Tolerogenic functions of plasmacytoid dendritic cells in T cell-medaited CNS autoimmunity

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Tolerogenic functions of plasmacytoid dendritic cells in T cell-mediated CNS autoimmunity

THESE

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ABBREVIATIONS

AICD	Activation-induced cell death
AIRE	Autoimmune regulator
Ag	Antigen
APC	Antigen presenting cell
APECED	Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy
APS1	Autoimmune polyendocrinopathy syndrome 1
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BBE	Bickerstaff brainstem encephalitis
BDCA-2	Blood dendritic cell antigen-2
BECs	Blood endothelial cell
BM	Bone marrow
BMDC	Bone marrow derived dendritic cells
BLB	Blood-leptomeningeal barrier
BST-2	Bone marrow stromal antigen-2
CCL	Chemokine ligand
CCR	Chemokine receptor
CD	Cluster of differentiation
cDC	Conventional dendritic cell
CDP	Common dendritic progenitor
CIS	Clinical isolated syndrom
CNS	Central nervous system
CRAMP	Cathelicidin-related antimicrobial peptide
CSF	Cerebrospinal fluid
cTECs	Cortical thymic epithelial cell
CTLA-4	Cytotoxic T-lymphocyte associated antigen
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor

DAMP	Domage-associated molecular pattern
DC	Dendritic cell
dLN	Draining lymph node
DMT	Disease-modifying therapies
DN	Double negative
DTR	Diphteria toxin receptor
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein Barr virus
eTAC	Extrathymic Aire expressing cell
FC	Facilitating cells
FRC	Fibroblastic reticular cell
Flt3L	Fms-like tyrosine kinase 3 ligand
FoxP3	Forkhead box P3
FS	Fisher syndrome
GA	Glaterimer acetate
GALT	Gut associated lymphoid tissues
GBS	Guillain-Barré-Syndrome
GCN2	General control nonderepressing 2
GM-CSF	Granulocytes macrophage-colony stimulating factor
GrB	Granzyme-B
GVDH	Graft versus host disease
HEV	High endothelial venule
HLA	Human leukocyte antigen
HMGB1	High mobility group box 1
HSC	Hematopoietic stem cell
IBD	Inflammatory bowel disease
IC	Immune complexe
ICAM-1	Intracellular adhesion molecule 1
ICOS	Inducible T-cell co-stimulator
ICOSL	Inducible T-cell co-stimulator ligand

IDO	Indolemine 2,3-dyoxygenase 1
IFN	Interferon
IFNAR	INF receptor
Ig	Immunoglobulin
IL	Interleukin
ILT-7	Immunoglobulin like transcript-7
iPC	IFN-producing cell
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy X-l
iTreg	Induced Treg
KO	Knock-out
LAG-3	Lymphocyte activation gene-3
LEC	Lymphatic epithelial cell
LN	Lymph node
LNSC	Lymph node stromal cell
LPS	Lipopolysacharide
MAIT	Mucosa-associated invariant T cells
MBP	Myelin basic protein
MHC	Major Histocompatibility complex
MMP	Matrix metalloproteinase
MOG	Myelin oligodendrocyte glycoprotein
MOBP	Myelin-associated Oligodendrocytic Basic Protein
MS	Multiple sclerosis
mTEC	Medullary thymic epithelial cell
NK	Natural killer
Nrp-1	Neuropilin-1
nTreg	Natural Treg
p-preDC	Plasmacytoid precursor DC
PAMP	Pathogen-associated molecular pattern
PD-L1	Programmed death-ligand 1
PD-1	Programmed death protein 1

pDCs	Plasmacytoid dendritic cell
PGE	Promiscuous gene expression
PLP	Myelin proteolipid Protein
PPMS	Primary progressive MS
PRR	Pattern recognition receptor
PSG-L1	P selectin glycoprotein-ligand-1
PTA	Peripheral tissue antigen
RUNX	Runt-related transcription factor
RRMS	Relapsing-remitting MS
SC	Spinal cord
SIGLEC	Sialic acid-binding immunoglobulin like lectin
SLO	Secondary lymphoid organ
SIRPα	Signal-regulatory protein α
Ss	Single strand
SLE	Systemic lupus erythematosus
SP	Single positive
SPMS	Secondary progressive form of MS
S1P	Sphingosine-1-phosphate
T1D	Type 1 Diabetes
TCR	T cell receptor
TGF	Transforming growth factor
TLR	Toll-like receptor
TLT	Tertiary lymphoid tissues
TNC	Thymic nurse cells
TNF	Tumor necrosis factor
Treg	Regulatory T lymphocyte
tRNA	transfer RNA
VCAM-1	Vascular cell adhesion protein 1
UV	Ultraviolet
Zeb2	Zinc finger E box–binding homeobox 2

ABSTRACT

T cell lymphocytes play an essential role in the adaptive immunity. They arise from the hematopoietic compartment and reach the thymus, where they achieve their development through specialized maturation and selection steps. Immune system comprises specific and complex mechanisms in the thymus and the periphery aiming at preventing the generation and the survival of self-reactive T cells that could lead to autoimmune disorders. These mechanisms are known as central and peripheral tolerance and rely on different cellular actors such as medullary thymic epithelial cells (mTECs), dendritic cells (DCs), lymph node stromal cells (LNSCs) and regulatory T and B cells. Self-reactive T cells escaping thymic central tolerance are kept in check in the periphery through specific mechanisms that include T cell anergy, T cell deletion and Treg induction. Although conventional DCs (cDCs) have been first in line in mediating peripheral tolerance, increasing evidence demonstrates the contribution of other actors such as plasmacytoid dendritic cells (pDCs) in this process.

pDCs are important linkers of the innate and adaptive immunity. They are characterized by their ability to secrete pro-inflammatory cytokines and large amount of type I interferon (IFN-I) upon pathogenic infections, but also express major Histocompatibility complex (MHC) and costimulatory molecules enabling them to interact with T cells. pDCs have been involved in the control of infections, but are also important actors of peripheral tolerance. The diversity of their functions, depending on the context in which they will evolve, has associated pDCs to pro-immunogenic and tolerogenic scopes. In the lab, we are principally interested in the contribution of pDCs in Multiple sclerosis, a progressive inflammatory demyelinating disease of the central nervous system (CNS). To address the role of pDCs in MS, we performed our studies in the murine model of Experimental autoimmune encephalomyelitis (EAE). Using

genetically modified mice harbouring specific abrogation of Ag-presenting function in pDCs, my co-workers previously demonstrated that myelin Ag presentation by pDCs promotes the expansion of regulatory T cells (Tregs) that inhibit encephalitogenic T_H1 and T_H17 cell priming in secondary lymphoid organs (SLOs).

In this manuscript I report our recent findings supporting that pDCs display tolerogenic functions during the priming and the effector phase of EAE development and are able to control and dampen the disease. The first study investigates the interplays between pDCs and Tregs and shows that Ag-specific MHCII interactions with Tregs licenced tolerogenic features in steady-state pDCs by inducing their expression of the indoleamine-2,3 dioxygenase (IDO1). In EAE context, Treg-educated IDO^+ pDCs are required to confer suppressive functions to Tregs which promote the inhibition of encephalitogenic T cell priming in draining LNs, resulting in attenuated EAE. In the second study, we explore the therapeutic effect of pDCs transfer in EAE mice after disease onset. We show that the transfer of immature MOG_{35-55} pre-loaded pDCs during EAE acute phase leads to substantial reduction of CNS inflammation and significant amelioration of disease clinical scores. We demonstrate that pDC-protection relies on the massive recruitment of endogenous immature pDCs in the inflamed spinal cord via the Chemerine/CMKLR1 axis. Endogenous pDC recruitment is required to down-modulate CNS inflammation, encephalitogenic T_H1 and T_H17 cell responses and EAE severity.

Overall this work supports previous findings showing the importance of pDCs in the regulation of CNS autoimmunity and unravel these cells for potential use, by targeting different particular functions, in the development of future therapies to treat MS patients.

RÉSUMÉ EN FRANÇAIS

Les lymphocytes T (LTs) ont un rôle prépondérant dans l'immunité adaptative. Issus du compartiment hématopoïétique, les précurseurs de LTs migrent ensuite dans le thymus où ils subissent différentes étapes de maturation nécessaires à l'achèvement de leur développement. Les LTs sont également soumis à une sélection intrathymique drastique ayant pour but d'éliminer les LTs auto-réactifs pouvant être à l'origine de maladies autoimmunes : c'est la tolérance centrale. Bien qu'efficaces, les mécanismes de la tolérance centrale ne sont pas infaillibles et laissent sortir des LTs potentiellement auto-réactifs en périphérie. D'autres mécanismes sont mis en place pour maintenir sous surveillance ces clones auto-réactifs afin qu'ils ne puissent pas être activés et proliférer lorsqu'ils rentrent en contact avec les antigènes (Ag) pour lesquels ils sont spécifiques : c'est la tolérance périphérique. Les mécanismes dépendants de la tolérance périphérique tels que l'induction d'anergie, la déplétion clonale ou la génération de lymphocytes T régulateurs (Tregs) nécessitent l'intervention d'acteurs cellulaires spécifiques comme les cellules dendritiques (DCs) et les cellules du stroma ganglionnaire. Bien que les DCs conventionnelles (cDCs) soient les principaux médiateurs de la tolérance périphérique en condition physiologique, de nombreuses études montrent que les DCs plasmacytoïques (pDCs) sont également largement impliquées dans ce processus en condition physiologique et sous inflammation.

Les pDCs ont un rôle important dans l'immunité innée et adaptative. Caractérisées par leur capacité à produire des cytokines pro-inflammatoires et de larges quantités d'Interféron de type I (IFN-I) lors d'infections pathogéniques, les pDCs expriment également à leur surface des molécules de complexe majeur d'histocompatibilité (MHC) et de co-stimulations leur permettant d'interagir avec les LTs. Selont le contexte dans lequel elles évoluent et de par la diversité de leurs fonctions, les pDCs peuvent être immunogènes ou tolérogènes. En effet, en dépit de leur implication dans le contrôle des infections, les pDCs contribuent également au

maintien de la tolérance périphérique. Dans notre laboratoire, nous nous intéressons principalement à l'implication de ces cellules dans la sclérose en plaque, qui est une maladie auto-immune pro-inflammatoire du système nerveux central (CNS) caractérisée par une démyélinisation progressive des gaines protégeant les axones des neurones. Afin de déterminer le rôle joué par les pDCs dans cette maladie, nous avons effectué nos études dans un modèle murin de sclérose en plaque appelé Encéphalomyélite auto-immune expérimentale (EAE). Grâce à l'utilisation de souris transgéniques dans lesquelles les fonctions de présentation antigénique des pDCs sont abrogées, une étude menée au sein de mon laboratoire, a permis de mettre en évidence le caractère tolérogène des pDCs dans le modèle d'EAE. Via la présentation d'Ags de myéline par le CMH II, les pDCs induisent une expansion de Tregs inhibant l'activation des LTs encephalitogéniques de types T_H1 et T_H17 dans les organes lymphoïdes secondaires (SLOs).

L'objectif de cette thèse a été de caractériser les mécanismes responsables des fonctions tolérogènes des pDCs dans ce modèle d'EAE. La première étude a permis de mettre en évidence l'importance des interactions spécifiques de l'Ag entre les pDCs et les Tregs. En condition physiologique, nous montrons que les Tregs induisent l'expression de l'indoléamine 2,3-dioxygénase 1 (IDO) par les pDCs, conduisant à l'acquisition de propriétés tolérogènes par ces dernières. Par ailleurs, en contexte EAE, nous démontrons que l'expression d'IDO par les pDCs est requise pour conférer aux Tregs leurs fonctions suppressives, et permet à ces derniers d'inhiber l'activation des LTs effecteurs auto-réactifs dans les SLOs. Dans la deuxième étude, nous avons exploré le potentiel thérapeutique des pDCs, lorsque celles-ci sont transférées à des souris immunisées pour l'EAE après l'apparition des symptômes de la maladie. Nous montrons que le transfert de pDCs immatures chargées en peptide de myéline, durant la phase aigüe de la maladie, réduit significativement l'inflammation au niveau du CNS

et améliore considérablement l'évolution de la maladie. Cette protection est notamment associée à un recrutement important de pDCs endogènes immatures dans les lésions inflammatoires de la moelle épinière, peu de temps après le transfert de pDCs exogènes. Le recrutement des pDCs endogènes dans le CNS est tributaire de l'engagement de la voie de signalisation chemerine/CMKLR1 et conduit à une diminution de l'inflammation et des LTs encéphalitogéniques dans le CNS, ainsi qu'à une baisse de la sévérité de l'EAE.

Dans son ensemble, ce travail contribue à démontrer l'importance des pDCs dans la régulation de l'auto-immunité au niveau du CNS et met en avant le potentiel thérapeutique de ces cellules pour de futurs traitements visant à soigner les patients atteints de sclérose en plaque.

I. INTRODUCTION

1. T CELL DEVELOPMENT AND IMMUNITY

1.1. THYMIC T CELL SELECTION

The thymus is a bilobed lymphoepithelial organ composed of the cortex and the medulla, two distinct anatomical areas, surrounded by a capsule. The cortex and the medulla are populated by different specific cell subsets that ensure a unique local microenvironment, allowing the achievement of specialized maturation and selection steps of T cell development. These cells arise from the hematopoietic compartment such as dendritic cells (DC), or belong to the stromal tissue as cortical thymic epithelial cells (cTEC) and medullary thymic epithelial cells (mTEC) (Nitta and Suzuki, 2016). Thanks to specific functions, which will be described below, these cells play a crucial role in the generation of functional, diverse, and self-tolerant T-cell repertoire.

1.1.1. INTRA-THYMIC LYMPHOCYTE PROGENITORS

Committed lymphoid progenitors arising from bone marrow (BM) haematopoietic stem cells reaching the thymus lose their potential to give rise to B-cell (B) or natural-killer cell (NK) and become double negative $CD4^-$ $CD8^-$ thymocytes. Their migration is punctuated by specific interactions mainly with cTECs, but also with DCs and fibroblasts allowing thymocyte proliferation and initiation of maturation processes intended to ensure T-cell receptor (TCR) development.

1.1.2. TCR FORMATION

The TCR structure is closely related to the one of immunoglobulins (Ig) and consists of two chains, each composed of variable (V) and constant (C) regions. While the C-region gene of

TCR chains encodes essentially for transmembrane proteins, V region genes code for the TCR part that recognizes antigenic peptides linked to major histocompatibility (MHC) molecules. In human, 95% of TCRs consist of the association of αβ chains, the rest being TCR γδ.

Genes coding for TCR-β are first rearranged and lead to the expression of a pre-TCR composed of a fully rearranged TCRβ chain and an invariant pTα chain, at the surface of DN thymocytes (von Boehmer 2005). At this stage, DN thymocytes undergo a first checkpoint for β-chain functionality known as β-selection. Successful TCR-β selection leads to a further step of proliferation and to the surface expression of CD4 and CD8 co-receptors. $CD4^+$ $CD8^+$ double positive (DP) thymocytes undergo TCRα chain rearrangement, giving rise to TCR $\alpha\beta^+CD4^+CD8^+$ thymocytes.

Then, DP thymocytes interact with cTECs, which express high density of MHC class I and MHC class II molecules associated with self-peptides, and enter the selection processes. More than 90% of DP thymocytes establish interactions with self-peptide MHC complexes that are too weak to receive survival signals and die by neglect. In contrary, DP thymocytes that display high affinity TCRs for self-ligands MHC complexes die by apoptosis (**Figure I-1**). This cortical positive selection is a first round of selection preventing the release of self-reactive cells and interestingly does not seem dependent on cTEC functions, but rather attributed to DCs, even though these cells are present in few number in the thymic cortex (Klein et al., 2014; McCaughtry et al., 2008). Finally, only cells that express TCRs recognizing self-ligands with moderate affinity, receive a positive survival signal transmitted by cTECs and go forward positive selection, thus giving rise to differentiated lineage-specific CD4+ or CD8+ simple positive (SP) thymocytes (Nitta and Suzuki, 2016) (**Figure I-1**).

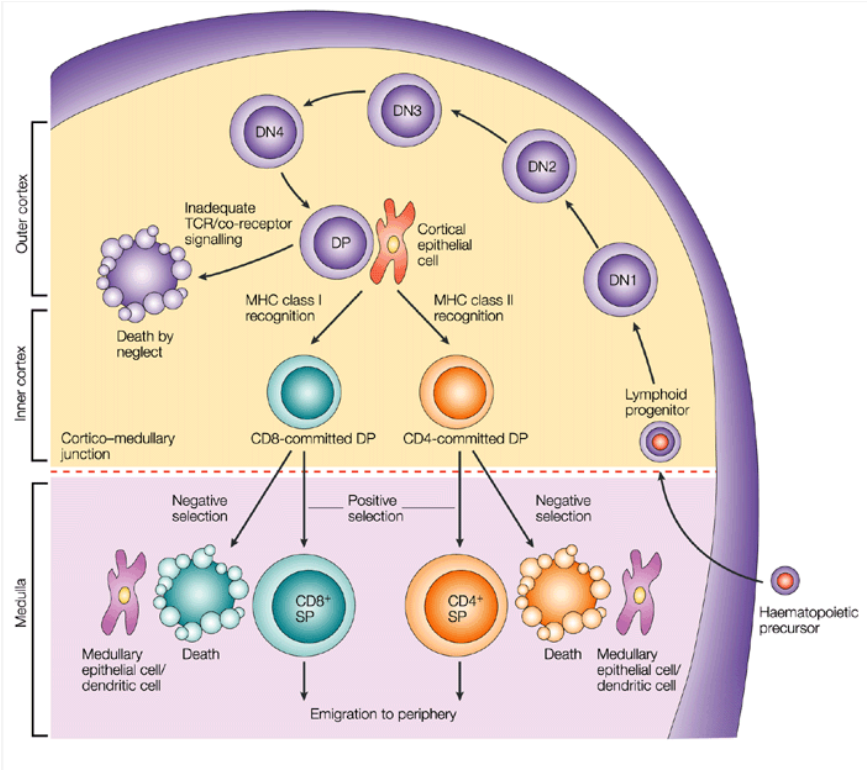


FIGURE I-1 ORGANIZATION OF T CELL DEVELOPMENT AND SELECTION IN THE THYMUS.

Commited lymphoid progenitors arise from bone marrow and enter the thymus via blood circulation. TCR maturation occurs in the cortex while thymocytes are double negative (DN) for CD4 and CD8. Maturation is a step by step process in which thymocytes progress from the inner cortex to the subcapsular area and undergo 4 different stages of differentiation (DN1, DN2, DN3 and DN4) characterized by diverse expression of the markers CD44 and CD25. Positive TCR-β selection allows DN4 thymocytes to proliferate and to become αβTCR$^+$ CD4$^+$ CD8$^+$ double positive (DP) cells. Then, DP thymocytes interact with cTECs and, depending on the signal transduced by TCR/self-antigen –MHC complex interaction, either die by neglect (weak signal) or by acute apoptosis (strong signal) or are positively selected (appropriate intermediate signal) and undergo maturation to single positive CD4$^+$ or CD8$^+$ thymocytes. Successful positive selection leads to the migration of SP cells to the thymic medulla within which they interact with mTECs and DCs. TCR specific interactions of thymocytes with peripheral tissue antigens (PTAs)-MHC complexes presented by mTECS and DCs allow the deletion of self-reactive clones and the approbation for non-self-cells to egress from the periphery as naive T cells. *Adapted from (Germain, 2002)*.

Then, SP thymocytes pursue their instruction in the thymic medulla through interactions with DCs and mTECs, and undergo negative selection aiming at preventing the survival of potential self-reactive lymphocytes and their release in the periphery.

1.2. CENTRAL TOLERANCE: NEGATIVE SELECTION IN THE THYMUS

Central tolerance refers to the thymic mechanisms permitting the deletion of self-reactive newly generated TCR^+ thymocytes from the T cell repertoire. These processes are dependent on specialized antigen presenting cells (APCs), the DCs and mTECs, which present a broad panel of peripheral tissue antigens (PTAs) also known as tissue restricted specific antigens (TRAs or TSAs). SP thymocytes reacting to PTAs are either deleted by apoptotic death or will alternatively give rise to regulatory T cells (Treg) depending on the strength of TCR/self-reactive pTAs interactions (**Figure I-1**). The deletional process is known as negative selection and prevents the survival of potential self-reactive lymphocytes that could lead to autoimmune disorders.

1.2.1. THYMIC APCS ENSURING THE CENTRAL TOLERANCE

Thymocytes displaying TCR with high-affinity for self-peptides MHC complexes undergo negative selection in the thymic cortex and the medulla. Medullary negative selection is driven by interactions of self-reactive SP thymocytes with both mTECs and DCs (Klein et al., 2009) that express a highly diverse set of PTAs (Gotter et al., 2004) presented through MHC molecules by different manners depending on the cell type.

Mature mTECs endogenously express PTAs via an original mechanism, termed promiscuous gene expression (PGE) that is in part controlled by the autoimmune regulator AIRE. AIRE is a nuclear regulatory protein whose binding to unmethylated histone regulates gene expression of PTAs and their presentation by MHC molecules (Peterson et al., 2008). Mutations of AIRE gene in mouse have been shown to affect not only PTAs expression but also clonal deletion (Anderson et al., 2005), medulla organization (Dooley et al., 2008; Milicevic et al., 2010), medullary DC frequency (Lei et al., 2011), maturation of thymocytes (Sha et al., 1988) and

subsequently results in multi-organ autoimmune disorders (Xing and Hogquist, 2012). In human, mutations of AIRE gene on both alleles are commonly linked to the autoimmune polyglandular syndrome type 1 (APS1) also known as polyendocrinopathy candidiasis ectodermal dystrophy syndrome (APECED). More recently, single AIRE point mutations on one allele have been described and related to an increased susceptibility for autoimmune diseases (Oftedal et al., 2015). Even though AIRE dependent mechanisms leading to PTA expression are not yet fully understood, together with recent findings revealing expression of AIRE-independent PTAs in mTECs (Takaba et al., 2015), AIRE appears as a cornerstone of mTEC mediated T cell tolerance.

Thymic DCs are also competent to present PTAs at their cell surface, but this phenomenon is not mediated through endogenous expression and consequently not directly dependent on AIRE. Three different subsets of DCs are found in the thymus: the intrathymic resident ($CD11c^{+}CD11b^{-}CD8a^{+}Sirp\alpha^{-}$) conventional DCs (cDCs), the migratory ($CD11c^{+}CD11b^{+}CD8a^{-}Sirp\alpha^{+}$) cDCs and the migratory ($CD11c^{int}PDCA1^{+}B220^{+}$) plasmacytoid DCs (pDCs) (Klein et al., 2009). Intrathymic resident DCs arise from intrathymic precursors and are able to either cross-present Ags provided by apoptotic mTECs (Koble and Kyewski, 2009) or present functional PTAs-MHC complexes acquired from mTECs (Millet et al., 2008). Immature migratory thymic DCs, which present myeloid characteristics, are mainly found in the medulla and enter the thymus after having trafficked in periphery. Consequently they present self-Ags acquired in periphery and therefore increased central tolerance mechanisms by widening the panel of intrathymic presented Ags (Bonasio et al., 2006). Although their role in central tolerance is less described, pDCs are found in human and mouse thymus, and as cDCs are involved in the T cell repertoire. Thymic pDCs arise from the blood circulation (Li et al., 2009) and are mainly localized in the thymic medulla (Bendriss-Vermare et al., 2001) where they present self-Ags

previously acquired in periphery (Hadeiba et al., 2012). Thymic pDCs are competent at mediating clonal deletion (Hadeiba et al., 2012) and could potentially induce nTreg development (Hanabuchi et al., 2010; Martin-Gayo et al., 2010), this last point remaining to be demonstrated *in vivo*.

1.2.2. MECHANISMS OF NEGATIVE SELECTION

CLONAL DELETION

The interaction of high affinity TCR displayed by autoreactive thymocytes with self-peptide-MHC complexes presented by mTECs and thymic DCs initiates the negative selection of the involved thymocytes by a process called clonal deletion, resulting in their rapid apoptosis (***Figure I-2***). Activation of apoptotic pathways dependent on the orphan nuclear receptor Nur77 and the pro-apoptotic member Bim, the antagonist of Bcl2, have been specifically associated with T cell deletion (Baldwin and Hogquist, 2007; Kovalovsky et al., 2010; Moran et al., 2011).

CLONAL DIVERSION

Another tolerogenic mechanism occurring in the thymus is called clonal diversion and results in the generation of Foxp3^{+} Tregs. Tregs are able to suppress immune responses through diverse mechanisms such as direct cell-cell contact, production of anti-inflammatory cytokines and modulation of DC activation state (Li et al., 2015). Thymocytes giving rise to Tregs display self-reactive TCR specificities and positively interact with self-peptide-MHC complexes presented by thymic APCs. However, the mechanisms by which they avoid clonal deletion and acquire suppressive and regulatory functions are still elusive. The strength and the duration of TCR interactions with self-peptide-MHC complexes could account for the outcome of self-

reactive thymocytes (***Figure I-2***). Alternatively, expression of CD28, IL-2 and TGF-β, all important for peripheral Treg differentiation and survival, could be as well linked to clonal diversion (Lio et al., 2010; Liu et al., 2008). On the other hand, it remains controversial whether clonal diversion depends on PTA presentation by mTEC and/or DCs. While Aschenbrenner *et al.* claimed that it is exclusively supported by mTEC-derived PTAs (Aschenbrenner et al., 2007), others demonstrated that thymic cDCs and pDCs are able to elicit Treg differentiation (Hadeiba et al., 2012; Hanabuchi et al., 2010; Martin-Gayo et al., 2010; Proietto et al., 2008). Finally, Lei et al. proposed another scenario in which mTEC and DCs interplay together to elicit clonal diversion (Lei et al., 2011).

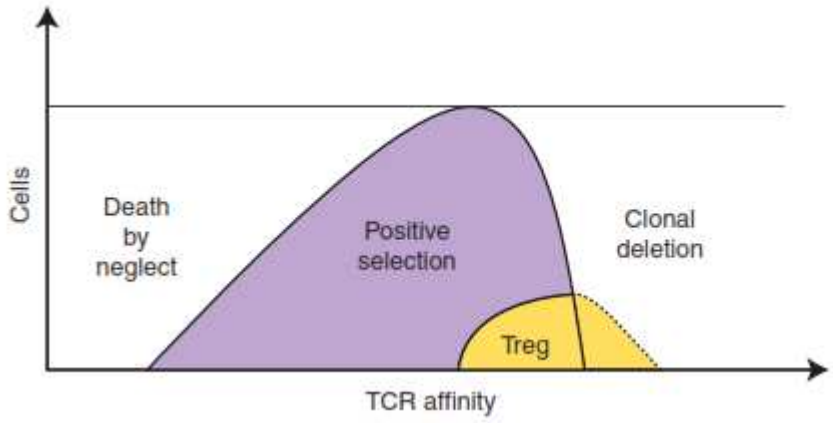


FIGURE I-2 THYMOCYTE OUTCOME IS DEPENDENT ON THE AFFINITY OF TCR FOR SELF-PEPTIDE-MHC COMPLEXES.

Thymocytes that display low affinity TCR for self-peptides presented by cTECs die by neglect whereas cells with intermediate affinity are positively selected. Thymocytes expressing highly self-reactive TCR can either die by apoptosis via clonal deletion or undergo clonal diversion and differentiate in regulatory T cells. *Adapted from (Xing and Hogquist, 2012).*

Mature SP thymocytes, having completed both cortical and medullary selection processes, egress from the thymus and are released into the blood circulation as mature naive T lymphocytes (Nitta and Suzuki, 2016).

1.3. T CELL HOMEOSTASIS

T cell homeostasis characterizes the complex mechanisms permitting the regulation and the maintenance of a mature T cell pool required to mediate adaptive immune responses. Specific TCR contacts with self-Ag-MHC complexes displayed by APCs in secondary lymphoid organs (SLOs) lead to the transduction of tonic homeostatic signals, which in conjunction with IL-7 and IL-15 expression, sustain the expression of anti-apoptotic molecules, such as Bcl-2 and allow prolonged T cell survival (Surh and Sprent, 2008).

Upon activation by cognate foreign Ag presented by APCs, T cells undergo massive expansion, subsequently associated with the acquisition of appropriate effector properties required to fight against invading pathogens (Surh and Sprent, 2008). While most of the effector T cells die within the next few days after their expansion, a small fraction survive and give rise to antigen-specific memory T cells. Memory T cell progeny survives almost indefinitely, but contrary to naive T cells, its survival relies mainly on IL-7 and IL-15 and is independent on TCR/self-peptide-MHC complex interactions (MacLeod et al., 2010). Memory T cell pool is one of the major asset of adaptive immunity relying on the ability to initiate more specific and more robust responses called secondary responses that rapidly control reinfections. Memory T cells are rapidly activated, require lower concentrations of Ag and co-stimulatory signals, and mediate more efficient effector responses (MacLeod et al., 2009).

1.4. T CELL RESPONSES

Because T cells represent one of the major elements of the adaptive immunity, the specificity and the efficiency of their responses notably carry weight in the immune system capacity to mediate and control inflammations and eradicate foreign pathogens. Their requirement is

especially important because they support the maturation and activation of other immune mediators from adaptive (B cells) and innate immunity (macrophages, basophils, eosinophils, mast cells).

1.4.1. PRIMING

- **ANTIGEN SPECIFIC INTERACTION WITH APCS**

Mature $CD4^+$ and $CD8^+$ naive T cells exiting the thymus recirculate via peripheral blood circulation and lymph and reach the SLOs including spleen, peripheral lymph nodes (LN), gut associated lymphoid tissues (GALT, such as Peyer's patches) and bronchus-asssociated lymphoid tissue (Fu et al., 2016). SLOs are very specialized structure characterized by specific tissue organizations that favour Ag-specific interactions between naive T cells and APCs. Specific Ags related to the infection either enter SLOs via migratory DC directly coming from the site of infection or are directly delivered to LN resident DC through the afferent lymph conducts (Sixt et al., 2005). To be primed and to achieve their differentiation, naive T cells require three specific signals provided by the APC: the engagement of the specific TCR by cognate peptide-MHC complexes displayed by APCs (signal I), the co-stimulation (signal II) and the sensing of inflammatory cytokines (signal III) (de Jong et al., 2005; Lafferty and Woolnough, 1977). While the signal I ensures the antigenic specificity of the immune response, the signals II and III delineate the polarization path that naive T helper (T_H) will take. In absence of signal II and III, TCR engagement by peptide-MHC complexes mediates a distinct T cell outcome that contributes to peripheral tolerance (Gallucci et al., 1999; Jenkins and Schwartz, 1987; Shakhar et al., 2005). Thus, depending on the signal they convey to T cells, APCs sorely control the balance between immunity and T cells.

- **POLARIZATION AND FUNCTIONALITY**

While activated $CD8^+$ T cells differentiate into cytotoxic cells able to directly kill infected cells, $CD4^+$ T cell priming can give rise to diverse type of T helper cell subsets exhibiting distinct functions. Polarization of naive $CD4^+$ T cells toward specific T_H subset is orchestrated by the expression of different "master regulators" genes, or specific transcription factors, and T_H subsets are characterized by their ability to secrete distinct cytokines (***Figure I-3***). For a long time persisted the notion that only two T_H sub-populations co-exist: T_H1 cells, which express Interferon-gamma (IFN-γ) and T_H2 cells that produce IL-4, IL-5 and IL-13. While T_H1 cells are important for the clearance of intracellular pathogens, T_H2 cells functionally target extracellular pathogens and parasites (Mosmann and Coffman, 1989). In the last decade, the simplistic conception of T_H1/T_H2 differentiation model has been revisited with the description of other T_H subsets, together with the emergence of a possible plasticity between the different phenotypes (***Figure I-3***). Nowadays, T_H family includes T_H17, T_H9, the follicular T_{FH} and the induced regulatory T cells iTreg. IL-17-producing T_H17 cells are mainly associated with immunological responses contributing to the clearance of extracellular pathogens and fungal infections (Korn et al., 2009). First identified in blood and gut tissue from Crohn's disease patients, it is now widely accepted that T_H17 are also highly involved in other autoimmune diseases (Bartlett and Million, 2015). The role of IL-9-secreting T_H9 and IL-22-producing T_H22 cells is still unclear. T_H9 cells appear to be important mediators of inflammation and have been related to autoimmunity and allergies (Kaplan et al., 2015). T_H22 cells have been associated with epidermal integrity, however they can exert either pathogenic or tolerogenic functions depending on the inflammatory contexts (Jia and Wu, 2014). T_{FH} cells produce IL-4 and IL-21, contribute to the germinal center reactions and are required for the production of high-affinity antibodies (Crotty, 2011).

Finally, Foxp3$^+$ iTregs are generated in periphery from naive CD4$^+$ T cells through the sensing of TGF-β and Retinoic acid (Coombes et al., 2007). Besides their role in tolerance, Tregs are important in the suppression of other effector T cell functions. Upon activation, Tregs exert direct killing of effector T cells and B cells through the expression of granzyme-B (GrB) and perforin (Cao et al., 2007; Zhao et al., 2006) and can mediate cell cycle arrest via the soluble or cell surface expression of Galectin-1(Garin et al., 2007). Because TGF-β is needed for the polarization of both T_H17 and iTregs and even if they exhibit totally distinct functions, these two subsets demonstrate a certain level of instability and plasticity, some iTregs being able to become T_H17 and vice-versa (Kleinewietfeld and Hafler, 2013). Lastly, a distinct subset of regulatory T cells, the Tr1 cells, exist and are characterized by the lack of Foxp3 expression, whereas they produce high levels of IL-10 and TGF-β (Groux et al., 1997). Tr1 are important for the suppression of tissue inflammation and autoimmunity (Clemente-Casares et al., 2016; Pot et al., 2011).

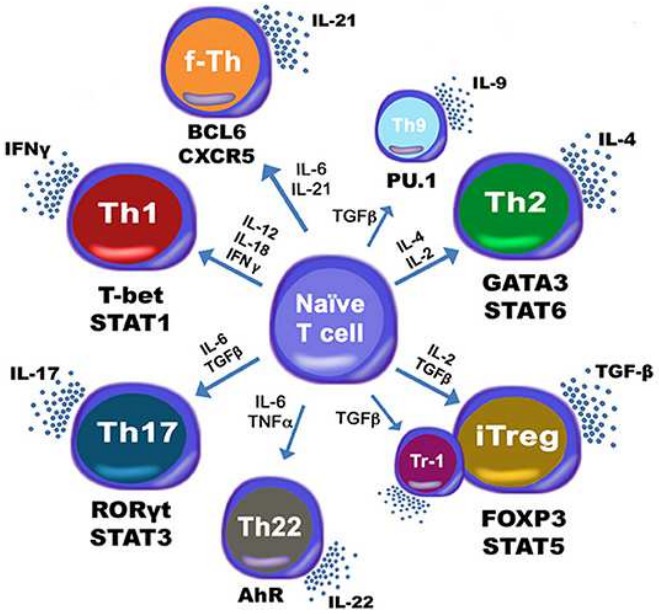


FIGURE I-3 POLARISATION OF CD4$^+$ T CELL SUBSETS.

Depending on the polarizing cytokine milieu, the priming of naive T cells by APCs conducts to distinct specific CD4$^+$ T cell subsets. Differentiation is mediated through the expression of specific transcription

factors depicted here. Each subset is characterized by the expression of cytokines. *Adapted from (Carbo et al., 2014).*

1.4.2. PERIPHERAL T CELL TOLERANCE

As described above, T cells are crucial actors of adaptive immunity, playing a central role in the initiation of immune responses by powerful recognition of foreign Ag fragment in the context of MHC presentation by APCs. Hence when directed to inappropriate targets, as self-Ags, T cells can lead to wide tissue damage and initiate auto-immune disorders. Central tolerance occurring during T cell development in the thymus represents the first line of surveillance allowing the deletion of auto-reactive T cells. However the fact that not all self-Ags are presented by thymic APCs allows the escape of autoreactive T cells from the thymic surveillance, their egress from the thymus and their contribution to the peripheral T cell pool. On the same scheme, food Ags, developmental Ags and Ags released during chronic infections are not presented in the thymus and T cells specifically recognizing these targets are also found in the periphery. Therefore tolerance mechanisms dedicated to pursue the vigilance in periphery are required to permit the control and the suppression of cells that could lead to inappropriate T cell responses.

- **ACTORS OF PERIPHERAL TOLERANCE**

In addition to their great ability to induce immune responses, peripheral DCs also play a crucial role in the induction and the maintenance of peripheral tolerance. DCs originate from the hematopoietic compartment and are represented by different subtypes whose main members are cDCs and pDCs. The role of pDCs in peripheral tolerance being specifically related to the present work, this will be subjected to a next part of this manuscript.

Under physiological conditions, cDCs are able to present Ags through MCH molecules and consequently to interact with steady-state antigen-specific T cells. However, differently to their activated state, they fail to deliver co-stimulatory signals and do not lead to T cell activation and proliferation (Gallucci et al., 1999; Shakhar et al., 2005). Instead, they dispense tolerogenic signals through 1) the production of soluble factors such as TGF-β, IL-10, corticosteroids or the indoleamine 2,3-dyoxygenase (IDO) (Morelli and Thomson, 2007), or 2) the engagement of co-inhibitory molecules such as programmed death-ligands (PD-L1 and PD-L2) (Brown et al., 2003; Okazaki and Honjo, 2006). Depending on the signal initiated, tolerogenic cDCs promote either T cell anergy, T cell deletion or Treg cell induction. In a pioneer study, Adler and colleagues identified that $CD4^+$ T cell tolerance induction to parenchymal self-Ag required Ag presentation by bone marrow-derived APCs (Adler et al., 1998). Then, the ability of DCs to induce T cell deletion and anergy in steady state was further characterized *in vivo* through the induction of specific Ag presentation by DCs via either DC-specific antibody (DEC-205) (Bonifaz et al., 2002; Hawiger et al., 2001) or using an inducible transgenic model (Probst et al., 2003). Ag presentation by DCs in steady state resulted in an intense tolerogenic response characterized by a massive reduction of Ag-specific $CD4^+$ and $CD8^+$ T cells (Bonifaz et al., 2002; Hawiger et al., 2001) and the unresponsiveness of remaining $CD4^+$ and $CD8^+$ T cells to subsequent systemic challenges (Hawiger et al., 2001; Probst et al., 2003). Using the same model of inducible Ag presentation by DCs, PD-1 and the cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) molecules were shown to act synergistically and to be required by resting DCs to promote peripheral CD8+ T cell tolerance (Probst et al., 2005). Lastly, $Foxp3^+$ Tregs have been shown to represent essential contributors to peripheral tolerance mediated by steady-state DCs (Schildknecht et al., 2010).

More recently the notion emerged that, besides their architectural functions, LN stromal cells (LNSCs) are involved in peripheral tolerance. Evidence suggests that fibroblastic reticular cells (FRCs) and lymphatic endothelial cells (LECs) endogenously express a broad set of peripheral tissue Ags (PTAs or TRAs) only partially redundant from the otherwise PTAs presented in the thymus by mTECs and DCs (Cohen et al., 2010; Fletcher et al., 2011). Direct presentation of those PTAs through MHC I molecules expressed by LNSCs has been described and relies on the deletion of PTA-reactive $CD8^+$ T cells. Moreover, LNSCs are also capable of acquiring functional Ag-MHC II complexes from DCs and induce $CD4^+$ T cell anergy, a mechanism recently identified by my co-workers in the lab (Dubrot et al., 2014). In addition, extrathymic AIRE-expressing cells (eTAC) have been identified in LNs and related to the induction of CD8+ T cell deletion and CD4+ T cell anergy (Gardner et al., 2008; Gardner et al., 2013).

- ## MECHANISMS OF PERIPHERAL T CELL TOLERANCE

T CELL ANERGY

Anergy is a tolerance mechanism affecting $CD4^+$ and $CD8^+$ T cell functionalities. Although first described as an *in vitro* mechanism of T cell tolerance, anergy was afterward characterized *in vivo* (Choi and Schwartz, 2007). T cell anergy can be initiated when signal I (TCR/peptide-MHC complex) is provided in absence of signals II and III. This can be the case under physiological conditions, when non-activated APCs express low level of co-stimulatory molecules and produce low amount of pro-inflammatory cytokines, or because T cells interact with cells, such as LNSCs, unable to provide the signals II and III.

In addition, the engagement of CTLA-4 or PD-1 molecules, both expressed by T cells, to their cognate receptors on APCs, is also associated with the initiation of anergy (Chen and Flies, 2013). Anergy leads to long-term hyporesponsiveness of T cells, which become insensitive to subsequent activation. Anergic T cells are characterized by their incapacity to proliferate and

to secrete cytokine, notably due to an active repression of both TCR signaling and IL-2 production (Choi and Schwartz, 2007; Schwartz, 2003).

PERIPHERAL T CELL DELETION

As in the thymus, peripheral deletion results in apoptotic cell death of self-reactive lymphocytes. Besides the Bim-dependent pathway, Fas/Fas-L-induced apoptosis is an important mechanism involved in peripheral deletion (Hughes et al., 2008; Kabelitz et al., 1993). Involvement of these two pathways in peripheral deletion have been demonstrated in transgenic mice following tissue specific Ag expression (Davey et al., 2002; Hernandez et al., 2001) and have been related to autoimmunity (Bouillet et al., 1999; Zhang et al., 2001).

REGULATORY T CELLS

Peripheral tolerance is as well maintained by Treg cells which exhibit the significant function of controlling immune response to self-Ags, tumor Ags, allergens and transplant. Depending on their origin, we can distinguish the natural Tregs (nTregs), which develop in the thymus, and the induced Tregs (iTregs) that are generated in periphery. Both subsets play a pivotal role in the maintenance of T cell homeostasis and tolerance (Feuerer et al., 2009). Because of the lack of marker that would truly allow the distinction between the two subsets, the biological features and the contributions of nTregs and iTregs in peripheral tolerance, tumor immunity and autoimmunity are difficult to clarify. It was however suggested that both subsets exhibit comparable suppressive functions but postulated that nTregs would be more dedicated to maintain self-tolerance and prevent auto-immunity, whereas iTregs, which are preferentially induced in LNs and gut associated lymphoid tissues, would be preferentially involved in the regulation of T cell responses and the maintenance of oral tolerance (Lin et al., 2013).

IMPORTANT INTRACELLULAR TREG MARKERS

Tregs are defined as $CD4^{+}CD25^{high}$ T cells expressing different intracellular markers playing crucial role in their development, maintenance and their acquisition of suppressive functions.

FOXP3. The transcription factor FOXP3 is considered as the master regulator for the development and the functions of $CD25^{+}$ $CD4^{+}$ Tregs, without however being expressed by IL-10-producing Tr1 regulatory T cells (Yagi et al., 2004). Foxp3 controls the expression of numerous genes regulating suppressive cytokine production and Treg surface molecules expression (Rudra et al., 2012). As described further, Foxp3 deficiency leads to very severe autoimmune syndrome (Wing and Sakaguchi, 2010).

