *in vitro* modified cell types as a strategy to induce donor-specific tolerance has proven to be effective to prolong allograft survival in a variety of animal models. Myeloid cells play a key role in transplantation. They are involved in both, tolerance and rejection. Therefore, the *in vitro* modification of myeloid cells for their use in cell therapy has gained interest those last years. The work developed during this thesis aimed at generating three regulatory myeloid cell types (tolerogenic dendritic cells, regulatory macrophages and myeloid-derived suppressive cells). *In vitro*, we studied their capacity to avoid T cell activation and the mechanisms underlying their suppressive activity. *In vivo*, we tested the potential of autologous regulatory myeloid cells to prolong allograft survival when injected in a mouse skin transplant model one day before transplantation and the *in vivo* mechanisms induced after their injection. We believe that the results obtained during this thesis will help to progress towards an efficient cell therapy and tolerance induction in the transplantation setting.

Key Words
transplantation, cell therapy, regulatory myeloid cells, autologous

Résumé

L'objectif en transplantation d'organes est d'induire une tolérance spécifique du greffon à long terme. Les thérapies actuelles sont efficaces pour contrôler le rejet aigu du greffon mais sont inefficaces pour prévenir le rejet chronique. De plus, elles peuvent induire à des effets secondaires importants, rendant les patients sensibles à d'autres maladies. De nouvelles thérapies sont ainsi en cours de développement. L'utilisation de différents types cellulaires modifiés *in vitro* comme stratégie pour l'induction d'une tolérance spécifique d'antigène a été démontré efficace pour prolonger la survie de l'allogreffe dans plusieurs modèles animaux. Les cellules myéloïdes jouent un rôle important en transplantation. Elles sont impliquées dans la tolérance, ainsi que dans le rejet de la greffe. La modification *in vitro* des cellules myéloïdes pour leur utilisation en transplantation a suscité un intérêt ces dernières années. Le travail développé pendant cette thèse a eu pour objectif la génération de trois types de cellules régulatrices myéloïdes (cellules dendritiques tolérogènes, macrophages régulateurs et cellules myéloïdes suppressives). *In vitro*, nous avons étudié leur capacité suppressive sur l'activation des lymphocytes T et les mécanismes impliqués dans cette suppression. *In vivo*, nous avons testé leur potentiel à prolonger la survie de l'allogreffe après injection autologue dans un modèle de greffe chez la souris ainsi que les mécanismes qu'elles induisent. Nous supposons alors que les résultats obtenus pendant cette thèse pourront aider à développer une thérapie cellulaire efficace pour l'induction d'une tolérance en transplantation.

Mots clés
transplantation, thérapie cellulaire, cellules régulatrices myéloïdes, autologue