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DIMET

MIGRATORY AND NOT LYMPHOID RESIDENT DENDRITIC CELLS MAINTAIN PERIPHERAL SELF TOLERANCE AND PREVENT AUTOIMMUNITY VIA INDUCTION OF iTreg CELLS

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CHAPTER 1 : INTRODUCTION

THE IMMUNE RESPONSE TO *NON SELF*

The immune response has evolved to protect the organism from a variety of pathogens (as viruses, bacteria, fungi and parasites) and from cancer. An efficient response to external harms is essential for the organism and the delicate equilibrium between a strong immune reaction to *non-self* and the need to preserve the *self* from being attacked and destroyed, is one of the most important and fascinating features of the immune system. Any failure in this balance could lead to severe pathologies such as autoimmune diseases, immune deficiencies, allergy and asthma.

The response to a pathogen involves both the first line of immune defence represented by the cells of the antigen (Ag) non-specific Innate Immunity, which are able to detect the pathogen and initiate the response, and the second line of the response represented by the Ag-specific adaptive immunity.

The first barrier a pathogen encounters is the epithelial and mucosal surfaces, which are very efficient in preventing microbe entry, however in some cases the pathogens manage to enter the body and an infection occurs. The first line of defence is represented by the innate immunity that features killer cells as NK cells, phagocytic cells, complement, other antimicrobial peptides and interferons. Most of the cellular component of the innate immunity is already present in tissues, ready to react to a pathogen intrusion. Cells like macrophages and dendritic cells have on their surfaces receptors that recognise exclusive specific microbial patterns called PAMPs (pathogen associated molecular patterns)(Kawai and Akira, 2007). The recognition of these PAMPs triggers multiple signalling pathways that lead to the activation of the inflammatory responses, recruitment of leucocytes and activation of antimicrobial effector mechanisms such as secretion of cytokines, activation of phagocytosis, production of ROS and other antimicrobial peptides. Some of these cells can also function as a link with the adaptive immunity and activate the antigen specific adaptive response.

Innate immunity also includes soluble non-cell-based system, like complement proteins that normally circulate in an inactive state and, after the encounter of a pathogen, are able to activate in a cascade that eventually ends in the opsonisation of the microorganism, which

facilitates the clearance mediated by phagocytes, or the lysis of the pathogen's cellular membrane.

ADAPTIVE IMMUNITY

The innate immune response is very effective as the first line of defence, and could be sufficient to eliminate invading microbes. However if the pathogen persists, adaptive immunity is triggered.

The main actors of adaptive immunity are T and B cells. Their main characteristic is the extreme specificity of action, based on the presence of different B and T cell clones capable of recognizing and selectively eliminating foreign microorganisms and molecules. The huge variety of different clones, each one with a characteristic antigen specific receptor, makes possible to recognise a virtually infinite number of antigens.

B cells are generated in the bone marrow and they exit as Naïve B cells. Their receptor is basically a membrane bound antibody with signalling intracellular domains. After encounter to a pathogen they become activated, differentiate into plasma cells and start secreting antibodies. They pass through different steps of differentiation such as somatic hypermutation and class switching and some of the activated cells eventually differentiate into memory B cell and migrate back to the bone marrow where they keep secreting antibodies.

T cells also arise in the bone marrow but their maturation process take part predominantly in the thymus where they arrange their T cell Receptor (TCR), pass through different steps of selection (which

will be described later on) and exit the thymus as naïve T cells. Differently from B cell receptor, which recognizes whole molecules, T cells recognize only peptides presented by the Major Histocompatibility Complex molecule. These molecules are present on the surface of specific “antigen presenting cells” (APCs), which are able to internalize and process the pathogen components and bind pathogen-derived peptides to the MHC molecule. TCR triggering by MHC-Peptide complex along with costimulatory signals, such as CD28 binding, trigger T cell activation, proliferation and cytokines production. T cells divide in two main groups, $CD4^+$ T cells which harbour the co-receptor CD4 for MHC class II, and therefore recognize only peptides coupled to MHC class II molecules, and $CD8^+$ T cells that recognize with the co-receptor CD8, MHC class I coupled peptides.

These two cell type give rise, when activated, to two different responses: $CD8^+$ cells differentiate in Cytotoxic Lymphocyte which exhibit killer activity by the release of cytotoxic molecules such as perforin and granzyme and IFN-gamma secretion. This cell type is fundamental in contrasting viral infections and intracellular bacteria. On the other hand, CD4 T-cells differentiate to a variety of different effector statuses, including the classical classes T helper 1 and Th2 and the most recently discovered Th17, Th9 and T follicular helper. They could also, under certain conditions, differentiate to cells with a regulatory activity such as Tr1 and T regulatory cells.