HELIOS. Initially described as a specific marker to discriminate between nTregs and iTregs, accumulating evidence showed that Helios, an Ikaros family transcription factor, is expressed by nTregs, iTregs and activated T cells (Akimova et al., 2011; Ross et al., 2014; Sugita et al., 2015). Besides its ability to promote Treg development and stability through the repression of IL-2 gene promoter, Helios is also associated to $Foxp3^{+}$ Tregs exhibiting higher suppressive capacity compared to $Foxp3^{+}$ $Helios^{-}$ Tregs in both human and mouse (Elkord et al., 2015; Getnet et al., 2010; Sugita et al., 2015). Importantly, a recent study reported that selective deletion of Helios in mice leads to progressive systemic immune activation, hypergammaglobulinemia and enhanced germinal center formation in the absence of organ-specific autoimmunity. While Helios-deficient Treg suppressor functions were not affected *in vitro* and potently inhibited inflammatory bowel disease (IBD) *in vivo*, these Tregs failed to control pathogenic T cells derived from scurfy mice and T_H1 response (Sebastian et al., 2016). Helios has an important role in some aspects of Treg-suppressive functions, and further characterization of Helios-mediated suppressive activity in Tregs will allow a better

understanding of the mode of action of this molecule and will reveal the real potential of Helios^{+} Treg in the regulation of specific aspects of T cell tolerance maintenance.

RUNX. The Runt-related transcription factor (RUNX) has been shown to play an important role in the maintenance and the development of Tregs. RUNX notably regulates the initiation of *Foxp3* gene expression in nTregs (Bruno et al., 2009). In addition, RUNX proteins are important for the maintenance of *Foxp3* gene expression in Tregs and consequently indirectly regulate the suppressive function of Tregs (Klunker et al., 2009). Accordingly, RUNX KO mice present important autoimmune disorders, although less severe than in Foxp3 deficient mice (Kitoh et al., 2009).

Besides the expression of intracellular markers, Tregs can also express extracellular markers such as CD69, CD103, ICOS, CTLA4, CD45RA, CD127 and others, whose expression varies depending on the activation state or their functions (Schmetterer et al., 2012).

TREG SUPPRESSIVE FUNCTIONS

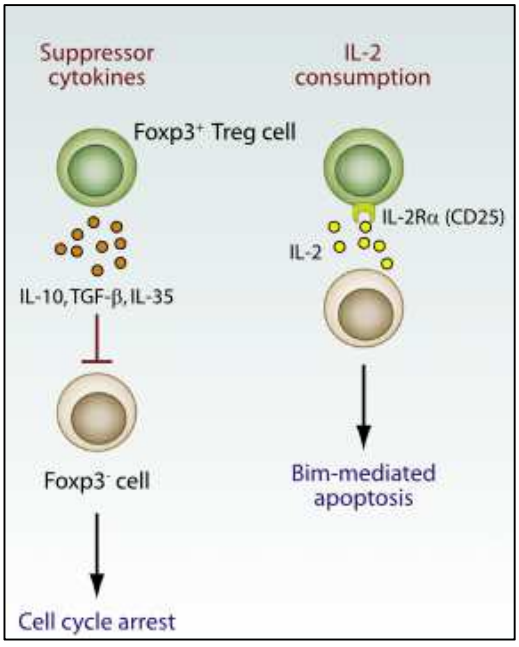
Treg suppressive functions rely on three major mechanisms: 1) their secretion of anti-inflammatory cytokines, 2) their consumption of metabolites, essential for target cells and 3) their ability to establish cell-cell contacts which will influence DCs and affect Foxp3^{-} lymphocytes.

Tregs can exert suppressive activity via the secretion of pro-inflammatory cytokines, such as IL-10, TGF-β and IL-35 (***Figure I-4***). The suppressive activity of these cytokines has mainly been identified in *vivo* either by antibody-mediated neutralization or by using knock-out mice, and they were identified as crucial players in Treg-mediated protection in many autoimmune diseases (Li et al., 2015).

Treg-mediated suppression can also be achieved through the disruption of IL-2, which is critical for the proliferation and the activation of effector T cells (***Figure I-5***). However, it is not clear if Treg-mediated IL-2 deprivation is related to an accrued consumption of IL-2 facilitated by high expression of IL-2 receptor (CD25) on Tregs surface (Pandiyan et al., 2007), or by direct inhibition of IL-2 mRNA transcription in Foxp3$^-$ T_H cells (Oberle et al., 2007). Treg-mediated IL-2 disruption leads to T cell apoptosis by activation of the Bim-dependent pathway (Pandiyan et al., 2007).

FIGURE I-4 DIRECT INHIBITION OF FOXP3$^-$ LYMPHOCYTES BY TREGS.

Tregs secrete anti-inflammatory cytokines that affect lymphocytes functions. Treg-mediated IL-2 disruption results in T cell apoptosis by activation of the Bim-dependent pathway. *Adapted from (Shevach, 2009)*



The third pathway involved in Treg-mediated suppression is achieved through direct interactions of Foxp3$^+$ Tregs with DCs and leads to the modulation of their activation and functions. Binding of the co-inhibitory molecule CTLA-4, which is highly expressed by Treg cells, to its cognate ligands CD80 and CD86 displayed by DCs, inhibits the interactions of DCs with the co-stimulatory molecule CD28 expressed by effector T cells (Walker, 2013) (***Figure I-5***). This mechanism relies not only on a competition between CTLA-4 and CD28, which share the same ligand receptors, but also on the physical excision and degradation of B7 molecules (CD80 and

CD86) from the DC surface by trans-endocytosis (Qureshi et al., 2011). In addition, upon binding to B7 molecules, CTLA-4 also up-regulates the expression of the tolerogenic protein IDO by splenic CD19+ DCs resulting in IDO-linked inhibition of effector T cell clonal expansion and apoptosis (Malapat 2007, Fallarino 2003, Mellor 2005, Baban 2005). Specific Treg-DC interactions also occurred through the binding of lymphocyte activation gene-3 (LAG-3) trans-membrane protein expressed by Tregs to MHCII molecules, leading to the inhibition of DC maturation and the suppression of their immunostimulatory capacity via decreased Ag presentation (Liang et al., 2008) (***Figure I-5***). In another anti-inflammatory mechanism dependent on CD39 expression, activated Tregs similarly influence DC maturation. Through its enzymatic activity, CD39 hydrolyses extracellular ATP that is released through cell damage. Thus, Tregs limit extracellular ATP sensing by DCs and inhibit their activation (Borsellino et al., 2007) (***Figure I-5***). Finally, Neuropilin-1 (Nrp-1), a specific receptor expressed by $Foxp3^{+}$Treg, promotes prolonged interactions of Tregs with immature DCs and therefore potentiates synapse formation between these two cell types (Sarris et al., 2008) (***Figure I-5***). This mechanism appears particularly important at limited Ag concentrations, giving advantages to Tregs compared to naive T cells for interacting with DCs. Anti-Nrp1 antibodies notably inhibit Treg-mediated suppression of T cells upon stimulation with low Ag concentration (Sarris et al., 2008). Therefore, Treg-mediated control of DC functions appears to be a key mechanism of tolerance maintenance through the simultaneous inhibition of distinct DC functions: Ag presentation, expression of co-stimulatory molecules and cytokine production. By preventing DC functions as a whole, Tregs significantly impair the expansion of self-reactive T cells and their differentiation into effector T cells such as T_H1, T_H2 or T_H17 cells.

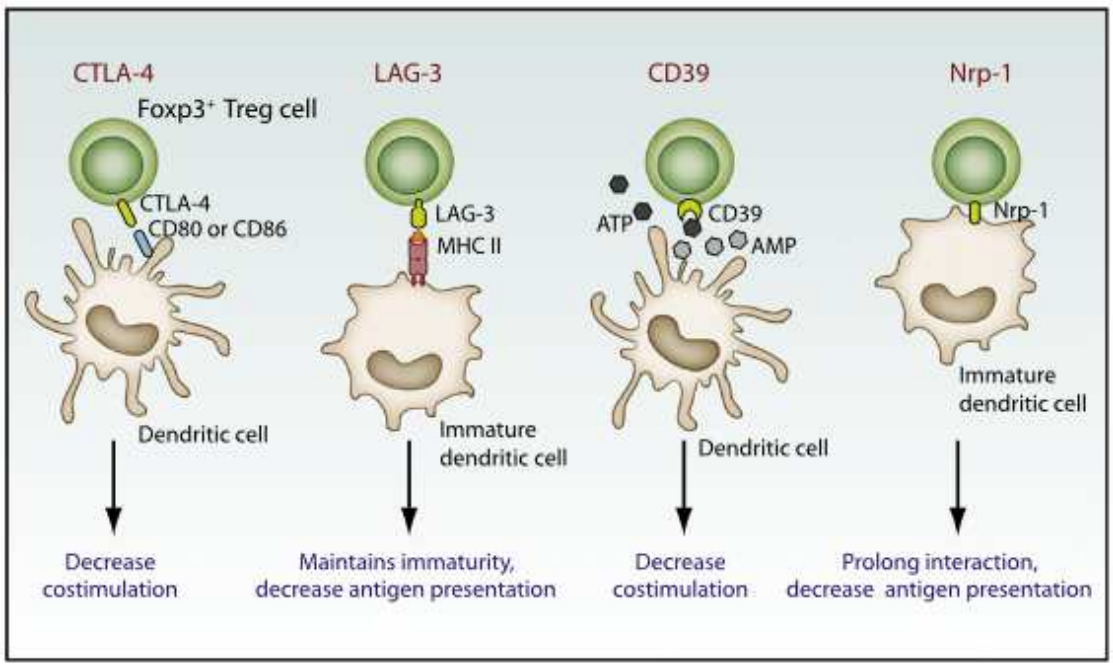


FIGURE I-5 SUPPRESSION OF DC FUNCTIONS BY TREG CELLS.

CTLA-4 prevents interaction of B7 molecules (CD80 and CD86) expressed by DCs either by competition with co-stimulatory molecules CD28 or by trans-endocytosis of CD80/86 molecules. Through interaction with MHCII molecules, LAG3 prevents the transduction of activation signal necessary for DC maturation. Following tissue destruction, ATP released by damaged cells is sensed by DCs and functions as an indicator, resulting in DC activation. Via its enzymatic activity, CD39 hydrolyses extracellular ATP to cAMP, limiting DC activation. Nrp-1 promotes prolonged interactions between Tregs and immature DCs. This mechanism gives advantages to Tregs relative to naive T cells for interacting with DCs, thus restricting naive T cells activation. *Adapted from (Shevach, 2009)*

Treg dysfunction has been extensively related to autoimmunity. In human, deficiency in Foxp3 genes results in lethal autoimmune disorders. Mutations in human Foxp3 are related to IPEX (immune dysregulation, polyendocrinopathy, enteropathy X-linked) syndrome, leading to early autoimmune disease development that could even start *in utero* (Wing and Sakaguchi, 2010). The majority of individuals with IPEX succumb to type 1 diabetes independently on their TCR affinity or HLA haplotype susceptibility (Gambineri et al., 2008), highlighting the crucial role of Tregs in mediating an intrinsic regulation of the immune system. In an elegant

study, Danke *et al.* showed that self-reactive T cells are constitutively present in peripheral blood of healthy patients, but are constantly repressed by Tregs. Indeed, following $CD25^+$ T cell depletion *in vitro*, stimulated peripheral blood T cells from healthy patients promote auto-reactive T cell expansion, directed toward various target organs involved in autoimmune disorders, and secreting pro-inflammatory cytokines. Addition of $CD25^+$ Tregs to the cell culture restored the repression of self-reactive cell proliferation (Danke et al., 2004). In another interesting study, comparison of autoreactive T cells, identified with Ag-specific tetramer reagents, from healthy donors and T1D patients showed similar capacity to respond *in vitro* to cognate Ag, but while autoreactive T cells from T1D patients exhibit a more memory T cells phenotype, those from healthy donors were mainly naive (Danke et al., 2005). Thus, more than their ability to suppress activated T cells, and to control DC activation, Tregs are also able to keep self-reactive T cells in a quiescent state.

In mice, Treg depletion conducts to chronic multiple organ autoimmune disorders. Wild type (WT) mice depleted of $CD4_+CD25_+$ T cells have developed inflammatory bowel disease (IBD) (Singh et al., 2001). Scurfy mice, lacking Foxp3 gene expression, exhibit severe lymphoproliferative autoimmune disease in early times after birth (Hadaschik et al., 2015). In addition, Tregs also play a primordial role in the control of the development of both colitis (Haribhai et al., 2009) and experimental autoimmune encephalomyelitis (EAE) (Irla et al., 2010; O'Connor and Anderton, 2008). On the other hand, mouse studies have also permitted to highlight the crucial function of CTLA-4 dependent and IL-2 dependent suppressive mechanism of Tregs in peripheral T cell tolerance. Indeed, contrary to other Treg mechanisms, specific neutralization of CTLA-4 and IL-2 dependent pathways leads to spontaneous autoimmunity in healthy mice and accrued disease development in autoimmune models. Inhibition of CTLA-4 by monoclonal antibody injection in healthy mice, promotes organ-

specific autoimmune disease and IBD (Takahashi et al., 2000), and is associated with exacerbated diabetes when administrated to NOD mice (Luhder et al., 1998). Moreover, mice with Treg-specific deficiency in CTLA-4 exhibit hyper-production of Ig-E and die of spontaneous lymphoproliferative autoimmune disorders (Wing et al., 2008). Finally, both WT mice in which IL-2 has been neutralized and IL-2 deficient mice are highly susceptible to autoimmunity (Sakaguchi et al., 2009; Setoguchi et al., 2005).

2. ROLE OF PLASMACYTOID DENDRITIC CELLS IN T CELL TOLERANCE AND AUTOIMMUNITY

2.1. PDCS: WHO ARE THEY?

Plasmacytoid dendritic cells (pDCs) have been observed for the first time in 1958 in human LNs and were described as clusters of lymphoid cells (Lennert and Remmele, 1958; Vollenweider and Lennert, 1983). Their plasma cell like morphology, their ability to produce type I IFN (IFN-I) as well as their expression of $CD4^+$ T cell marker earned them to be termed plasmacytoid monocytes, IFN-producing cells (iPCs) and plasmacytoid T cells (Chehimi et al., 1989; Toonstra and van der Putte, 1991; Vollenweider and Lennert, 1983). The identification of their DC nature came only in 1997 with the work of Grouard *et al.* (Grouard et al., 1997), and they had to wait until the end of the 20^{th} century to be characterized as a unique distinct cell type (Cella et al., 2000; Siegal et al., 1999).

2.1.1. PDCS GENERATION IN THE BONE MARROW

pDCs belong to the heterogeneous family of DCs and constitute with conventional DCs one of the major subset of this family (Merad et al., 2013). As cDCs, pDCs develop in the BM from hematopoietic early committed progenitors under the influence of specific growth factors. Under the sensing of FMS-related tyrosine kinase 3 ligand (FLt3L), common dendritic progenitors (CDP) arising from either the lymphoid or the myeloid pool, generate pre-DCs or immature pDCs (D'Amico and Wu, 2003; Karsunky et al., 2003) (***Figure I-6)***. Expression of transcription factors such as IRF8, E2-2, STAT3 and 5, SpiB and Ikaros supports pDC development and definitively inhibits the differentiation of these cells into cDCs (Allman et al., 2006; Cisse et al., 2008; Nagasawa et al., 2008) (***Figure I-6)***. Importantly, IRF8 and E2-2 are specifically required for pDC lineage commitment from CDP cells, and a continuous expression of E2-2 ensures pDC homeostasis (Reizis et al., 2011). Recently Runx2, a transcription factor belonging to Runt family was highlighted as essential for the expression of CCR2 and CCR5 chemokine receptors required for the release of mature pDCs from the BM to the periphery (Sawai et al., 2013) (***Figure I-6)***.. Mature pDCs travel via the blood circulation by which they mainly reach the SLOs, and to less extent, the thymus.

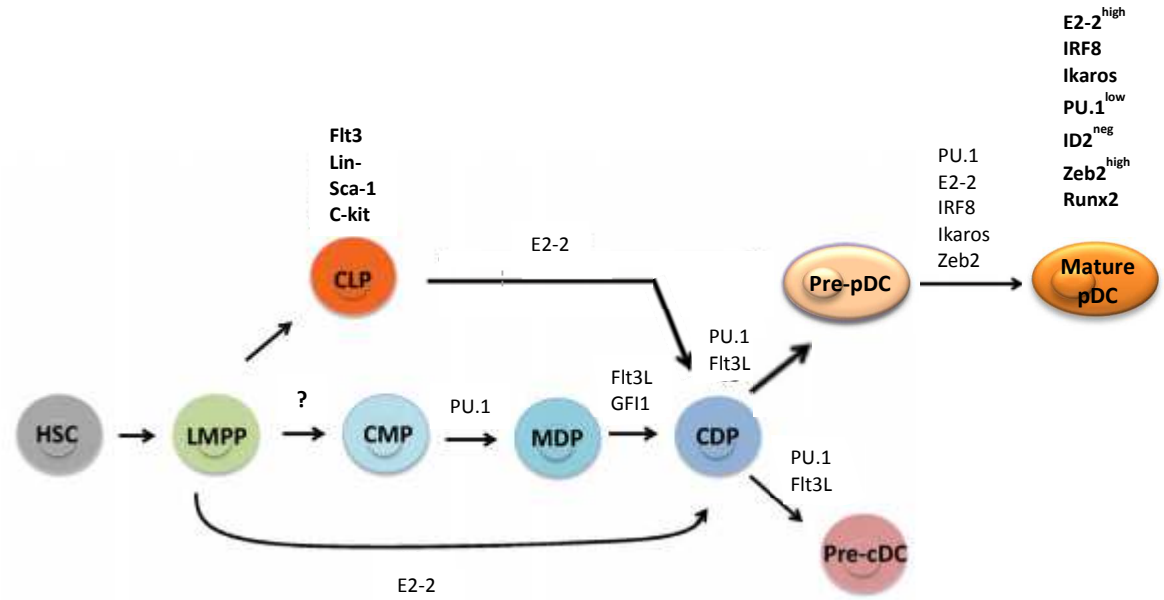


FIGURE I-6 MAIN TRANSCRIPTION AND GROWTH FACTORS GOVERNING PDC SUBSET DEVELOPMENT IN THE BONE MARROW

This graph represents the key transcription and growth factors required for pDC development from myeloid or lymphoid BM precursors. pDCs and cDCs subsets derived from CDP. Flt3L is the main growth factor leading to pDC development. E2-2 plays a fundamental role in the maintenance of pDC development and phenotype. Recently, it has been shown that the transcription factor zinc finger E box–binding homeobox 2 (Zeb2) is expressed in pre-pDC and, by repressing ID2, contributes to the differentiation of pre-pDCs to mature pDCs (Scott et al., 2016). CDP, common DC progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; Flt3L, Flt3 ligand; GFI1, growth factor independent 1; HSC, hematopoietic stem cells; LLMP, lymphoid-primed multipotent progenitor; MDP, macrophage and DC progenitor. *Adapted from (Galicia and Gommerman, 2014).*

2.1.2. PHENOTYPE OF PDCS

Human and mouse pDCs represent less than 0.5% of peripheral blood cells and are mainly found in SLOs. In steady state, they display low cell surface levels of MHCII and co-stimulatory molecules (Reizis et al., 2011). As a result of their expression of different cell type markers as well as the diversity of their functions, pDCs have been difficult to characterize for many years. In the last decade, the identification of relatively specific markers for pDCs in human and mouse permitted to specifically delineate their own functions and to widely investigate their role in immunity and tolerance.

- **HUMAN PDCS**

Human pDCs express IL3Rα (CD123), the T cell marker CD4 and the B and T cell marker CD45RA, whereas they did not display other B cell, T cell, NK cell and myeloid cell linage markers and are negative for the integrin CD11c (Reizis et al., 2011). In human, pDCs can be in part discriminated by the expression of the blood dendritic cell antigen-2 (BDCA-2 or CD303), a member of the type C lectin family involved in the capture and the internalization of Ags (Dzionek et al., 2001). However upon activation or when cultured *in vitro*, human pDCs lose the expression of this marker which consequently cannot be used to follow and discriminate them *in vivo*. Human pDCs also express BDCA-4 (CD304 or neuroplilin-1), which contrary to BDCA-2, is up-regulated after *in vitro* activation (Dzionek et al., 2000). More recently, the immunoglobulin-like transcript-7 (ILT-7), which belongs to the family of inhibitory and stimulatory cell surface immune receptors, has been identified as a novel marker for human pDCs (Cao et al., 2006) (***Figure I-7***).

- **MOUSE PDCS**

Identified in 2001, murine pDCs are positive for the leukocyte common antigen CD45, the T and B cell markers $CD4^+$ and $B220^+$, the monocyte marker Ly6C, and display low surface levels of CD11c (Asselin-Paturel et al., 2001). Like human pDCs, they are characterized by weak expression of MHCII and co-stimulatory molecules under physiological conditions (Reizis et al., 2011). In steady state, pDCs are the only cell type characterized by the expression of the tetherin bone marrow stromal antigen-2 (BST-2 or PDCA-1), which can however be up-regulated by other immune cells upon inflammation (Blasius et al., 2006). Murine pDCs are also positive for the sialic acid-binding immunoglobulin like lectin-H (SIGLEC-H) which is linked to pDC endocytic functions (Zhang et al., 2006). Finally, fully differentiated pDCs emerging

from the BM can be discriminated from immature pDCs by the acquisition of the specific receptor Ly49q (Kamogawa-Schifter et al., 2005) (***Figure I-7***).

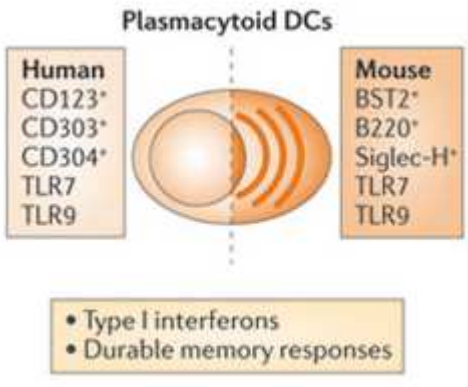


FIGURE I-7 MAJOR MARKERS DIFFERENTIATING HUMAN AND MOUSE PDCs.

Human pDCs are characterized by the expression of CD123, CD303 (BDCA-2), CD304 (BDCA-4 or neuropilin-I). Mouse pDCs express the B cell marker B220, BST2 (PDCA-1) and Siglec-H at their surface. Both human and mouse pDCs express the endosomal markers TLR7 and TLR9. *Adapted from (Collin et al., 2011).*

2.1.3. pDC FUNCTIONS

Peripheral pDCs express several patterns recognition receptors (PRRs) that sense pathogen-associated molecular patterns (PAMPs) released by viruses and bacteria, and damage-associated molecular patterns (DAMPs) discharged by host cells, and have a preponderant role in tissue injury detection. In addition, they also express MHCI, MCHII and co-stimulatory molecules, allowing them to modulate T cell responses. Hence, this two-faced profile manages the diversity of their functions that consequently influence immune responses of distinct natures.

- **pDC INNATE FUNCTIONS**

Primary named IFN producing cells, pDCs have the ability to rapidly secrete abundant amounts of IFN-α and IFN-β, which can be up to thousand fold more important than in other cells (Liu, 2005). Type I IFN (IFN-I) secretion can be initiated following activation of PRRs and particularly the endosomal acid-sensing Toll like receptors TLR7 and TLR9 recognizing single-stranded DNA

(ssDNA), single-stranded RNA (ssRNA), and synthetic ligands containing unmethylated CpG motifs (Asselin-Paturel et al., 2001; Cella et al., 2000; Liu, 2005; Siegal et al., 1999), or by the activation of the *toxoplasma* profiling-specific TLR12 (Koblansky et al., 2013). Importantly, it has been shown that pDCs free themselves from the traditional autocrine IFN-α receptor (IFNAR) pathway responsible for IRF7 expression, the master regulator of IFN genes transcription. Although the IFNAR pathway is functional in pDCs, naive pDCs constitutively express IRF7, and IRF7 activation is further potentiated in pDCs by post-transcriptional mechanisms dependent on the TLR signaling adaptor MyD88 (Colina et al., 2008; Honda et al., 2005; Ito et al., 2006). Therefore, pDCs isolated from $IFNAR^{-/-}$ mice exhibit the capacity to produce type I IFN similar to WT pDCs (Barchet et al., 2002).

Besides the production of IFN-I, TLR7/9 activation as well as TLR2 stimulation by bacterial polysaccharide A, leads to pDC phenotypic and functional maturation. This includes MHII up-regulation, the expression of the co-stimulatory molecules CD80, CD86 and CD83, and the production of inflammatory cytokines such as TNF-α, IL-12, IL-6 and chemokines including CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CXCL8 (IL-8) and CXCL10 (IP-10) (Dasgupta et al., 2014; Osawa et al., 2006).

The secretion of type I IFN as well as the production of cytokines and chemokines by pDCs induces direct or indirect alterations of different functions on a broad range of immune cells (myeloid DC, monocytes, NK cells, T and B lymphocytes), including their activation, their survival and/or their recruitment (Swiecki and Colonna, 2010). Moreover, IFN-I acts in an autocrine manner on pDCs by favouring their survival and by regulating their migratory capacity through the up-regulation of CCR7 (Asselin-Paturel et al., 2005; Ito et al., 2001). Importantly, a recent study related that IFN-I promotes fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS) and therefore affects cellular metabolism. In pDCs, TLR9

activation by CpGA promotes the production of IFN-I which, through an autocrine IFNAR sensing, increases FAO that subsequently enhances OXPHOS and results in critical changes in gene expression. This pathway induces a metabolic reprogramming in pDCs, including an increase in the availability of cellular ATP, which is critical for their functional activation (Wu et al., 2016).

Through their innate functions, pDCs play a pivotal bridge between innate and adaptive immunity and are hence involved in the clearance of viral infections and in the development of auto-immunity (Swiecki and Colonna, 2015) (***Figure I-9***).

- **ADAPTIVE FUNCTIONS OF pDCs**

Besides their important role in the innate immunity, pDCs enable Ag specific interactions with T cells, and are consequently involved in the regulation of adaptive immune responses.

Human and murine pDCs possess all the machinery to capture, process and present Ag through MHCI and MHCII (Tel et al., 2012; Villadangos and Young, 2008). Exogenous Ag capture is mainly fulfilled thanks to endocytic receptors such as Siglec-H and BST-2 for murine pDCs (Loschko et al., 2011a; Loschko et al., 2011b; Viswanathan et al., 2011; Zhang et al., 2006) or BDCA2 and the c-type lectin receptor DCIR expressed at the surface of human pDCs (Dzionek et al., 2001). Several mechanisms, depending on the endocytic receptors engaged, are used by pDCs to internalise exogenous Ag and are subsequently associated to the inhibition of type I IFN production (Blasius et al., 2006; Meyer-Wentrup et al., 2008). In addition, human and mouse pDCs are as well able to cross-present extracellular Ag on MHCI, and therefore contribute to the activation of $CD8^+$ T cell responses important for anti-viral and

anti-tumoral immunity (Di Pucchio et al., 2008; Kool et al., 2011; Liu, 2005; Meyer-Wentrup et al., 2008; Tel et al., 2012).

Because the majority of the *in vivo* studies aiming at determining the role of pDCs in different inflammatory contexts were performed through antibody-mediated or genetic cell depletion, it has been difficult for years to decipher the relative contribution of innate and adaptive pDC functions. Recently, *in vitro* and *in vivo* studies using pDC specific Ag-targeting or selective deletion of MCHII expression in pDCs have widely support the direct impact of this specific DC subset on T cell responses (Guery et al., 2014; Irla et al., 2010; Loschko et al., 2011a; Loschko et al., 2011b) (***Figure I-8***).

While in steady state, pDCs exhibit mainly tolerogenic functions. Once activated, they promote both immunogenic or tolerogenic responses. Upon inflammation, pDCs are massively recruited to inflamed tissues and importantly in particular to the LN T cell area, supporting their involvement in T cell priming (Cella et al., 1999; Irla et al., 2010). Their activation is also characterized by the acquisition of DC morphology and the up-regulation of MHCII and co-stimulatory molecules at their surface. *In vitro*, Ag-specific activation of mouse and human pDCs supports $CD4^+$ T cell priming and differentiation in T_H1 cells (Cella et al., 2000; Krug et al., 2001) and T_H17 (Yu et al., 2010). *In vivo*, Ag-specific delivery to TLR-activated pDCs promotes T_H1 cells inhibiting viral infection and tumor growth (Loschko et al., 2011b). In tumor context, CpG-B activated pDCs induced tumor-specific T_H17 cells which impact tumor growth and are required for intratumoral Cytotoxic T lymphocytes (CTL) recruitment (Guery et al., 2014; Xu et al., 2012). In addition, in mouse models of CNS autoimmunity, pDCs have been shown to promote Tregs and control disease development (Irla et al., 2010; Loschko et al., 2011a).

A human study published this year identified in human peripheral blood a new pDC subset expressing Siglec-1, an adhesion molecule usually displayed by macrophages-monocyte lineage and known to promote T cell activation and pro-inflammatory cytokine secretion (Wilhelm et al., 2016). Siglec-1 expressing pDCs exhibit a semi-mature phenotype characterized by the expression of HLA-DR, CD80, CD86 but not CD83 and reduced levels of BDCA-2. In addition, contrary to Siglec-1^- pDCs, Siglec-1^+ pDCs are unable to produce IFN-α production *in vitro* following TLR7 and TLR9 stimulation, thereby supporting adaptive rather than innate function of this pDC subset (Wilhelm et al., 2016). Because Siglec-1 has been shown to play a role in anergy induction (Kirchberger et al., 2005), the authors hypothesized that Siglec-1^+ pDCs could be specialized in the maintenance of self-tolerance (Wilhelm et al., 2016). This hypothesis is supported by the fact that Siglec-1^+/Siglec-1^- pDC ratio is increased in peripheral blood of patients with systemic lupus erythematosus, suggesting that Siglec-1^- pDCs secreting IFN-I would migrate from the periphery to the inflamed tissues, whereas tolerogenic Siglec-1^+ pDCs would be retained in the periphery (Wilhelm et al., 2016). Although this theory is interesting, it remains hypothetic and worth to be supported by future studies.

Therefore, through their capacity to interact with T cells, pDCs are involved in immunity, auto-immunity and tolerance (Cervantes-Barragan et al., 2012; Ochando et al., 2006; Swiecki and Colonna, 2015).

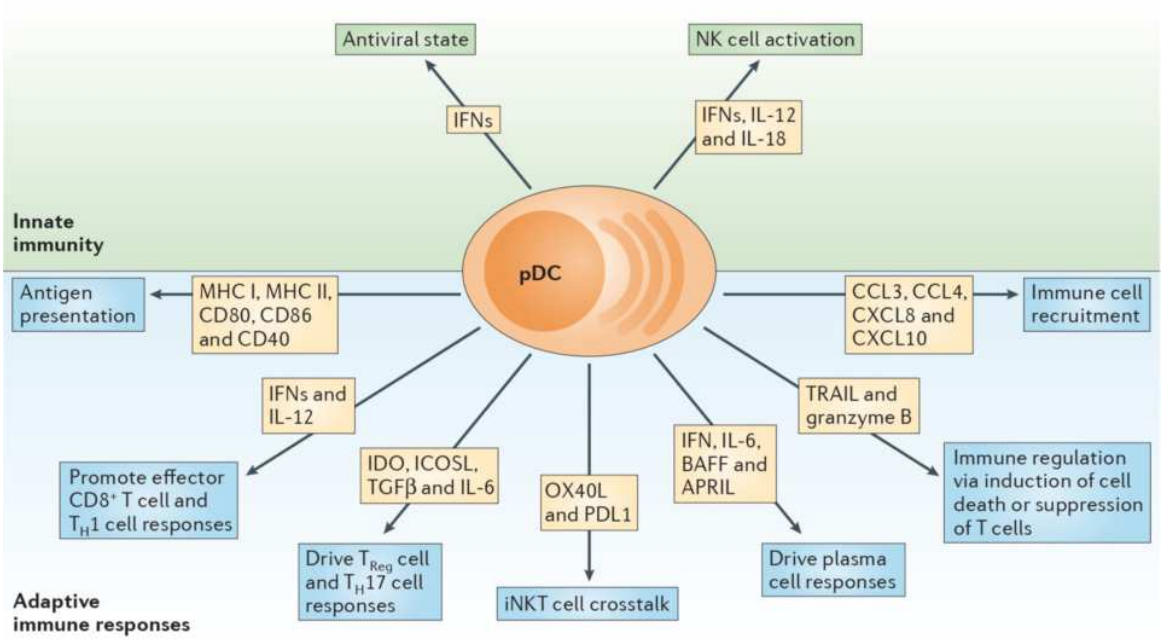


FIGURE I-8 PDCS LINK INNATE AND ADAPTIVE IMMUNITY THROUGH DIVERSE FUNCTIONS.

Upon viral infection, pDCs produce large amounts of IFN-I, promoting the induction of interferon stimulated gene expression and resulting in apoptosis of infected cells. Via IFNs, IL-12 and IL-18, pDCs activate NK cells to induce target cell lysis. Ag-presentation through MHC I and MHC II molecules and co-stimulatory molecule expression by pDCs allow them to interact with $CD8^+$ and $CD4^+$ T cells. IFN- and IL-12-secreting pDCs enhance $CD8^+$ T cell effector functions and contribute to the polarization of naive T cells into T_H1 cells. Expression of IDO, ICOSL along with the secretion of TGF-β and IL-6 elicit pDCs to promote either Treg or T_H17. pDCs interplay with invariant NKT (iNKT) cells either through OX40/OX40L or PD-1/PD-L1 interactions and regulate antiviral adaptive responses. Production of IFN-I, IL-6, BAFF and APRIL by pDCs promotes B cell differentiation into antibody-producing plasma cells. Upon TRAIL and granzyme B production, pDCs suppress tumor and infected cells. Secretion of chemokines such as CCL3, CCL4, CXCL8 and CXCL10 by pDCs leads to the recruitment of immune cells on inflammatory or infected sites. *Adapted from* (Swiecki and Colonna, 2015)

2.2. Role of pDCs in T cell tolerance and autoimmunity

Besides their involvement in infections, pDCs play important roles in tolerance induction, anti-tumoral responses, allergies and autoimmunity. Through the diversity of their functions and

depending on the context in which they will evolve, pDCs may be associated to pro-immunogenic and tolerogenic scopes.

In the next paragraphs I will tackle the most described immune contexts for which pDCs have been shown to be important. Notably, we will see that, contrary to cDCs, their ability to mediate tolerance *versus* immunity is not only dependent on their activation state but as well relies on their localisation and their environment.

2.2.1. *pDC-MEDIATED TOLEROGENIC T CELL RESPONSES IN STEADY-STATE AND STERILE-INFLAMMATION CONTEXT*

Despite their relative poor ability to present Ag, immature pDCs promote, in particular contexts, T cell deletion, T cell anergy and mediate Treg differentiation and expansion. Through these mechanisms, they have been involved in oral tolerance and play an important role in alloimmune tolerance and tolerance to cancer.

- **pDCs IN MUCOSAL TOLERANCE**

Oral tolerance represents an immune unresponsiveness state that prevents inappropriate immune responses against inhaled or innocuous Ags. Two major studies have highlighted a preponderant role of pDCs in the induction and the regulation of oral tolerance. In mouse, pDCs prevent T cell priming following Ag feeding with proteins and haptens, their depletion resulting in delayed-type hypersensitivity (DTH) responses associated with Ag-specific $CD8^+$ T cell priming (Goubier et al., 2008). In the same insight, transfer of Ag-loaded immature pDCs, originated from the liver of Ag-fed mice, into naive mice induce the anergy and the deletion of Ag-specific $CD4^+$ and $CD8^+$ T cells (Goubier et al., 2008). Moreover, in a model of allergic contact dermatitis mediated by hapten-specific cytolytic $CD8^+$ T cells, pDCs promote the deletion of Ag-specific T cells in gut-associated lymphoid tissues through the induction of Tregs

(Dubois et al., 2009). These last observations support a role for Ag presentation by pDCs in oral tolerance, which likely occurs in the mesenteric LN, the Peyer's patches and the liver.

Peyer's patches DCs contribute also to the induction of IgA class switch recombination in B-cells through the production of critical factors such as IL-6, retinoic acid, BAFF and APRIL in response to TLR ligation (Tezuka et al., 2007; Uematsu et al., 2008). IgA are important suppressor of pro-inflammatory intestinal T cells responses and largely influence the maintenance of gut homeostasis. Although Peyer's patches cDCs and pDCs have been proposed to contribute to the IgA response, a recent study relates that pDCs are dispensable for this mechanism (Moro-Sibilot et al., 2016). Using transgenic mice allowing *in vivo* pDC depletion (BDCA2-DTR and $CD11c^{Cre}E2\text{-}2flox$ mice), the authors demonstrate that pDCs do not impact B-cell priming and T cell independent IgA class switch recombination after oral immunization with NP hapten coupled to ficoll. Therefore, this study suggests that pDCs ensure gut immune homeostasis independently on the induction of IgA switch recombination in Ag-specific B cells (Moro-Sibilot et al., 2016).

In another mouse study, pDCs have been shown to uptake inhaled Ags in the lung and present them to T cells in draining LNs. Indeed, pDC depletion following Ag-inhalation elicits IgE sensitization, airway eosinophilia infiltration, goblet cell hyperplasia and Th2 cell cytokine production. In addition, adoptive transfer of pDCs prevents the generation of effector T cells responsible for asthma development (de Heer et al., 2004).

Together these studies show that pDCs are important for the maintenance of oral tolerance and the prevention of unwanted inflammatory responses to harmless Ags. In addition, these works elicit pDCs as potential therapeutic targets for the treatment of mucosal and systemic inflammatory diseases.

- **pDCs IN ALLO-IMMUNE TOLERANCE**

In the last decade, several studies have related the involvement of pDCs in the promotion of allogeneic engraftment. First, TLR-activated plasmacytoid precursor DCs (p-preDCs) have been described as necessary components of the facilitating cells (FC) BM population favouring allogeneic hematopoietic stem cell (HSC) engraftment (Fugier-Vivier et al., 2005). Through the induction of Ag-specific Treg cells, p-preDCs significantly enhance HSC engraftment and reduce graft versus host disease (GVHD) (Cardenas et al., 2011; Huang et al., 2011). In a recent interesting review, Yolcu *et. al.* discussed the mechanisms relative to $CD8^+/TCR^-$ FC population, which includes p-preDCs, in the facilitation of HSC engraftment (Yolcu et al., 2015). Notably, they described that human p-preDCs belong to a specific FC population expressing the surface marker CD56 (Huang et al., 2016) and produce specific factors promoting the migration and the durable engraftment of HSC in the hematopoietic niche (Huang et al., 2016; Yolcu et al., 2015).

In another model of allogeneic $CD4^+$ T cell transfer into irradiated mice, immature CCR9+ pDCs are potent inducers of $Foxp3^+$ Tregs able to inhibit Ag-specific immune responses and suppress GVHD (Hadeiba et al., 2008; Hanabuchi et al., 2010) presumably through ICOS-ICOSL (Collin et al., 2011; Ogata et al., 2013) or IDO (Lu et al., 2012) dependent signalisation pathway.

In addition, pDCs promote tolerance in organ transplantation. They have been identified as phagocytic APCs required for tolerance in a transplantation model of vascularized cardiac allografts. Alloantigen-presenting recipient pDCs migrate from the graft to the draining LNs and induce alloantigen-specific $Foxp3^+$ Tregs which promote allograft tolerance. Both pDC depletion and prevention of pDC homing to LNs inhibit Treg development and tolerance induction, whereas the adoptive transfer of tolerized pDCs prolonged graft survival (Ochando et al., 2006). In another cardiac allograft transplantation model, the lack of pDCs in LN from

CCR7$^{-/-}$ mice leads to Treg impairment and graft rejection, two phenomena counteracted by the transfer of syngenic pDCs (Liu et al., 2011). In a heart transplant model of tolerance in rat, by injecting anti-CD40L antibody and donor-specific blood cells, pDCs accumulate in the graft and, in an IDO-dependent manner, promote CD8^{+} Tregs which suppress allo-reactive CD4^{+} T cell responses (Li et al., 2010).

Importantly human studies support these observations and demonstrated that pDCs promote CD8^{+} Treg in an IDO-dependent manner and suppress alloimmune responses (Boor et al., 2011).

- **pDC-MEDIATED IMMUNE REGULATION IN CANCER**

The tumoral microenvironment comprising cancer cells and stromal cells is the scene of a complex ballet in which protective and tumor-promoting immune cells are constantly counteracting. Through their ability to orchestrate innate and adaptive immunity, activated pDCs represent powerful actors of antitumor immunity. However, when recruited to the tumor, pDCs are often restricted to an unactivated tolerogenic state and hence encourage tumoral immunosuppression (Demoulin et al., 2013). Several human studies brought evidence that pDCs can infiltrate the tumoral microenvironment, as they have been detected in primary carcinomas, cutaneous melanoma or lymphomas (Vermi et al., 2011). Tumor-infiltrating pDCs generally exhibit an immature phenotype characterized by the low expression of co-stimulatory molecules and a weak production of IFN-I (Hartmann et al., 2003; Perrot et al., 2007; Sisirak et al., 2012; Sisirak et al., 2013; Vermi et al., 2011). Several publications have documented that immature tumor-infiltrating pDCs promote tumor-specific T cell anergy and Treg cell development (Faget et al., 2012; Huang et al., 2014; Pedroza-Gonzalez et al., 2015; Sisirak et al., 2012; Zou et al., 2001) and cDC function inhibition (Zou et al., 2001). Consequently, pDCs are generally associated with poor prognosis in different type of cancers

(Aspord et al., 2014; Faget et al., 2013; Labidi-Galy et al., 2012; Sisirak et al., 2013; Treilleux et al., 2004; Vermi et al., 2011).

In the last few years, interesting investigations have been conducted to identify the appropriate activator signals that could lead to pDC re-programming into efficient modulators of anti-tumor responses. Thus, the topical administration of Imiquimod, a TLR7 synthetic ligand (ALDARA), on skin neoplasms leads to the recruitment of IFN-I-producing pDCs in dermal lesions, resulting in significant tumor regression (Stary et al., 2007; Urosevic et al., 2005). Recently, an elegant study performed in melanoma-bearing humanized mice reported similar conclusions and described that Imiquimod-activated pDCs also impede tumoral neovascularization supporting long-term and anti-metastatic effects (Aspord et al., 2014). In a mouse model of melanoma, topical administration of Imiquimod led to intratumoral recruitment of tumor-killing effector pDCs able to suppress tumor growth through the secretion of TRAIL and granzyme B (Drobits et al., 2012). Furthermore, in an orthotopic murine mammary tumor model, intratumoral administration of different TLR-7 ligands restore the activation of tumor associated pDCs, leading to anti-tumoral effects (Le Mercier et al., 2013).

The exploitation of Ag-presenting pDC functions to induce tumor-specific T cell immunity has also been explored. In humans, intranodal injections of activated pDCs loaded with tumor Ag-associated-peptides have been associated with antitumor specific T-cell responses in melanoma patients (Tel et al., 2013). In murine models, specific Ag delivery to pDCs using BST2 in combination with TLR agonists induces a protective immunity and inhibits tumor growth (Loschko et al., 2011b). Recently, our group demonstrated that, via their Ag-presenting functions, CpG-B-activated pDCs prime Th17 cells that control tumor growth by promoting the recruitment of specific CTL into the tumors (Guery et al., 2014).

Altogether these studies show the importance of pDCs in peripheral T cell tolerance and highlight how the inflammatory and regulatory functions of those cells may be manipulated for therapeutic purposes.

2.2.2. pDCs in Autoimmunity

Autoimmunity development is a consequence of self-tolerance breakdown and leads to aberrant immune responses targeting self-tissues. In the past, autoimmune diseases were mainly studied as target organ associated disorders. However, recent advances in immune knowledge have permitted to identify and better understand the mechanisms shared by different organ-specific pathogenesis. These studies have strengthened the concept of multi-factorial immune dysregulations relying on plural actors either promoting or regulating inflammation. Thanks to their strategic ability to influence both innate and adaptive immunity, pDCs have been broadly described as involved in autoimmune disorders. Their role in autoimmunity is widely dependent on the inflammatory context and can either support pathogenicity or conversely tolerance. The next paragraph will discuss the positioning of pDCs in distinct autoimmune diseases for which the role of these cells has been the most characterized. The role of pDCs in multiple sclerosis (MS) being closely related to my research, it will be presented in details in the third part of the introduction.

- **pDCs in systemic Lupus erythematous**

Systemic lupus erythematous (SLE) is an autoimmune disease characterized by the production of anti-self-nucleic acid antibodies that form immune complexes (IC). IC deposits in tissues generate inflammation and damage in skin, kidney, joints and central nervous system (CNS). Innate recognition of self-nucleic acids ensures the production of cytokines and particularly

high levels of IFN-I secretion by pDCs, which widely contribute to disease development (Tang et al., 2008).

Self-nucleic acid ICs bind the Fc receptor CD32 expressed by pDCs, and are subsequently internalised and released in the endosomal compartment, where they activate and trigger the TLR9 pathway, resulting in IFN-I production (Means et al., 2005; Swiecki and Colonna, 2015). Under physiological and normal conditions, self-nucleic acid sensing by pDCs is prevented by high concentrations of extracellular DNases, low levels of unmethylated CpG motifs within mammalian DNA, and the non-internalization of self-nucleic acids that therefore do not reach subcellular TLR9. Tolerance breakdown in SLE is in part promoted by the release of anti-microbial peptides such as LL37 and HMGB1 that bind self-DNA fragments discharged from dying cells. Anti-microbial peptides and self-DNA fragments form aggregates insensitive to extracellular DNase-mediated degradation, and can be delivered into early endosomes in pDCs (Matta et al., 2010; Swiecki and Colonna, 2015).

Several studies in SLE patients have reported that pDCs are present at low frequencies in peripheral blood but massively infiltrate inflamed tissues (Kim et al., 2015). IFN-I plays a preponderant role in SLE development by supporting the differentiation of normal monocytes into inflammatory cDCs that promote the priming of effector T cells (Blanco et al., 2001) and the generation of auto-antibody producing plasma cells (Jego et al., 2003).

As mentioned before, Wilhelm and colleagues recently described that Siglec-1^+/ Siglec-1^- pDC ratio is significantly increased in peripheral blood of SLE patients compared to healthy subjects, suggesting a greater migration of Siglec-1^- pDCs, able to produce IFN-I, from the periphery to the inflamed tissue and the retention of “tolerogenic” Siglec-1^+ pDC in the periphery. In addition, the imbalanced Siglec-1^+/ Siglec-1^- pDC ratio found in peripheral blood of SLE patients correlates with an intense disease activity (Wilhelm et al., 2016). As discussed

before and as mentioned by the authors, the specific of Siglec-1$^+$ pDCs in SLE and other autoimmune diseases remains to be addressed.

In an elegant study, Zhou *et. al.* recently related that peripheral pDCs, coming from a broad panel of lupus-prone mouse strain, exhibit an increased propensity to respond to TLR7 and TLR9, including an higher production of IFN-a compared to WT pDCs (Zhou et al., 2016). Moreover, impairment of the ability of pDCs to respond to TLR9 ligands significantly ameliorates murine SLE-like disease through the inhibition of anti-DNA antibodies and the reduction of glomerulonephritis (Sisirak et al., 2014).

- **pDCs in psoriasis**

Psoriasis is an inflammatory T-cell mediated autoimmune disease affecting particularly the skin and the joints. Psoriasis pathogenesis relies notably on adaptive autoreactive responses mediated by IL-17-producing CD8$^+$ and CD4$^+$ T cells infiltrating the epidermis and the dermis, respectively (Saadeh et al., 2016). pDCs have been clearly associated with early events of the pathogenesis through IFN-I production and subsequent activation of innate immunity cascade as well as activation and expansion of pathogenic T cells driving psoriatic inflammation (Nestle et al., 2005). As for lupus, pDCs are highly decreased in peripheral blood of psoriatic patients, while augmented in lesions (Nestle et al., 2005). Interestingly, pDC recruitment in psoriatic skin seems strictly dependent on the chemerin/ChemR23 axis, which is transiently active during psoriatic plaque development (Albanesi et al., 2009). As for lupus, pDCs are activated through TLR9-dependent sensing of self-DNA fragments forming aggregates with anti-microbial peptides overexpressed in psoriatic lesions (Lande et al., 2015; Saadeh et al., 2016).

- **pDCs in Type 1 Diabetes**

Type 1 diabetes (T1D) is a T_H-1 mediated autoimmune disease mediated by the destruction of insulin producing pancreatic β cells, conducting patients to be dependent on exogenous

insulin supply. While auto-reactive T cells targeting pancreatic β cells are the major actors of this pathology, infection by certain viruses has been linked to increased risk of T1D development. In particular, innate components, such as IFN-I, are important for the initiation and the modulation of the islet-specific adaptive immune response (Zipris, 2008). In addition, in several studies, IFN-α therapy given to patients with hepatitis C has been reported to induce T1D in autoantibody positive subjects (Bosi et al., 2001; Fabris et al., 2003; Fattovich et al., 1996; Okanoue et al., 1996).

The role of pDCs in T1D is however not so simple and worth to be further investigated. While the frequency of pDCs was initially found lower in peripheral blood of T1D patients compared to healthy individuals (Chen et al., 2008b), two other studies claim the contrary and relate increased levels of pDCs in peripheral blood of T1D patients at different stages of the disease (Allen et al., 2009; Xia et al., 2014). In addition, Xia *et al.* also report increased level of IFN-I in peripheral blood of T1D patients and correlate the ability of peripheral blood pDCs to produce IFN-I with the intensity of islet-specific T_H1 response (Xia et al., 2014).

In non-obese diabetic (NOD) mice, IFN-α has been shown to trigger early processes of T1D pathogenesis, and neutralisation of its receptor IFNAR using monoclonal antibody results in almost complete impeding of T1D induction (Li and McDevitt, 2011). In addition, pDC depletion in young NOD mice significantly delays disease onset and reduces disease incidence (Li and McDevitt, 2011). Recently, an important study identified that spontaneous T1D in NOD mice is induced by specific mechanisms relying on the interactions between B cells, neutrophils and pDCs, these three cell types being strictly required for the initiation of diabetogenic T cell responses (Diana et al., 2013). In addition, they reported that IFN-I production by pDCs is triggered through the activation of the TLR9 pathway by self-DNA bound to the cathelicidin-related anti-microbial peptide (CRAMP) (Diana et al., 2013). Interestingly,

one year later, the same group described that pDCs in pancreatic draining LNs are also critical for the recruitment of Treg cells in pancreatic islets and the prevention of T1D development (Beaudoin et al., 2014). Furthermore, in the two T1D models mentioned above, the interplay between iNKT cells and pDCs in pancreatic draining LNs promotes TGF-β production by pDCs, leading to the conversion of naive anti-islet T cells into Foxp3^{+} Tregs, thereby preventing T1D development (Diana et al., 2011). Another important study in NOD mice also delineates the tolerogenic role of pDCs during T1D development showing that, via an IDO dependent mechanism, pDCs prevent insulitis acceleration (Saxena et al., 2007). Finally, IDO expression and its catalytic function have been shown to be defective in pDCs from NOD mice. Importantly, the restoration of IDO production by NOD pDCs recapitulates their immunoregulatory functions (Pallotta et al., 2014).

Altogether, these studies highlight the countervailing role of pDCs in T1D, which seems to be related to disease time course and dependent on their maturation state. Indeed, while these cells seem to display pathogenic functions via IFN-I production in pancreatic islets during the initiation phase of the disease, they may have important roles in the control of insulitis, later on during disease development, potentially through their adaptive functions and the promotion of Treg cells in pancreatic draining LNs.

2.2.1. MOLECULAR MECHANISMS RELYING ON PDC TOLEROGENICITY

Tolerogenic functions of pDCs rely mainly on their ability to promote Treg cells either by inducing their differentiation or by activated them and favouring their proliferation.

In contrast to cDCs that present stable Ag-MHC II complexes at their surface following their activation, pDCs constantly internalize and ubiquitinate Ag-MHC II complexes, leading to a rapid turnover of Ag-MHC II complexes (Villadangos and Young, 2008). Besides the interesting

advantages that represent this turnover by allowing a continuous update of the T cell repertoire activated during viral infection, it also results in a low level of Ag presented at pDC surface. Consequently, and similarly to steady state cDCs, pDCs provide a weak TCR engagement, enabling Treg induction even upon maturation (Kang et al., 2007; Turner et al., 2009).

In addition to the strength of TCR engagement, the outcome of T cell responses is highly dependent on the balance between co-stimulatory and co-inhibitory signals provided at the APC surface.

In human, freshly isolated pDCs from the blood constitutively display low level of inducible co-stimulator ligand (ICOS-L) expression, while CD80 and CD86 are absent (Ito et al., 2007). Upon activation through IL-3/CD40L, TLR-dependent pathways or IFN-α and IL-6 stimulation, pDCs up-regulate ICOS-L expression and rapidly display high levels of this molecules at their surface, whereas the up-regulation of CD80 and CD86 leading to pDC maturation is gradual and slower (Ito et al., 2007). Interactions between human pDCs and T cells involve ICOS-ICOSL engagement (Janke et al., 2006), leading to the priming of $Foxp3^+$ Tregs (Conrad et al., 2012; Gilliet and Liu, 2002b) and Tr1 cells producing high levels of IL-10 (Colvin et al., 2009; Ito et al., 2007; Ogata et al., 2013). More than promoting their priming, pDC-mediated ICOSL signaling also contribute to the expansion and the survival of $Foxp3^+$ $ICOS^+$ Treg cells, as well as the maintenance of their IL-10 production (Faget et al., 2012; Gilliet and Liu, 2002b; Huang et al., 2014).

Even if less clear, the co-inhibitory molecule programmed death ligand-1 (PD-L1 or B7-H1) has also been involved in pDC-mediated Treg cell proliferation and tolerance induction. In human, pediatric liver transplanted patients tolerant to the graft exhibit a high PD-L1/CD86 ratio on

pDCs correlating with an elevated frequency of Tregs in peripheral blood (Tokita et al., 2008). Moreover, a mouse study demonstrated that compared to WT pDCs, PD-L1$^{-/-}$ pDCs induce high proliferation of CD4+ T cells *in vitro*. In addition, in a mouse model of cardiac allograft, host derived pDCs down-regulate alloreactive T cell responses and prolong engraftment through the PD-L1/PD-1 signaling pathway (Matta et al., 2010). IL-27 stimulated pDCs from mouse liver up-regulate PD-L1 and mediate Treg proliferation (Matta et al., 2012). Finally, in a mouse model of allergic asthma, CpG activated pDCs display anti-inflammatory responses through PD-L1/PD-1 interaction, resulting in the regulation of airway inflammation (Kool et al., 2009).

Importantly, IDO expression by pDCs has been correlated with the suppression of effector CD4$^+$ and CD8$^+$ T cells through the starvation of tryptophan in the surrounding milieu, the production of immunoregulatory kynurenines or the generation of Treg cell. Human pDCs incubated *in vitro* with HIV induce the differentiation of naive CD4+ T cells into Treg suppressing CD4$^+$ T cell proliferation in an IDO-dependent mechanism (Boasso et al., 2007; Manches et al., 2008; Puccetti and Fallarino, 2008).

In mouse, it is commonly accepted that IFN-γ, IFN-α or the engagement of B7 molecules by CTLA-4 or OX-2 (CD200) promote IDO expression and activate its enzymatic properties in pDCs (Fallarino et al., 2004; Mellor et al., 2003; Pallotta et al., 2011). More recently, Pallotta and colleagues identified that IDO can also acts as a signal-transducing molecule, independently on its enzymatic function, specifically in TGF-β treated pDCs. Through the activation of this pathway, pDCs acquire a stably regulatory phenotype characterized by the amplification of IDO and TGF-β production, and resulting in Treg induction (Pallotta et al., 2011).

In mouse, tumor-draining LN pDCs potently inhibit anti-tumor T cell responses via IDO expression (Munn et al., 2004a) via PD-L1$^+$ Tregs (Sharma et al., 2007). Accordingly, IDO inhibition in pDCs promotes the conversion of Tregs into T_H17 cells that efficiently inhibit

tumor growth (Sharma et al., 2009). Upon CpG-activation, a minor splenic $CD19_+$ population of pDCs has also been described in mouse to block Treg conversion into TH17 like cells in an IDO dependent manner (Baban et al., 2009). Another study depicts as well these splenic $CD19^+$ pDCs as the only pDCs subset expressing IDO following B7 ligation by CTLA-4-Ig (Baban et al., 2005). A study in NOD mice demonstrates that IDO expression by pDCs is required for these cells to display immunoregulatory functions (Pallotta et al., 2014). Recently, a new role for IDO^+ pDCs has been identified in the protection of atherosclerosis via the induction of $IL10^+$ Treg cells in mice (Yun et al., 2016). Ag-presentation by pDCs promotes the expansion of Ag-specific Tregs in atherosclerotic aorta, and the selective depletion of pDCs in BDCA2-DTR mice leads to decreased number of Tregs and reduced IL-10 levels in aorta, resulting in exacerbated atherosclerosis. Importantly, this study related similar observations in human aorta showing pDCs in the intima of healthy tissues and co-localization with Treg cells in human atherosclerotic aortas (Yun et al., 2016).

In addition to $CD4^+$ Treg cell induction, CD40L and IL-3 stimulated human pDCs promote the generation of anergic $CD8^+$ regulatory T cells characterized by a weak cytotoxic activity and high production of IL-10. These CD8+ regulatory T cells respond to a second Ag stimulation and strongly inhibit allogeneic target cells (Gilliet and Liu, 2002a). Even though the molecular pathways accounting for their regulatory functions have not been described yet, $CD8^+$ regulatory T cells expressing more CTLA-4 and ICOS compared to other $CD8^+$ T cells (Churlaud et al., 2015), we can postulate that interactions with pDCs are dependent on at least one of these two molecules.

3. CNS AUTOIMMUNITY

3.1. MULTIPLE SCLEROSIS

Multiple Sclerosis (MS) is a progressive inflammatory demyelinating disease of the central nervous system (CNS). MS represents the CNS autoimmune disorder with the highest prevalence, affecting approximately 2.5 million people worldwide, mostly in young and middle-aged adults and especially women (Compston and Coles, 2008). MS distribution is uneven throughout the world with most incidence in temperate areas, specifically in countries with large proportion of Northern European originated people such as Canada, United States, New Zealand and Australia (Milo and Kahana, 2010).

High heterogeneity exists from a patient to another regarding disease course and symptomatology, leading to define different subtypes of MS. Relapsing-remitting MS (RRMS) is the most common form and affects 85% of MS patients. First signs of the disease are generally characterized by an initial episode of neurological dysfunctions named as clinical isolated syndrome (CIS) that lasts at least 24 hours and which is followed by a complete or partial clinical recovery. Then recurring bouts of relapse occur intermittently with remitting periods (Dendrou et al., 2015). Relapses matched with focal acute inflammatory demyelination of CNS white matter and with grey matter atrophy (Roman and Arnett, 2016), both visible using magnetic resonance imaging (MRI). 80% of RRMS patients go on to develop, in the 25 years following diagnosis, a secondary progressive form of MS (SPMS) characterized by gradual accumulation of irreversible disabilities (Miller and Leary, 2007). Around 10% of MS patients present a gradually progressive course of the disease from the onset called primary progressive MS (PPMS) (Dendrou et al., 2015).

3.1.1. CAUSES OF MULTIPLE SCLEROSIS

The exact initiating factors of the disease are not well defined, but substantial evidence highlights that a combination of genetically susceptibilities and environmental factors lead to MS pathogenesis (Dendrou et al., 2015).

- **GENETIC FACTORS**

MS is not a hereditary disease in itself, but it is well established that predisposing genetic factors exist. Indeed, the risk to develop the disease for relatives of MS patients is around 5% for direct descendants and siblings (Hemminki et al., 2009), and reaches 25% for monozygotic twins (Hawkes and Macgregor, 2009). Pioneer studies established that variants in the human leukocyte antigen (HLA) genes from the MHC are associated with MS susceptibility (Jersild et al., 1972; Naito et al., 1972). Following this discovery, intensive researches have been carried out to better characterize the importance of HLA alleles in MS aetiology. Recent works based on new genetic approaches such as large-scale genome-wide association studies (GWASs) and ImmunoChip-based studies not only revealed that non-HLA single nucleotide polymorphisms (SNPs) account for the majority of MS-associated genes (International Multiple Sclerosis Genetics et al., 2013), but also permitted the identification of more than 100 distinct genetic regions associated with MS susceptibility, and showed that almost all are primarily related to immunity (International Multiple Sclerosis Genetics et al., 2013; Nylander and Hafler, 2012). Indeed, as reviewed by *Dendrou et al*., susceptibility variants for MS embrace genes implicated in central tolerance mechanisms, homeostatic cell proliferation, cytokine production and importantly, genes related to T helper cell differentiation (International Multiple Sclerosis Genetics et al., 2007). Some epigenetics studies aiming at addressing whether DNA methylation, a strong regulator of gene activity, could account for MS susceptibility have also been performed. However, obtained results diverged from one study to another, and didn't permit to clarify this question. Nevertheless, these issues pointed out the importance of large

scale analysis to get valuable insights (Bos et al., 2016). Substantial progresses in the understanding of MS genetic architecture have been done during the last decade, paving the way toward the identification of new therapeutic targets. However, genetic factors accounting for only 30% of the overall disease risks, the contribution of other factors in MS penetrance is important enough to warrant the attention of the scientific and medical communities.

- **ENVIRONMENTAL FACTORS**

Environmental factor influence has been strongly correlated to MS aetiology and is supported by immigration studies reporting that MS incidence varies depending on immigrant populations. As reviewed by Milo and Kahana (Milo and Kahana, 2010), these studies suggest that the migration from a high to a lower MS prevalence area correlates with a decrease of MS rate, whereas migration in the opposite direction tends to retain the low MS incidence characteristics of the country of origin. Moreover, it seems that, besides the ethnicity belonging, MS prevalence depends on the age of the person immigrated and on the exposure to certain environmental factors before adulthood (Alter et al., 1978; Dean, 1967; Dean and Elian, 1997; Gale and Martyn, 1995). To date, the strongest evidence showing influences of environmental factors on MS incidence relies on sunlight exposure and vitamin D production, Epstein-Barr virus (EBV) infections, and cigarette smoking.

SUNLIGHT EXPOSURE

The more obvious explanation of a latitudinal gradient in MS frequency is certainly the sunlight intensity. Immunosuppressive effects of ultraviolet (UV) radiations have been pointed out previously and were associated with a modulation of the adaptive immune response (Fisher and Kripke, 1977). Higher sunlight exposure during adolescence has been associated with a reduced risk of MS (Alonso et al., 2011; Dalmay et al., 2010; van der Mei et al., 2003).

Intriguingly, skin cancers, which are strongly related to sun exposure, appear to be significantly less common in patients with MS, whereas the frequency of other cancer is similar (Goldacre et al., 2004). Like some MS therapies, including glatiramer acetate, fingolimod, IFNβ and natalizumab, UVs act by modulating immune responses after initiation of molecular and cellular events. As reviewed by Marsh-Wakefield and Byrne, UVB exposure induces IL-10 secretion by Tregs and Bregs (Marsh-Wakefield and Byrne, 2015). Interestingly, UV-induced regulatory DCs, derived from BM precursors of UV-irradiated mice, have been identified both *in vivo* (Ng et al., 2013b) and after BM derived *in vitro* differentiation (Ng et al., 2013a). In both conditions, UV-BMDCs displayed reduced priming abilities leading to poor immunogenicity and *in vivo* long-lasting systemic immunosuppressivity, as demonstrated by re-challenges with inflammatory agents. Moreover, progeny of UV-irradiated mothers present reduced BMDC immunogenicity (Ng et al., 2013b). In the light of these results, it would be interesting to assess if immunosuppressive UV-induced BMDCs impact the priming of encephalitogenic T cells in a mouse model of MS and, of course, to determine whether these UV-induced regulatory DCs exist in human and could support MS protection.

UVs and Vitamine D

Another plausible explanation of UV-induced suppressive effects is the involvement of UV radiations in Vitamin D release. UV radiations permit the conversion of cutaneous 7-dehydrocholesterol to pre-vitamin D3, the major precursor of the activated form $1,25(OH)_2D_3$ Vitamin D which circulate in the blood. Some studies showed that intake of Vitamin D is inversely associated risks of MS (Munger et al., 2004). In addition, people with high levels of circulating $25(OH)D_3$ (> 100 nmol/L) exhibit lower risks to develop MS compared to people

presenting low levels of 25(OH)D_3 (<75 nmol/L) (Shaygannejad et al., 2010). Moreover, genetic polymorphisms of Vitamin D receptors have been correlated with MS susceptibility (Smolders et al., 2009). Potential mechanisms proposed to support beneficial effects of Vitamin D in MS prevention include the inhibition of IL-2 genes transcription by T cells and subsequently the reduction of T cell proliferation and clonal expansion (Alroy et al., 1995). Furthermore, modifications of Ag-presenting DC toward a more tolerogenic phenotype could favour the differentiation of iTreg instead of inflammatory T_H1 and T_H17 cells (Adorini, 2003; Adorini and Penna, 2009; Griffin et al., 2001). All together, these studies provide evidence that low exposure to sunlight and low serum levels of Vitamin D are correlated with increased risks of MS.

EBV CONTRIBUTION

While the exact role of EBV infection in MS pathogenesis is still not fully understood, there is large evidence supporting the idea that EBV is a plausible causative agent of the disease. EBV is a double-stranded DNA γ-herpes virus transmitted by saliva and causing infections in the most part of the world adult population. EBV infects follicular DCs and B cells through the binding of the viral protein gp350 to its receptor CD21 present at the cell surface. As other lymphocryptoviruses, EBV has the ability to activate and expand B cells, and drive the appearance of latently infected resting memory B cells (Pender and Burrows, 2014). EBV infection is usually asymptomatic during the first years of life, but leads to infectious mononucleosis during adolescence. Epidemiologic features of MS, including latitudinal variation in prevalence, correlation with higher socio-economic status, reduced occurrence in Asian/African populations. In addition, MS incidence changes in migrating populations, can be consistently correlated with late primary EBV infection occurring during the adolescence or young adulthood (Haahr and Hollsberg, 2006). Studies report that EBV seropositivity is higher

in MS patients than in the rest of the population (Ascherio and Munger, 2007a; Sumaya et al., 1980). Moreover, history of infectious mononucleosis has also been associated with a higher risk of MS development (Disanto et al., 2013). Different hypotheses have been proposed to explain the role of EBV in MS development. The most prevalent one relates that cross-reactivity between EBV proteins and CNS Ags would lead to the priming of CNS antigen-specific T cells (Lang et al., 2002; Wucherpfennig and Strominger, 1995). Another hypothesis postulates that EBV induces lymphoid-cell surface expression of αB-crystallin, a small heat-shock protein, which would be recognized by mistake as a microbial Ag by the immune system. $CD4^+$ T cell responses would be therefore initiated and target αB-crystallin derived from oligodendrocytes, resulting in demyelination (van Noort et al., 2000). A third hypothesis, which could be extended to all human chronic autoimmune diseases, proposes that EBV could infect auto-reactive B cells and lead to the accumulation of these cells in target organs, the CNS for MS. Infected autoreactive B cells would produce autoantibodies and act as self-Ag presenting APCs, allowing the re-activation of T cells trafficking through the target organ. Auto-reactive B cells could therefore ensure the maintenance in the target organ of auto-reactive T cells, which would have die by activation-induced apoptosis in absence of re-activation(Pender, 2003).

CIGARETTE SMOKING

Finally, cigarette smoking has been correlated with a higher risk of developing MS (Ascherio and Munger, 2007b; Handel et al., 2011), and even if less clear, has also been shown to accelerate the conversion of CIS to established RRMS (Di Pauli et al., 2008), and of RRMS to SPMS (Healy et al., 2009). Some tobacco smoke components such as cyanides, nitric oxide and metabolites are known to be neurotoxic and could explain the negative impact of smoking on MS.

3.1.2. IMMUNOPATHOLOGY OF MS

Because the predominant factors initiating the disease are not well defined yet, it is still an open question whether MS is initiated in the periphery or in the CNS. For the ones who classified MS as an autoimmune disorder, disease results from a dysregulation of the immune system outside the CNS.

- **PRIMING OF AUTO-REACTIVE CELLS IN SLOS**

Autoreactive T cells, escaping the central tolerance deletion, egress from the thymus and are released into the periphery. Tolerance breakdown, such as reduced Treg functions (Viglietta et al., 2004) or resistance to suppressive mechanisms, would permit these autoreactive T cells to bypass the different peripheral tolerance checks, and to be activated in SLOs by APCs to differentiate into pathogenic CNS-directed effector cells. Molecular mimicry, novel auto-Ag presentation, recognition of peripheral CNS Ag normally sequestered, or bystander activation (such as presentation of αβ-crystallin antigen as mentioned earlier (Dendrou et al., 2015)) could possibly account for the activation of encephalitogenic T cells. Autoreactive T cells found in MS patients recognize a broad panel of myelin proteins such as Myelin Oligodendrocyte Glycoprotein (MOG), Myelin Basic Protein (MBP), Myelin proteolipid Protein (PLP), and Myelin-associated Oligodendrocytic Basic Protein (MOBP) (Wu and Alvarez, 2011).

Several evidence showed that T_H1 and T_H17 lymphocytes are involved in MS physiopathology. However, the relative importance of one subtype to the other in disease development is not clearly defined. Myelin-reactive peripheral $CD4^+$ T cells found in MS patients exhibit both T_H1 and T_H17 profiles and express the CC-chemokine receptor 6 (CCR6) required for homing into the CNS (Cao et al., 2015). In addition, both T_H1 and T_H17 subsets remain sequestered in SLOs after Fingolimod treatment, which prevents the egress of immune cells from LNs (Chun and

Hartung, 2010). This suggests that both T_H subsets are originally primed in SLOs. However, T_H17 cells are preferentially expanded in peripheral blood of MS patients during acute relapses compared to T_H1 (Durelli et al., 2009; Kebir et al., 2009). Through the secretion of matrix metalloproteinase (MMP9 and MMP2) and radical oxygen species, T_H17 contribute to the blood brain barrier (BBB) permeability (Huppert et al., 2010), while T_H1-derived TNF-α and IFN-γ induce the expression of adhesion molecules such as the vascular cell adhesion protein 1 (VCAM-1) and the P-selectin by endothelial cells, favouring the transmigration of auto-reactive cells into the CNS (Minagar et al., 2006). More importantly, via their secretion of cytokines, T_H1 and T_H17 contribute to the recruitment and the activation of other cell types including $CD8^+$ T cells, B cells and macrophages in SLOs (Dendrou et al., 2015). Recently evidence suggests that the secretion of the granulocyte macrophage colony-stimulating factor (GM-CSF) by autoreactive T cells is required for the initiation of CNS autoimmune inflammation. Important studies, report that mice lacking GM-CSF secretion by autoreactive T_H cells failed to initiate neuroinflammation despite the expression of IL-17A or IFN-γ (Codarri et al., 2011). In EAE, T_H17 cells seem to be the major source of GM-CSF, and this production is promoted by IL-1β and IL-23 (El-Behi et al., 2011). In MS, recent findings show that GM-CSF-expressing $CD4^+$ and $CD8^+$ T cells are increased in peripheral blood of untreated MS patients compared to coth healthy individuals and INF-treated MS patients (Rasouli et al., 2015). In vitro, IFN-β treatment reduces GM-CSF production by T cells from MS patients. However in MS, which cells among T_H1 and T_H17 are the most competent to secrete GM-CSF it is still a matter of debate (Dendrou et al., 2015; Noster et al., 2014; Rasouli et al., 2015)

In MS patients, a notable proportion of $CD8^+$ T cells producing IL-17, probably mucosa-associated invariant T cells (MAIT), can be found in active lesions (Willing et al., 2014). $CD8^+$ MAIT cells present in PB of MS patients express the CNS homing molecule CCR6 and

upregulate the integrin very late antigen-4 (VLA-4), which is required for their migration into the CNS (Ifergan et al., 2011; Willing et al., 2014).

It is well established that B cells, via the production of auto-antibodies, are important players of MS, even though their role varies throughout disease progression. The detection of antibodies in the CSF of patients is notably a hallmark of the diagnosis and the increased numbers of antibody-secreting plasma cells is closely associated with clinical progression (Frischer et al., 2009). For a long time, B cells were thought to be directly activated in the CNS where they accumulate. However, new high-throughput sequencing technologies recently allowed the identification of CNS specific B cell clonotypes in the periphery and in the CSF of MS patients, indicating that B-cell maturation can occur in cervical LNs before their migration to the CNS (Palanichamy et al., 2014; Stern et al., 2014).

Finally, peripheral Treg cells are affected in MS patients. nTreg isolated from peripheral blood of RRMS and SPMS patients fail to suppress autoreactive effector T cells *in vitro*. In addition, the frequency of $CD31^+$ memory Treg cells is largely decreased in PB of early MS patients (Venken et al., 2008). Moreover, myelin-specific $CCR6^+$ T cells isolated from peripheral blood of MS patients secrete less IL-10 compared to cells coming from healthy controls (Cao et al., 2015). Functional abnormalities in Tr1 regulatory T cells have also been reported in MS patients. Indeed, following *in vitro* stimulation with anti-CD3 and anti-CD46 antibodies, known to induce Tr1 cells, $CD4^+$T cells isolated from peripheral blood of MS patients failed to differentiate into Tr1 secreting- IL-10 cells (Astier et al., 2006). In the light of these studies, we can postulate that the emergence of autoreactive responses in MS patients may be a consequence of defective functions of $Foxp3^+$ Tregs and/or Tr1 cells. Nevertheless, we can state that functional abnormalities in $Foxp3^+$ Tregs and Tr1 cells widely compromise the

regulation of autoreactive responses, therefore contributing to the expansion of the auto-inflammation.

- **MIGRATION TO THE CNS**

The CNS is qualified as an immune privileged organ, to which access is strictly restricted, due to endothelial and epithelial barriers such as the BBB in the CNS microvessels and the blood-leptomeningeal barrier (BLMB) localized at the surface of the brain and the spinal cord (SC) (Engelhardt and Ransohoff, 2012). Until the recent identification of functional lymphatic vessels lining the dural sinuses of brain in mouse (Louveau et al., 2015), the CNS was considered, in absence of any BBB breach, to be isolated from the adaptive immune system. CNS-intrinsic immune surveillance was attributed to patrolling memory T cells trafficking through the CSF and interacting with Ag presenting blood-derived innate immune cells appropriately localized within the brain to present CNS-restricted Ags.

It is assumed that MS is initiated by the migration of few activated autoreactive lymphocytes through one of the blood barriers restricting CNS access. VLA-4 is expressed at the surface on most leukocytes except granulocytes, and is composed by two integrin chains. The interaction of VLA-4 with its cognate ligand VCAM-1 expressed on activated endothelial cells of the BBB permits the firm adhesion of immune cells to the barrier, and initiates immune cell crawling on the endothelium (Baron et al., 1993) (***Figure I-9***). Crawling process allows lymphocytes scanning of the endothelium for an area permissive for diapedesis. Transmigration of immune cells across the BBB is then insured by different diapedesis processes which subsequently lead to the entry of autoreactive cells into the perivascular space of the CNS (Engelhardt and Ransohoff, 2012). Importantly, interference with one of the molecules involved in these

processes, ends up lymphocyte transmigration and results in the return of the cells to the blood circulation (Carrithers et al., 2000). By targeting VLA-4 on immune cells surface, the blocking antibody Natalizumab impedes its interaction with VCAM-1 and therefore prevents the entry of auto-reactive lymphocytes in the CNS (Schwab et al., 2015).

Even if controlled, T cell extravasation across the BLMB seems faster and less constrained than transmigration across the BBB. Indeed, while intravenously injected encephalitogenic labelled T cells fail to cross the BBB in naive mice, they are detectable in the leptomeningeal compartment neighbouring BLNB (Carrithers et al., 2000). T cell migration across the BLMB does not rely on VLA-4/VCAM-1 interactions but depends on the interplay between the lymphocyte-expressed P selectin glycoprotein-ligand-1 (PSGL-1) and the P-selectin, which, in contrast to VCAM-1 that is only induced upon inflammation, is constitutively expressed by endothelial cells (Carrithers et al., 2000).

- **INVASION OF THE CNS**

IFN-γ and IL-17 are present in the CNS lesions and in the CSF of MS patients (Dhib-Jalbut et al., 2006; Matusevicius et al., 1999; Pashenkov et al., 2001; Selmaj et al., 1991). Both pro-inflammatory cytokines induce the activation of macrophages and microglial cells localized in the parenchymal and leptomeningeal compartments (Kawanokuchi et al., 2008; Strachan-Whaley et al., 2014; Yamasaki et al., 2014). GM-CSF is produced by encephalitogenic $CD4^+$ and $CD8^+$ T cells in MS lesions (Rasouli et al., 2015), supports the recruitment of monocytes and $CD103^+$ DCs, and increases MHCII expression and cytokine production, by local APCs (Dendrou et al., 2015; King et al., 2010). Activated CNS APCs in turn contribute to the local re-activation of autoreactive effector T cells and consequently favour the chronic inflammation. On the other hand, T-cell activated macrophages and microglial cells enhance the release of pro-

inflammatory mediators, oxygen species and nitric oxide radicals in the milieu, ultimately resulting in demyelination and axonal loss (Strachan-Whaley et al., 2014). On the other hand, axonal damage have been shown to correlate with the number of IL-17 producing-CD8+ T cells and macrophages within active lesions (Bitsch et al., 2000).

B cells clonally expand in the meninges and the parenchyma of MS patients and the detection of CSF plasmablasts has been correlated with active brain lesions visualised by MRI (Grigoriadis et al., 2015; Lucchinetti et al., 2000). Autoantibodies secreted by plasma cells do not only target myelin-rich components but also neurons and glia (Fraussen et al., 2014).

Importantly, post-mortem analysis of MS brains reveals the presence in cerebral meninges of tertiary lymphoid tissues (TLTs) characterized by the aggregation of plasma cells, B cells, T cells and DCs. These lymphoid structures widely support immune cell activation and expansion (Howell et al., 2011; Pikor et al., 2015; Serafini et al., 2004). TLTs have been associated with cortical damage and disease progression (Magliozzi et al., 2010) and are present in 40% of patients with SPMS (Magliozzi et al., 2007). Via IL-17 secretion, T_H17 cells contribute to the ectopic formation of these tertiary lymphoid structures (Howell et al., 2011).

All these mechanisms result in severe white matter pathologies and contribute to continuous gray matter damage (Mallucci et al., 2015).

In RRMS patients, remitting phases coincide with the clearance of dead cells and debris. This function is in particular attributed to activated microglial cells and is required for the resolution of inflammatory processes (Czeh et al., 2011). Moreover, both microglial cells and macrophages drive the maturation of oligodendrocyte progenitors, which are responsible for the remyelination of naked axons (Miron et al., 2013).

3.1.3. MS TREATMENTS

As mentioned previously, MS is a multifactorial pathogenesis staging numerous cellular actors interacting with each other and driving the activation of many different molecular signaling pathways. Disease complexity is likely the cause of diverse physiopathological impacts, which depend on the patient and the disease course, and result in a broad clinical heterogeneity. That being said, we can imagine that MS is not an easy treatable disease. To date, twelve disease-modifying therapies (DMTs) are available to treat RRMS, but no approved efficient medication exists for the treatment of SPMS and PPMS (Grossman et al., 2016). DMTs are successful in reducing MS relapses, however, none has been effective in all patients, and many are associated with serious adverse effects.

DMTs are mainly associated with immunomodulatory or immunosuppressive modes of action and mostly impact T- and B-cell functions. IFN-β1 treatments, the monoclonal antibody Natalizumab, and the Fingolimod, act on lymphocyte trafficking and migration across the BBB. While Natalizumab selectively inhibits VLA-4 integrin expressed on leukocyte surface, IFN-β1 decreases the expression of VCAM-1 located at the surface of endothelial cells and reduces the expression of its ligand VLA-4 on T cells. Both treatments inhibit VLA-4 mediated adhesion to VCAM-1, and therefore prevent lymphocyte trans-migration across the BBB (Kasper and Reder, 2014; Schwab et al., 2015). Fingolimod is a Sphingosine 1-phosphate receptor agonist that, after binding, induces the internalisation and the degradation of its receptor in leukocytes, thus preventing the egress of auto-reactive lymphocytes from lymphoid tissues (Chun and Hartung, 2010). On the other hand, IFN-β treatment reduces pathogenic GM-CSF-producing $CD4^+$ and $CD8^+$ T cells in the periphery (Rasouli et al., 2015).

Glatiramer acetate (GA), a random polymer composed of 4 amino acids commonly found in MBP, has a mode of action which is not well defined yet (Ziemssen and Schrempf, 2007). Given its antigenic nature, GA may act by competing with myelin Ag for MHC binding. The presentation of GA via MHCII molecules on APCs has been associated with a shift from T_H1/T_H17 pro-inflammatory to T_H2/Treg anti-inflammatory phenotype in both the periphery and the CNS (Ben-Nun et al., 2014; Neuhaus et al., 2000; Ziemssen and Schrempf, 2007).

Mitoxantrone and Teriflunomide are two treatments affecting T and B cell proliferation. Mitoxantrone is a cytotoxic agent inhibiting the DNA repair enzyme topoisomerase II, leading to the suppression of T-, B- cell and macrophage proliferation and functions (Fox, 2004). Teriflunomide is a pyrimidine synthesis inhibitor exerting its cytotoxic effect on proliferative cells such as T and B cells (Bar-Or et al., 2014).

Recently, a new generation of treatments, aiming at deviating the lymphocyte population in MS patients from an autoreactive and aggressive to an inert pattern, emerged. Alemtuzumab is a humanized monoclonal antibody directed against CD52, a surface glycoprotein expressed by mature circulating lymphocytes (Hartung et al., 2015). This treatment, generally used for chronic lymphocytic leukaemia, causes massive anti-body dependent T and B cell depletion, followed by the repopulation of lymphocyte subsets with different qualitative antigenic patterns (Hartung et al., 2015). Presently tested in clinical trials in Europe, this treatment is promising because provides an efficacy superior to other disease-modifying therapies (Hartung et al., 2015).

Finally, great hopes are granted to anti-CD20 monoclonal antibodies. Rituximab, Ofatumumab and the humanized antibody Ocrelizumab are B-cell depleting antibodies currently tested in clinical trials. Their efficacy relies not only on reducing the relapses of RRMS patients but also

on significant reduction of active lesions visualized by MRI. Importantly these treatments could be administrated not only to RRMS patients but also to SPMS and PPMS patients (Gasperini et al., 2013; Sorensen and Blinkenberg, 2016).

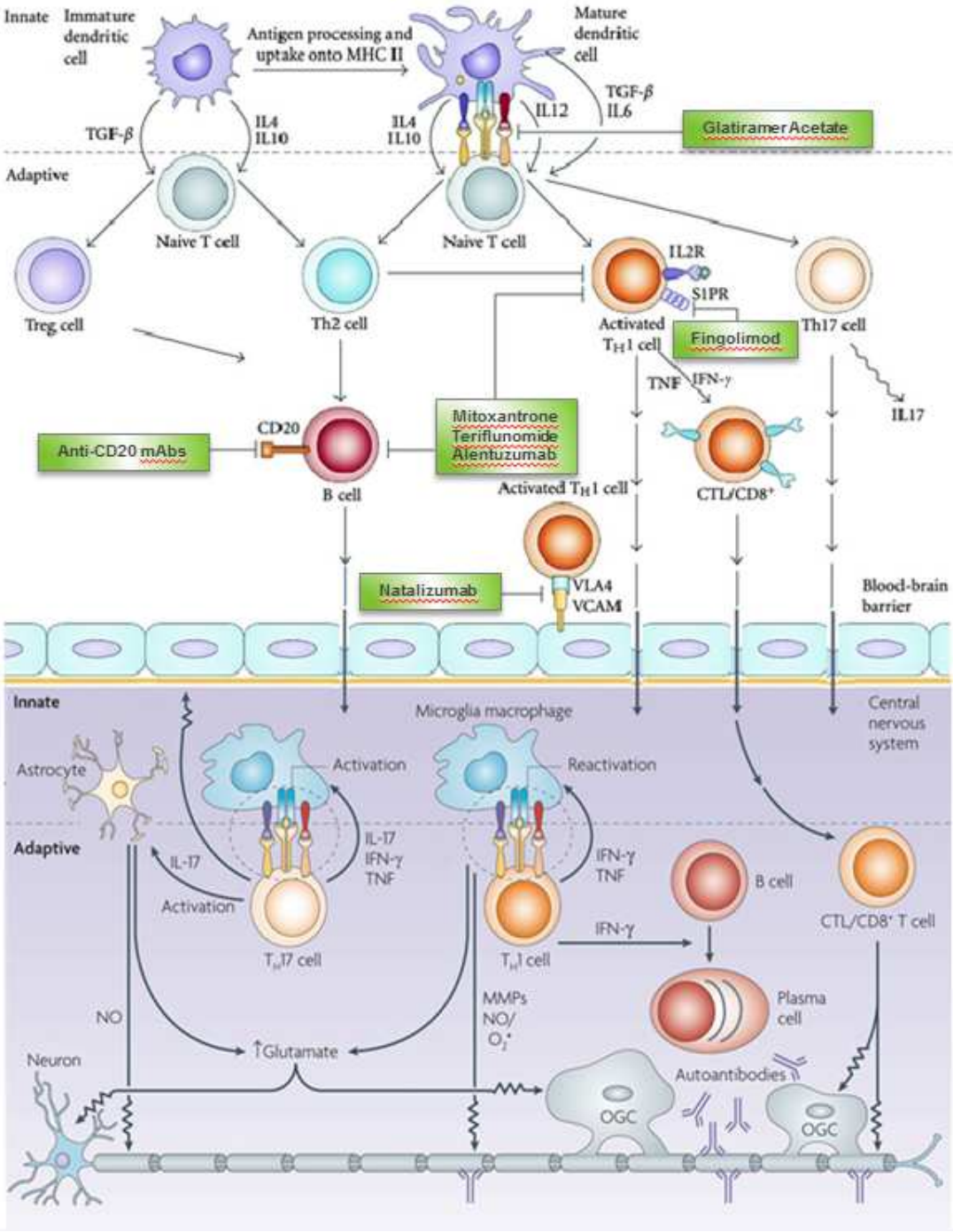


FIGURE I-9 IMMUNOPATHOLOGY OF MULTIPLE SCLEROSIS AND CELLULAR TARGETS OF CURRENT TREATMENTS.