The skewing toward a particular Th subtype is governed mainly by the cytokine environment and in some cases from the strength of the TCR-pMHC interaction. Th1 cells differentiate in the presence of IL12 and, once differentiated are massive producer of IFN gamma which activates macrophages and CTL thus favouring a cell mediated response. Th2 cells differentiate in the presence of IL-4 and secrete a pattern of cytokines that favours the humoral immune response such as IL-3, IL-4, IL-5 and IL-13. Th17 cells were initially associated with autoimmune disease and chronic inflammation but have also been shown to mediate responses to extracellular bacteria and fungi through the production of IL-17A, IL-17F, and IL-22. TGF-β and IL-6 were shown to induce IL-17 production from naive T cells (Ghoreschi et al., 2011). Th9 are a newly discovered subtype of T cell with the unique ability to secrete IL-9 involved in the immune response to helmints as well as allergy (Zhou et al., 2009b). T follicular helpers have also been discovered recently and play a pivotal role in helping B cells in the follicular zone. They are implicated in promoting B cell activation, class switch and have a not yet fully understood role in promoting Germinal Center formation (Fazilleau et al., 2009).

$CD4^+$ T cells could also differentiate in cells with a regulatory activity. The main regulatory T cell type is represented by Treg cells, those cells express the transcription factor Foxp3 that regulates a number of genes linked to the suppressive activity. Due to their pivotal role in maintaining peripheral homeostasis they will be described extensively later on.

A small number of antigen-specific long-lasting B and T cells survive after antigen or pathogen challenge and constitute a pool of memory cells. Memory B cells reside in the bone marrow and secrete antibodies into the bloodstream. Memory T cell divides in two groups: Central Memory T cells, which reside in central lymphoid organs, and Effector Memory T cells that reside in the tissues ready to give rise to an early and powerful adaptive response at the site of infection.

DENDRITIC CELLS: TRANSLATING INNATE TO ADAPTIVE IMMUNITY

The initiation of an adaptive response is not independent of innate immunity. While B and T cell can give rise to a very potent antigen specific response, they are not able to initiate it and they need the support of Antigen Presenting Cells. Among the APCs, dendritic cells are the most specialized and they are necessary to efficiently stimulate T and B cells also shaping the type of the immune response.

The driving force for the first sighting of dendritic cells in 1972 was to understand immunogenicity. The antigen processing and presentation arm of immunogenicity was not known when DCs where discovered in 1972. Nobel price Ralph Steinman and colleagues were searching for accessory cells that enhanced immunity when they spotted unusual stellate cells in mouse spleen that where named dendritic cells. Subsequent seminal studies conducted by Steinman and colleagues and the work from Paul Langerhans contributed on describing dendritic cells (DCs) as following a life cycle usually referred to as the 'Langerhans cell paradigm'. According to this paradigm, DCs are present in peripheral tissues in an immature state that is specialized for sampling the environment using various endocytic mechanisms, but is characterized by low levels of expression of MHC molecules and T-cell co-stimulatory molecules.

Immature DCs are well equipped with a series of receptors for pathogen-associated molecular patterns (PAMPs) and for secondary inflammatory compounds, such as Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD) proteins, RIG-I-like receptors, C-type lectin receptors, cytokine receptors and chemokine receptors. Signalling through these receptors triggers DCs migration towards the secondary lymphoid organs. On reaching these organs, DCs develop into a mature state, which is characterized by high levels of expression of MHC and T-cell co-stimulatory molecules, and the ability to present antigen captured in the periphery to T cells. According to this pathway, DCs would provide the necessary link between the probable points of pathogen entry and the lymph nodes, bringing in and presenting antigens that T cells would otherwise not be able to detect. In this context, the phenomenon of maturation was defined as the series of phenotypic changes that enabled DCs to initiate immunity.

The picture is actually more complicated than the Langerhans model; DCs are in fact a heterogeneous group of cells with many subtypes each one with a different phenotype, localization and function. dendritic cells play also a pivotal role in inducing tolerance to self antigens and to regulate also the innate immune response, for example by the interaction with NK cells.

DC SUBTYPES.

There are two main categories of DCs: plasmacytoid DCs and conventional DCs. Plasmacytoid DCs circulate through the blood and lymphoid tissues and only acquire the typical DC morphology after activation, which is accompanied by the release of type I interferons (IFNs). Their ability to process and present antigens to T cells is poor and their major role seems to be to produce large amounts of interferons during the early phase of viral infections (Colonna et al., 2004; Sun et al., 2007a)). A few studies have linked pDCs to tolerance induction, particularly with the induction of regulatory Tcells in a cardiac transplant model (Moseman et al., 2004; Ochando et al., 2006).