Under physiological context peripheral tolerance is maintained by immature DC and regulatory T cells. In MS patients, abnormal activation of Ag-presenting DCs occurs in SLOs and leads to tolerance breakdown. Presentation of myelin-specific self-Ags by DCs in SLOs promotes the clonal expansion of self-reactive T cells which differentiate in encephalitogenic $CD4^+$ effector T_H1, T_H2, T_H17 or Treg, depending on the co-stimulatory molecules engaged and the cytokinic environment. Via cytokine secretion, T_H1 and T_H17 contribute to the recruitment and the activation of other cell types including $CD8^+$ T cells, B cells and macrophages in SLOs. Activated autoreactive leukocytes traffic via the blood and penetrate the CNS through to VLA-4/VCAM-1 interactions that support transmigration by diapedesis through the BBB. In the CNS, self-reactive T_H1, T_H17 and CTLs are reactivated by local APCs and amplify the inflammatory response via cytokine release, thus promoting 1) B cell activation and differentiation in auto-antibody-producing plasma cells, and 2) abnormal activation of astrocytes and microglial cells leading to the production of reactive oxygen species, NO, MMPs and glutamate. Altogether, inflammatory cytokines, reactive oxygen species, NO, MMPs, autoantibodies, cytotoxicity and excitotoxicity lead to demyelination, axonal damage and neuronal loss. By its antigenic nature, Glatiramer acetate (GA) competes with myelin Ag for MHC binding, and GA presentation via MHCII molecules on APCs seems to induce a shift from T_H1/T_H17 pro-inflammatory toward T_H2/Treg anti-inflammatory phenotype. Fingolimod targets (S1P) receptors including S1P-1 expressed at the cell surface of T and B cells. Fingolimod effects include the internalisation and the degradation of its receptor in leukocytes, thus preventing the egress of auto-reactive lymphocytes from lymphoid tissues. Mitoxantrone and Teriflunomide are two treatments affecting specifically T and B cell proliferation. Alemtuzumab, a humanized monoclonal antibody targets CD52, a surface glycoprotein expressed by mature circulating lymphocytes leading to massive depletion of T and B cell. Natalizumab selectively inhibits VLA-4 integrin expressed on the leukocytes surface, impeding VLA-4 mediated adhesion to VCAM-1, and therefore preventing lymphocyte transmigration across the BBB. Finally, antibodies targeting CD20 (Rituximab and Ofatumumab) drive specific B cell depletion. *Adapted from (Brinkmann et al., 2010).*

3.2. pDC IMPLICATION IN MS

Whether pDCs rather exhibit tolerogenic or immunogenic functions in MS is still not well defined and contradictory evidence have been reported depending on the studies. Rarely present in the CNS under nonpathologic conditions, pDCs are increased in the CSF of MS

patients (Lopez et al., 2006; Pashenkov et al., 2001) and are recruited and accumulate in white matter lesions and leptomeninges of MS patient brains (Lande et al., 2008). pDCs from RRMS patients exhibit a less mature phenotype compared to pDCs from healthy donors, characterized by a significant lower expression of CD86 and CD137 (Stasiolek et al., 2006). Upon stimulation, pDCs from PBMCs of MS patients display an impaired maturation, with significant alterations in the up-regulation of CD86, CD40, CD137 and CD83 expression (Bayas et al., 2009; Lande et al., 2008; Stasiolek et al., 2006). Furthermore, decreased IFN-α secretion, together with altered capacity to induce Treg by pDCs from MS patients compared to healthy individuals, were also reported (Hirotani et al., 2012; Stasiolek et al., 2006). On the other hand, the expression of the chemokine receptor CCR7, which allow homing to SLOs, was shown increased in pDCs from RRMS patients following TLR9 stimulation by viral and bacterial ssRNA (Aung et al., 2010).

In human, phenotypically and functionally different pDC subsets have been described (Schwab et al., 2010). pDC type 1 (pDC1) display high levels of CD123 and low levels of CD86 and TLR2 and induce IL-10 producing T cells. pDC type 2 (pDC2) exhibit low expression of CD123 and high levels of CD86 and TLR2, and promote IL-17 producing cells. While pDC1 are more abundant than pDC2 in the blood of healthy donors, MS patients are characterized by an inverted pDC1/pDC2 ratio in their peripheral blood (Schwab 2010). Importantly, some RRMS treatments seem to influence pDC phenotype and functions. Indeed, IFN-β treatment reverses the aberrant pDC1/pDC2 distribution by increasing the number of tolerogenic pDC1 (Schwab et al., 2010) and GA partially restores the mature phenotype and the function of pDCs in MS patients (Stasiolek et al., 2006).

3.3. OTHER CNS AUTOIMMUNE DISORDERS

MS is not the only described autoimmune disease of the CNS. Indeed, although less frequent, Fisher syndrome (FS), Bickerstaff brainstem encephalitis (BBE) and Guillain-Barré-Syndrome (GBS) are also reported as CNS autoimmune diseases. These disorders are associated in most cases with anti-GQ1b-IgG antibodies targeting gangliosides, which are sialic acid-containing glycosphingolipids abundantly expressed in the CNS (Kamm and Zettl, 2012). Studies performed in ganglioside-deficient mice highlight the prominent role of these molecules not only as structural components of biomembranes but also as cell-cell recognition and adhesion molecules, and signal transduction mediators (Yu et al., 2011). Usually, FS, BBE and GBS occur after a prior viral infection and share similarities in clinical and pathological outcomes, so that several experts in the field consider them as a spectrum of the same clinical entity (Kamm and Zettl, 2012). Few studies have been done so far to investigate the role of DCs in these different pathologies. A single pioneer study highlights that pDC numbers are increased in the peripheral blood of GBS patients during acute phases, and that blood pDC frequency can be positively correlated to clinical severity (Wang et al., 2015).

4. SCIENTIFIC INTEREST OF MY PROJECT

During my PhD, I have focused on a better characterization of the role of pDCs in MS, their interactions with T cells and the molecular mechanisms employed by these cells to mediate their functions in this particular context of auto-inflammation. To address this, I performed my studies in mice in both steady state and in a context of CNS autoimmunity relying on the experimental model of autoimmune encephalomyelitis (EAE).

4.1. SCIENTIFIC CONTEXT

pDCs represent a specific subset of DCs playing a crucial role in the mediation of innate and adaptive immunity. They are important innate sensors, well known as substantial producers of type I interferon (IFN-I) and to pro-inflammatory cytokines following their activation through TLR signaling by microbial peptides or abnormal release of self-DNAs during inflammation. In addition, they exhibit APC functions and consequently directly impact T cell responses. The specificity of their phenotypic and functional features, in steady state and under activation, allows them to significantly impact T cell peripheral tolerance. As cDCs, immature pDCs strictly exhibit tolerogenic functions and therefore contribute to the maintenance of T cell tolerance in steady state. Upon inflammation they can, however, display either immunogenic or tolerogenic functions depending on the nature of the inflammatory context.

On the other hand, pDCs are influential actors of the autoimmunity. Indeed, pDCs can promote pathogenicity through the production of IFN-I and inflammatory cytokines inducing the differentiation of cytotoxic $CD8^+$ T cells and $CD4^+$ effector T cells. Conversely, pDCs are also strong tolerogenic actors mediating $CD8^+$ T cell deletion, $CD4^+$ T cells anergy, as well as Treg differentiation and expansion.

Since pDCs represent the main producers of IFN-I and because IFN-β1 constitutes one of the first line treatments of MS, we would have expected that the involvement of pDCs in MS would have been extensively studied. However, their role has been poorly addressed and not clearly defined yet. Notably, low frequencies of pDCs in peripheral tissues make their characterization and the study of their functions difficult.

Schwab and colleagues reported that both tolerogenic pDC1 and immunogenic pDC2 populations can be found in peripheral blood of MS patients but exhibit an inverted ratio compared to healthy individuals characterized by more pDC2 than pDC1 in periphery (Schwab

et al., 2010). This observation is interesting but difficult to interpret. It could correlate either with a defect of pDC1 function in MS patients that would contribute to the pathogenesis. Alternatively, it could reflect an increased migration of pDC1 into the CNS, hence inverting the peripheral pDC1/pDC2 ratio supporting a protective role of pDCs (Schwab et al., 2010). Both hypotheses can be correlated with the observations in MS patients reported by Hirotani *et al.*, Stasiolek *et al.* and Lande et *al.*. Compared to PBMCs pDCs from healthy individuals, pDCs isolated from PBMCs of MS patients exhibit a significant dysregulation in cytokine production following *in vitro* CpG-DNA stimulation (Hirotani et al., 2012; Stasiolek et al., 2006). Indeed, pDCs from MS patients produce decreased amounts of IFN-α and IL-10, and in contrast, increased levels of IL-12p70, IFN-γ and IL-17. This suggests that pDCs from MS patients either exhibit impaired functions or that the ratio immunogenic pDC2/tolerogenic pDC1 is elevated in the peripheral blood of MS patients, a phenomenon that may be attributed to intensified migration of pDC1 into the brain. Accordingly, immature pDCs are present in CSF of MS patients and accumulate in white matter lesions of leptomeninges (Lande et al., 2008), supporting that indeed pDCs could migrate into the CNS and be present on the site of inflammation. Nevertheless the current knowledge on pDC positioning in MS is not sufficient to understand whether these cells rather contribute to the development of the pathology or conversely promote of tolerogenic mechanisms.

An alternative option to better characterize pDCs functions in MS is to address their role in the mouse model of experimental autoimmune encephalomyelitis (EAE). In the lab, we developed several murine tools in order to address the role of pDCs in the priming and the effector phases of the disease.

THE MODEL OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE)

EAE is the most common animal model for MS and is mediated by the induction of myelin-specific autoreactive $CD4^+$ T cells in SLOs that migrate to the CNS and induce a local inflammation and extensive brain damage. EAE can be either initiated by active immunisation of C57Bl/6 or SJL/J mice with myelin components, respectively MOG_{35-55} peptide or PLP-1, mixed with Complete Freund Adjuvant (CFA) injected subcoutaneously, or by the adoptive transfer of already primed MOG_{35-55} autoreactive $CD4^+$ T cells in C57Bl/6 recipient mice. Both protocols also require intravenous injections of *pertusis* toxin which contribute to the permeabilization of the BBB and therefore allow the infiltration of pathogenic cells in the CNS. Active immunization with myelin derived components induces the presentation of myelin peptide by MHCII molecules in SLOs and consequently the priming of encephalitogenic $CD4^+$ T cells. Autoreactive MOG_{35-55}-specific $CD4^+$ effector T cells migrate via the blood circulation and reach the CNS, where they are reactivated by local APCs displaying cognate Ag-MHCII complexes. This local reactivation drives the effector phase of the disease, characterized by an acute CNS inflammation leading to demyelination, axonal degeneration and neuronal breakage. Adoptively transfered MOG_{35-55} autoreactive effector $CD4^+$ T cells, called passive EAE, bypass the priming phase in SLOs and lead directly to the achievement of the effector phase in the CNS. In both case, disease development is characterized by a progressive paralysis of the animal, which can be evaluated with clinical scores.

Obviously, although EAE represents an essential tool for clinical research, it remains a mouse model displaying several discrepancies with MS pathology. First, the course of MOG_{35-55} induced EAE is often monophasic, with an acute phase chracterized by severe clinical syptoms, followed by a stabilisation phase with stagnating clinical scores and a remission phase correllating with a reduction of clinical syptoms. Therefore, contrary to the PLP-dependant EAE model induced in SLJ mice, the MOG_{35-55}-dependent EAE model does not allow

observations on relapsing and remitting MS cycles. Moreover, due to the MHCII restricted peptide used for the immunization, T cell response is heavily biased toward $CD4^+$ T cells, obscuring $CD8^+$ T cell responses which are important as well in MS. In addition, disease development induced after MOG35-55 immunization does not require B cells, which is different compared to MOG protein-induced EAE, or MS. Lastly, as in any animal models, interspecies immune differences exist and results obtained in mice do not always exactly reflect what is happenning in humans.

Despite these differencies, EAE represents a useful model to study cell migration, cellular functions and interactions *in vivo* in a inflammatory context that is still closed to the one observed in MS patients.

WHAT IS KNOWN ABOUT PDCS IN EAE

Several research groups have used the EAE model to address the role of pDCs in this particular inflammatory context. However, as in humans, their low frequencies in peripheral tissues and the diverse nature of their functions have rendered difficult to study the role of pDCs in EAE, which therefore remains elusive. The next paragraph reports the major findings.

Upon EAE inflammation, pDCs can be found in SLOs as well as in the CNS (Bailey-Bucktrout et al., 2008; Galicia-Rosas et al., 2012; Irla et al., 2010; Orsini et al., 2014), likely suggesting an implication in the immune response generated after immunization with myelin components.

Contrasting roles of pDCs in the priming phase of EAE

Two different studies claim that pDCs are involved in EAE initiation. First, Isaksson and colleagues show that the specific depletion of pDCs by administration of anti-PDCA1

antibodies prior immunization leads to attenuated EAE development and lower IFN-I levels in peripheral blood compared to non-treated mice (Isaksson et al., 2009). However, while the clinical scores indeed appeared reduced in the acute phase, EAE onset was not affected by the depletion of pDCs. More recently, using the same depleting antibody, Ioannou *et al.* show that ablation of the pDC population during MOG_{35-55} challenge results in delayed EAE onset, decreased frequencies of myelin specific T cells in draining LNs and attenuated disease (Ioannou et al., 2013). They postulate that during EAE priming phase, pDCs inhibit MCP-1-dependent mobilization in draining LNs of myeloid-derived suppressor cells (MDSCs), potent suppressor of $CD4^+$ T cells, and consequently support the priming of the inflammatory response in SLOs (Ioannou et al., 2013). On the other hand, in the study mentioned above, Isaksson and colleagues also demonstrate that the neutralization of IFN-I prior disease onset ameliorates the early phase of the disease but does not impact EAE severity as the whole (Isaksson et al., 2009).

These results suggest that during EAE priming phase, pDCs play rather an immunogenic role and sustain the priming of encephalitogenic T cells in SLOs, potentially through the secretion of IFN-I. However, because these studies were performed using pDC specific depleting antibodies, they do not allow the discrimination of the role played by pDCs in EAE through their distinct innate and adaptive functions, which can be strikingly different.

The impact of pDC adaptive functions on the priming phase of EAE was elegantly addressed a few years ago by Loschko and colleagues and by Irla and colleagues. The first group demonstrated that specific delivery of MOG_{35-55} peptide to pDCs using Siglec-H antibody targeting strategy efficiently delays EAE disease onset and reduces disease severity by inducing the anergy of autoreactive T cells in draining LNs (Loschko et al., 2011a). In the same line of outcomes, Irla et al. also related a tolerogenic role of pDCs during EAE priming phase

by using knockout mice exhibiting a specific ablation of MHCII expression in pDCs. This study demonstrates that myelin-Ag presentation by pDCs in SLOs during EAE priming phase promotes the expansion of Treg cells required to inhibit encephalitogenic T_H1 and T_H17 cell priming in draining LNs. pDC-mediated Treg expansion correlates as well with a significant decrease of auto-reactive T cells in the CNS and results in dampened EAE. Furthermore, they showed that BM derived MOG_{35-55} peptide loaded pDCs transferred in mice one day prior EAE immunization significantly delayes disease onset and progression through mechanisms dependent on MHCII restricted Ag presentation by pDCs. Importantly, they relate that Tregs primed during EAE significantly control disease development only when pDCs can present self-Ags (Irla et al., 2010). These two studies demonstrate that pDC adaptive functions play a tolerogenic role in SLOs during the T cell priming phase of the disease and are important to control EAE development.

Therefore, through their innate or adaptive functions, pDCs seem to mediate opposit influences on EAE initiation.

One of my objectives was to address the specific mechanisms relying on the promotion of suppressive Tregs by pDCs during the priming phase of EAE.

Probable tolerogenic role of pDCs in the effector phase of EAE

As in MS patients, pDCs infiltrate the CNS and accumulate in the SC and the brain of EAE mice (Bailey-Bucktrout et al., 2008; Galicia-Rosas et al., 2012; Orsini et al., 2014). Using the PLP-dependant EAE model, Galicia-Rosas and colleagues identified that pDC infiltration in the CNS of EAE mice is two days subsequent to T cell entry and coincides with a reduction of pDCs in peripheral blood (Galicia-Rosas et al., 2012). In addition, pDC depletion during the acute phase of MOG_{35-55}-induced EAE (Isaksson et al., 2009), as well as during both acute and relapse

phases in PLP-induced EAE (Bailey-Bucktrout et al., 2008) leads to increased disease severity. In the PLP model, exacerbated EAE is mediated through intensified activation of encephalitogenic T_H1 and T_H17 in the CNS (Bailey-Bucktrout et al., 2008). Interestingly, the protective effect of S1P-1R-agonist treatment (AUY954 in mice and Fingolimod in human), known to reduce lymphocyte migration into the CNS of EAE mice and MS patients respectively, relies on pDC migration in the CNS. Indeed when pDCs are depleted, the protective effect of S1P1R-agonist treatment is completely impeded hereby re-establishing lymphocyte infiltration and normal EAE clinical development (Galicia-Rosas et al., 2012). Lastly, Orsini and colleagues reported that in EAE mice, the presence of Tregs in the CNS at peak disease correlates with high IDO expression, high pDC frequency in the CNS and accelerated EAE remission (Orsini et al., 2014).

Altogether, these studies point out the therapeutic value of pDCs relying on their potency to reach the CNS during EAE acute phase, therefore paving the way of new therapeutic strategies for MS treatment.

Because we previously showed that prophylactic transfer of BM-derived pDCs one day prior EAE immunization significantly delayed and moderately decreased EAE development, we wondered whether the therapeutic transfer of pDCs during the acute phase of the disease could lead to disease amelioration.

4.2. Hypotheses and Work axis

Following the observations done by Irla *et al.* relating tolerogenic features of pDCs during EAE priming phase, **we analysed the molecular mechanisms underlying with pDC-mediated promotion of suppressive Tregs in draining LN during EAE priming phase.**

Because the immunomodulatory enzyme IDO has been implicated in pDC tolerogenicity (Baban et al., 2005; Fallarino et al., 2004; Munn et al., 2004b; Pallotta et al., 2011) and Treg induction (Belladonna et al., 2009; Pallotta et al., 2011; Yang et al., 2014), and has been related to EAE amelioration (Kwidzinski et al., 2005; Orsini et al., 2014; Sakurai et al., 2002), we investigated whether pDC-mediated tolerogenic effect occurring in SLOs during EAE priming phase could relies on IDO expression by pDCs. In a more general context, we questioned if and how IDO is induced in LN pDCs during steady-state and upon EAE inflammation. Furthermore we wondered whether and how the expression of this molecule by pDCs in these two contexts could impact Treg responses.

The works of Bailey-Bucktrout and Galacia-Rosas point out the therapeutic potency of pDCs in the acute phase of EAE once they have reached the CNS. Because we previously showed that prophylactic transfer of BM-derived pDCs one day prior EAE immunization significantly delayed and moderately decreased EAE development, **we wondered whether therapeutic transfer of pDCs during the acute phase of the disease could lead to disease amelioration.**

In this second work, we studied address the therapeutic potential of pDC transfer after disease onset on EAE development. This specific point was assessed through disease clinical score evaluation and the analysis of CNS inflammation and damage following pDC transfer. We determined whether transferred pDCs reach the CNS and the migration pathway(s) involved, as well as the processes relying on disease protection mediated by pDC after transfer.

II. MATERIEL AND METHODS / RESULTS

1. SECTION 1

IDO-ORCHESTRATED CROSSTALK BETWEEN PDCS AND TREGS INHIBITS AUTOIMMUNITY

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INTRODUCTION

Few years ago, my colleagues demonstrated that through their ability to present Ag, pDCs exert a substantial tolerogenic effect during priming phase of EAE. Indeed, self-Ag presentation of myelin peptide by pDCs promoted Treg expansion in draining LNs of EAE mice and was associated with a significant inhibition of auto-reactive T_H1 and T_H17 in SLOs. Consequently encephalitogenic T_H1 and T_H17 cells were decreased in the CNS, resulting in dampened CNS inflammation and EAE clinical scores. Here we demonstrate that in steady-state LNs, IDO is preferentially expressed by pDCs compared to other LN cells. We show that steady state IDO expression in LN pDCs relies on MHCII-mediated interactions with Tregs. Furthermore during EAE, IDO expression by MHCII competent pDCs is required to confer suppressive functions to pDC-induced Tregs that inhibit encephalitogenic T cell priming in draining LNs and result in reduced disease severity. Overall, we identified a crucial interplay between pDCs and Tregs that favours T cell tolerance.

OBJECTIVES

In this study, we addressed the mechanisms relying on tolerogenic functions of pDCs, particularly their ability to express the immunoregulatory enzyme IDO, and the effect of this molecule on Treg cells during the priming phase of EAE.

CONTRIBUTION TO THIS WORK

This study constitutes the main part of my thesis project. I am the principal investigator of this work and consequently designed, performed and analysed the experiments shown in this part. I also contributed to the writing of the manuscript.

IDO-orchestrated crosstalk between pDCs and Tregs inhibits autoimmunity

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Abbreviations used: Ag, antigen; cDCs, conventional dendritic cells; BM, bone marrow; CNS, central nervous system; cTECs, cortical thymic epithelial cells; dLN, draining lymph node; EAE, experimental autoimmune encephalomyelitis; IDO, indoleamine-2,3-dyoxygenase; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; pDCs, plasmacytoid dendritic cells.

Abstract

Plasmacytoid dendritic cells (pDCs) have been shown to both mediate and prevent autoimmunity, and the regulation of their immunogenic versus tolerogenic functions remains incompletely understood. Here we demonstrate that pDCs are the predominant source of Indoleamine-2,3-dioxygenase (IDO) in steady-state lymph nodes (LNs). IDO expression by LN pDCs was closely dependent on MHCII-mediated, antigen-dependent, interactions with Treg. We further established that IDO production by pDCs was necessary to confer suppressive function to Tregs. During EAE development, IDO expression by pDCs was required for the generation of Tregs capable of dampening the priming of encephalitogenic T cell and disease severity. Thus, we describe a novel crosstalk between pDCs and Tregs: Tregs shape tolerogenic functions of pDCs prior to inflammation, such that pDCs in turn, promote Treg suppressive functions during autoimmunity.

Keywords

Regulatory T cells, Plasmacytoid dendritic cells, Indoleamine 2,3-dyoxygenase, Antigen presentation, Tolerance, Experimental autoimmune encephalomyelitis

Introduction

Plasmacytoid dendritic cells (pDCs) are important sensors of non-self-nucleic acids derived from bacteria or viruses and are crucial mediators of innate anti-microbial responses through the production of inflammatory cytokines and type-I IFNs (Cervantes-Barragan et al., 2007; Colonna et al., 2004). In addition, pDCs have been implicated in the development of several autoimmune diseases, including lupus, psoriasis, multiple sclerosis (MS) and type-1 diabetes (Chan et al., 2012; Kared et al., 2005; Longhini et al., 2011; Nestle et al., 2005; Reder and Feng, 2013). Following abnormal release of self-DNA during inflammatory processes, pDCs are activated through TLR signalling and subsequently produce type-I IFN (Gilliet et al., 2008). Importantly, a few years ago, the notion emerged that pDCs act not only as innate sensors but can also function as *bona fide* antigen (Ag) presenting cells (APCs) and directly impact T cell responses (Swiecki and Colonna, 2015). It was shown that pDCs capture and process Ags (Villadangos and Young, 2008), and load antigenic peptides onto MHC class I (MHCI) (Di Pucchio et al., 2008) and MHC class II (MHCII) molecules (Hoeffel et al., 2007; Sapoznikov et al., 2007; Young et al., 2008). The modulation of Ag-presenting pDC functions led to important consequences on T cell immunity, the outcome being highly dependent on the cytokine microenvironment (Guery and Hugues, 2013).

Many studies, including those investigating oral tolerance and allograft models, suggest that steady-state Ag-presenting pDCs exclusively promote T cell tolerance (Goubier et al., 2008; Liu et al., 2011; Ochando et al., 2006). Although the nature of the factors controlling distinct pDC functions remains to be established, once activated, pDCs exhibit both immunogenic and tolerogenic functions. For example, using mice exhibiting a specific loss of MHCII expression by pDCs, we showed that CpG-B activated pDCs present Ag and promote effector Th17 cell

differentiation, a property that can be exploited for anti-tumor vaccines (Guery et al., 2014). Pro-pathogenic Ag-presenting pDCs were similarly described in a mouse model of atherosclerosis in which pDCs induced pathogenic Th1 cells (Sage et al., 2014). In addition, BST-2 mediated specific Ag delivery to CpG-activated pDCs led to cytotoxic T lymphocyte (CTL) and Th1 cell differentiation and triggered protective immunity against viral infection and tumor growth (Loschko et al., 2011b). In contrast, in the context of EAE, Ag targeting to pDCs via Siglec-H promoted $CD4^+$ T cell anergy and inhibited CNS inflammation (Loschko et al., 2011a). We previously demonstrated that in EAE, pDCs present myelin Ags on MHCII molecules to induce the expansion of suppressive Tregs, a phenomenon correlated with disease amelioration (Irla et al., 2010).

Indoleamine 2,3-dioxygenase (IDO) is an immunomodulatory enzyme involved in the initial and the rate-limiting step of tryptophan catabolism. Upon inflammation, IDO production has been shown to compromise T cell proliferation, promote T cell anergy and Tregs (Baban et al., 2009; Chen et al., 2008a; Yang et al., 2014). Depending on the experimental context, IDO can be induced either by IFN-γ, IFN-α/β, or TGF-β. CTLA-4 binding to cell-surface expressed costimulatory molecules promotes IDO production by pDCs through IFN-γ or IFN-α/β signalling (Fallarino et al., 2003; Grohmann et al., 2002; Manlapat et al., 2007; Mellor et al., 2003). Furthermore CD200-Ig binding to his cognate receptor induces IDO in an IFN-α/β dependent signalling pathway (Fallarino et al., 2004). Both IFN-γ and IFN-α/β pathways result in IDO^+ immunosuppressive effects which are closely dependent on the catalytic activity of the enzyme.

CTLA-4-binding also promote IDO in tumor contexts, but the enzyme has reveal activity in only a minor DC subpopulation expressing the marker CD19, but none of the pDC classical markers

(Baban et al., 2005; Munn et al., 2004b). IDO enzymatic functions in tumor dLN-sorted pDCs have been correlated to *in vitro* Treg differentiation and suppressive functions (Baban et al., 2009; Sharma et al., 2009). More recently, Pallotta and colleagues described that IDO^+ pDCs induced long-lived Tregs by using a TGF-β-dependent pathway distinct from the catalytic activity of the enzyme. In mouse a model of skin delayed-type hypersensitivity, they shown that whereas IFN-γ-dependent IDO enzymatic activity in pDCs leads to T cell anergy, TGF-β induced IDO phosphorylation results in increased Treg frequencies (Pallotta et al., 2011).

It is so far unknown whether IDO expression in naïve pDCs pre-exists, and how it would be regulated in steady-state LNs. In contrast, recent work has implicated IDO expression in pDC immunoregulatory functions, including Treg induction, in inflamed LNs. Furthermore, IDO production by tumor-associated pDCs has been correlated to *in vitro* Treg-mediated suppression. However, the nature of the cells expressing IDO, as well as the impact on Treg functions in chronic inflammatory diseases, such as autoimmune disorders, remain undetermined.

Here we show that in steady-state lymph nodes (LNs), IDO is highly expressed by pDCs compared to other LN resident cells. We further established that IDO expression is positively regulated in steady-state pDCs following MHCII-mediated interactions with Tregs. During autoimmune disorders, such as EAE, IDO expression by MHCII competent pDCs is mandatory to confer suppressive functions to pDC-induced Tregs. IDO-competent Ag-presenting pDCs promote Tregs that inhibit autoimmune effector T cell responses in LNs, resulting in reduced disease severity. Therefore, we have identified a bidirectional interaction between pDCs and Tregs that favours self-tolerance.

Materials and methods

Mice

All mice had a pure C57BL/6 background and were bred and maintained under SPF conditions at Geneva medical school animal facility and under EOPS conditions at Charles River, France or at the National Institutes of Health, Bethesda, US. DEREG (Lahl et al., 2007), Ubiquitin-eGFP (Schaefer et al., 2001), pIII+IV$^{-/-}$ (Reith et al., 2005), IDO$^{-/-}$ (Baban et al., 2004), BDCA2-DTR (Swiecki et al., 2010), MARILYN Rag2$^{-/-}$, OTII Rag2$^{-/-}$ (Barnden et al., 1998), AND Rag2$^{-/-}$ (Kaye et al., 1989), SMARTA Rag1$^{-/-}$ (Oxenius et al., 1998), Rag2$^{-/-}$, Scurfy (Means et al., 2000), CD45.1 (Charles River, France), and 2D2 (Bettelli et al., 2003) mice have been previously described. WT C57BL/6 mice were purchased from Harlan laboratories (France) or Taconic (US). All procedures were approved by and performed in accordance with the guidelines of the animal research committee of Geneva or of the NIH.

Generation of BM chimeric mice

BM chimeric mice were generated as described (Guery et al., 2014). Briefly, BM cells were recovered from tibia and femurs of donor mice. 5 to 7 x 10^6 cells were injected intravenously into sub-lethally irradiated recipient mice (two consecutive doses of 450 cGy). Reconstitution was assessed by analysing blood cells by flow cytometry after 6-8 weeks. For mixed BM chimeras, CD45.2 WT eGFP and CD45.2 pIII+IV$^{-/-}$ BM cells were simultaneously transferred into irradiated CD45.1 WT recipient mice in a 1:1 ratio.

EAE experiments

Active EAE was induced by immunizing mice, subcutaneously in both flanks, with 100 µg of MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK, Biotrend) emulsified in incomplete Freund's adjuvant (BD Diagnosis) supplemented with 500 µg/ml *Mycobacterium tuberculosis* H37Ra

(BD Diagnosis). At the time of immunization and 48h later, mice also received 300 ng of pertussis toxin (Sigma-Aldrich) into the tail vein. Mice were monitored daily for disease clinical symptoms, and blindly scored as follows. 1, flaccid tail; 2, impaired righting reflex and hind limb weakness; 3, complete hind limb paralysis; 4, complete hind limb paralysis with partial fore limb paralysis; 5, moribund.

For passive EAE induction, encephalitogenic $CD4^+$ T cells were generated *in vitro* from LN and spleen cells of 2D2 mice as described (Domingues et al., 2010). 1 - 2 x 10^6 total cells were injected *i.p.* into recipient mice. Mice received 67 ng of pertussis toxin at the day of cell injection and 48h later. Mice were monitored daily for disease clinical symptoms as described above.

Adoptive transfers of Treg cells were performed as follows. WT→WT, pIII+$IV^{-/-}$→WT and $IDO^{-/-}$ →WT chimeric mice were immunized or not with MOG_{35-55} + CFA. $CD4^+$ $CD25^{hi}$ T cells were harvested from total skin LNs (naïve) or dLNs (day 10 after EAE immunization) and 1-5 x 10^5 $CD4^+$ $CD25^{hi}$ T cells were injected intravenously into tail vein of recipient mice. EAE was induced by active immunization the day after Treg transfer.

Adoptive transfers of pDCs were performed as follows. 1.2-1.5 x 10^6 BM derived pDCs loaded with 10µg/mL of MOG_{35-55} were injected intravenously into tail vein of recipient mice, and EAE was induced by active immunization the day after.

In some experiments, EAE mice were treated *i.p*. at indicated time points with DT (100 ng/mouse for BDCA2-DTR and 1 µg/mouse for DEREG).

Ex vivo cell isolation

Treg cells were isolated from total skin LNs of naïve mice or from dLNs of EAE mice (day 10 after immunization). LNs were scratched and LN cells were subjected to $CD4^+$ T cell enrichment using $CD4^+$ T cell isolation kit (Miltenyi biotec). $CD4^+$ $CD25^{hi}$ Treg cells were next sorted using a MoFlowAstrios (Beckman Coulter).

For qPCR experiments, pDCs, cDCs, B cells and macrophages were recovered from LN after digestion with an enzymatic mix containing collagenase D (1 mg/mL) and DNAse I (10 µg/mL) (Roche) in HBSS. B cells were isolated using anti-CD19 beads (Miltenyi Biotech), macrophages, cDCs and pDCs were then purified, after $CD3^+$ cells depletion (Miltenyi Biotech), by flow cytometry as $CD11c^-CD11b^+Ly6C^+$ for macrophages, $CD11c^+PDCA1^-$ for cDCs and $CD11c^{int}PDCA1^+SiglecH^+$ for pDCs, using a MoFlowAstrios (Beckman Coulter) or BD FACSAria (BD Biosciences).

In vitro BM derived pDC generation

pDCs were generated from BM of WT, pIII+$IV^{-/-}$ and $IDO^{-/-}$ mice as previously described (Irla et al., 2010). Briefly, BM cells were recovered from tibia and femurs of mice and cultured, after red cell lysis, for 7 days in complete RPMI medium (10% heat-inactivated fetal bovine serum, 50 mM 2-βMercaptoethanol, 100mM sodium Pyruvate and 100 µM of Penicillin/Streptomycin) supplemented with 100 ng/mL of murine Flt3L (PeproTech).

Co-cultures

$CD4^+$ T cells were recovered from scratched total skin LNs of 2D2 mice and purified with $CD4^+$ T cell isolation kit (Miltenyi Biotec) according to manufacturer's instructions. Both cell types purity was assessed by flow cytometry using a Cyan™ ADP (Beckman Coulter) and exceeded 90%.

WT and Rag2$^{-/-}$ pDCs were recovered from total skin LN and spleen after digestion with the enzymatic mix described above. pDCs were isolated using the Plasmacytoid Dendritic Cell isolation kit II (Miltenyi Biotec) according to manufacturer's instructions. pDCs were loaded or not with MOG_{35-55} peptide (10 µg/mL). 300.000 pDCs were seeded in 48 well plates with 200.000 2D2 CD4$^+$ T cells. Cells were co-cultured in complete RPMI medium for 16h and pDCs were isolated again using the Plasmacytoid Dendritic Cell isolation kit II (Myltenyi Biotech) according to manufacturer's instructions.

WT BM-derived pDCs were generated *in vitro* for 7-8 days and purified using the Plasmacytoid Dendritic Cell isolation kit II (Miltenyi Biotec) according to manufacturer's instructions. Cell purity was assessed by flow cytometry using a Cyan^{TM} ADP (Beckman Coulter) and exceeded 90%. pDCs were loaded or not with MOG_{35-55} peptide (10 µg/mL). 500 000 pDCs were seeded in 24 well plates with 300 000 2D2 CD4$^+$ T cells or 300 000 pDCs were seeded in 48 well plates with 100 000 2D2 CD4$^+$ T cells or 100 000 2D2 CD4$^+$ CD25$^-$ cells or 100 000 2D2 CD4$^+$ CD25$^+$ cells. Cells were co-cultured in complete RPMI medium for 16h and pDCs were isolated again using the Plasmacytoid Dendritic Cell isolation kit II (Miltenyi Biotec) according to manufacturer's instructions.

Flow cytometry

Monoclonal antibodies used for flow cytometry were from: Biolegend; anti-CD11c (N418), anti-Ly6C (HK1.4); from eBioscience: anti-CD4 (GK1.5 and RM4-5), anti-CD69 (H1.2F3), anti-IL10 (JES5-16E3) anti-TER119 (TER-119), anti-Foxp3 (FJK-16s), anti–IL-17 (ebio17B7), anti-IFN-γ (XMG1.2) anti-Siglec-H (ebio440c), anti-Ki67 (SolA15), anti-CD45.1 (A20), anti-CD11b (M1/70), anti-CD11c (N418), anti-TCR (H57-597), anti-ICOS (C398.4A), anti-CD103 (2E7), anti

CD16/32 (93), anti-CD5 (53-7.3); from BD: anti-CD25 (PC61), anti-CD19 (1D3), anti-CD3 (145-2C11), anti-Ki67 (B56) and anti–IFN-γ (XMG1.2).

For flow cytometry analysis, single cell suspensions were incubated with FcBlock (anti-CD16/32 FcγRII-RIII) for 10 min, at 4°C and stained with antibodies. Intracellular cytokine stainings were done using the Intracellular Fixation & Permeabilisation buffer set (eBioscience) or with the Fix & Perm kit (BD Biosciences) for IL-10. Cell proliferation was assessed by flow cytometry using anti–mouse Ki67 and respective isotype control (Rat IgG2a, kappa). For IFN-γ, IL-17 and IL-10 staining, cells were re-stimulated in complete RPMI containing PMA/ionomycin, and incubated 4h at 37°C, 5% CO2. Golgi stop solution (BD Biosciences) was added to the last 2.5h of culture. Data were acquired with a Cyan^{TM} ADP or a Gallios (Beckman Coulter) and analyzed using FlowJo software (FlowJo company). cDCs were defined as CD11c^{hi}PDCA-1^{-} and pDCs as CD11c^{int}PDCA-1^{+} or CD11c^{int}Siglec-H^{+}. B cells and macrophages were defined respectively as CD19^{+} and CD11b^{+}Ly6C^{+}.

Quantitative RT-PCR

Total RNA was isolated and prepared with TRIzol reagent (Invitrogen) and RT-PCR were performed as described (Irla et al., 2010). cDNA was synthesized with random hexamers and M-MLV Reverse Transcriptase (Promega). PCR were performed with CFX Connect Real-time System (Bio rad) and iQ SYBR green Super-mix (Bio-Rad Labolatories). GAPDH mRNA was used for normalization. Primer sequences were as follows: IDO, forward, 5'-GGG ATG ACG ATG TTC GAA AG-3' and reverse 5'-CAG GAC ACA GTC TGC ATA AG-3'; GAPDH, forward, 5'- CCC GTA GAC AAA ATG GTG AAG -3' and reverse 5'- AGG TCA ATG AAG GGG TCG TTG -3'.

Statistics

Significance was assessed by two-tailed Mann-Whitney test or by one-way ANOVA with Bonferroni post Hoc test. EAE incidence was analyzed using two-way ANOVA with Bonferroni post Hoc test. All statistical analyses were done using Prism 5.0 software (GraphPad Software). * $P < 0.05$, ** $P< 0.01$, *** $P< 0.001$, **** $P< 0.0001$, NS= Non significant

Results

3.1 IDO expression by pDCs in steady-state LN is dependent on MHCII restricted Ag-specific interactions with Treg

We analysed IDO mRNA expression in different cell subtypes sorted from steady-state LNs. IDO mRNA was expressed to far greater levels (>5 fold) by pDCs ($CD11c^{int}PDCA\text{-}1^{+}$), than by cDCs ($CD11c^{hi}PDCA\text{-}1^{-}$), B cells ($CD11c^{-}CD19^{+}$), and macrophages ($CD11c^{-}CD11b^{+}LyC6^{int/+}$ (**Fig. 1A**). Consistently, IDO preferential expression by steady-state LN pDCs compared to cDCs was also observed in Balb/c mice, ruling out a mouse C57BL/6 strain restricted effect (**Supplementary Fig. 1A**). IDO expression by pDCs was shown to be induced by T-cell produced cytokines, such as IFN-γ and TGF-β (Fallarino et al., 2007; Pallotta et al., 2011). To determine whether, in steady state, T cells are involved in the regulation of IDO expression by pDCs, we measured IDO mRNA in $Rag2^{-/-}$ mice, which are devoid of T cells. Compared to WT mice, pDCs from steady-state LNs of $Rag2^{-/-}$ exhibited a significant reduction of IDO mRNA, which was *in fine* comparable to levels obtained in $IDO^{-/-}$ pDCs (**Fig. 1B**). We next tested whether interactions with T cell were required for IDO induction in pDCs of naïve mice. We examined IDO expression in pDCs from LNs of mice expressing a monoclonal population of $CD4^{+}$ T cells specific to OVA peptide, OT-II $Rag2^{-/-}$ mice. IDO expression was significantly reduced and was comparable to what was seen in $Rag2^{-/-}$ mice (**Fig. 1B**). Thus, OT-II transgenic $CD4^{+}$ T cells were insufficient at inducing IDO in pDCs.

This result might be explained by a low TCR self-reactivity for MHCII molecules of the OTII transgenic $CD4^{+}$ T cells (Mandl et al., 2013). To test the possibility that distinct TCR self-reactivity would exhibit differential ability to induce IDO expression in pDCs, we isolated pDCs from different $CD4^{+}$ TCR transgenic $Rag1^{-/-}$ mice that express distinct levels of CD5, which reflects clone-specific strength self-reactivity (Mandl et al., 2013). Each TCR transgenic T cell

population display a specific surface amount of CD5 that covered a range from low (Marilyn, TCR specific for HY), medium (Smarta, TCR specific for LCMV), to high (AND, TCR specific for Pigeon Cytochrome c) (Figure 1C). IDO mRNA expression was reduced in LN pDCs isolated from all TCR transgenic $Rag^{-/-}$ mice compared to WT mice (**Fig. 1C**), showing that IDO induction in pDCs is independent of TCR self-reactivity. An alternative hypothesis which might explain why IDO expression in negligible is a potential specific requirement of Tregs in regulating IDO expression in pDCs, this population being almost absent in LNs of the TCR transgenic T cell mice tested (**Fig. 1D**). Consistently, MOG_{35-55}-loaded $Rag2^{-/-}$ pDCs, exhibit a significant restoration of IDO expression when co-cultured with 2D2 TCR tg $CD4^+$ T cells, which contain a low but significant frequency of $Foxp3^+$ cells (**Fig. 1E**). Moreover, pDCs isolated from LNs of Scurfy mice, which are devoid of $Foxp3^+$ Tregs (Means et al., 2000), presented a significant reduction of IDO mRNA expression compared to WT pDCs (**Fig. 1F**). Thus, our data suggest that Tregs significantly contribute to induce IDO expression by pDCs in steady-state LN. We next evaluated whether Ag-specific pDC-T cell contacts were required to promote IDO expression in pDCs. To do so, cultures were done using *in vitro* generated BM-derived pDCs, for which IDO mRNA levels were found to be negligible, reinforcing the idea that a crosstalk with T cells is indeed required for pDCs to competently express IDO (**Fig. 1G**). When co-cultured with 2D2 TCR tg $CD4^+$ T cells, a modest increase in IDO mRNA expression was observed in BM-derived pDCs (**Fig. 1G**). IDO expression was further increased by 2 fold when pDCs were previously loaded with MOG_{35-55} peptide, reaching similar levels of expression as pDCs isolated from WT LNs (**Fig. 1G**), Therefore, MHCII-restricted Ag-specific interactions with $CD4^+$ T cells significantly contribute to the induction of IDO expression in pDCs. To test a role for Tregs in this process, we repeated co-culture experiments using 2D2 TCR tg $CD4^+$ T cells that were separated into in $CD25^{hi}$ and $CD25^{neg}$ populations. While 2D2 TCR tg $CD4^+CD25^{neg}$

cells were incompetent at inducing IDO, 2D2 TCR tg $CD4^+CD25^{hi}$ cells significantly enhanced IDO mRNA levels in pDCs loaded with MOG_{35-55} (**Fig. 1H**), confirming that Tregs are mandatory at promoting IDO expression in pDCs. pDCs co-cultured with total 2D2 TCR tg $CD4^+$ T cells exhibit a slight, but not significant increase in IDO mRNA levels compared to pDCs co-cultured with 2D2 TCR tg $CD4^+CD25^{hi}$ cells, suggesting that although Tregs are required to promote IDO, non-Treg $CD4^+$ T cells might also contribute to this process (**Fig. 1H**).

To clearly demonstrate that MHCII-restricted Ag specific interactions between pDCs and T cells are necessary to induce IDO in pDCs, we used genetically deficient mice that selectively lack MHCII expression by pDCs. These mice have been described before and are deficient for the promoters III and IV (pIII+IV) of CIITA, the master regulator for MHCII expression (Reith et al., 2005). In mice, CIITA is under the control of cell specific promoters, pI, pIII and pIV (LeibundGut-Landmann et al., 2004a; LeibundGut-Landmann et al., 2004b) (**Supplementary Fig. 2**). Absence of pIV leads to MHCII abrogation on cortical thymic epithelial cells (cTECs), resulting in the lack of $CD4^+$ T cell positive selection. To restore $CD4^+$ T cell thymic positive selection by MHCII competent cTECs, bone marrow (BM) cell precursors from $pIII+IV^{-/-}$ mice need to be injected into irradiated WT recipients ($pIII+IV^{-/-}$→WT) and compared to WT→WT controls. $pIII+IV^{-/-}$→WT mice exhibiting genetic deficiencies compared to WT→WT, it is possible that distinct immunological environments will affect IDO expression by pDCs. Therefore, to immerse MHCII competent and deficient pDCs in an identical milieu, we performed mixed BM chimeric mice using BM cells from Ubi-eGFP WT and $pIII+IV^{-/-}$ mice (ratio 1:1) that were co-injected into irradiated recipient mice expressing the congenic marker CD45.1 (**Fig. 2A**). LN cells were sorted 2 months later as pDCs and cDCs from donor BM cells (gated on $CD45.2^+$) and further separated as WT ($eGFP^+$) or $pIII+IV^{-/-}$ ($eGFP^-$) cells (**Fig. 2A and B**). We confirmed

that cDCs expressed little IDO mRNA (Figure 2C). IDO expression by MHCII deficient pDCs was impaired compared to MHCII competent pDCs in mixed BM chimeric mice (**Fig. 2C**). Since cells were isolated from the same LNs, decreased IDO expression in absence of MHCII expression by pDCs was not related to different cytokine expression profiles, but linked to a defective MHCII expression by pDCs. An alternative explanation is that CIITA directly acts as a transcription factor regulating IDO gene expression. However, we observed a similar reduction of IDO mRNA in pDCs isolated from H2-Db$^{-/-}$ mice (not shown), ruling out this hypothesis. Altogether, our data demonstrated that MHCII-restricted Ag specific interactions with Tregs are required for the induction of IDO expression by pDCs in steady-state LNs.

3.2 IDO expression in LNs is restricted to pDCs during EAE, and is impaired in MHCII$^{-/-}$ pDCs

We next evaluated whether, as in steady-state LNs, pDCs remains the major source of IDO in LNs in a model of chronic inflammation. IDO has been shown to exert a protective role in EAE using either IDO$^{-/-}$ mice or IDO blocking antibodies (Kwidzinski et al., 2005; Sakurai et al., 2002). Therefore, we have quantified IDO mRNA expression in different cell types sorted from LNs draining the site of EAE immunization (day 10) in wild type (WT) mice. IDO was predominantly (> 5 fold) expressed by pDCs compared to other LN cells (**Fig. 3A**). Levels of expression were comparable to steady-state LN pDCs (**Supplementary Fig. 3A**). Next, we analysed IDO expression by MHCII deficient pDCs during EAE. pDCs and cDCs were sorted from draining LNs of WT→WT and pIII+IV$^{-/-}$→WT mice 10 days after EAE induction. Again, cDCs expressed little IDO mRNA (**Fig. 3B**). We observed a substantial reduction in IDO expression by pDCs sorted from pIII+IV$^{-/-}$→WT compared to WT→WT chimeras (**Fig. 3B**). Differential IDO expression by MHCII competent and MHCII deficient pDCs might be explained by distinct inflammatory cytokinic environments in WT→WT and pIII+IV$^{-/-}$→WT mice, as the latter developed more

severe EAE (Irla et al., 2010). Therefore, we performed mixed BM chimeric mice using as before BM cells from Ubi-eGFP WT and pIII+IV$^{-/-}$ mice (ratio 1:1) that were injected into irradiated CD45.1 recipient mice. EAE was induced in mixed BM chimeric mice, and LN cells were sorted 10 days after immunization as pDCs and cDCs from donor BM cells (gated on CD45.2^{+}) and further separated as WT (eGFP^{+}) or pIII+IV$^{-/-}$ (eGFP^{-}) cells. IDO expression was significantly impaired in MHCII deficient compared to MHCII competent pDCs isolated from LN of EAE mice (**Fig. 3C**). Therefore, decreased IDO expression in absence of MHCII expression by pDCs was not related to different cytokine expression profiles between WT→WT and pIII+IV$^{-/-}$→WT chimeras during EAE, but rather linked to a defective MHCII expression by pDCs. Altogether, our data suggest that IDO expression by LN pDCs is dependent on their expression of MHCII molecules, independent on the inflammatory status of the mice, and reflects a more general regulation of IDO protein expression at the mRNA level.

3.3 IDO deficiency leads to exacerbated encephalitogenic T cell priming

To investigate the role of pDC-induced Treg in EAE pathogenesis, we have induced EAE in mice lacking Treg and MHCII expression by pDCs. We backcrossed DEREG mice, in which Foxp3^{+} Tregs can be selectively depleted using diphtheria toxin (DT) injection (Lahl et al., 2007), with pIII+IV$^{-/-}$ mice and generated DEREGxpIII+IV$^{-/-}$→WT chimeras (**Supplementary Fig. 4 A-C**). In untreated mice, as previously described (Irla et al., 2010), EAE was exacerbated (**Supplementary Fig. 4D**) when pDCs did not express MHCII. Moreover, EAE clinical course was similarly aggravated in DEREG→WT treated with DT and DEREGxpIII+IV$^{-/-}$→WT chimeras injected or not with DT (**Supplementary Fig. 4D**). Encephalitogenic Th1 and Th17 CD4^{+} T cell frequencies were increased in DT-injected DEREG→WT and DEREGxpIII+IV$^{-/-}$→WT mice compared to un-injected DEREG→WT mice (Figure S4E). This demonstrates that the depletion

of Tregs and the absence of MHCII expression by pDCs similarly enhance pathogenic T cell priming and disease severity. Altogether, our data show that pDC-instructed Tregs inhibit the priming of encephalitogenic T cells in LNs during EAE.

We next evaluated whether IDO was implicated in the regulation of encephalitogenic T cell priming during EAE. Knowing that MHCII deficient pDCs exhibit impaired IDO expression both in steady-state and during EAE (**Fig. 2C and 3C**), we analysed EAE development in WT→WT, pIII+IV$^{-/-}$→WT and IDO$^{-/-}$→WT chimeric mice. As before, disease was exacerbated in mice lacking MHCII on pDCs (pIII+IV$^{-/-}$→WT) compared to control WT→WT animals (Figure 4A). This could be consequent to either an absence of Ag-presentation by pDCs and/or a resulting defect in IDO expression by pDCs from pIII+IV$^{-/-}$→WT mice. Consistently with previously published data (Kwidzinski et al., 2005; Sakurai et al., 2002), mice deficient for IDO (IDO$^{-/-}$→WT) developed aggravated EAE compared to WT controls. Importantly, clinical scores of IDO$^{-/-}$→WT were comparable to mice lacking MHCII on pDCs (**Fig. 4A**). Notably, we did not notice any significant impact of IDO deficiency during EAE induced by transferring MOG_{35-55}-specific 2D2 CD4^{+} effector T cells (Figure S5), suggesting a critical role for IDO in the modulation of pathogenic T cell priming in SLOs during EAE. To confirm this hypothesis, we measured effector cytokine production by T cells before clinical symptom appearance (day 9) in IDO$^{-/-}$→WT, pIII+IV$^{-/-}$→WT and WT→WT chimeras. Elevated IFN-γ and IL-17 producing encephalitogenic CD4^{+} T cell frequencies were observed in LNs of IDO deficient mice (IDO$^{-/-}$→WT) and mice lacking MHCII expression by pDCs (pIII+IV$^{-/-}$→WT) compared to WT→WT controls, (**Fig. 4B**). Foxp3^{+}CD25^{hi} Treg proliferation was impaired in pIII+IV$^{-/-}$→WT mice, whereas it was unaffected in IDO$^{-/-}$→WT mice (**Fig. 4C, left**), showing that MHCII-mediated Ag presentation, but not IDO expression by pDCs, promoted Foxp3^{+} Treg proliferation. The

proliferation of Foxp3$^+$ Tregs exhibiting a suppressive phenotype (CD103$^+$ICOS$^+$) (Barthlott et al., 2015) (**Fig. 4C middle**), as well as the frequency of IL-10 expressing Foxp3$^+$ Tregs (**Fig. 4C right**), were significantly decreased in LNs from both pIII+IV$^{-/-}$→WT and IDO$^{-/-}$→WT compared to WT→WT mice. Our results identify a new role for IDO in impacting the ability of Tregs to suppress encephalitogenic T cells in LNs.

3.4 IDO deficiency in MOG$_{35-55}$-presenting pDCs leads to EAE exacerbation

We have shown that IDO mRNA was preferentially expressed by pDCs compared to other LN cells both in steady-state and during EAE (**Fig. 1A and 3A**). However, whether restricted IDO expression by pDCs is sufficient and required to inhibit disease development still need to be clarified. To address this, we used BDCA-2 DTR mice in which endogenous pDCs were depleted following DT injection (Rowland et al., 2014). It has been demonstrated that active immunization induces DT toxicity, and may therefore confound experiments in DTR transgenic mice for neuroinflammatory models, such as EAE (Meyer Zu Horste et al., 2010). To avoid this problem, we generated BM chimeric mice by injecting BM cells from BDCA-2 DTR into irradiated WT recipients. In these chimeric mice, no signs of toxicity or lethality in DT-treated mice upon EAE induction were observed. Moreover, efficient depletion of endogenous pDCs in the blood and LNs of BDCA-2 DTR→WT chimeras was achieved after DT injection (not shown). We transferred (i.v.) MOG$_{35-55}$-loaded WT or IDO$^{-/-}$ pDCs into DT-treated BDCA-2 DTR→WT chimeras one day prior to EAE induction. In these settings, IDO was selectively supplied - or not - by adoptively transferred pDCs. Following EAE induction, only WT, but not IDO$^{-/-}$ pDCs significantly inhibited disease development (**Fig. 5A**). Foxp3$^+$CD25$^+$ Treg proliferation was increased in LN upon pDC transfer compared to control BDCA-2 DTR→WT EAE mice, whether transferred pDCs expressed IDO or not (**Fig. 5B**), confirming that IDO

expression by pDCs is not involved in MHCII-dependent, pDC-mediated, Treg expansion. In contrast, the frequencies of suppressive Tregs co-expressing CD103 and ICOS (**Fig. 5C**), expressing high levels of CD25 (Figure 5D), or upregulating the activation marker CD69 (**Fig. 5E**), were significantly reduced in LN from BDCA-2 DTR→WT EAE mice transferred with $IDO^{-/-}$ pDCs compared to WT pDCs.. These results demonstrate that during EAE, IDO expression by pDCs is required to elicit pDC-mediated suppressive Tregs.

3.5 IDO is required for in vivo Treg-mediated EAE suppression

We next tested the *in vivo* suppressive activity of Tregs primed during EAE in $pIII+IV^{-/-}$→WT, $IDO^{-/-}$→WT and WT→WT chimeric mice. Purified $CD4^+CD25^+$ cells, which are predominantly $Foxp3^+$ (**Fig. 6A**), were transferred into WT hosts one day prior to EAE induction (**Fig. 6A and B**). Tregs primed in WT mice reduced EAE incidence, delayed disease onset, and conferred significant protection as we have previously reported (Irla et al., 2010). In contrast, Tregs primed in mice lacking MHCII expression by pDCs did not exhibit any suppressive activity (**Fig. 6B and C**). Strikingly, Tregs primed in absence of IDO did not confer any delay in disease onset nor reduced EAE severity (**Fig. 6B and C**). Our data demonstrated that IDO provided by pDCs is critical for the acquisition of *in vivo* Treg suppressive functions. In addition, only Tregs activated following EAE immunization in WT mice efficiently inhibited EAE incidence and severity (day 14), whereas neither Tregs isolated from naïve WT, nor Tregs isolated from naïve or immunized $IDO^{-/-}$ mice, conferred any protection upon transfer into EAE mice (**Supplementary Fig. 6**). EAE-mediated activation is consequently mandatory to confer IDO-dependent Treg suppressive functions.

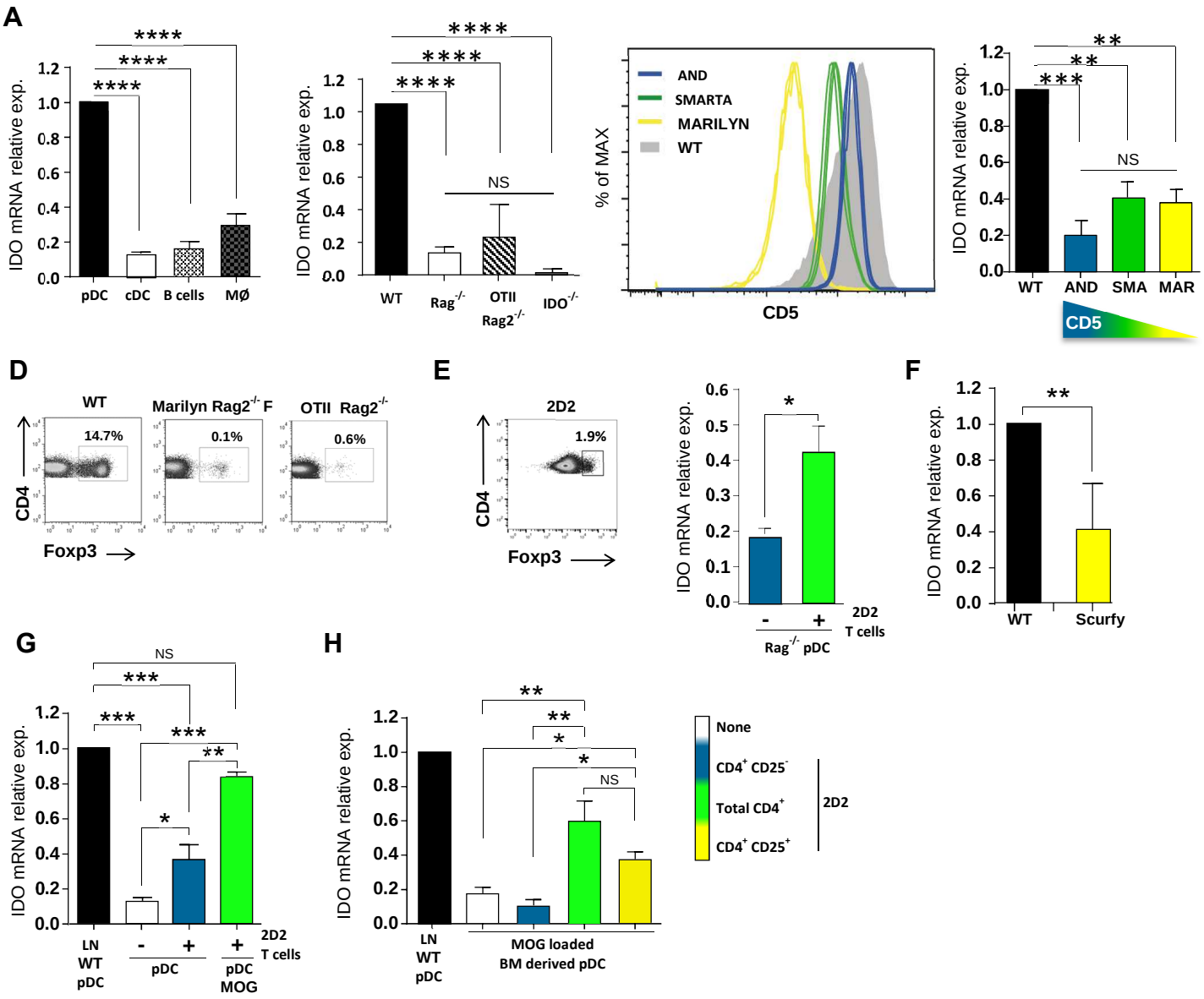


Figure 1. IDO expression by LN pDCs is induced after Ag-specific interactions with Tregs. (A) IDO mRNA expression in B cells, cDCs, pDCs and macrophages (MØ) sorted from total skin LNs of naïve WT. (B) IDO mRNA in pDCs isolated from total skin LNs of naive WT, Rag2$^{-/-}$, OTII Rag2$^{-/-}$ and IDO$^{-/-}$ mice. (C) CD5 expression levels by AND Rag2$^{-/-}$, Smarta Rag1$^{-/-}$ (SMA) and Marilyn Rag2$^{-/-}$ (MAR) CD4^{+} TCR tg T cells (left), and IDO mRNA levels in pDCs isolated from skin LN of AND, SMA, MAR and WT mice (right). (D) Foxp3^{+}CD4^{+} Treg frequencies in LNs of OTII Rag2$^{-/-}$ and female Marilyn Rag2$^{-/-}$ mice. (E) Foxp3^{+}CD4^{+} Treg frequencies in LN cells of 2D2 TCR tg mice (left), and IDO mRNA levels in pDCs isolated from skin LN of Rag2$^{-/-}$ mice and co-cultured *in vitro* with 2D2 TCR tg CD4^{+} T cells and MOG_{35-55} peptide for 16h (right). (F) IDO mRNA levels in *ex-vivo* pDCs sorted from skin LN of 3wk-old WT, pIII+IV$^{-/-}$ and Scurfy mice. (G) IDO mRNA levels of *ex vivo* WT pDCs and WT BM derived pDCs loaded or not with MOG_{35-55} and co-cultured *in vitro* with 2D2 CD4^{+} T cells for 16h. (H) IDO mRNA levels of *ex vivo* WT pDCs and WT BM derived pDCs loaded with MOG_{35-55} and co-cultured *in vitro* for 16h with 2D2 CD4^{+} CD25^{-} cells, 2D2 CD4^{+} T cells or 2D2 CD4^{+} CD25^{+} Treg cells. (A-H) Results are representative of at least 2 independent experiments. Error bars depict mean ± SEM. (A, B, C, G, and H) One-way ANOVA with Bonferroni post Hoc test or (E and F) two-tailed Mann-Whitney test was used. * $P <$ 0.05, ** $P<$ 0.01, *** $P<$ 0.001, **** $P<$ 0.0001, NS = Non significant.

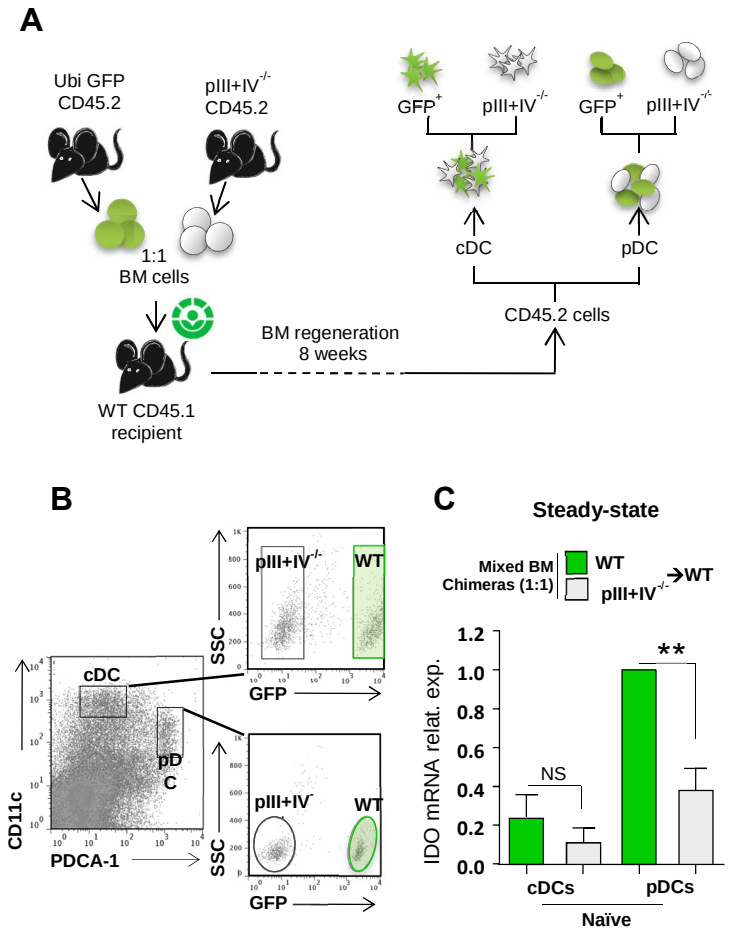


Figure 2. IDO expression by LN pDCs is dependent of MHCII-TCR interactions. (A-C) Mixed BM chimeric mice were generated by co-transferring (1:1) CD45.2 GFP+ WT and CD45.2 pIII+IV-/- BM cells in lethally irradiated CD45.1 WT mice. (A) Experimental design. (B) WT GFP+ cDCs and pDCs, and pIII+IV-/- cDCs and pDCs were sorted from total skin LN of naïve mice based on CD45.2, CD11c, PDCA-1 and GFP markers. (C) IDO mRNA expression level in indicated cells is represented. Results are representative of at least 3 independent experiments. Error bars depict mean ± SEM. One-way ANOVA with Bonferroni post Hoc test was used. ** P< 0.01, NS = Non significant.

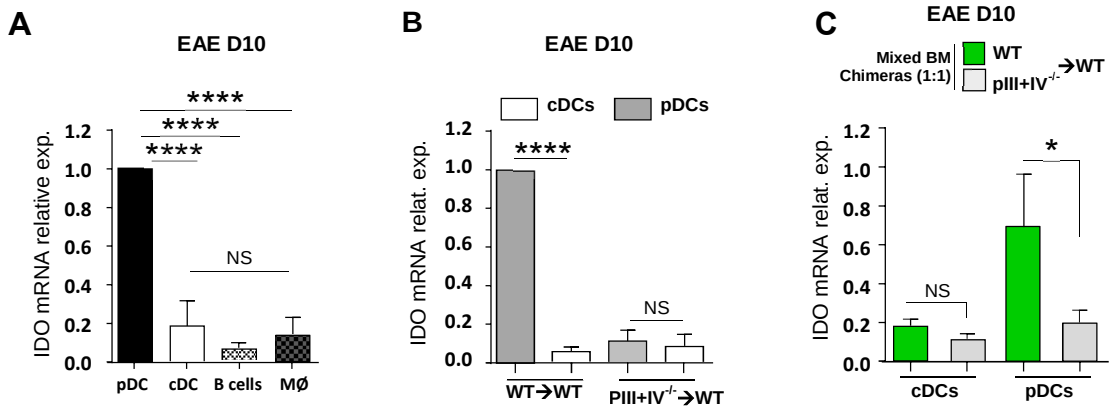


Figure 3. MHC-II competent pDCs express IDO during EAE. (A) IDO mRNA expression in B cells, cDCs, pDCs and macrophages (MØ) sorted from dLNs of WT EAE mice 10 days after immunisation. (B) EAE was induced in WT→WT and pIII+IV$^{-/-}$→WT chimeric mice, and IDO mRNA was measured in cDCs and pDCs sorted from dLN 10 days after immunization. (C) Mixed BM chimeric mice were generated by co-transferring (1:1) CD45.2 GFP^{+} WT and CD45.2 pIII+IV$^{-/-}$ BM cells in lethally irradiated CD45.1 WT mice. WT GFP^{+} cDCs and pDCs, and pIII+IV$^{-/-}$ cDCs and pDCs were sorted from dLNs 10 days after EAE induction based on CD45.2, CD11c, PDCA1 and GFP markers. IDO mRNA expression level in indicated cells (A-C) Results are representative of at least 3 independent experiments. Error bars depict mean ± SEM. One-way ANOVA with Bonferroni post Hoc test was used. * $P < 0.05$, **** $P< 0.0001$, NS = Non significant.

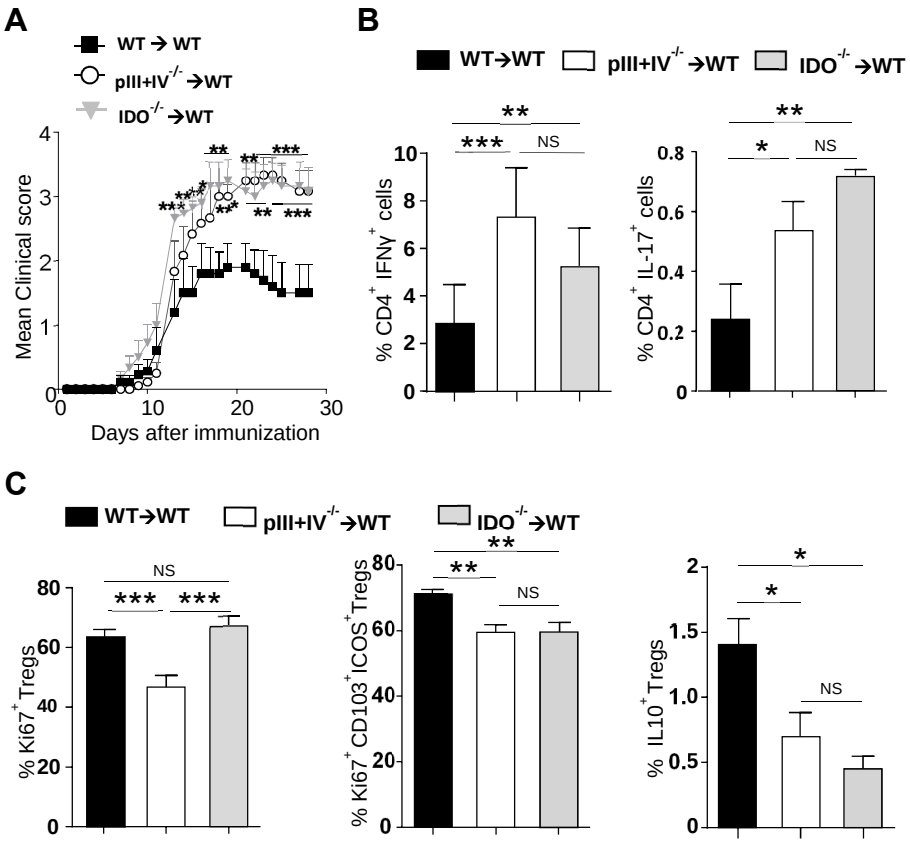


Figure 4. IDO regulates EAE priming phase. (A-C) EAE was induced in WT→WT (■), pIII+IV$^{-/-}$→WT (○) and IDO$^{-/-}$→WT (▼) BM chimeras. (A) Clinical scores were followed daily (two-way ANOVA with Bonferroni post Hoc test). Frequencies of (B) IFN-γ^{+} (left) and IL-17^{+} (right) CD4^{+} T cells, (C) Ki67^{+} (left), Ki67^{+}CD103^{+}ICOS^{+} (middle) and IL10^{+} (right) among CD25^{hi}Foxp3^{+}CD4^{+}Tregs in dLN at d9 (one-way ANOVA with Bonferroni post Hoc test). (A-C) Results are representative of at least 3 independent experiments with 8 mice per group. Error bars depict mean ± SEM. * P < 0.05, ** P< 0.01, *** P< 0.001, NS = Non significant.

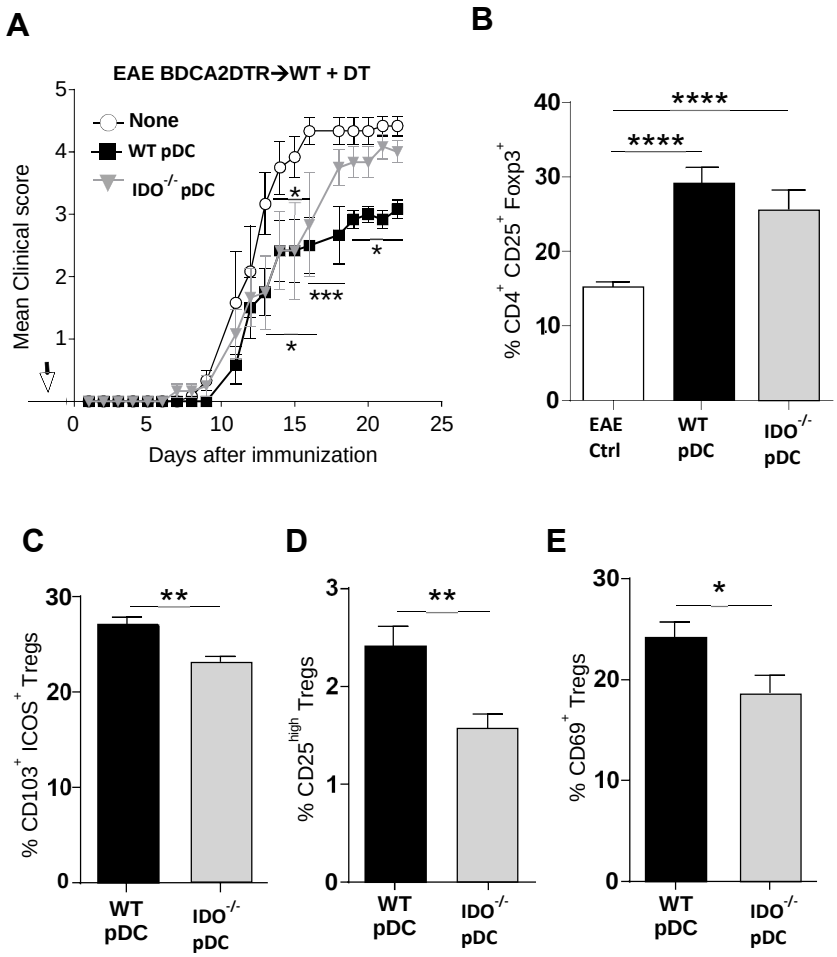
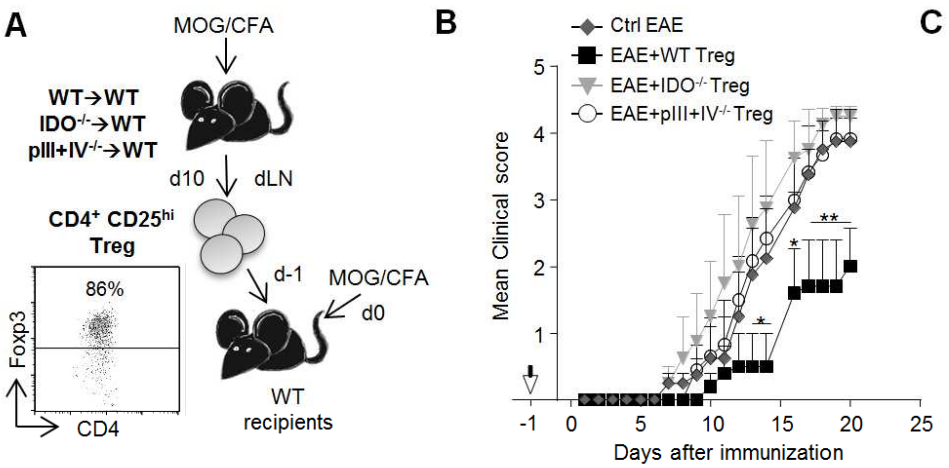


Figure 5. IDO sufficient pDCs drive suppressive Tregs during EAE. (A-E) MOG_{35-55} loaded BM-derived pDCs from WT (■) and $IDO^{-/-}$ (▼) were transferred (arrow) or not (○) into BDCA2-DTR→WT BM chimeras and EAE was induced 1 day after. Mice received 5 consecutive DT injections every 3-4 days. (A) Clinical scores were followed daily (two-way ANOVA with Bonferroni post Hoc test). Frequencies of (B) $CD4^+CD25^+$ $Foxp3^+$ Treg cells (one-way ANOVA with Bonferroni post Hoc test), (C) $CD103^+ICOS^+$ Tregs, (D) $CD25^{high}$ Tregs and (E) $CD69^+$ Tregs from dLNs are represented at d10 after EAE immunisation (two-tailed Mann-Whitney test). (A-E) Results are representative of at least 2 independent experiments with 6-8 mice per group. Error bars depict mean ± SEM. * $P < 0.05$, ** $P< 0.01$, *** $P< 0.001$, **** $P< 0.0001$.



	Mean clinical score	Cumulative clinical score
Ctrl EAE	1.32 ± 0.32	25.13
EAE + WT Treg	**** 0.57 ± 0.17	10.80
EAE + IDO$^{-/-}$ Treg	1.70 ± 0.39	32.25
EAE + pIII+IV$^{-/-}$ Treg	1.36 ± 0.36	25.88

Figure 6. Tregs induced by IDO sufficient pDCs *in vivo* control EAE development. (A-C) CD4^{+}CD25^{hi} cells were purified from dLNs of WT→WT, pIII+IV$^{-/-}$→WT and IDO$^{-/-}$→WT BM chimeras 10 days after EAE induction, and transferred (arrow) into WT recipients further immunized for EAE the day after. (A) Foxp3 expression in sorted cells. Experimental design is represented. (B) Clinical scores were followed daily in control mice (♦) and in mice transferred with WT Treg (■), IDO$^{-/-}$ Tregs (▼) or pIII+IV$^{-/-}$ Tregs (○) (two-way ANOVA with Bonferroni post Hoc test). (C) Mean and cumulative clinical scores are indicated (two-way ANOVA with Bonferroni post Hoc test). (A-C) Results are representative of at least 2 independent experiments. Error bars depict mean ± SEM. * $P < 0.05$, ** $P< 0.01$, *** $P< 0.001$, **** $P< 0.0001$.

Supplementary Informations

IDO-orchestrated crosstalk between pDCs and Tregs inhibits Autoimmunity

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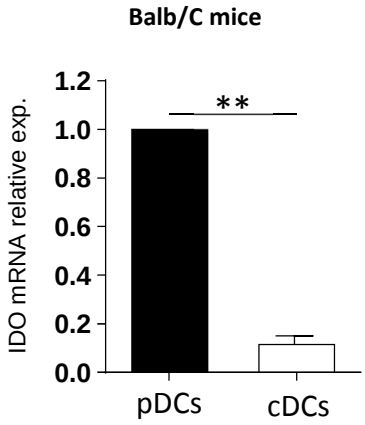
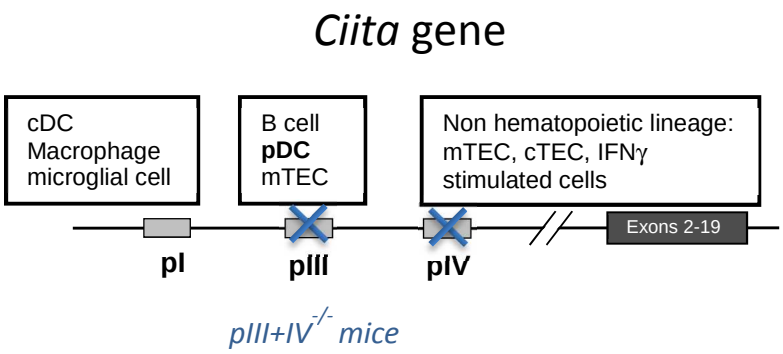
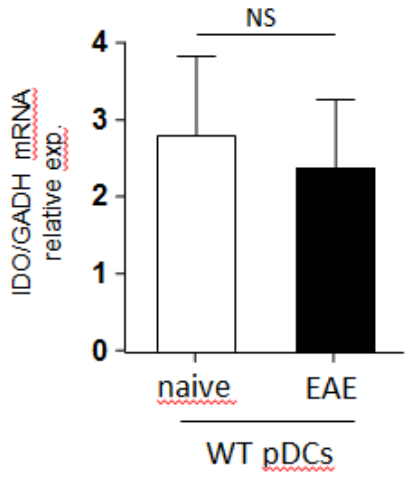


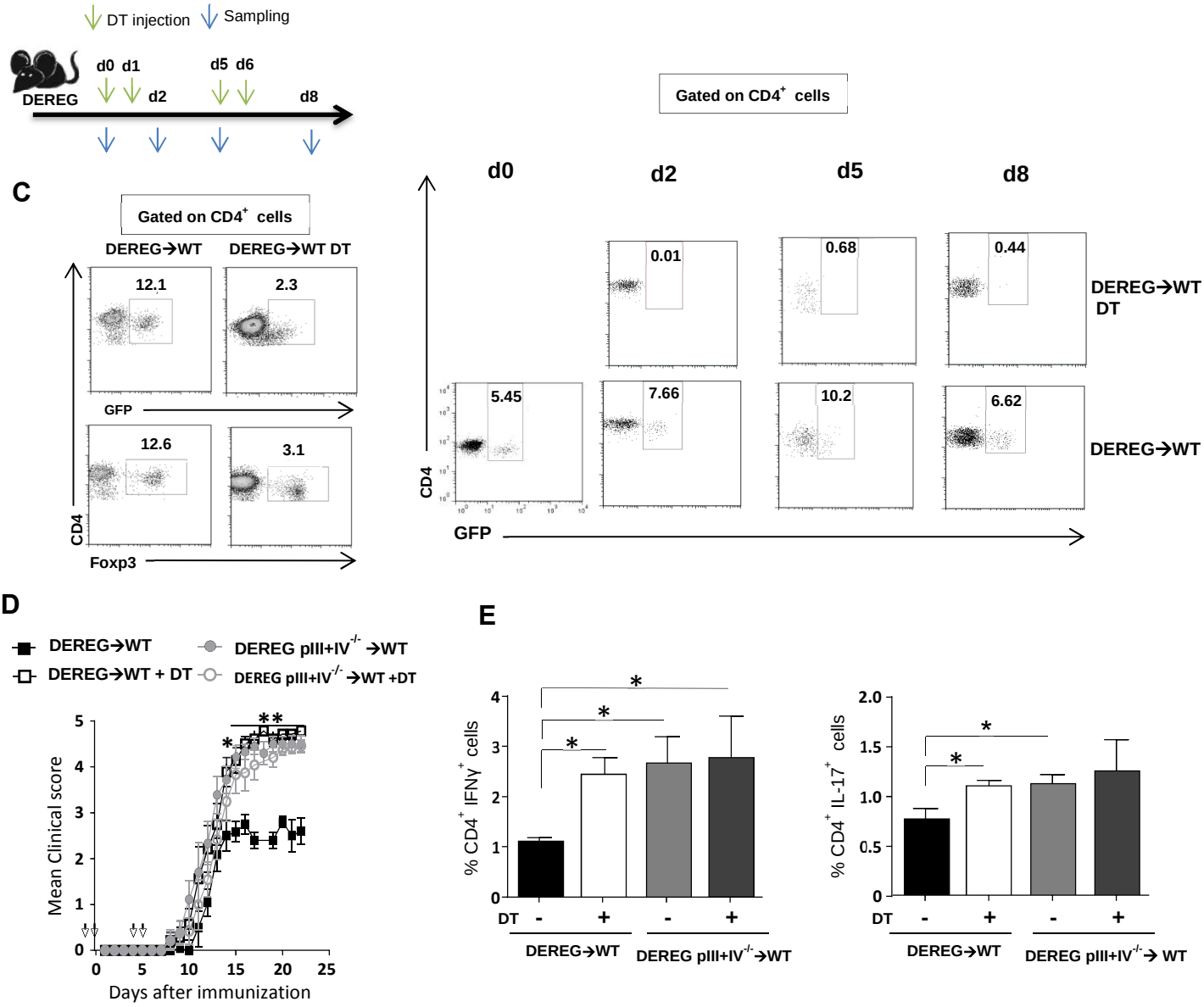
Supplementary Figure 1. IDO expression by pDCs sorted from LNs of Balb/c mice. Related to Figure 1. (A) pDCs and cDCs were selectively sorted from total skin LNs of Balb/C mice by flow cytometry and mRNA extraction was performed. IDO mRNA expression in pDCs and cDCs was determined by qPCR. Error bars depict mean ± SEM. Two-tailed Mann-Whitney test was used. ** $P< 0.01$.



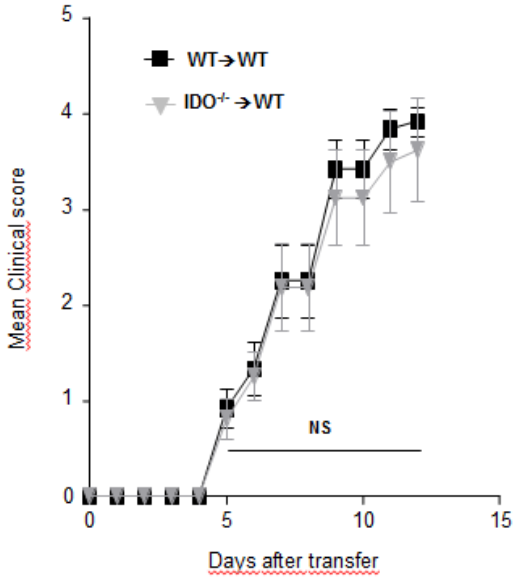
Supplementary Figure 2. Mice selectively lacking MHCII expression on pDCs. Related to Figure 2 and 3. In mice, CIITA gene expression is driven by three different cell specific promoters pI, pIII and pIV. pIII+IV$^{-/-}$ mice carry the deletion of promoters pIII and pIV. mTECs: medullary thymic epithelial cell, cTECs: cortical TEC. (B-E) WT→WT and pIII+IV$^{-/-}$→WT chimeric mice were generated by transfer of either WT or pIII+IV$^{-/-}$ bone marrow precursor cells in lethally irradiated WT recipient mice.



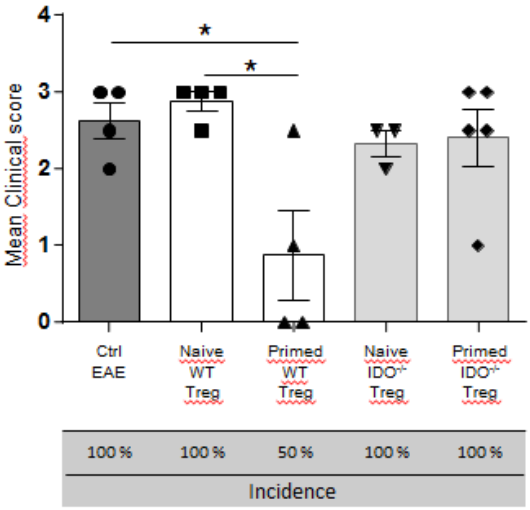
Supplementary Figure 3. IDO expression by pDCs sorted from LN of naïve and EAE mice. pDCs were selectively sorted by flow cytometry from total skin LNs of naive C57Bl/6 WT mice or from draining LNs of EAE C57Bl/6 WT mice at day 10 after immunization. IDO mRNA expression in pDCs was determined by qPCR and expressed relatively compare to GAPDH expression in the same cells. Error bars depict mean ± SEM. Two-tailed Mann-Whitney test was used. NS = Non significant.



Supplementary Figure 4. pDC-induced Tregs suppress encephalitogenic T cell priming in lymph nodes. Related to Figure 3. DT was injected or not in DEREG→WT BM chimeric mice at d0, d1, d5 and d6. (A) Experimental design is shown. Treg depletion was assessed (B) in dLN at d8 based on $CD4^+$ $Foxp3^+$ staining and (C) in blood at d0, d2, d5 and d8 by following $CD4^+$ GFP^+ cells. Flow cytometry data are shown. (D and E) EAE was induced in DEREG→WT chimeric mice treated (☐) or not (■) with DT (arrows) and DEREG x pIII+$IV^{-/-}$→WT chimeric mice treated (○) or not (●) with DT as before (D) Clinical scores were followed daily (two-way ANOVA with Bonferroni post Hoc test). (E) Frequency of $IFN\gamma^+$ and $IL\text{-}17^+$ $CD4^+$ T cells in dLN at d9 (one-way ANOVA with Bonferroni post Hoc test). (A-E) Data are representative of at least 2 independent experiments with 6-8 mice per group. Error bars depict mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



Supplementary Figure 5. IDO deficiency does not affect EAE effector phase. Related to Figure 4. Passive EAE was induced by transferring MOG_{35-55} primed 2D2 effector T cells in WT→WT (■) and $IDO^{-/-}$ →WT (▼) BM chimeras. Clinical scores were followed daily. Error bars represent mean ± SEM (2-way ANOVA with Bonferroni's post-Hoc test). NS = Non significant.



Supplementary Figure 6. Antigen specific activated Tregs primed by IDO-sufficient pDCs inhibit EAE. Related to Figure 6. CD4+CD25hi cells were purified from total skin LNs of naïve WT→WT and IDO-/-→WT BM chimeras or from dLNs of WT→WT and IDO-/-→WT BM chimeras 10 days after EAE induction. CD4+CD25hi cells were transferred into WT recipients further immunized for EAE the day after. Clinical scores and incidence (d14) are depicted. Data are representative for 2 experiments. Error bars represent mean ± SEM. One-way ANOVA with Bonferroni post Hoc test was used. * $P < 0.05$

Discussion

Whether pDCs exhibit tolerogenic or immunogenic APC functions in different immunogical context is an important matter of debate, and might depend on their own ability to produce inflammatory cytokines, as suggested by previous studies (Swiecki and Colonna, 2015). pDC tolerogenicity has been correlated to their expression of IDO in different settings (Baban et al., 2009; Fallarino et al., 2004; Pallotta et al., 2011). In a mouse model of skin inflammation, IDO was induced in pDCs either after IFN-γ or TGF-β stimulation, the first pathway promoting IDO enzymatic activity-dependent tryptophan depletion, the second one implicating IDO phosphorylation and subsequent Treg induction (Pallotta et al., 2011). Here we show that already in steady-state, pDCs are the major source of IDO in LNs. We further provide evidence that IDO expression in naïve pDCs is regulated through antigen-specific MHCII-restricted interaction with Foxp3$^+$ Tregs. First, pDCs isolated from TCR tg mice in which CD4$^+$ T cells all recognize a non-expressed antigenic peptide, exhibited substantially reduced IDO expression, independently of the self-reactivity of the TCR. Second, in absence of an existing Treg population in those TCR tg mice, LN pDCs exhibited a dramatic reduction in IDO expression. In addition, pDCs isolated from LN of Treg-deficient Scurfy mice expressed very little IDO mRNA. We cannot exclude that in Scurfy mice, the non-Treg CD4$^+$ T cell population, which is strongly biased toward a pro-inflammatory autoimmune repertoire compared to WT mice, might impact IDO expression. Nevertheless, IDO expression by pDCs from T cell deficient mice was induced after co-culture with a CD4$^+$ T cells only when the later contained a descent Treg population. In addition, IDO restoration was further dependent on the presence of the cognate antigenic peptide. The contribution of Treg in inducing IDO in pDCs has been suggested before (Mellor and Munn, 2004), however without real evidence for a requirement of MHCII-restricted, Ag-specific interactions between pDCs and Tregs. Whether a particular Treg subpopulation would be specifically involved in this crosstalk with pDCs to induce IDO expression, and possibly the expression of other genes as well, remains to be determined. A role for IL-10 has been demonstrated in IDO expression stabilization (Munn et al., 2002). Therefore, it is possible that IL-10 producing Tregs might be more competent to induce IDO up-regulation in pDCs. Our results reinforce the idea that Treg exhibit their Ag-specific immunomodulatory roles by impacting different cellular targets, not only by inhibiting effector T cells (Grant et al., 2015), inducing tumor-associated DC death (Boissonnas et al.,

2010) but also by promoting pDC tolerogenic functions. Future investigations will determine whether other pDC-specific tolerogenic features similarly depend on interactions with Tregs.

IDO expression by DCs has also been correlated with high levels of ICOS (Coquerelle et al., 2009) and CTLA-4 expression by T cells, and might depend, or not, on IFN-γ production (Baban et al., 2005; Fallarino et al., 2004; Grohmann et al., 2002; Mellor et al., 2004; Munn et al., 2004b). In mixed BM chimeric mice containing both MHCII sufficient and deficient pDCs, IDO is selectively abolished in MHCII deficient pDCs, whereas the same Treg populations expressing a certain level of ICOS, CTLA-4 or other molecules are present. Therefore, although ICOS and CTLA-4 might be required to promote IDO in pDCs, cells nevertheless need to express MHCII molecules.

During EAE finally, mice lacking MHCII on pDCs developed exacerbated disease, with increased IFN-γ and reduced TGF-β production in LNs compared to WT mice (Irla et al., 2010). MHCII-deficient pDCs nevertheless still exhibit a strong impairment in IDO expression upon EAE. Therefore, neither IFN-γ, nor TGF-β, was sufficient to induce IDO in absence of MHCII expression by pDCs. In addition, we show that pDCs tolerogenicity in EAE context is dependent on their expression of IDO. First, in IDO deficient mice, encephalitogenic T cell frequency was increased, and as a consequence, disease was exacerbated. Second, adoptively transferred Tregs inhibited EAE only when isolated from actively MOG_{35-55} immunized WT, but not $IDO^{-/-}$ mice, suggesting that Tregs need to be primed in an IDO sufficient microenvironment to acquire their suppressive functions. Third, in LNs, IDO was predominantly expressed by pDCs, in both naïve and EAE mice. Finally, in an experimental setting in which we controlled IDO expression exclusively in pDCs, we observed an inhibition of EAE, as well as an increase in suppressive Tregs, only when pDCs expressed IDO. Notably, Treg induced after Ag-specific interaction with IDO^{+} pDCs are necessary to inhibit pathogenic T cells in LNs. Altogether, our results demonstrate that IDO expression by pDCs is required for the generation of suppressive Tregs that, upon activation, competently inhibit encephalitogenic T cells during EAE priming phase. In conclusion, we showed that naïve pDCs are the primary contributors of IDO production in the LN, a property acquired after a cross-talk with $Foxp3^{+}$ Tregs. By expressing IDO, pDCs become competent to confer suppressive functions to Tregs. In the context of EAE, Ag-presenting IDO^{+} pDCs promote suppressive Tregs which inhibit the priming of encephalitogenic Th1 and Th17 cells and dampen CNS autoimmunity. Future investigations

will determine whether other pDC features and functions, apart from IDO expression, are impacted following interactions with Tregs. Together, our data have identified a new regulatory role for Tregs in shaping Ag-presenting pDC functions toward tolerogenicity in autoimmunity.

Competing Interests

The authors have no competing financial interests.

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2. SECTION 2

pDC THERAPY INDUCES RECOVERY FROM EAE BY RECRUITING ENDOGENOUS pDC TO SITES OF CNS INFLAMMATION

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INTRODUCTION

In a previous study, my colleagues reported that adoptive transfer of pDCs in mice one day prior immunization for EAE significantly delayed disease onset and controlled disease development. This effect was dependent on MHCII restricted Ag presentation by pDCs, hereby the transfer of MHCII deficient pDCs, failed to impact disease progression. Here, we investigated whether adoptive transfer of pDCs could be used in a therapeutic way after disease onset. In this paper we related that transfer of pDCs, during the acute phase of the disease drastically reduces EAE clinical scores and dampens CNS inflammation. Although the molecular mechanisms responsible for pDC-mediated protection are still unclear, we observed that pDC exogenous transfer drives the recruitment of immature endogenous pDCs to the CNS via the ChemR23/Chemerin axis. Immature endogenous pDCs modulate CNS inflammation, including the reduction of MCHII expression by microglial cells and the decrease of encephalitogenic T_H1 and T_H17.

OBJECTIVE

This study investigates the therapeutic properties of pDCs when these cells are transfer *in vivo* during the acute phase of EAE. The impact of pDC transfer on clinical score development and

on CNS pathogenesis, as well as the mechanisms relying on pDC-mediated EAE protection were addressed.

CONTRIBUTION TO THIS WORK

This study was carried out in collaboration with another PhD student in the lab, Fernanda Duraes. We equally contributed to this work, and most experiments and results were done together.

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pDC therapy induces recovery from EAE by recruiting endogenous pDC to sites of CNS inflammation

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ABSTRACT

Plasmacytoid dendritic cells (pDCs) exhibit both innate and adaptive functions. In particular they are the main source of type I IFNs and directly impact T cell responses through antigen presentation. We have previously demonstrated that during experimental autoimmune encephalomyelitis (EAE) initiation, myelin-antigen presentation by pDCs is associated with suppressive Treg development and results in attenuated EAE. Here, we show that pDCs transferred during acute disease phase confer recovery from EAE. Clinical improvement is associated with migration of injected pDCs into inflamed CNS and is dependent on the subsequent and selective chemerin-mediated recruitment of endogenous pDCs to the CNS. The protective effect requires pDC pre-loading with myelin antigen, and is associated with the modulation of CNS-infiltrating pDC phenotype and inhibition of CNS encephalitogenic T cells. This study may pave the way for novel pDC-based cell therapies in autoimmune diseases, aiming at specifically modulating pathogenic cells that induce and sustain autoimmune inflammation.

1. Introduction

Plasmacytoid dendritic cells (pDCs) are the main producers of type I interferons (IFN-I) in response to foreign nucleic acids, thereby indirectly influencing immunity. They can also differentiate into antigen presenting cells (APCs) to directly stimulate and modulate T cell responses [1]. pDCs have been implicated in the pathogenesis of many human inflammatory diseases and their corresponding mouse models. For example, chronic pDC activation and subsequent IFN-I production can promote autoimmune diseases, such as lupus erythematosus (SLE) [2] and psoriasis [3].

Multiple Sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). In MS patients, pDCs are present in the cerebrospinal fluid (CSF), leptomeninges and demyelinating lesions [4,5]. The exact role of pDCs in the pathogenesis of MS is controversial, as they might have both pathogenic and protective functions. In one hand, pDC-derived cytokines, including type-I IFN and IL-6 induces pro-pathogenic Th1 and Th17 cells, which are implicated in MS pathogenesis [6,7]. On the other hand, pDCs can exert a protective role in MS through the production of type-I IFN [8]. For instance, relapsing patients treated with IFN-β exhibit reduced disease severity [9]. In experimental autoimmune encephalomyelitis (EAE), the rodent model of MS, IFN-β and IFNAR-deficiency exacerbates disease severity [10,11]. Furthermore, one study suggests that pDCs inhibit pro-pathogenic conventional DCs (cDCs) functions in the CNS and, consequently locally inhibit encephalitogenic Th17 cells [12]. In addition to a controversial local role in the CNS, pDCs have also been implicated in the modulation of autoimmune Th1 and Th17 priming in secondary lymphoid organs (SLOs) during early phases of EAE development. It was suggested that pDCs promote Th17 priming [13], whereas we have demonstrated that the selective abrogation of MHCII expression by pDCs leads to increased MOG-specific Th1 and Th17 and impaired Treg proliferation. As a result, lack of MHCII on pDCs correlates with exacerbated EAE [14].

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Thus, although many questions remain to be elucidated, modulation of pDC functions as an approach to treat MS is currently an axis of intense investigation [15]. We have previously demonstrated that the adoptive transfer of MHCII-sufficient pDCs prior to EAE induction significantly dampened disease severity, whereas MCHII-deficient pDC had no effect [14]. Here we investigated whether and how pDCs may modulate the course of EAE after disease onset. We found that when transferred during acute EAE phase, pDCs led to dramatic disease remission. Protection was pDC-dependent, correlated with reduced CNS inflammation, and decreased encephalitogenic Th1 and Th17 cells. Our results demonstrated that pDC transfer induced the recruitment of resting endogenous pDCs to the CNS via a chemerin dependent mechanism, and confer a tolerogenic environment, which, together with local myelin peptide delivery, inhibits encephalitogenic T cells and results in disease improvement. Therefore, modulation of CNS inflammation by pDCs in EAE mice is an interesting axis of investigation to ameliorate disease clinical outcome. Up to date, almost all current therapies in autoimmune diseases are based on the systemic suppression of immune functions and are not curative. Our work reinforces and validates the relevance of emerging therapeutic concepts regarding the use of cell therapies for autoimmune diseases.

2. Materials and methods

2.1. Mice

H2-Aa$^{-/-}$ (MHCII$^{-/-}$) [16], Ubiquitin-GFP [17], IFN-β$^{-/-}$ [10], IFNAR$^{-/-}$ [18], BDCA2-DTR [19], CD45.1 (Charles River, France) and 2D2 [20] mice were in a C57BL/6 background. WT C57BL/6 mice were purchased from Harlan laboratories. Mice were bred and maintained under SPF conditions at Geneva medical school animal facility and under EOPS conditions at Charles River, France. Bone marrow (BM) chimeric mice were generated as described [14]. All procedures were approved by and performed in accordance with the guidelines of the animal research committee of Geneva.

2.2. EAE induction

Active EAE was induced as described by immunizing mice with 100 μg of MOG_{35-55} peptide (Biotrend) emulsified in incomplete Freund's adjuvant (BD Diagnosis) supplemented with 500 μg/ml *Mycobacterium tuberculosis* H37Ra (BD Diagnosis). At the time of immunization and 48 h later, mice also received 300 ng of pertussis toxin (Sigma–Aldrich) into the tail vein. For passive EAE induction, encephalitogenic CD4$^+$ T cells were generated *in vitro* from LN and spleen cells of 2D2 mice as described [21]. 1–2 × 10^6 total cells were injected *i.p.* into recipient mice. Mice received 67 ng of pertussis toxin at the day of cell injection and 48 h later. Mice were monitored daily for disease clinical symptoms, and blindly scored as follows. 1, flaccid tail; 2, impaired righting reflex and hind limb weakness; 3, complete hind limb paralysis; 4, complete hind limb paralysis with partial fore limb paralysis; 5,moribund. In some experiments, EAE mice were treated at indicated time points with α-NETA (Abcam) (10 mg/kg/daily), DT (100 ng/mouse), or 10 μg/mL of MOG_{35-55} peptide in PBS.

2.3. BM-pDC and cDC generation

cDCs and pDCs were generated as described [22] from BM of WT for cDCs and WT, MHCII$^{-/-}$, Ubi-GFP, CD45.1, and IFN-β$^{-/-}$ mice for pDCs. For some experiments, pDCs were treated with 1 μg/mL CpG-B (Invivogen) for the last 24 h of culture.

2.4. pDC and cDC adoptive transfer

pDCs and cDCs were generated and purified from BM cell cultures. pDCs were enriched from BM cell cultures after 7 days or purified ex-vivo from BM using a pDC isolation kit (Mylteniy biotec) according to manufacturer's instructions. Purity generally exceeded 95%. cDCs were purified by cell sorting from BM-cell cultures as CD11c^{hi}PDCA-1$^-$, using a MoFlowAstrios (Beckman Coulter). cDCs and pDCs were loaded or not with 10 μg/mL MOG_{35-55} or $OVA_{323-339}$ peptide and 10 × 10^6 cells were injected i.v. into EAE recipient mice, 10–12 days after MOG_{35-55} + CFA immunization.

2.5. Ex vivo DC isolation

DCs were isolated from Lymph nodes (LN), spleen, liver and Spinal cord (SC). by digesting organ fragments with an enzymatic mix containing collagenase D (1 mg/mL) and DNAse I (10 μg/mL) (Roche) in HBSS [14]. For liver and SC, single cell suspensions were further centrifuged through a discontinuous 30:70% percoll (Invitrogen) gradient.

2.6. Antibodies and flow cytometry

Monoclonal antibodies used for flow cytometry were from: Biolegend; anti-CD11c (N418), anti–I-Ab (AF6-120.1), anti-CD11b (M1/70), anti-CD86 (P03), anti-PD-1 (10F.9G2) (PMP1-30); anti-CD8 (53–6.7); anti-VLA-4 (HMB1-1), anti-Ly6C (HK1.4), anti-ICOS-L (HK5.3); from eBioscience: anti-CD4 (GK1.5), anti-CD69 (H1.2F3), VEGFR2 (FLK1), anti-TER119 (TER-119); anti-CMKLR1 (BZ194), Anti-Foxp3 (FJK-16s), anti–IL-17 (ebio17B7), anti-FLT3 (CD135) (A2F10), anti-SIGLEC H (ebio440c); from BD: anti–PDCA-1 (BST-2, CD317), anti-CD45 (30F11), anti-CD16/32 FcyRIII (clone 2.4G2), anti-CD45R/B220 (RA3-6B2), anti-CD19 (1D3), anti-CD3 (145-2C11), anti-c-kit (CD117) (2B8), anti-Sca-1 (D7), Ki67 (B56) and anti–IFN-γ (XMG1.2). Anti-Ly49q (clone 2E6) was from MBL.

For flow cytometry analysis of DCs, single cell suspensions were incubated with FcBlock (anti-CD16/32 FcγRIII) for 10 min, at 4 °C and stained using antibodies against CD11c and PDCA-1 or Siglec-H. cDCs were defined as CD11c^{hi}PDCA-1$^-$ and pDCs as CD11c^{int}PDCA-1$^+$ or CD11c^{int}Siglec-H$^+$. For hematopoietic progenitor analysis, red cells from BM were lysed with $NH_4ClNaHCO_3$ buffer. BM cells were stained with the following biotinylated lineage markers: anti-CD3, anti-B220, anti-CD19, anti-CD11b, anti-TER119, anti- Ly6C followed by streptavidin FITC conjugated staining. LSK BM progenitors were defined as Lin$^-$Sca-1$^+$c-kit$^+$FLT3$^-$. Microglial cells were defined as CD11c$^-$CD11b$^+$ CD45^{int}. Data were acquired in a Cyan™ ADP (Beckman Coulter) and analysed using FlowJo software (Tree Star).

Intracellular cytokine stainings were done with the Cytofix/Cytoperm kit (BD) for IFN-γ and IL-17 staining. Foxp3 staining was performed with the eBioscience kit, according to manufacturer's instructions. Cell proliferation was assessed by flow cytometry using anti–human Ki67 and respective isotype control. For IFN-γ and IL-17 staining, SC, LN and spleen cells were cultured in RPMI containing 10% heat-inactivated fetal bovine serum, 50 mM 2-mercaptoethanol, 100 mM sodium pyruvate, and 100 μM penicillin/streptomycin at 37 °C, 5% CO2. Cells were stimulated for 18 h with PMA/ionomycin and Golgi stop solution (BD) was added to the last 4 h of culture.

2.7. Immunofluorescence microscopy

Mice were transcardiacally perfused with PBS followed by 4% paraformaldehyde (PFA). Cryopreserved SC, post-fixed with 4% PFA and embedded in OCT (Sakura Finetek), were cut into 10 μm-thick sections, and stained using primary antibodies against CD45 and

PDCA-1 appropriate species-specific Alexa555-labelled, Cy3-, or Cy5-conjugated secondary antibodies (all from Jackson ImmunoResearch Laboratories, Inc.) with DAPI (Sigma–Aldrich) counterstaining. Sections were mounted with Mowiol fluorescent mounting medium (EMD). Images were acquired with a confocal microscope (LSM 700; Carl Zeiss, Inc.)

2.8. Histological analysis

Mice were transcardiacally perfused with PBS followed by 4% PFA and post-fixed overnight. Brain and SC, dehydrated and embedded in paraffin, were cut into 2 μm thick sections and stained with Mayer's hemalum, differentiated in acidic-alcohol and co-stained with eosin and coverslipped with DePeX mounting medium (Serva Electrophoretics GmbH, Heidelberg, Germany) using Tissue Tek Prisma slide stainer (Sakura Seiki C.o., Nagano, Japan).

2.9. Statistical analysis

Statistical significance was assessed by the two-tailed unpaired Student's *t* test or 1-way ANOVA with Bonferroni post Hoc test. EAE incidence was analysed using the 2-way ANOVA with Bonferroni post Hoc test, using Prism 5.0 software (GraphPad Software).

3. Results

3.1. Adoptive transfer of pDCs during EAE acute phase inhibits CNS inflammation and induces disease remission

In order to explore a potential therapeutic role for pDCs in EAE, we adoptively transferred *in vitro* bone marrow (BM) derived WT MOG_{35-55} loaded pDCs into C57BL/6 mice after disease onset, 12 days after immunization with MOG_{35-55} + CFA. Strikingly, whereas clinical scores of control non pDC-injected mice continued to rise, those of pDC-injected mice stayed stable for few days post-pDC transfer, and then rapidly decreased, resulting in a significant inhibition of EAE (Fig. 1A). Clinical symptoms almost completely disappeared 10 days after pDC injection (Fig. 1A). Moreover, mice presenting a clinical score of 3 at the time of pDC transfer either significantly recovered or exhibited stabilized disease development, whereas scores of control animals maintained their progression (Supplementary Fig. 1A, B). Consistent with an amelioration of clinical scores, as early as four days post-pDC transfer, inflammatory foci were reduced in spinal cord (SC) of pDC-transferred mice, compared to control EAE mice (Fig. 1B). In agreement with reduced CNS inflammation, microglial cells downmodulated the expression of MHCII molecules (Fig. 1C). In spite of this, four days after pDC transfer, we did not observe any differences regarding the frequency of $CD4^+$ and $CD8^+$ T cells infiltrating the SC (Supplementary Fig. 2A), nor in the expression of VLA-4 by T cells (Supplementary Fig. 2B) between pDC transferred and control EAE mice. Consequently, T cell migration from SLOs to SC was not altered upon pDC transfer. However, the expression of PD-1, a molecule that delivers negative signals to T cells [23], was increased on SC-infiltrating $CD4^+$ T cells one day after pDC transfer (Fig. 1D). Remarkably, encephalitogenic Th1 (at day 4 and 9 after pDC transfer) and Th17 frequencies (at day 9 after pDC transfer) were substantially decreased in SC of pDC-transferred mice (Fig. 1E). These data demonstrate that adoptive transfer of BM-derived pDCs after EAE onset induces a strong and rapid improvement of disease clinical symptoms and CNS inflammation. This effect appeared to be pDC-specific, since transfer of MOG_{35-55}-loaded cDCs did not significantly impact EAE development (Supplementary Fig. 3A). In addition, although most of our experiments were done using $5–10 \times 10^6$ of transferred pDCs, a similarly efficient therapeutical effect was observed by using only 1×10^6 cells (Supplementary Fig. 3B). Importantly, adoptively transferred MOG_{35-55} loaded *ex vivo* BM resident pDCs efficiently induced EAE amelioration (Fig. 1F), demonstrating that *in vivo*, terminally differentiated pDCs efficiently inhibit the disease.

3.2. pDC transfer induces the selective recruitment of endogenous pDCs to the CNS and inhibits EAE effector phase

To determine the mechanisms accounting for pDC-mediated EAE amelioration, we analysed the body distribution of injected BM-pDCs derived from Ubiquitin-GFP mice. Two days post-injection, GFP^+ pDCs were detectable in LN, spleen and inflamed SC (Fig. 2A). In addition, a very low frequency of GFP^+ pDCs was observed in the liver and the BM (data not shown). In situ immunofluorescence staining of SC revealed that, GFP^+ pDCs localized within EAE lesions (Fig. 2B, top), expressed the pDC-specific marker PDCA-1 (yellow arrows) and were surrounded by endogenous infiltrating pDCs (white arrows) (Fig. 2B, bottom). While the frequencies of cDCs, macrophages and microglial cells were similar in SC of pDC-transferred and control EAE mice (Supplementary Fig. 4), we observed a massive (six-fold) increase of total pDC frequencies in SC of pDC-transferred EAE mice (Fig. 2C). Injected pDCs (GFP^+) represented only 25% of total SC pDCs (Fig. 2D), suggesting that endogenous pDCs were recruited to the SC following exogenous pDC transfer. In agreement, increased endogenous pDC frequencies in SC of pDC-transferred animals did not result from local pDC proliferation (data not shown). Therefore, we hypothesized that they would be recruited from the BM, where pDCs originate from. Accordingly, four days post-pDC transfer into EAE mice, we observed in the BM a substantial increase of total pDC frequencies, with only a minor proportion consisting of transferred GFP^+ pDCs (Supplementary Fig. 5A, B). These results suggest that the increase in endogenous pDC frequency could be due to active proliferation or enhanced *de novo* generation. Ki67 staining revealed a negligible proliferation rate of pDCs in the BM, in both control and pDC-transferred EAE mice (Supplementary Fig. 5C). In contrast, we observed a significant increase in LSK (lineage-negative, $SCA1^+$, c-Kit^+) progenitor's frequencies in the BM, one-day post-pDC transfer (Supplementary Fig. 5D), suggesting a general enhancement of hematopoietic cell generation. In line with this notion, both cDC and pDC frequencies were significantly increased in LN, 4 days after pDC-transfer (Supplementary Fig. 5E). Furthermore, at the same time point, BM-resident pDCs, from pDC-transferred mice, exhibited an upregulation of Ly49q, a marker of fully differentiated pDCs [24] compared to control mice (Supplementary Fig. 5F). Altogether, these data show that pDC transfer in EAE mice increases hematopoietic cell generation in the BM, leading to enhanced cDC and pDC frequencies in SLOs, and resulting in the selective recruitment of pDCs to the CNS.

Increased pDC frequencies in SC of EAE mice following pDC transfer suggests that these cells might play a protective role locally in the CNS. To test this hypothesis, we induced passive EAE by injecting *in vitro* differentiated MOG_{35-55}-specific 2D2 $CD4^+$ T cell effectors [14]. Upon both mild (1×10^6 2D2 effectors, Fig. 2E, left) and strong (2×10^6 2D2 effectors, Fig. 2E, right) EAE induction, pDCs injected at early disease clinical symptoms onset were able to inhibit disease development, with significantly reduced EAE incidence and clinical scores. These results demonstrated that pDC transfer inhibits CNS-local effector phase of EAE.

3.3. Recruitment of endogenous pDCs to the inflamed CNS mediates EAE protection

Our data strongly suggests that endogenous pDCs recruited to

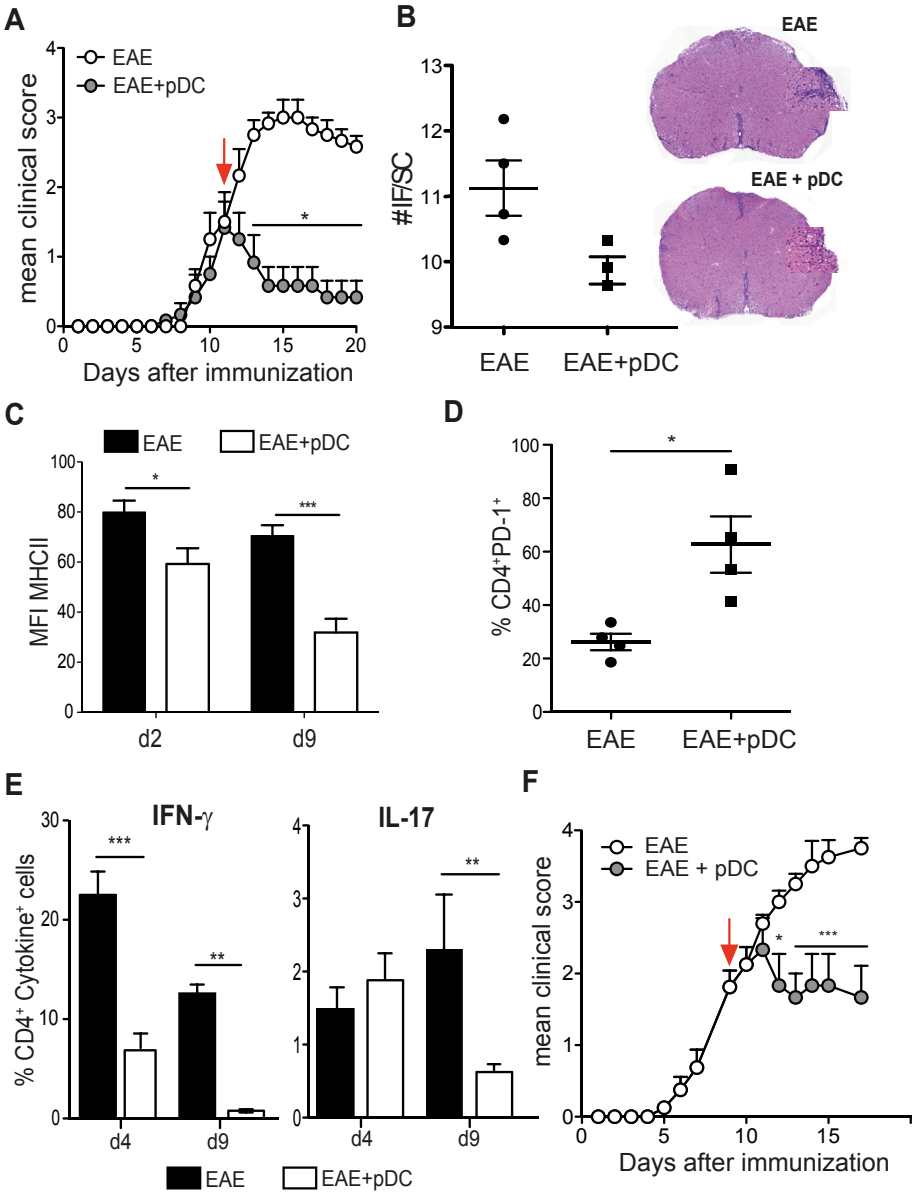

Fig. 1. pDC adoptive transfer during disease acute phase inhibits EAE. EAE was induced in WT mice by immunization with MOG_{35-55}. (A–E) EAE mice were injected i.v. (EAE + pDC) or not (EAE) with MOG_{35-55}-loaded WT BM-pDCs during disease acute phase (arrow). (A) Clinical scores were followed daily. (B) SCs of indicated mice were harvested four days after transfer, processed and stained for H&E. Graph represents the mean number of inflammatory foci (IF) per SC from 3 to 4 individual mice per group (left). Representative SC images (right). (C) MHCII MFI on microglial cells in SC of EAE mice at day 2 and 9 after pDC transfer. Frequencies of (D) PD-1 expressing $CD4^+$ T cells at day 1, and (E) IFN-γ (left panel) and IL-17 producing (right panel) $CD4^+$ T cells in SC at day 4, and day 9 after pDC transfer. (F) EAE clinical scores of WT mice transferred (EAE + pDC) or not (EAE) during EAE acute phase (arrow) with 5×10^6 ex-vivo purified from the BM, MOG_{35-55}-loaded, WT pDCs. (A–F) Data are representative of at least 2 experiments with minimum 5 mice per group. Data represent mean ± SEM. (A and F) 2-way ANOVA with Bonferroni post hoc test; (B–E) Standart two-tailed Student's *t* test; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

the CNS following pDC transfer are implicated in EAE amelioration. Indeed, exogenous MOG_{35-55} loaded cDCs, which did not significantly ameliorate EAE upon transfer (Supplementary Fig. 3A; Fig. 3A), also did not induce endogenous pDC recruitment to SC (Fig. 3B). To firmly demonstrate that endogenous pDC recruitment to SC was necessary for disease amelioration following exogenous pDC transfer, we used a genetic mouse model to deplete endogenous pDCs. For this purpose, CD45.1 WT pDCs were transferred into BDCA-2 DTR EAE mice [19], and mice were injected or not with DT at the same time pDCs were transferred. Endogenous pDCs were efficiently depleted in the spleens of DT-treated mice (not shown) [25]. However, exogenous ($CD45.1^+$) pDC numbers reaching the SC were not affected (Fig. 3C, left), but as a consequence of BDCA-2 DTR pDC depletion, endogenous pDC recruitment to the SC was substantially impaired in DT treated, compared to untreated animals (Fig. 3C, right). Importantly, EAE amelioration following MOG_{35-55} loaded pDC transfer was totally abrogated in DT treated mice (Fig. 3D). Therefore, endogenous pDC recruitment to SC after pDC transfer is mandatory to confer EAE protection.

3.4. Chemerin-CMKLR1 axis mediates endogenous pDC recruitment to the CNS following pDC transfer

Although a specific receptor mediating pDC migration to

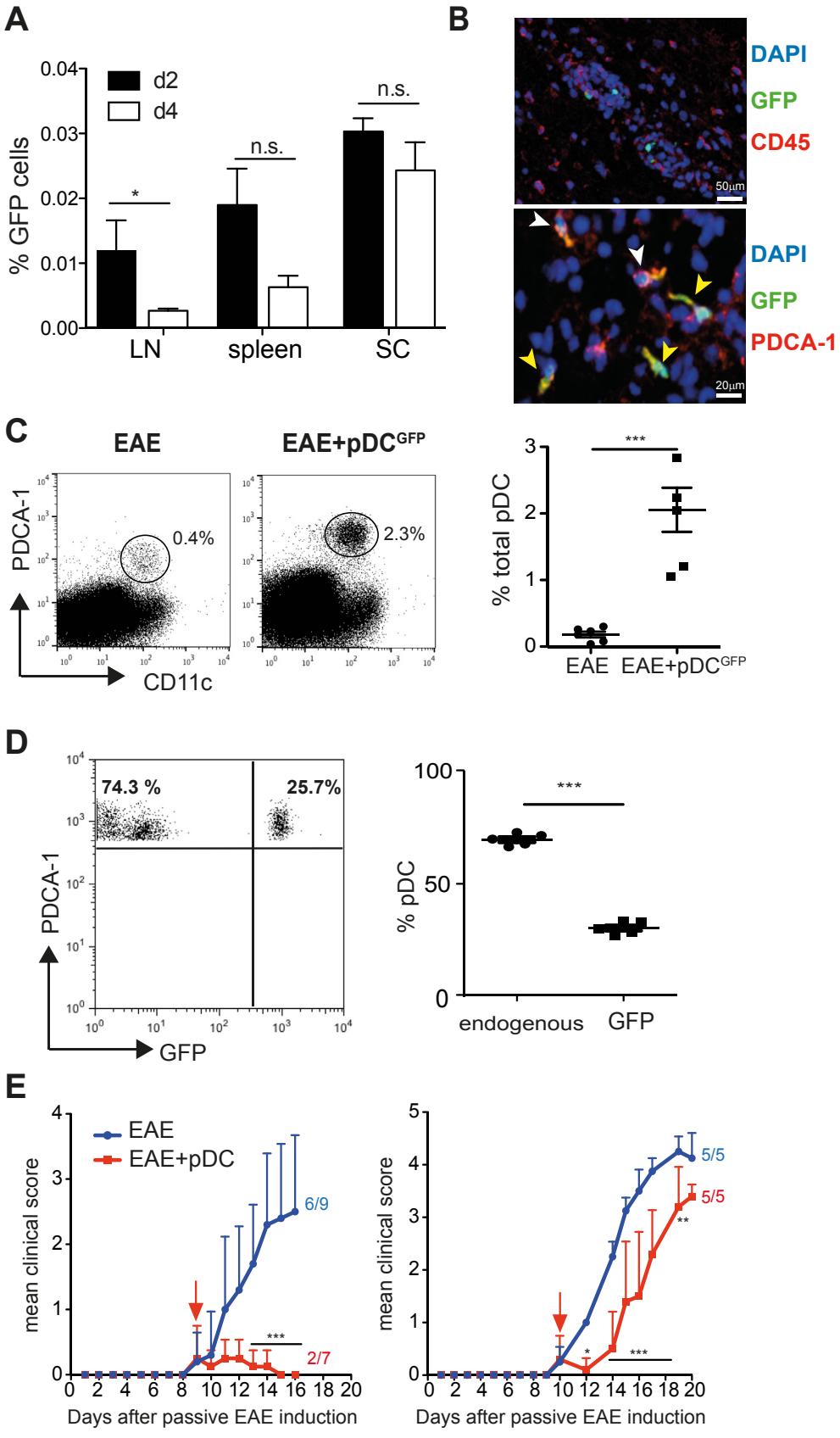

Fig. 2. pDC transfer induces the selective recruitment of pDCs to SC and inhibits EAE effector phase. (A–D) EAE was induced in WT mice and BM-derived, MOG_{35-55}-loaded, pDCs from Ubi-GFP mice were transferred into mice during EAE acute phase. SC cells from control EAE (EAE) or GFP^+pDC transferred EAE (EAE + pDC^{GFP}) mice were analysed after pDC transfer. (A) Graph shows the frequency of $CD11c^{int}$ $PDCA\text{-}1^+$ GFP^+ transferred pDCs infiltrating the LNs, spleen and SC at indicated time after pDC transfer. (B) Representative confocal images of SC from GFP^+pDC transferred mice (day 4), show GFP^+ cells among $CD45^+$ cells (top) and PDCA-1 co-localization with GFP^+ cells (bottom). Arrows depict $PDCA\text{-}1^+GFP^-$ cells (white) and $PDCA\text{-}1^+GFP^+$ cells (yellow). (C) Representative FACS profile (left) and quantification (right) of total pDCs in SC from control (EAE) or pDC transferred (EAE + pDC^{GFP}) EAE mice (day 4). (D) Representative FACS profile (left) and quantification (right) of GFP^+ (exogenous) and GFP^- (endogenous) pDC frequencies among total pDCs in SC (day 4). (A–D) Data are representative of 4 independent experiments with 6–8 mice/group. (E) EAE was induced in WT mice by adoptive transfer of 1×10^6 (left) or 2×10^6 (right) effector MOG_{35-55}-specific 2D2 transgenic $CD4^+$ T cells. BM-derived WT pDCs were transferred i.v. into EAE mice at the appearance of clinical symptoms (arrows). Clinical scores were followed daily. Incidence is indicated for each group. Data are representative of 2 experiments with at least 5 mice/group. Data represent mean ± SEM. (A, C and D) Standard two-tailed Student's *t* test; (E) 2-way ANOVA with Bonferroni post hoc test $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

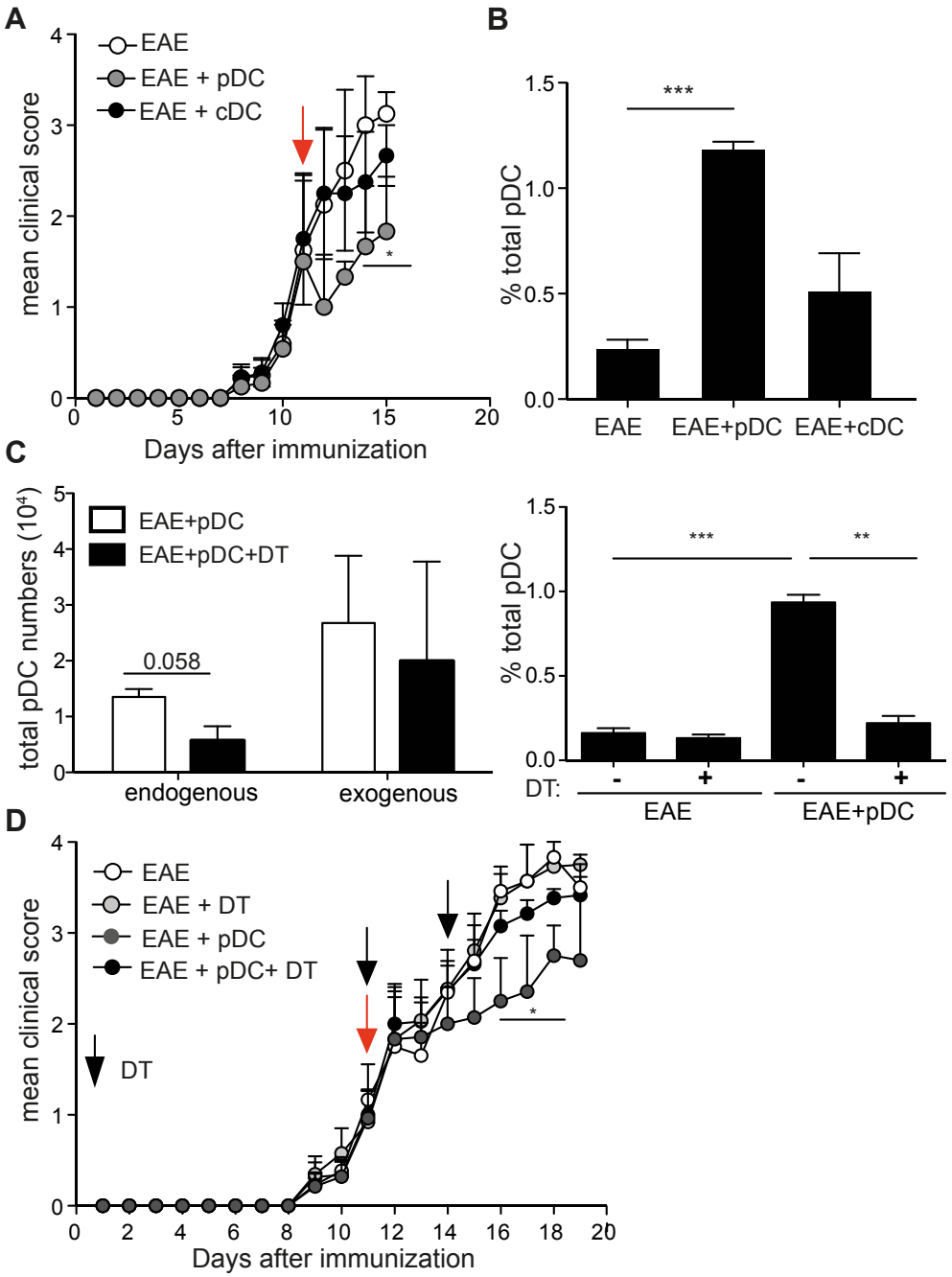

Fig. 3. Endogenous pDC recruitment to the SC following pDC transfer is mandatory for EAE protection. (A, B) EAE was induced in WT mice and BM-derived, MOG_{35-55}-loaded, WT pDCs (EAE + pDC) or cDCs (EAE + cDC) were transferred or not (EAE) into mice during disease acute phase (arrow). (A) Clinical scores were followed daily. (B) Total pDC frequencies were analysed in SC of EAE mice four days after cell transfer. (C, D) EAE was induced in BDCA2-DTR:WT BM chimeric mice and BM-derived, MOG_{35-55}-loaded, CD45.1 WT pDCs were transferred into EAE mice during EAE acute phase (D, red arrow, day 11). When indicated, mice were also injected with DT (D, black arrows, day 11 and day 14 after EAE induction). (C) Endogenous (gated on CD45.2 cells) and exogenous (gated on CD45.1 cells) pDC numbers (left) and total pDC frequencies (right) were analysed in SC of EAE mice four days after pDC transfer. (D) Clinical scores were followed daily. (A–D) Data are representative of at least 2 independent experiments with 8 mice per group. Data represent mean ± SEM. (A and D) 2-way ANOVA with Bonferroni post hoc test; (B, C right panel) 1-way ANOVA with Bonferroni post hoc test $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

inflamed tissues has not yet been identified, different chemokines and chemotactic factors, as well as its receptors have been related to pDC homing to peripheral tissues under pathological conditions [26]. In order to identify possible mechanism(s) responsible for pDC recruitment to CNS, we analysed the implication of Chemerin/CMKLR1 (chemerin receptor), an axis linked to pDC migration in humans [27]. First, and as previously observed [28], we found that SC of EAE mice contained increased chemerin mRNA levels, compared to naive SC (Fig. 4A). In addition, in spleens of EAE mice, pDCs expressed high levels of CMKLR1, compared to cDCs, irrespective of pDC transfer (Fig. 4B). Interestingly, the frequency of pDCs expressing CMKLR1 was substantially increased in SC as early as one day after pDC transfer, and was sustained four days after transfer (Fig. 4C). In contrast, CMKLR1 expression remained low and unaltered on SC infiltrating cDCs from both pDC-transferred and control EAE mice (Fig. 4C). In order to test a possible involvement of the Chemerin/CMKLR1 axis in the recruitment of endogenous pDCs to the SC following pDC transfer, we treated pDC

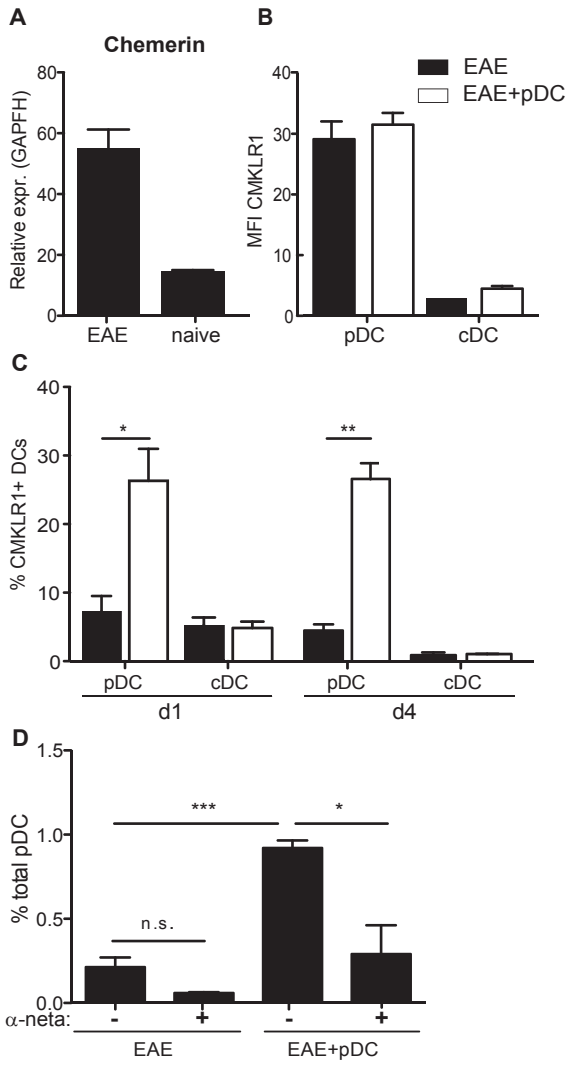

Fig. 4. Chemerin-CMKLR1 axis mediates pDC recruitment to SC. EAE was induced or not in WT mice and BM-derived, MOG_{35-55}-loaded, pDCs were transferred (EAE + pDC) or not (EAE) into mice during EAE acute phase. (A) Chemerin mRNA levels were analysed in SC of naïve and EAE mice day 12 after immunization. (B, C) CMKLR1 expression levels by cDCs and pDCs (B) in spleens one day after pDC transfer and (C) in SC one and four days after pDC transfer. (B) Graphs show the MFI and (C) the frequency of expressing cells. (D) EAE was induced in WT mice and WT BM-pDCs were transferred or not into mice during EAE acute phase. α-NETA was injected *i.p.* or not one day following pDC transfer for 3 consecutive days. Frequency of total pDCs was analysed in SC 4 days after pDC transfer. (A–D) Data are representative of 2 independent experiments with 4 mice per group each. Data represent mean ± SEM.(A–C) Standard two tailed Student's *t* test; (D) 1-way ANOVA with Bonferroni post hoc test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

transferred or control EAE mice with a CMKLR1 small molecule antagonist, α-naphthoyl ethyltrimethylammonium iodide (α-NETA), known to block $CMKLR1^{+}$ cell migration [29]. α-NETA treatment, for 3 consecutive days following pDC transfer, significantly blocked pDC recruitment to SC of EAE mice (Fig. 4D). These results demonstrate that pDC recruitment to SC following pDC transfer is mediated by the chemerin-CMKLR1 axis. However, given that this molecule not only interferes with pDC homing but also affects migration of additional key effector cells in EAE, such as macrophages and microglial cells (Supplementary Fig. 6) [29], we could not use this experimental approach to evaluate the impact of blocking endogenous pDC recruitment to the CNS on disease evolution.

3.5. Transferred pDCs provide myelin antigen and modulate pDC activation state in the CNS

pDCs are important producers of type-I IFN upon viral infections. In addition, IFN-β is widely used as a treatment for MS. However, the precise mechanisms involved in IFN-β mediated disease amelioration are still incompletely understood. In order to check whether IFN-β was involved in pDC-mediated protection in EAE, we transferred IFN-β^{ko} or WT MOG_{35-55} loaded pDCs into EAE recipient mice. Both WT and IFN-β^{ko} pDCs mediated the same level of disease inhibition, thus excluding a role for IFN-β production by exogenous pDCs in disease amelioration (Fig. 5A). Similarly, WT pDCs induced the same level of disease amelioration (Fig. 5B, left), as well as a similar increase in SC pDC frequencies (Fig. 5B, right), when transferred into either WT or IFN-I receptor deficient ($IFNAR^{-/-}$) EAE recipient mice. Altogether, these results firmly rule out a role for IFN-I in disease amelioration induced by pDC transfer. We have previously showed that when transferred prior to EAE induction, pDC-mediated protection was MHCII dependent [14]. However, in post-EAE settings, disease inhibition was as efficient using either MHCII sufficient or deficient pDCs (Fig. 5C), suggesting a MHCII independent role for injected MOG_{35-55} loaded pDCs in regulating CNS-inflammation. A requirement for MHCII expression by endogenous pDCs, that are recruited to the SC after pDC injection, was also ruled out, since EAE protection after WT pDC transfer was not altered in mice selectively lacking MHCII expression by pDCs (μMTxpIII + $IV^{-/-}$:WT BM chimeric mice, as described before) [14] (Supplementary Fig. 7A). Surprisingly, EAE protection was abolished when transferred pDCs were either unloaded or loaded with an irrelevant peptide ($OVA_{323-339}$), prior to their injection (Fig. 5D). Importantly, in contrast to high peptide concentrations (100–200 μg/mL) that have been described to confer EAE amelioration [30], intravenously injection of 10 μg/mL of MOG_{35-55} peptide, the concentration used to load pDCs, did not confer any disease improvement (Supplementary Fig. 7B), emphasizing the requirement of pDCs for therapeutic effect. Notably, disease amelioration in EAE mice injected with MOG_{35-55}-loaded WT or $MHCII^{-/-}$ pDCs correlated with pDC recruitment to SC (Fig. 5E). In contrast, pDC frequencies were not increased in SC of EAE mice injected with unloaded pDCs, in which no disease protection was observed (Fig. 5D, E), again supporting the need of endogenous pDC recruitment to SC for pDC therapeutic effect.

In order to investigate the features related to pDC tolerogenic effect, we analysed pDC phenotype in SC of EAE mice. Irrespective of pDC transfer, CNS pDCs expressed lower levels of CD86, compared to cDCs (Fig. 6A). Conversely, the expression of CD69 by endogenous pDCs was substantially downregulated upon pDC transfer (Fig. 6B). These findings suggest that the inflammatory status of pDCs in SC from EAE mice was markedly altered following pDC transfer. To determine whether the lack of pDC activation is involved in the pDC transfer-therapeutic effect, we treated pDCs with CpG-B prior to their injection. CpG-treated pDCs did not induce *in situ* upregulation of CMKLR1 by pDCs (Fig. 6C), neither the recruitment of endogenous pDCs to SC of EAE mice (Fig. 6D). Importantly, we further observed that injection of CpG-B activated MOG_{35-55} loaded pDCs did not confer any significant disease amelioration, compared to untreated MOG_{35-55} loaded pDCs (Fig. 6E), supporting a role for steady-state pDCs in disease amelioration. Altogether, these results demonstrate that steady-state pDC-transfer promotes a pDC-dependent tolerogenic environment in SC of EAE mice, resulting in the general suppression of EAE pathogenicity and clinical symptoms.

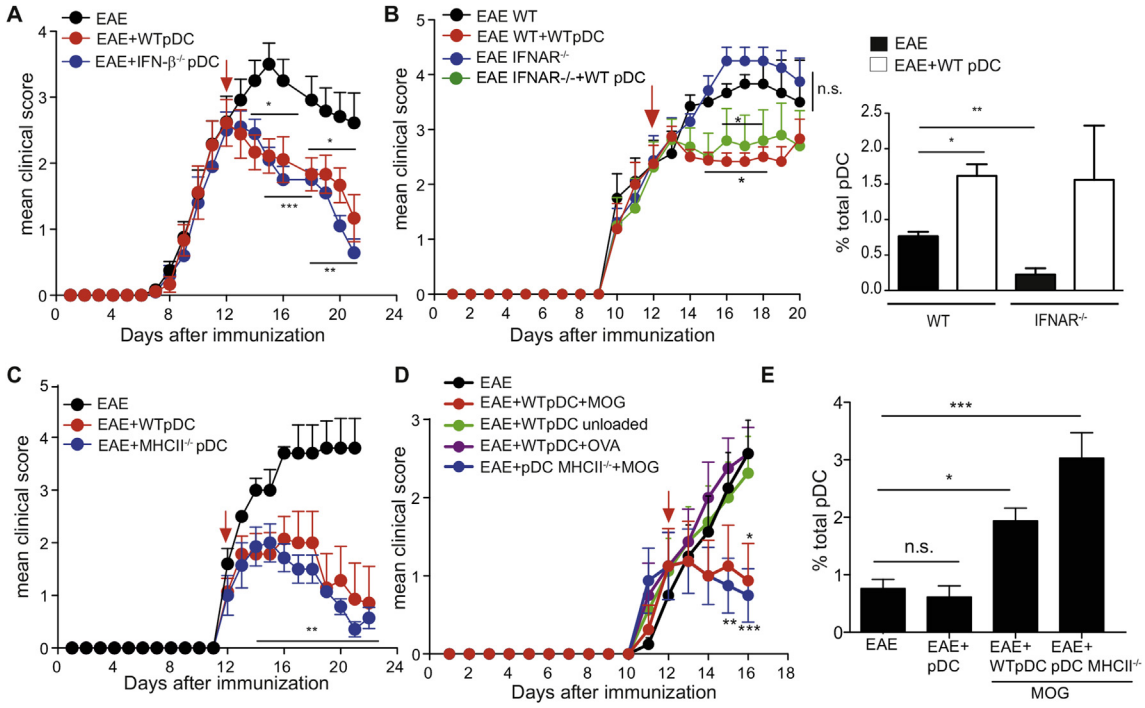

Fig. 5. Transferred pDCs deliver myelin antigen to SC. (A–C) EAE was induced in indicated mice and BM-derived, WT or IFN-$\beta^{-/-}$ (A), WT (B) WT or MHCII$^{-/-}$ (C) MOG_{35-55}-loaded pDCs were transferred into mice during EAE acute phase (red arrows). Clinical scores were followed daily. (A) EAE clinical scores in WT mice transferred with WT or IFN-$\beta^{-/-}$ pDCs. (B) EAE clinical scores from WT or IFNAR$^{-/-}$ mice transferred or not with WT pDCs (left). Total pDC frequencies were analysed in SC of mice four days after pDC transfer (right). (C) EAE clinical scores from WT mice transferred or not with WT or MHCII$^{-/-}$ pDCs. Data are representative of 5 independent experiments with 8 mice/group each. (D, E) EAE was induced in WT mice and BM-derived WT or MHCII$^{-/-}$ pDCs, loaded or not with MOG_{35-55} or $OVA_{323-339}$ as indicated, were transferred into mice during EAE acute phase. (D) Clinical scores were followed daily. (E) Total pDC frequencies were analysed four days after pDC transfer in SC of mice from. (A–E) Data are representative of 3 independent experiments with 8 mice/group each. Data represent mean ± SEM. (A, B left panel, C, D) 2-way ANOVA with Bonferroni post hoc test; (B right panel) Standart two-tailed Student's *t* test; (E) 1-way ANOVA with Bonferroni post hoc test $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

4. Discussion

In EAE and MS, whether pDCs have a prominent pathogenic or tolerogenic role is still controversial. Activated pDCs, through the production of pro-inflammatory cytokines, such as TNF and IL-6, have been claimed to be pathogenic and promote disease progression, both in mice and humans. In agreement, pDCs were shown to be pathogenic in EAE by inducing Th17 responses [13]. On the other hand, constitutive or antibody mediated pDC depletion during peak disease correlates with EAE exacerbation [12,31]. We have previously demonstrated that pDCs can present myelin-derived autoantigens via MHCII to CD4^{+} T cells in an EAE context and promote the expansion of suppressive Treg cells [14]. In the present work, in order to explore pDC tolerogenic potential in a therapeutic EAE setting, we performed adoptive transfer of syngenic pDCs after disease onset. Strikingly, pDCs induced a rapid and substantial amelioration of both CNS inflammation and EAE clinical scores upon transfer into sick mice. Transferred pDCs rapidly reached the SC, localized in lesion areas in the CNS, and inhibited already primed encephalitogenic MOG_{35-55}-specific 2D2 CD4^{+} T effector cells, pointing out to a local immunoregulatory role of these cells during EAE effector phase. However, we cannot rule out a possible systemic impact on immune cells in other organs following pDC transfer, and future studies will determine whether this might be beneficial in other inflammatory diseases.

The idea that few exogenous pDCs reaching the SC could confer such strong disease amelioration seemed rather puzzling. When investigating the cellular compartments in the SC after pDC transfer, we observed a tremendous increase in total pDC frequency, but not of other cell types such as cDCs, macrophages or microglial cells, suggesting a specific recruitment of endogenous pDCs to the SC. Importantly, selective depletion of endogenous pDCs completely abrogated disease amelioration following pDC adoptive transfer. Thus, the accumulation of endogenous pDCs in the CNS of EAE mice following pDC transfer accounts for EAE protection. Interestingly, we observed a significant increase in *de novo* HSC progenitors' generation in the BM of pDC transferred compared to control EAE mice. One possibility is that upon transfer, few exogenous pDCs reach the BM and locally produce a factor promoting HSC generation. In agreement, it has been recently demonstrated that angiopoietin-like 7, which is produced by pDCs, regulates the expansion and repopulation of human hematopoietic stem and progenitor cells [32]. Alternatively, LSK progenitor increase in BM might reflect a physiologic demand for pDC generation, as an indirect consequence of pDC-recruitment to the CNS following pDC transfer. Newly generated BM-pDCs would then be specifically recruited to the inflamed CNS through chemerin/CMKLR1 axis, an inflammatory chemotactic factor that is involved in pDC migration [33]. *In vivo*, chemerin expression correlated with pDC infiltration into peripheral tissues during autoimmune diseases, such as SLE [26]. Most importantly, chemerin was detected in intralesional cerebrovascular endothelial cells of MS patients, and its receptor is expressed by pDCs [34]. In agreement, we observed an increase in chemerin expression in the SC of EAE mice compared to naïve mice. Furthermore, we found a significant and specific increase in the expression of CMKLR1 (or Chem23R) by pDCs in the SC of pDC-transferred mice. Blockade of CMKLR1 induced cell migration abrogated pDC recruitment to the SC, demonstrating that following pDC transfer, endogenous pDC homing to inflamed CNS is mediated by the chemerin/CMKLR1 axis. However, microglia cells

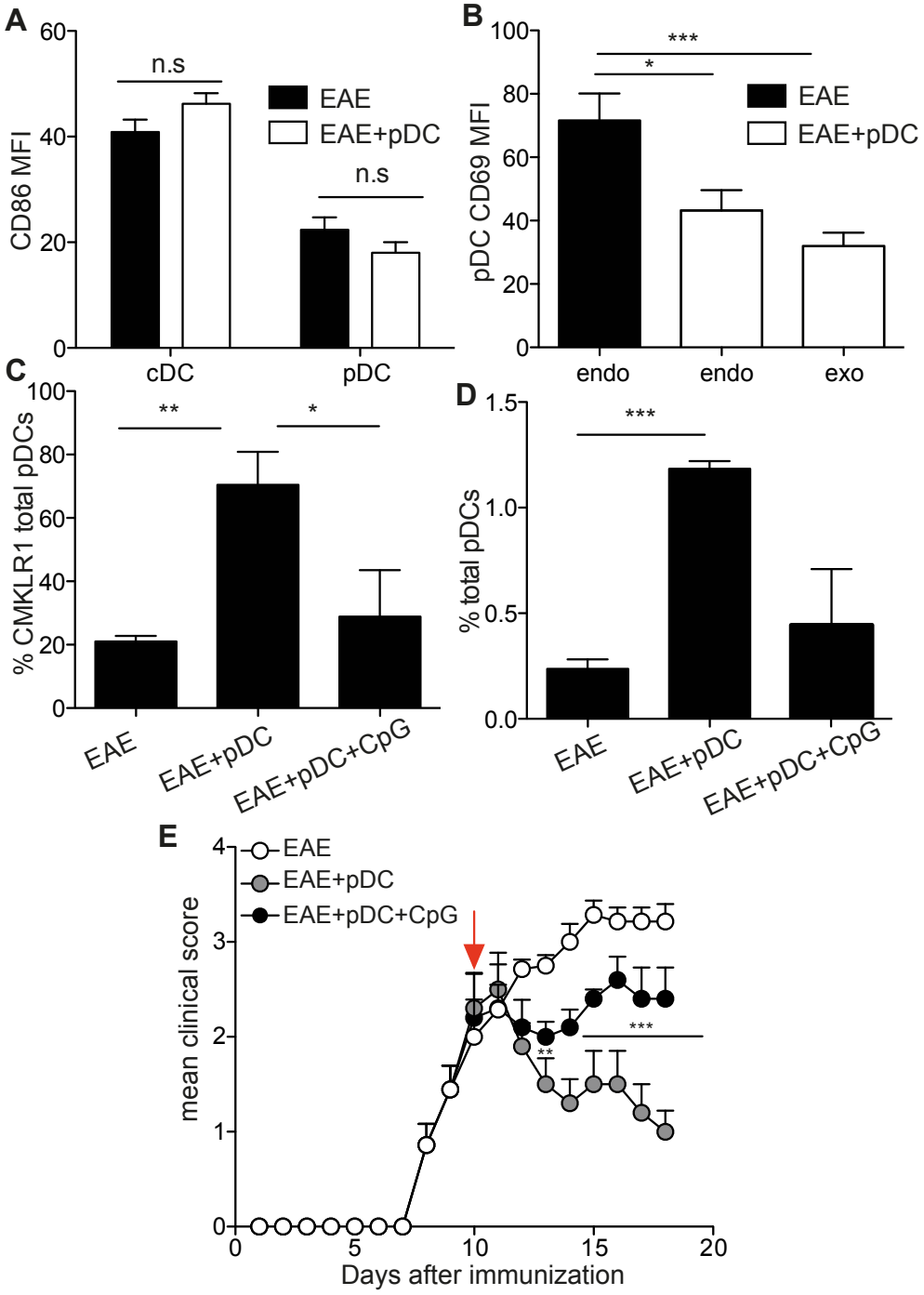

Fig. 6. EAE amelioration is mediated by resting pDCs. (A, B) EAE was induced in WT mice and BM-derived, MOG_{35-55}-loaded, CD45.1 WT pDCs were transferred into mice during EAE acute phase. DC phenotype was analysed in SC of EAE mice four days after pDC transfer. (A) CD86 MFI on cDCs and pDCs. (B) CD69 MFI on endogenous pDCs (EAE), and on endogenous and exogenous pDCs (EAE + pDC). (C–E) EAE was induced in WT mice and BM-derived, MOG_{35-55}-loaded, WT pDCs, previously treated (EAE + pDC + CpG) or not (EAE + pDC) with CpG-B, were transferred or not (EAE) into mice during EAE acute phase (arrow). Frequencies of (C) CMKLR1 expressing pDCs and (D) total pDCs were analysed in SC of EAE mice 4 days after pDC transfer. (E) Clinical scores were followed daily. (A–E) Data are representative of 2 independent experiments with 6–8 mice per group each. Data represent mean ± SEM. (A) Standart two-tailed Student's *t* test; (B–D) 1-way ANOVA with Bonferroni post hoc test; (E) 2-way ANOVA with Bonferroni post hoc test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

and CNS-infiltrating myeloid dendritic cells also express CMKLR1, and EAE clinical and histological disease was found less severe in CMKLR1 deficient mice when compared to WT counterparts [28]. One possibility is that under inflammatory EAE condition, activated CNS pDCs indeed promote disease pathogenesis, and that in CMKLR-1 deficient mice, pDC recruitment to the CNS is impaired and mice consequently develop attenuated EAE. In our settings of pDC transfer in EAE mice, endogenous pDCs would be deflected from pro-pathogenic to pro-tolerogenic functions after down-modulation of their activation state.

In our system, pDC protective role was not related to type I IFN production. Rather, a “non-activated” state for pDCs seemed to be

required. Accordingly, CpG-B activated pDCs did not induce endogenous pDC recruitment to the CNS of EAE mice, neither did they conferred any significant level of disease amelioration, when compared to steady-state pDCs. Strikingly, resting pDCs transferred in EAE mice downmodulated the activation of local pDCs. Notably, expression of CD69, a marker for activated pDCs [35], was substantially reduced on endogenous pDCs recruited to SC after pDC transfer. Therefore, the combination of several pDC-specific features in the CNS: i) increased pDC frequencies; ii) modulation of pDC activation status; iii) ability to provide myelin antigen; iv) production of a state permissive for tolerance, altogether likely contribute to pDC protective role in EAE. MHCII deficient pDCs, were able to induce the same degree of disease inhibition compared to WT pDCs, ruling out a role for MHCII mediated antigen presentation by exogenous pDCs in this protective setting. Our data support a local inhibition of encephalitogenic effector T cells in the CNS of EAE mice after pDC transfer. First, as early as 1 day post-pDC injection, T cells up-regulated the inhibitory molecule PD-1, and second, after 4 days, we observed reduced frequencies of encephalitogenic Th1 and Th17 cells in the SC.

Current available antibody based therapies target various immune cell populations mediating the autoimmune chronic reaction for the treatment of MS, rheumatoid arthritis and other autoimmune diseases. Autologous stem cell transplantations are currently tested as immunotherapy for autoimmune diseases, including MS [36]. Although visionary, therapies using terminally differentiated cells for the treatment of autoimmune diseases are emerging and are up to date mainly focused on the adoptive transfer of Tregs. Both preclinical and clinical data in support for Treg infusional therapy suggest that *ex vivo* expanded, autologous Tregs might have a beneficial effect in patients for T1D [37] and SLE [38,39]. In MS, the rationale for Treg therapy is less clear. Indeed, while Tregs infused prior EAE induction confer protection, their therapeutic value is considerably diminished when infused after disease initiation [40,41]. Accordingly, Tregs immersed in inflamed CNS of EAE mice lost their suppressive activity [42]. Whether Tregs acquire suppressive functions and are directly linked to EAE protection after pDC-transfer will require further investigation.

Adoptive transfer of tolerogenic APCs for MS therapies has also been considered. If these approaches efficiently suppress EAE when injections were performed prior disease induction [43], tolerogenic APCs however impact mainly the priming of encephalitogenic T cells, and no efficacy was consequently proven when effector T cell responses and disease symptoms are already established. These observations make our results even more attractive in the sense that pDC suppressive effect on established EAE is not APC dependent, and directly impact disease effector phase in the CNS. Together, our results highlight pDCs as important mediators in MS immunotherapy. Manipulation of pDC numbers as well as enhancement of pDC potential immunoregulatory properties could be exploited in order to treat MS patients.

Competing interests

The authors have no competing financial interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaut.2015.08.014.

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pDC therapy induces recovery from EAE by recruiting endogenous pDC to sites of CNS inflammation

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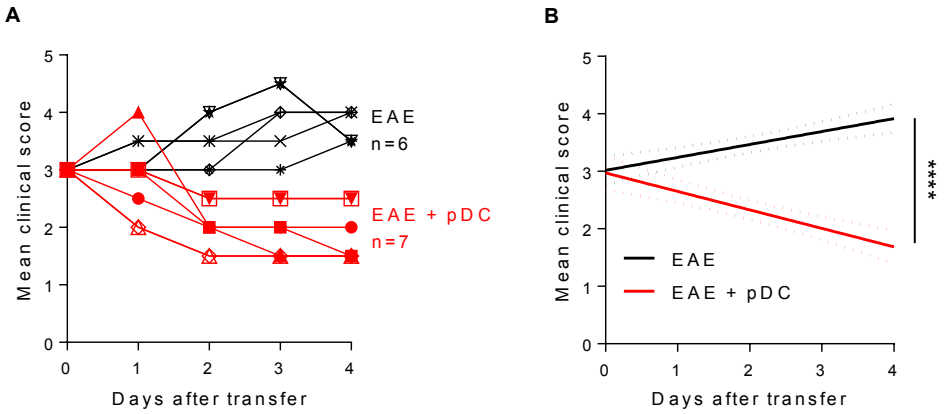
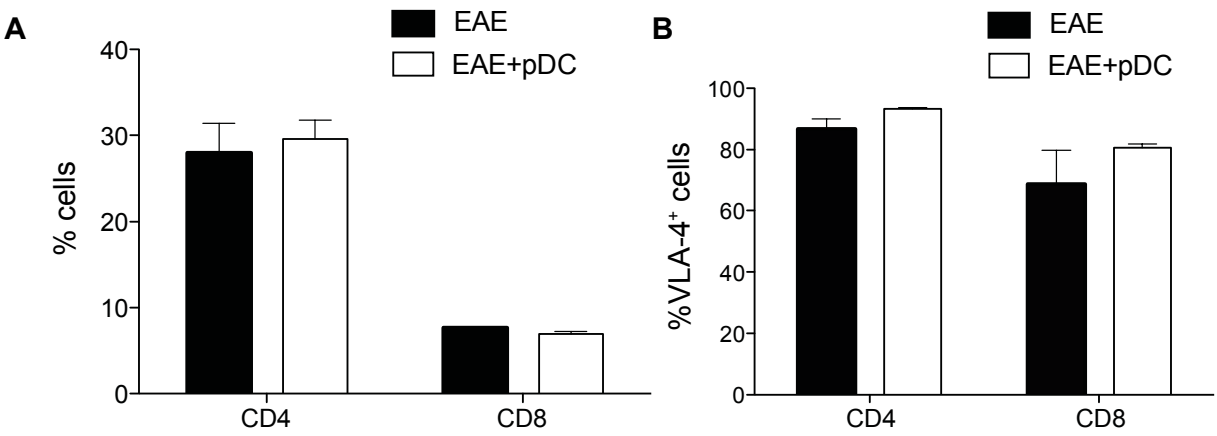
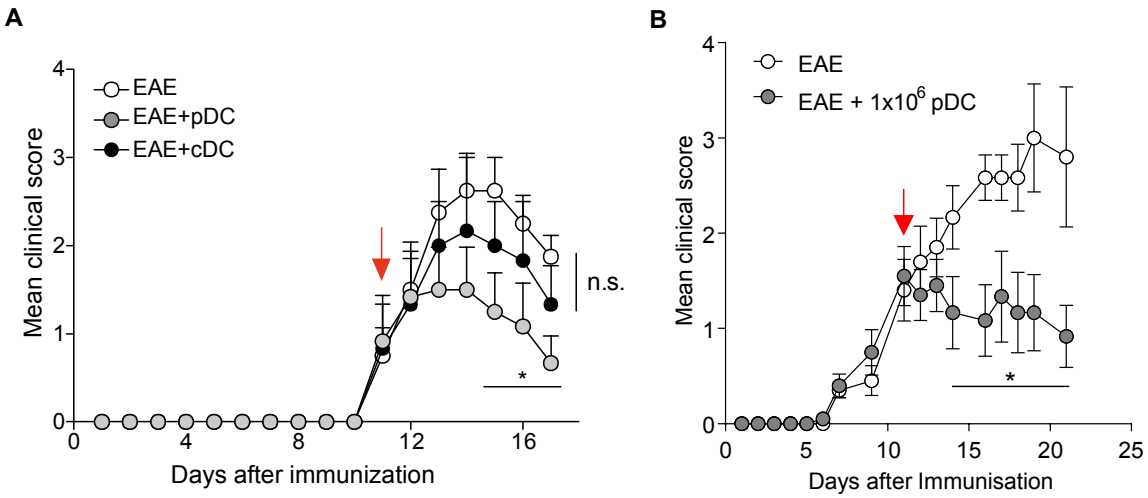


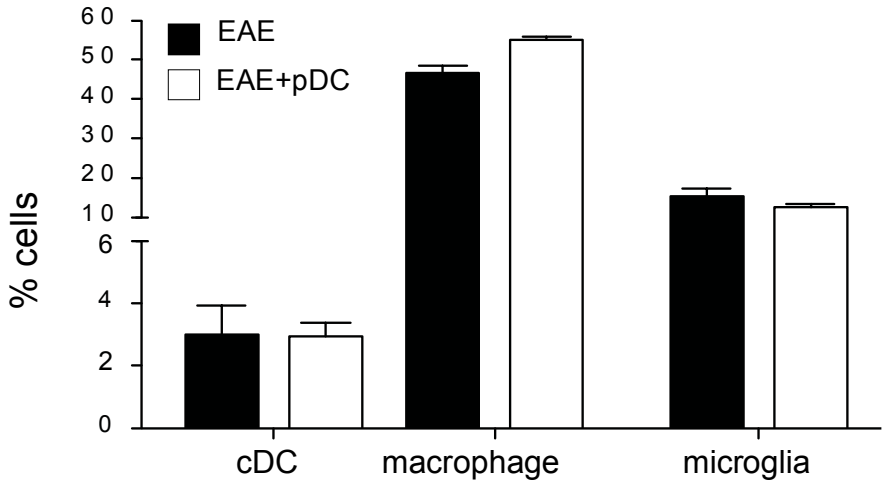
Supplementary Fig. 1: (**A, B**) EAE was induced in WT mice by immunization with MOG_{35-55}. EAE mice exhibiting a clinical score of 3 were injected i.v. (EAE+pDC) or not (EAE) with MOG_{35-55}-loaded BM-pDCs). Clinical scores were followed every day for four days after pDC transfer. (**A**) Individual clinical scores. (**B**) Linear regression of mean clinical scores for each group. Standard two-tailed Student's *t* test. *****P* < 0.0001



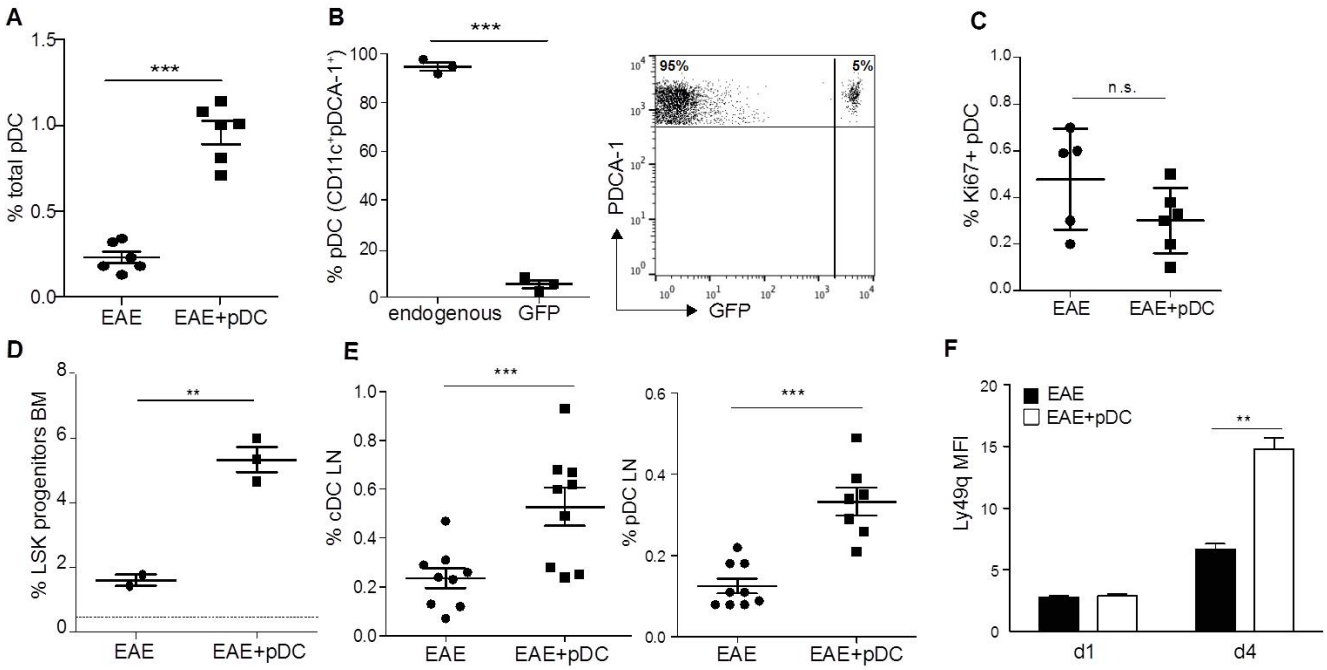
Supplementary Fig. 2: (**A, B**) EAE was induced in WT mice by immunization with MOG_{35-55}. EAE mice were injected i.v. (EAE+pDC) or not (EAE) with MOG_{35-55}-loaded BM-pDCs during disease acute phase (between days 10-12). Frequencies of (**A**) $CD4^+$ and $CD8^+$ T cells, and (**B**) VLA-4^+ cells among $CD4^+$ and $CD8^+$ T cells in SC of EAE mice 4 days after pDC transfer. Data are representative of four individual experiments with 6-8 mice/group.



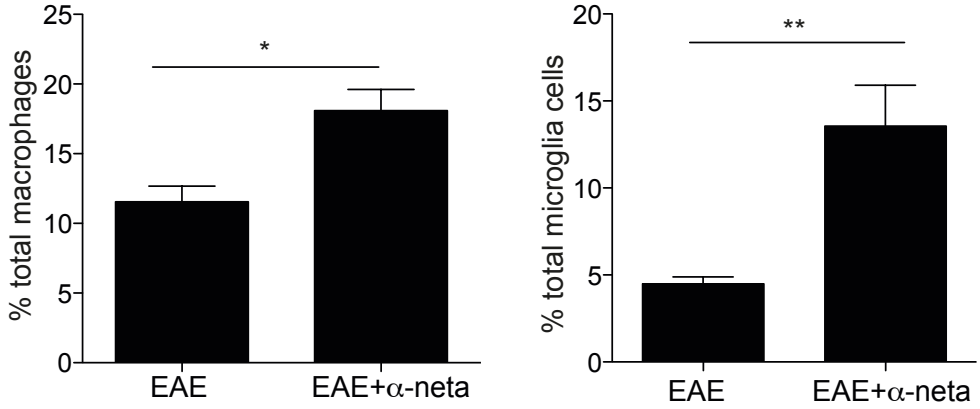
Supplementary Fig. 3: (**A**) EAE was induced in WT mice and $5x10^6$ BM-derived, MOG_{35-55}-loaded, WT pDCs (EAE+pDC) or cDCs (EAE+cDC) were transferred or not (EAE) into mice during disease acute phase (arrow). (**B**) EAE was induced in WT mice and $1x10^6$ BM-derived, MOG_{35-55}-loaded, WT pDCs (EAE+pDC) were transferred or not (EAE) into mice during disease acute phase (arrow). (**A,B**) Clinical scores were followed daily. Data are representative of 2 independent experiments. 2-way ANOVA with Bonferroni post hoc test; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.



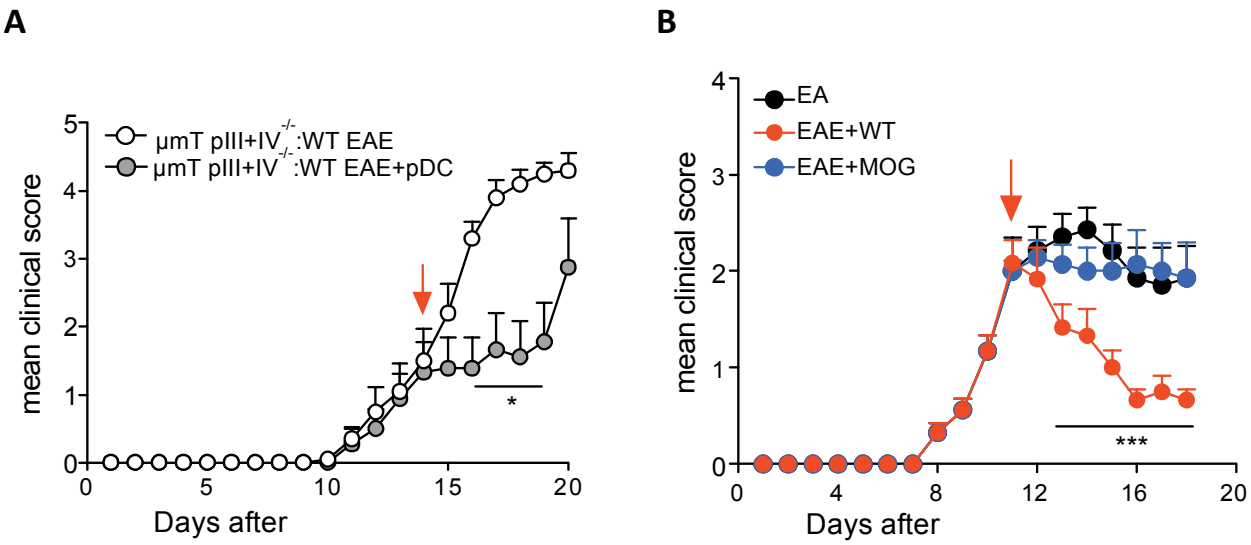
Supplementary Fig. 4: EAE was induced in WT mice and BM-derived, MOG_{35-55}-loaded, pDCs from WT mice were transferred into mice during EAE acute phase. SC cells from control EAE (EAE) or pDC transferred EAE (EAE+pDC) mice were analysed 4 days after pDC transfer. Graph shows the frequency of cDCs (gated as $CD45^{hi}CD11c^{hi}PDCA\text{-}1^{neg}$), macrophages (gated as $CD45^{hi}CD11b^{hi}PDCA\text{-}1^{neg}$) and microglial cells (gated as $CD45^{low}CD11b^{int}PDCA\text{-}1^{neg}$) in SC. Data are representative of 3 independent experiments with 6 mice/group.



Supplementary Fig. 5: pDC transfer induces *de novo* generation of protective pDCs. (**A-F**) EAE was induced in WT mice and BM-derived, MOG_{35-55}-loaded, GFP^+pDCs were transferred into mice during EAE acute phase. (**A-E**) Cells from control (EAE) or GFP^+pDC transferred (EAE+pDC) EAE mice were analysed in indicated organs by flow cytometry either one or four days after pDC transfer. Graphs show frequencies of (**A**) total pDCs in BM, or (**B**) endogenous and GFP^+ transferred pDCs among total pDCs in BM from pDC transferred mice (left). Representative FACS profile shows the frequency of GFP^+ (exogenous) and GFP^- (endogenous) pDCs among total pDCs (right) in BM. (**C**) Frequency of Ki67+ proliferating cells among total pDCs in BM. (**D**) Frequency of BM-LSK progenitors one day after pDC transfer. (**E**) Frequencies of cDCs (left) and pDCs (right) in draining LN of control EAE (EAE) and pDC transferred mice (EAE+pDC) four days after pDC transfer. (**F**) Ly49q expression by total pDCs in BM at one and four days after pDC transfer. Data are representative of 5 independent experiments with 4-6 mice/group each. (**A-F**) Standard two-tailed Student's *t* test. *P< 0.05; **P < 0.01; ***P < 0.001.



Supplementary Fig. 6: EAE was induced in WT mice and a-NETA was injected i.p. or not from day 11 to day 15. Frequency of macrophages and microglial cells were analysed in SC 4 days after pDC transfer. Data are representative of 2 independent experiments with 4 mice/group each. Data represent mean ± SEM. Standard two tailed Student's *t* test; **P*< 0.05; ***P* < 0.01; ****P* < 0.001.



Supplementary Fig. 7: (**A**) EAE was induced in indicated μMT pIII+IV$^{-/-}$:WT chimeric mice and BM-derived, WT MOG_{35-55}-loaded pDCs were transferred into mice during EAE acute phase (red arrows). Clinical scores were followed daily. Data are representative of 3 independent experiments with 8 mice/group each. (**B**) EAE was induced in WT mice and BM-derived MOG_{35-55} loaded WT pDCs (EAE+WTpDC), or MOG_{35-55} peptide alone (EAE+MOG) were injected or not (EAE) during EAE acute phase (red arrows). Clinical scores were followed daily. Data are representative of 2 independent experiments with 3-4 mice/group. (**A-B**) Data represent mean ± SEM. 2-way ANOVA with Bonferroni post hoc test; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

III. DISCUSSION AND PERSPECTIVES

pDCs have been described as crucial actors of the innate immunity through their ability to sense nucleic acids from pathogens and subsequently produce inflammatory cytokines and IFN-I (Cervantes-Barragan et al., 2012; Cervantes-Barragan et al., 2007; Swiecki and Colonna, 2010). Few years ago, the notion emerged that pDCs can also function as bona fide APCs and therefore impact adaptive responses through direct interactions with T cells (Di Pucchio et al., 2008; Sapoznikov et al., 2007; Villadangos and Young, 2008). Being preponderant linkers of the innate and the adaptive immunity, pDCs extensively modulate immune responses, and in particular autoimmunity (Swiecki and Colonna, 2015). However, whether pDCs will contribute to the maintenance of self-tolerance or, in contrast, will promote autoimmunity seems to largely depend on the autoimmune context, and relies on unelucidated mechanisms.

Although several studies point out the implication of pDCs in MS and in EAE, the prominent pathogenic or tolerogenic role these cells play in these two contexts is still controversial. pDCs can be found in SLOs as well as in the CNS of EAE mice (Bailey-Bucktrout et al., 2008; Galicia-Rosas et al., 2012; Irla et al., 2010; Orsini et al., 2014). During EAE priming phase, pDCs produce IFN-I and pro-inflammatory cytokines, such as TNF and IL-6, and therefore rather sustain the priming of encephalitogenic T cells in SLOs (Ioannou et al., 2013; Isaksson et al., 2009). Interestingly, the neutralization of IFN-I prior disease onset ameliorates the early phase of the disease but does not impact EAE severity as a whole (Isaksson et al., 2009). On the other hand, we and others previously demonstrated that, through their APC functions, pDCs promote the anergy of autoreactive T cells (Loschko et al., 2011a) and the expansion of suppressive Tregs in SLOs during EAE priming phase (Irla et al., 2010). Importantly, constitutive or antibody mediated pDC depletion during EAE acute or relapse phases leads to intensified activation of

encephalitogenic T_H1 and T_H17 in the CNS of EAE mice and results in disease exacerbation (Bailey-Bucktrout et al., 2008; Isaksson et al., 2009). Moreover, the protective effect of S1P-1R-agonist treatment (AUY954 in mice and Fingolimod in human), known to reduce lymphocyte migration into the CNS of both EAE mice and MS patients, relies on pDC migration in the CNS (Galicia-Rosas et al., 2012). Lastly, in EAE mice, the presence of Tregs in the CNS at peak disease correlates with high IDO expression and high pDC frequency in the CNS, as well as accelerated EAE remission (Orsini et al., 2014).

This manuscript reports the results of two experimental studies I carried out during my PhD, which support tolerogenic functions for pDCs in both the priming and the effector phases of EAE. The first findings describe how tolerogenic features of pDCs during EAE priming phase rely on their pre-expression of the immunomodulatory enzyme IDO at steady state. Consequently, myelin Ag presentation by IDO^+ pDCs negatively impacts pathogenic T cell priming and EAE development by promoting suppressive Tregs. The second study relates the ability of pDCs, when transferred after EAE onset, to dampen CNS inflammation and inhibit disease development, opening potential new therapeutic approaches in MS treatment.

IDO-ORCHESTRATED INTERPLAYS BETWEEN PDCS AND TREGS INHIBIT AUTOREACTIVE T CELLS PRIMING IN SLOS DURING EAE

IDO is an immunomodulatory protein involved in diverse biological pathways. Pioneer work by Munn and Mellor first identified IDO as an immunosuppressive enzyme expressed by placenta cells to prevent foetal rejection *in utero* (Mellor et al., 2002). Since its discovery, IDO has been further described to take part in diverse immunological contexts, regulating immune

responses by compromising T cell proliferation, promoting T cell anergy or Treg induction (Baban et al., 2009; Chen et al., 2008a; Yang et al., 2014).

- **SUMMARY AND MAIN CONCLUSIONS OF OUR STUDY**

The first study shows that pDCs are the major producers of IDO in steady-state and EAE draining LNs. Importantly, we identify that IDO expression in naive pDCs is induced through Ag-specific MHCII-restricted interactions with $Foxp3^+$ Tregs. Indded, we demonstrate that IDO expression in pDCs isolated from $Rag^{-/-}$ LNs or generated *in vitro* from BM derived precursors, is negligible compared to pDCs from WT LNs. IDO can however be induced by culturing pDCs with $CD4^+$ T cells containing Tregs together with the cognate Ag. In the light of these observations, we hypothesized that, in physiological conditions, Tregs promote a "ready-to-be" tolerogenic state in pDCs, characterized by basal IDO expression levels.

In the lab, we previously reported that self-Ag presentation of myelin peptide by pDCs promotes Treg expansion in draining LNs of EAE mice and is associated with a significant inhibition of auto-reactive T_H1 and T_H17 in SLOs. Consequently, encephalitogenic T_H1 and T_H17 cells were decreased in the CNS, resulting in reduced CNS inflammation and dampened EAE clinical scores.

Here, we further described that in EAE, IDO expression by pDCs is required for the generation of suppressive Tregs that, upon activation, significantly inhibit the priming of encephalitogenic T cells in SLOs. The protective role of IDO in EAE occurs preferentially during the priming phase and does not seem to affect disease effector phase, since WT and $IDO^{-/-}$ mice develop similar EAE following adoptive transfer of 2D2 MOG_{35-55} effector T cells. Importantly, we demonstrate that IDO expression by LN pDCs during EAE priming phase is not required for pDC-mediated Treg expansion but is instead necessary to confer suppressive functions to Tregs. Accordingly,

only Tregs primed in an IDO competent context potently control EAE disease development after adoptive transfer.

- ## DISCUSSION AND PERSPECTIVES

IDO EXPRESSION BY PDCS : INDUCTION OR POTENTIALIZATION?

Numerous studies relate the contribution of IDO expression in tolerogenic DCs. However, some studies did not discriminate between cDCs and pDCs, leading to some confusion in the mechanisms mediating IDO expression and the subsequent function of this protein in each cell subtypes. Nevertheless, some groups attached a particular attention to pDCs and the tolerogenic role IDO expression confers to them (Baban et al., 2009; Fallarino et al., 2004; Mellor et al., 2003; Munn et al., 2004a; Pallotta et al., 2014; Pallotta et al., 2011). In all of these studies, pDCs originate from the peripheral compartment and particularly from the spleen or tumor draining LNs. Thus, Mellor and colleagues reported that CTLA-4-Ig injection in CBA x C57Bl/6 F1 mice promotes IDO expression in splenic $CD11c^+B220^+CD19^-$ pDCs, as well as in $CD11c^+$ $CD8\alpha^+$ $B220^-$ cDCs, and correlates with an inhibition of T cell clonal expansion *in vivo* (Mellor et al., 2003). Munn and colleagues subsequently reported that in tumor draining LNs (TdLNs), both $CD19^+$ and $CD19^-$ $CD11c^+$ $B220^+$ pDC subsets express IDO. However, the catalytic activity of the enzyme seems only functional in $CD19^+$ pDCs and mediates potent suppression of antitumor T cell responses (Munn et al., 2004a). In addition, Fallarino et al. suggested that $CD11c^+$ $B220^+$ $120G8^+$ $(PDCA\text{-}1)^+$ pDCs isolated from spleen of DBA2 mice treated with CD200-Ig *in vitro* overexpress IDO in a IFNα/β dependant pathway, and exhibit increased tolerogenic properties in an IDO catalytic activity dependent manner (Fallarino et al., 2004). More recently, Pallotta and colleagues described that IFN-γ and TGF-β both promote IDO expression

in splenic pDCs by respectively using different signaling pathways, the first one being dependent on the catalytic activity of IDO and the second one relying on the phosphorylation of IDO (Pallotta et al., 2011).

Relatively to our results, we think that basal expression of IDO by pDCs pre-exists in steady-state and is required for further IDO upregulation promoted by previously described signals such as IFN-γ, TGF-β or B7 ligation by CTLA-4.

Therefore, in the different studies relating that IDO is expressed by pDCs under different stimuli such as IFN-γ, TGF-β or CPGs, IDO upregulation by pDCs was possible only because pDCs were isolated from SLOs, hence already expressing a basal level of IDO (Baban et al., 2009; Pallotta et al., 2011). Accordingly, Mellor and colleagues already reported that, in physiological conditions, IDO expression by splenic DCs was not null but low (Mellor et al., 2003). In addition, some of our recent preliminary data show that *in vitro* IFN-γ treatment potentializes IDO mRNA expression by MOG_{35-55}-loaded BM derived pDCs only when co-cultured for 16h with cognate 2D2 T cells containing Tregs. In contrast, IFN-γ treatment does not promote IDO expression in BM derived pDCs when T cells are absent from the culture (***Figure III-1 A***).

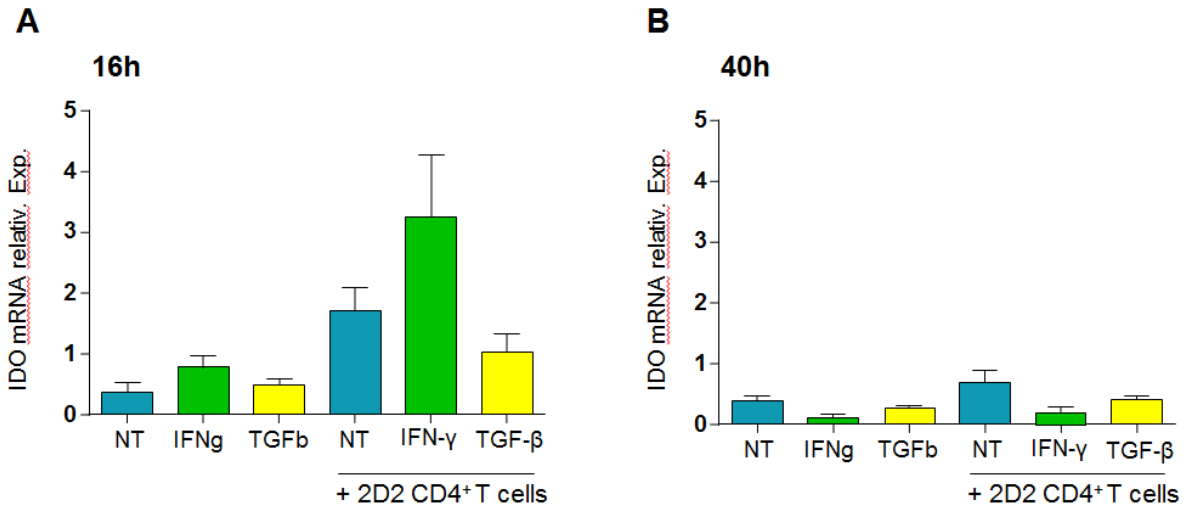


FIGURE III-1 EFFECTS OF IFN-γ AND TGF-β TREATMENTS ON THE EXPRESSION OF IDO BY pDCS.

BM derived pDCs were loaded with MOG_{35-55} and co-cultured with or without LNs-isolated 2D2 MOG specific $CD4^+$ T cells. IFN-γ or TGF-β were added or not. pDCs were isolated after (A) 16h or (B) 40h in cultured and IDO mRNA expression was determined by qPCR. Graphs depict relative expression of IDO compared to GAPDH RNA expression.

Surprisingly, in our experimental settings, TGF-β does not influence IDO expression by pDCs neither when 2D2 $CD4^+$ T cells are absent nor present. IDO induction through TGF-β signaling pathway might last longer. Therefore, we quantified IDO mRNA expression by pDCs at a later time point. After 40h, IDO expression by pDCs is quasi null in all conditions, suggesting that IDO expression by pDCs is only transiently induced in these *in vitro* conditions (***Figure III-1 B***). In addition, the treatment with TGF-β may not be optimal in term of time points or cytokine stability. Nevertheless, these data are preliminary and should be examined in more details, in particular by co-culturing pDCs with 2D2 Tregs.

CTLA-4-Ig ligation to B7 molecules at the surface of pDCs has been shown to promote IDO expression in pDCs (Mellor and Munn, 2004), suggesting a possible implication of Tregs in this mechanism. However, whether direct ligation of CTLA-4 expressed by Tregs with B7 molecules on pDCs mediate IDO expression has not been demonstrated. In addition, it has never been assessed if IDO induction in pDCs specifically requires MHCII-restricted Ag-specific interactions between pDCs and Tregs. Therefore, we have identified a novel mechanism which promotes the expression of IDO in pDCs.

As for IFN-γ or TGF-β treatments, we hypothesized that IDO promotion in pDCs through B7 molecule ligation by CTLA-4 requires as well Ag-specific MHCII-restricted interactions with

Foxp3^{+} Tregs. Even though CTLA-4-Ig has been shown to promote IDO expression *in vitro* in pDCs, the experiments were performed either *in vivo* in a context in which pDCs can interact with Tregs, or *in vitro* using splenic pDCs that pre-express basal IDO levels. It would be interesting to compare *in vitro* the effect of CTLA-4-Ig treatment on IDO expression by BM derived or Rag$^{-/-}$ pDCs that lack basal level of IDO, with *ex vivo* LN pDCs.

Nevertheless, basal expression of IDO by pDCs relies on Ag-specific MHCII interactions with Tregs which would either enable B7 / CTLA-4 interactions, or strictly initiate a specific molecular signaling downstream Ag-MHCII complex engagement.

These two hypotheses could be tested by injecting CTLA-4-Ig in our pIII+IV$^{-/-}$:WT mice which lack MHCII expression by pDCs, and determine whether, in absence of MHCII-restricted interaction between pDCs and T cells, CTLA-4-Ig promotes IDO expression in pDCs or not. If not, we can postulate that Ag-specific MHCII interactions with Tregs lead to a MHCII downstream signaling in pDCs and drive IDO expression. In that case, IFN-γ, TGF-β, CD200-Ig and CTLA-4-Ig ligation on pDC surface receptors would act more as potentializers rather than inducers of IDO expression in pDCs. Lastly, we can imagine that defective MHCII downstream signaling in pDCs would affect, in addition of IDO, the expression of other genes that could as well impede their tolerogenic functions. To address this hypothesis, it would be interesting to perform RNA sequencing on *ex-vivo* pDCs sorted from pIII+IV$^{-/-}$:WT chimeras or WT:WT controls to determine whether the modulation of gene expression correlates with molecular signaling pathways known to be important for pDC tolerogenicity.

DOES THE ACQUISITION OF TREG SUPPRESSIVITY DURING EAE PRIMING PHASE RELY ON THE CATALYTIC ACTIVITY OF IDO EXPRESSED BY PDCS?

We bring several pieces of evidence that pDCs are the major producers of IDO in LNs during EAE priming phase and are linked to EAE protection. However, we did not firmly demonstrate

that selective IDO expression by pDCs is crucial to confer suppressive functions to Tregs in EAE context. This issue could be addressed by testing the suppressivity of Tregs primed *in vitro* using IDO^+ or IDO^- pDCs.

Whether pDC-mediated, IDO-dependent, acquisition of suppressive functions by Tregs cells during EAE priming phase relies or not on IDO catalytic activity has not been addressed. As mentioned before, Pallotta and colleagues described that both IFN-γ and TGF-β promote IDO expression in splenic pDCs. While IFN-γ pathway activates IDO enzymatic activity and results in tryptophan starvation and kynurenine production, TGF-β signaling pathway induces and sustains Treg responses through IDO phosphorylation, independently on its enzymatic function (Pallotta et al., 2011). Moreover, Baban and colleagues previously showed that *in vivo* administration of high doses of CpGs promotes IDO expression and activation in $CD19^+$ splenic pDCs and leads to Treg induction through a mechanism dependent on the catalytic IDO activity (Baban et al., 2009). Therefore, both enzymatic catalytic activity-dependent and independent IDO pathways can positively impact Treg responses. We injected 1-Methyl-D-tryptophan, an inhibitor of IDO catalytic activity, in WT:WT and or $pIII+IV^{-/-}$:WT mice immunized for EAE. However, 1-MT treated WT:WT and $pIII+IV^{-/-}$:WT EAE mice exhibited severe side effects, and died prematurely during EAE course, precluding any reliable conclusion. An alternative option would be to reiterate *in vitro* cocultures of MOG_{35-55} specific 2D2 T cells and MOG_{35-55} loaded IDO^+ or IDO^- pDCs by adding or not 1-MT and to evaluate in vitro Treg suppressivity.

Lastly, the contribution of IDO catalytic activity in pDC ability to promote Treg suppressivity in EAE could have been assessed using $GCN2^{-/-}$ mice. The general control nonderepressible 2 (GCN2), is a serine/threonine-proteine kinase sensing amino acid deficiency through the binding of uncharged transfer RNA (tRNA). GCN2 activation leads to phosphorylation of

eukaryotic initiation factor 2 (eIF2a) that blocks the ribosomal translation of most mRNA and selectively enhances the translation of a small number of transcripts (Munn and Mellor, 2013) (***Figure III-2***). Because IDO catalytic activity induces tryptophan starvation, this process activates GCN2 in surrounding cells and results in different outcomes depending on the T cell subtype. In effector T cells, IDO-mediated tryptophan deprivation sensed by GCN2 drives T cell suppression (Munn and Mellor, 2013). In contrast, by acting synergistically with kynurenine metabolites, activated GCN2 mediates *de novo* differentiation of $CD4^+$ naive T cells into $Foxp3^+$ Tregs (Fallarino et al., 2004; Munn and Mellor, 2013) (***Figure III-2***).

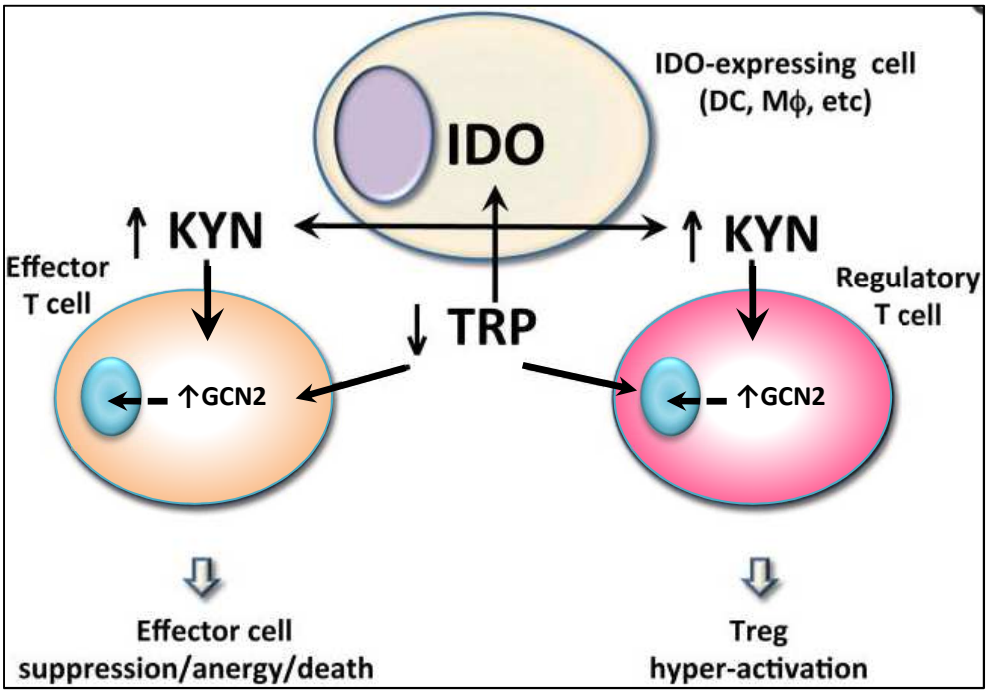


FIGURE III-2 IDO-DEPENDENT METABOLIC CONTROL OF T CELL AND TREG RESPONSES RELYING ON GCN2 EXPRESSION

Activation of IDO enzymatic activity in DCs and macrophages leads to tryptophan (TRP) catabolism and kynurenine (KYN) metabolite secretion. TRP starvation and increased KYN metabolites in the surrounding milieu of IDO-expressing cells affect neighbouring T cells and activates GCN2. GCN2 activation in effector T cells leads to cell-cycle arrest and functional anergy, whereas in naive T cells it promotes de novo generation of Treg differentiation, activation of mature Tregs and blocks T_H17 differentiation. *Adapted from (Munn and Mellor, 2013).*

Although GCN2 activation via IDO enzymatic activity seems implicated in EAE remission phase (Orsini et al., 2014), whether it also affects EAE priming phase is unknown. We could have investigated whether Treg expansion and suppressive activity, along with the frequency of autoreactive T_H17 and T_H1, are affected in SLOs of $GCN2^{-/-}$ mice during EAE priming phase.

DOES IDO EXPRESSION BY PDCS PREVENT TREG CONVERSION INTO TH17-LIKE CELLS IN EAE?

Two studies report that IDO expression by pDCs prevents Treg conversion into T_H17-like cells. First, Baban and colleagues show that in physiological settings, systemic administration of high doses of CpGs induces the expression of IDO through TLR9 ligation in a minor population of splenic pDCs expressing the surface marker CD19. Following CpG treatment, purified IDO-expressing $CD19^+$ pDCs induce Tregs that suppress effector T cell responses *in vivo* when transferred together with activated CFSE labelled OT-1 $CD8^+$ effector T cells in mice constitutively expressing OVA as a tissue alloantigen (Baban et al., 2009). Furthermore, inhibition of IDO catalytic activity not only inhibits Treg activation but also promotes T_H17 cell differentiation following systemic CPG treatment. Lastly, in mice treated with CPGs, inhibition of IDO catalytic activity leads to the secretion of IL-6 by splenic $CD19^+$ pDCs and correlate with the production of IL-17 by 80% of $Foxp3^+$ Treg cells (Baban et al., 2009). In another study, Sharma and colleagues reported that in tumor draining LNs, IDO catalytic activity in pDCs inhibits their capacity to secrete IL-6 by activating GCN2-kinase pathway that blocks *IL6* gene transcription. Thus, IDO expressing-pDCs in tumor draining LNs prevent the conversion of mature Tregs into T_H17 cells and contribute to tumor tolerance (Sharma et al., 2009). Therefore, in two very different contexts, both studies relate that IDO expression is important in the control of tolerogenic features of pDCs and the prevention of Treg cell conversion into T_H17-like cells. This specific pathway requires IDO catalytic activity, activating GCN2-kinase

pathway and subsequently blocking *IL6* gene transcription (Baban et al., 2009; Sharma et al., 2009).

In the light of these results, we can postulate that, during EAE priming phase, IDO expression by LN pDCs prevents Treg cell conversion into T_H17-like cells by using the mechanisms described below. This hypothesis could be tested by using RORγt-GFP x Foxp3-RFP double reporter mice, in which the conversion of Treg into T_H17, and vice-versa, can be followed by expression of distinct fluorescence (Lochner et al., 2008; Wan and Flavell, 2005). Naive RFP^+ Treg cells isolated from LNs of RORγt-GFP x Foxp3-RFP mice could be transferred in WT:WT or $pIII+VI^{-/-}$:WT chimeric mice and check for their potential conversion into GFP^+ T_H17 cells in draining LNs during the priming phase of EAE (10 days) and in SC and LNs at later time points, when mice exhibit clinical symptoms (15 days). The comparison of RFP^+ Tregs and GFP^+ T_H17 frequencies in EAE WT:WT and $pIII+VI^{-/-}$:WT mice will determine whether IDO impacts Treg-T_H17 conversion during EAE priming phase. In addition, analysis of IL-6 expression in draining LN-pDCs will determine whether, as described in the contexts of tumor development and alloresponses, IDO inhibits pDC ability to produce this cytokine during the course of EAE.

Is IDO selectively expressed by a minor population of pDCs?

In the Baban's study, IDO-dependent blocking of Treg-T_H17 conversion relies on IDO expression in a specific and minor splenic population of pDCs characterized by the expression of CD19 (Baban et al., 2009). In contrast, Sharma et. *al.* do not specify that this mechanism is restricted to a particular pDC population in tumor draining LNs (Sharma et al., 2009). Whether only $CD19^+$ pDCs express a functional IDO protein is a matter of debate. Prior to these two papers, the same collaborators showed that, if both $CD19^+$ and $CD19^-$ pDCs are present in tumor draining LNs and express IDO, only $CD19^+$ pDCs express a functional catalytic activity of

IDO enzyme. They further documented that $CD19^+$ IDO competent pDCs representing 0.3% of the total tumor draining LN cells are really difficult to isolate, overlapping with B cells and lacking the classical pDC markers such as PDCA-1 and Siglec-H. They finally demonstrated that this particular population is sufficient to potently suppress tumor Ag-specific $CD8^+$ effector T cells by inducing anergy (Munn et al., 2004a). Although interesting, these data do not provide a clear demonstration that this rare LN cell subset belongs to a real pDC population.

In our study, we did not discriminate between $CD19^+$ and $CD19^-$ pDC populations. However, it is unlikely that lower frequencies of $CD19^+$ pDCs in LNs of $pIII+IV^{-/-}$ mice account for decreased IDO expression in $pIII+IV^{-/-}$ pDCs compared to WT mice. Indeed, we did not observe any difference in frequencies or absolute numbers of $CD19^+$ and $CD19^-$ pDC populations in LNs from both mouse strains. In addition, experiments of pDC adoptive transfers in BDCA2-DTR mice were performed with WT or $IDO^{-/-}$ pDCs purified using magnetic isolation removing $CD19^+$ cells. WT pDCs devoid of $CD19^+$ cells still control EAE development in BDCA2-DTR mice, whereas $IDO^{-/-}$ pDCs did not. Therefore, IDO dependent, pDC-mediated protection during EAE priming phase does not rely on $CD19^+$ pDCs.

In addition, the identification by Pallotta and colleagues of a new IDO tolerogenic pathway which is independent on the catalytic activity of the enzyme, challenges the theory that, in particular contexts, only $CD19^+$ pDCs are tolerogenic. Therefore, the $CD19^-$ pDC population, which also expresses IDO could consequently use TGF-β-dependent pathway to regulate T cell responses.

- **CONCLUDING REMARKS**

An important study in human relates that human pDCs express high levels of IDO when treated with CpG-ODN *in vitro* and that IDO-catalytic activity-dependent pathway is required for pDC-driven Treg generation from $CD4^+$ $CD25^-$ T cells (Chen et al., 2008a) . As previously discussed, results obtained in mice do not always reflect what is happening in humans. Altogether however, these studies show that pDCs can impact Treg generation via IDO expression. Therefore, we could imagine that peripheral blood pDCs from MS patients could be manipulated to highly express IDO and be retransferred during relapses in MS patients in order to dampen the priming of new autoreactive T cells in SLOs.

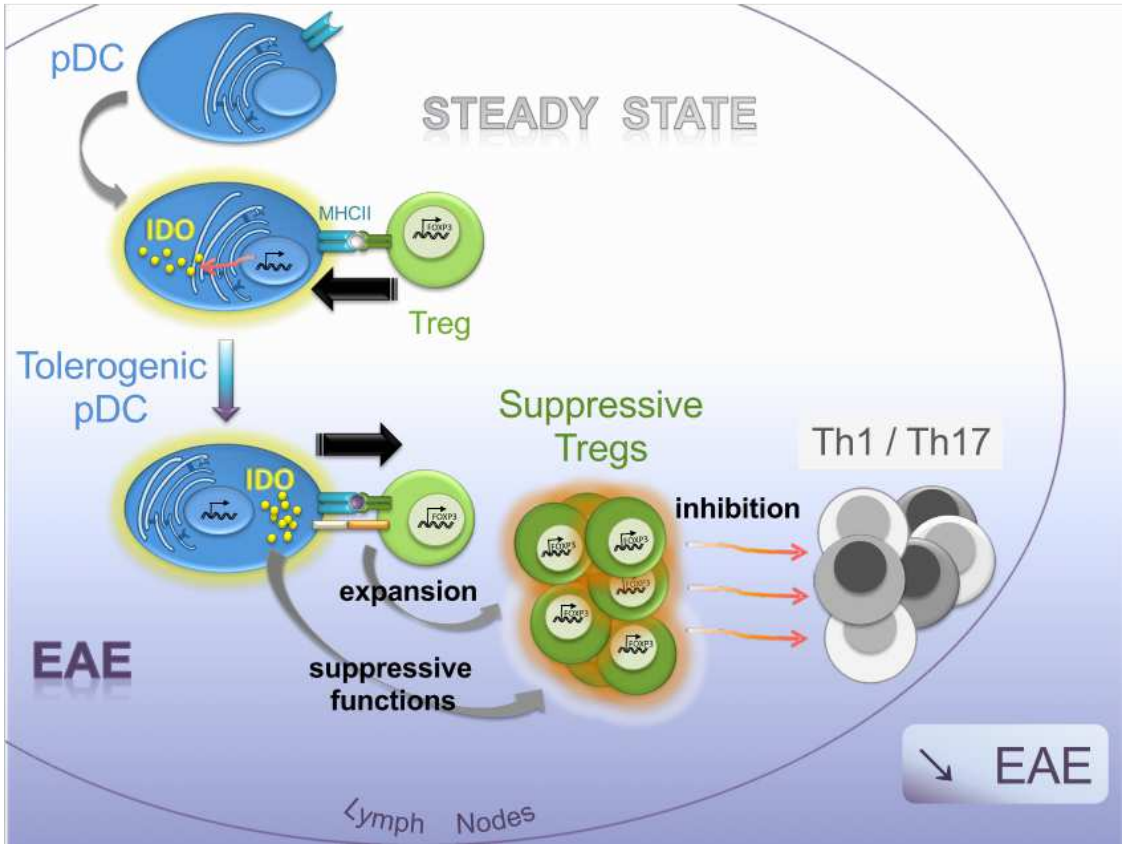


FIGURE III-3 IDO-ORCHESTRATED CROSSTALK BETWEEN PDCS AND TREGS INHIBITS CNS AUTOIMMUNITY

In steady-state LNs, Ag-specific MHCII-dependent interactions between pDCs and $Foxp3^+$ Tregs induce basal expression of IDO by pDCs conferring them a tolerogenic phenotype. In EAE context, myelin self-Ag presentation by IDO^+ pDCs in SLOs leads to suppressive Treg expansion that inhibit encephalitogenic T_H1 and T_H17 priming and results in dampened CNS inflammation.
Adapted from Lippens et al. Manuscript in revision in the Journal of Autoimmunity

THERAPEUTIC TRANSFER OF SYNGENIC PDCS AMELIORATES EAE

The second part of this study focuses on the therapeutic ability of pDCs to control and reduce EAE development.

Several previous studies showed that pDCs can be manipulated to modulate immune responses in inflammatory models by impacting effector T cell responses and Treg generation. Thus, in different cancer models, the topical administration of the TLR-7 ligand Imiquimod promotes the recruitment of pDCs producing either IFN-I- (Stary et al., 2007; Urosevic et al., 2005) or TRAIL- and Granzyme B (Drobits et al., 2012), resulting in tumor regression. In addition, we and others demonstrated that Ag-presenting pDC functions can be exploited for the induction of tumor-specific T cell immunity. Indeed, tumor Ag delivery to pDCs using BST2 targeting in combination with TLR agonists elicits a protective anti-tumor immunity and inhibits tumor growth (Loschko et al., 2011b). Recently, our group demonstrated that, via their Ag-presenting functions, CpG-B-activated pDCs prime Th17 cells that control tumor growth by promoting the recruitment of specific CTL into the tumors (Guery et al., 2014). In autoimmunity, the modulation of pDCs functions can also be beneficial. For instance, in a murine model of SLE, impairment of pDC ability to respond to TLR9 ligands induces a reduction of anti-DNA antibodies and dampens glomerulonephritis, therefore leading to disease amelioration (Sisirak et al., 2014). In T1D model, the restoration of IDO production by NOD pDCs, along with TGF-β treatment, recapitulates their immunoregulatory functions and suppresses *in vivo* the presentation of pancreatic β-cell auto-antigens (Pallotta et al., 2014).

- **SUMMARY AND MAIN CONCLUSIONS OF OUR STUDY**

In the lab, we previously demonstrated that adoptive transfer of pDCs in mice one day prior EAE immunization significantly delays disease onset and controls disease development. This effect is dependent on MHCII restricted Ag-presentation by pDCs, hereby the transfer of MHCII deficient pDCs fails to impact disease progression (Irla et al., 2010). In the light of these results, we investigated whether adoptive transfer of pDCs could be used in a therapeutic way after disease onset and impact CNS inflammation and EAE clinical score development. Strikingly, we show that following transfer during EAE acute phase, pDCs rapidly and strongly ameliorate CNS inflammation and EAE clinical scores. Transferred pDCs reach the CNS in few hours, localize in lesion areas and inhibit encephalitogenic T_H1 and T_H17 cells locally, through, among others, the down-modulation of MHCII expression by microglial cells and the up-regulation of PD1 expression by SC infiltrating $CD4^+$ T cells. Importantly, we show that pDC transfer has also a therapeutical effect when EAE is induced via the injection of 2D2 MOG_{35-55} effector T cells, pointing out to a local immunoregulatory role of these cells during EAE effector phase. Accordingly, pDC depletion during the acute phase of MOG_{35-55}-induced EAE (Isaksson et al., 2009), as well as during both acute and relapse phases in PLP-induced EAE (Bailey-Bucktrout et al., 2008) leads to increased disease severity.

Deepen investigations of the SC cellular compartment show that pDC transfer leads to a tremendous increase in total pDC frequency, while cDCs, macrophages and microglial cells frequencies in SC of transferred mice are similar to the ones observed in control EAE mice. The depletion of endogenous pDCs in EAE BDCA-DTR mice abrogates the therapeutical activity of transferred pDCs, demonstrating that the recruitment of endogenous pDCs to the CNS induced by exogenous pDCs is essential to ameliorate the disease. We further determined that endogenous pDC recruitment to the SC was mediated by the chemerin/CMKLR1 axis. Indeed, blockage of CMKLR1 using α-NETA abrogates the recruitment of endogenous pDCs to the SC

following exogenous pDC transfer. The implication of CMKLR1 in the migration of pDCs in different inflamed tissues has been extensively described in the discussion of the published manuscript and will not be further argued here.

- **DISCUSSION AND PERSPECTIVES**

In EAE, pDC migration to the CNS has previously been associated with disease amelioration (Galicia-Rosas et al., 2012; Orsini et al., 2014). In addition, CNS accumulation of pDCs has also been described in MS patients. Indeed, pDCs are increased in the CSF of MS patients (Lopez et al., 2006; Pashenkov et al., 2001) and are recruited and accumulate in white matter demyelinating lesions and leptomeninges of MS patients (Lande et al., 2008).

IFN-β is used as a standard therapeutic treatment for RRMS and efficiently reduces MS relapses, decreases the formation of novel inflammatory lesions in the CNS and extends remission periods of MS patients (Schwid and Panitch, 2007). In EAE, IFN-I and particularly IFN-β treatments potently suppress disease development, and mice lacking IFN-β exhibit exacerbated disease severity (Prinz and Knobeloch, 2012; Teige et al., 2003). Nevertheless, in our study, we demonstrate that the protective role of transferred pDCs, which happens locally in the CNS, is not related to IFN-I. In the line of this result, Prinz and colleagues suggest that, in EAE, IFN-I mainly acts on peripheral myeloid cells and regulates priming phase of EAE by limiting T cell activation in SLOs (Prinz and Kalinke, 2010).

In our system, the protection conferred by the transfer of pDCs relies on a “non-activated” state of both exogenous and endogenous pDCs. Indeed, compared to steady-state pDCs, the transfer of CpG-B activated pDCs does not induce any endogenous pDC recruitment to the CNS of EAE mice, nor does confer any significant disease amelioration. In addition,

endogenous pDCs recruited in the SC of EAE mice transferred with steady-state pDCs exhibit significantly lower expression of the activation marker CD69 compared to control EAE mice. Interestingly, Schwab and colleagues, described that, in humans, “tolerogenic” pDC1 display a non-activated phenotype, characterized by high levels of CD123 and low levels of CD86 and TLR2, and induce IL-10 producing T cells. In contrary, “immunogenic” pDC2 exhibit low expression of CD123 and high levels of CD86 and TLR2, and promote IL-17 producing cells. Strikingly, the pDC1/pDC2 ratio is inverted in peripheral blood of MS patients compared to healthy individuals and is characterized by more pDC2 than pDC1 in periphery. As discussed previously, this observation reflects either a defect of pDC1 tolerogenicity in MS patients that would contribute to the pathogenesis, or an increased migration of pDC1 into the CNS, hence inverting the ratio pDC1/pDC2 in periphery. We can imagine that extensive CNS inflammation induce the recruitment of pDC1 into the CNS, thus promoting a local tolerogenic effect, similarly to our experimental model. Supporting this hypothesis, Lande et al. reported that pDCs infiltrating the white matter lesions and leptomeninges of MS brains display an immature phenotype with low expression of MCHII and co-stimulatory molecules (Lande et al., 2008). It is puzzling to think that “tolerogenic”pDC1 could be increased into CNS of MS patients, hence inverting the peripheral pDC1/pDC2 ratio. However, it is conceivable that CNS infiltrating pDCs have a tolerogenic role locally in the CNS, partially controlling to some extent the inflammation, and becoming overtaken and ineffective from a certain level of inflammation. Aiming at promoting the infiltration of immature “tolerogenic” pDC1 in the CNS, as in our study, could be a way to overcome the runoff of pDCs occurring once the inflammation becomes too severe.

Unfortunately, in our study, we have not uncovered the precise mechanism accounting for pDC-protective effect in the CNS. We performed the screening for a broad panel of RNA

transcripts, and qPCR on total extracts of SC from EAE mice transferred or not with pDCs. However, apart from the identification of the chemerin pathway as involved in the migration of pDCs in the CNS, we did not identify any molecule that we could relate to a plausible implication in the tolerogenic effect we have observed following pDC transfer. Nevertheless, this strategy does not allow the identification of mediators specifically expressed by pDCs. One option would have been to sort endogenous pDCs from the SC of EAE mice transferred with pDCs to assess their gene expression profile. However, we definitively ruled out this procedure because 1) of the high number of cells required by the procedure and 2) we could not find an appropriate benchmark.

On the other hand, we described that EAE protection mediated by pDCs in the CNS mainly relies on a local modulation of the inflammation. Indeed, MHCII expression appears down-regulated in microglial cells, and PD-1 up-regulated $CD4^+$ T cells, and these observations correlate with an inhibition of encephalitogenic T_H1 and T_H17 in the CNS promptly following pDCs transfer. PD-1/PD-L1 axis has been previously related to pDC-mediated Treg cell proliferation and tolerance induction (Tokita et al., 2008). Orsini and colleagues also reported that, in EAE mice, the presence of Tregs in the CNS at peak disease correlates with high IDO expression, high pDC frequency in the CNS and accelerated EAE remission (Orsini et al., 2014). Moreover, Koutrolos et al. demonstrated that in EAE mice, Tregs control autoreactive T cells in the CNS by limiting their proliferation and motility and by regulating their cytokine production (Koutrolos et al., 2014). In our study, we did not observe any difference in term of $Foxp3^+$ Treg frequency and expansion in draining LNs and SC of EAE mice transferred or not with pDCs. However, we cannot exclude that pDCs modulate Treg functions through the expression of IDO or other molecules. It would have been interesting to evaluate the expression of activation markers such as CD69 and, the TGF-related latency-associated-

peptide (LAP), as well as the co-expression of the suppressive markers ICOS and CD103, in CNS infiltrating Tregs after pDC transfer. Moreover, in humans, “tolerogenic” pDC1 promote IL-10 expressing T cells (Schwab et al., 2010), hereby it would have been interesting to evaluate the frequencies of IL-10 producing cells in the SC in EAE mice transferred with pDCs. Concerning IDO, we did not observe any differential IDO mRNA expression in total extracts of SC from EAE mice transferred or not with pDCs. Nevertheless, we cannot totally rule out a possible involvement of IDO in the clinical amelioration observed in EAE mice after pDC transfer. Indeed, IDO expression has been demonstrated in activated microglial cells and macrophages in the CNS of EAE mice (Kwidzinski et al., 2005). Moreover, IDO-related effects seem to occur strictly to the cells neighbouring IDO-producing cells. Overall, it suggests that, even if CNS infiltrating pDCs express IDO, it would be difficult to detect any IDO mRNA variation in a pool of total cells. Once again, the optimal experiment would have been to sort pDCs infiltrating the CNS after pDC transfer and to quantify IDO mRNA in this specific cell subset. Nevertheless, as mentioned before, we would have faced the same issues concerning the weak number of cells and the lack of benchmark cells.

Finally, because a single pDC transfer during EAE acute phase induces a fast and strong reduction of disease severity with long lasting therapeutic effect, it would have been very exciting to determine whether a similar protection can be observed in the PLP-induced relapsing EAE model, and assess whether this strategy could also dampen the severity or increase the intervals between relapsing-remitting cycles.

IV. CONCLUSION

Together, our studies contribute to elucidate how pDCs acquire a tolerogenic phenotype and highlight the importance of these cells in the regulation of autoimmune inflammatory responses in EAE. First, we demonstrate that Tregs promote basal IDO expression by pDCs in steady-state LNs via Ag-specific MHCII-dependent interactions. Treg-licenced IDO^+ pDCs hence acquire a "ready-to-be" tolerogenic status which is thereafter necessary to these cells to promote Treg responses under inflammatory settings. Indeed, during EAE development, IDO expression in pDCs is required to confer potent suppressive functions to expanded Tregs, enabling the inhibition of encephalitogenic T cell priming in SLOs. Future investigations will determine whether pDCs require other molecular pathways along with Ag-specific MHCII-dependent interactions with Tregs to mediate basal IDO expression in pDCs. In addition, it is likely that, more than IDO expression in pDCs and acquisition of Treg suppressive functions, pDC-Treg crosstalk will impact other important features in these two cell types that may contribute to their respective tolerogenic functions in inflammatory contexts. While we established that, during disease priming phase, tolerogenic properties of pDCs in EAE are dependent on their APC functions, we demonstrate in a second study that during disease acute phase, pDCs substantially impact CNS inflammation through distinct mechanisms. Thus, tolerogenic functions of pDCs can be exploited in EAE after disease onset to control and reduce CNS inflammation. When adoptively transferred in EAE mice, immature pDCs migrate in CNS inflammatory lesions where they potently drive the recruitment of endogenous tolerogenic pDCs that subsequently modulate the local inflammation, leading to substantial disease amelioration. A better understanding of pDC-specific mediator(s) relying on their protective effect in EAE will provide new strategies for MS treatments.

Overall this work highlights pDCs as potent immunoregulatory mediators in EAE, and supports the potential use of these cells for the development of future therapies to treat MS patients.

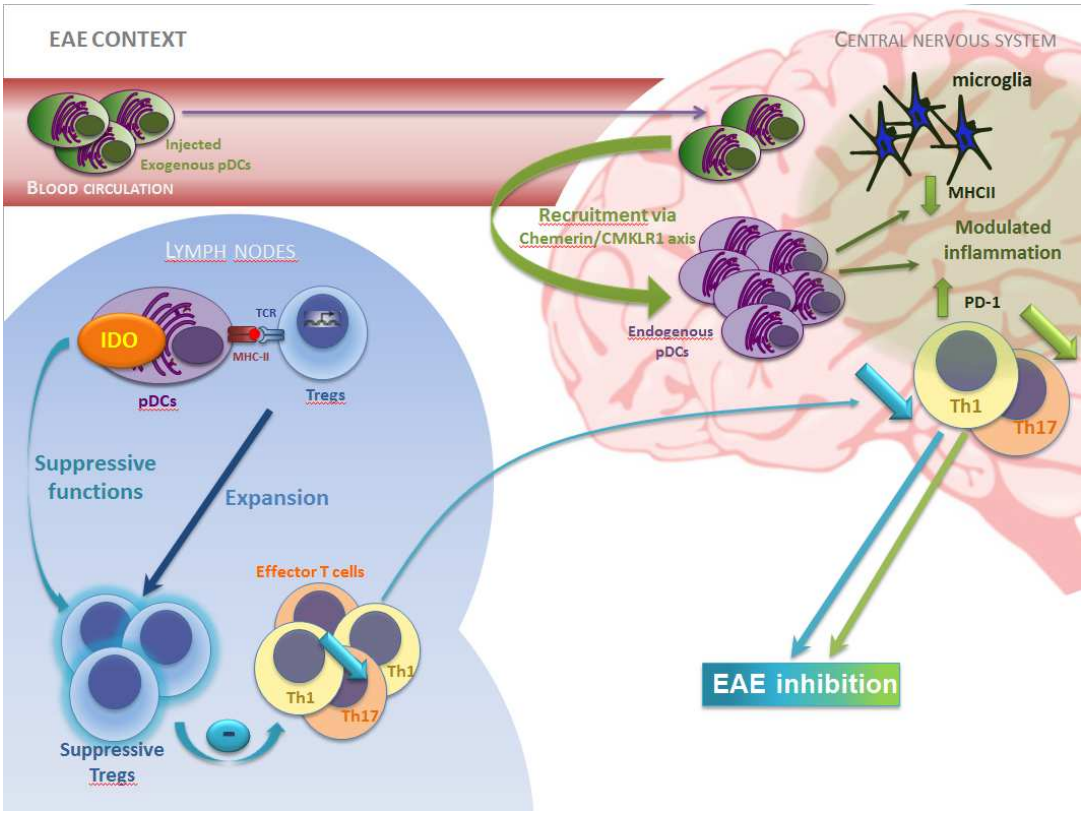


FIGURE IV-1 TOLEROGENIC FUNCTIONS OF PDCS IN EAE

Upon EAE pDCs exhibit tolerogenic functions in periphery and in the CNS. Myelin self-Ag presentation by pDCs in draining LNs promotes Treg expansion, through Ag-MHCII-TCR interactions (Irla et al., 2010), and confers to them suppressive ability through IDO-dependant mechanisms (Lippens et al., in revision). Expanded suppressive Tregs in SLOs inhibit autoreactive T_H1 and T_H17 cell priming (Lippens el al., in revision) and correlate with decreased of encephalitogenic T cells in the CNS. Adoptively transferred myelin loaded-pDCs in EAE mice after disease onset leads to their migration in the CNS, where they actively recruit endogenous pDCs via the chemerine/CMKLR1 axis. Accumulation of endogenous pDCs in the CNS locally modulates inflammation and correlates with immature pDC phenotype, decreased MHCII presentation by microglial cells, up-regulation of PD-1 by $CD4^+$ T cells and decreased of encephalitogenic T_H1 and T_H17 in the CNS(Duraes et al., 2016). Both the regulation of the T cell priming in SLOs and the modulation of the inflammatory environment in the CNS by pDCs result in dampened EAE.

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