Conventional dendritic cells can be further divided in many different subsets with different phenotypes, location and functions. DCs present in lymph nodes, spleen and thymus can be divided in two main groups that are distinguished by the paths they follow to access the lymphoid organs. The first category corresponds to the migratory DCs, which develop from earlier precursors in peripheral tissues and travel through the afferent lymphatics to reach the local draining lymph nodes, where they constitute approximately 50% of all lymph-node DCs (Villadangos and Schnorrer, 2007) This group of DCs is largely absent from the spleen and thymus because these organs do not receive afferent lymph. In subcutaneous lymph nodes, part of the migratory set of DCs is represented by Langerhans cells, which

migrate from the skin epidermis. The other migratory are the so-called interstitial DCs (TABLE 1). The interstitial DCs contained in subcutaneous lymph nodes migrate from the dermis and are often termed dermal DCs. There are several subtype of these cells, that can be divided by the expression of the three markers CD207, CD103, CD11b. Bernard Malissen's group has recently described 4 populations of migratory dermal DCs (mDDCs): $CD207^{+}CD103^{-}$, $CD207^{+}CD103^{+}$, $CD207^{-}CD11b^{-}$, $CD207^{-}CD11b^{+}$(Henri et al., 2010). Migratory cells are also found in the gut where $CD103^{+}$ DCs represent the major subset migrating from the mucosa to the mesenteric Lymph nodes (Agace and Persson, 2011b) and in the lungs (del Rio et al., 2007; GeurtsvanKessel et al., 2008) where they show cross presenting capabilities in both inflammatory and non inflammatory settings. Migratory DCs follow the life cycle described by the Langerhans cell paradigm: they traffic from peripheral tissues to the lymph nodes, where they exhibit a mature phenotype with upregulation of MHC II and costimulatory molecules (Villadangos and Schnorrer, 2007). The lymph nodes of mice or rats that are maintained in germ-free conditions, or are deficient in both of the TLR signalling molecules MyD88 (myeloid differentiation primary-response gene 88) and TRIF (TIR-domain-containing adaptor protein inducing IFN-β ; also known as TICAM1) contain a similar proportion of migratory DCs to normal mice (Villadangos and Schnorrer, 2007). Furthermore, in these mice the migratory DCs appear mature, as in normal mice. This implies that migration and maturation of these DCs types proceeds constitutively and independently of pathogens and

TLR signalling, perhaps being triggered by compounds that are released in low amounts by peripheral tissues. For instance Jagged1, a compound constitutively present in tissues, can stimulate dendritic cells via Notch to produce IL-2, and in some cases it has ben reported to confer a mature phenotype to DCs (Bugeon et al., 2008; Weijzen et al., 2002). The paucity of DCs in the efferent lymph has led to the idea that DCs die in the lymph nodes.

The second major category of lymphoid-organ DCs, are the blood-derived or resident DCs, which constitute the second half of lymph node DCs and all the splenic and thymic DCs. They can be subdivided into three types that are distinguished by their expression of CD4 and CD8: $CD4^+$ DCs, $CD8^+$ DCs and $CD4^-CD8^-$ DCs (TABLE 1) (Villadangos and Schnorrer, 2007) The lymphoid-organ-resident DCs do not conform to the Langerhans cell paradigm; they develop from bone-marrow precursors within the lymphoid organs without previously trafficking through peripheral tissues (Liu et al., 2007a). Furthermore, in the absence of infection, the resident DCs maintain an immature phenotype throughout their entire lifespan, so they can be distinguished from migratory DCs in the lymph nodes by their lower cell-surface expression of MHC class II and T-cell co-stimulatory molecules (Villadangos and Schnorrer, 2007).

Features	Lymphoid-organ-resident DC subsets			Migratory DC subsets		Monocyte derived
	$CD4^+$ DCs	$CD8^+$ DCs	DN DCs	Interstitial DCs	Langerhans cells	
Location						
Spleen	Yes	Yes	Yes	No	No	Sites of inflammation
Subcutaneous lymph nodes	Yes	Yes	Yes	Yes	Yes	
Visceral lymph nodes	Yes	Yes	Yes	Yes	No	
Thymus	Yes	Yes	Yes	No	No	
Surface markers						
CD11c	+++	+++	+++	+++	+++	+++
CD4	+	–	–	–	–	–
CD8	–	++	–	–	–/+	–
CD205	–	++	–/+	+	+++	–/+
CD11b	++	–	++	+/-	++	++
Langerin	–	+	–	–	+++	–
CD24	+	++	+	ND	ND	ND
SIRPα	+	–	+	+	+	ND
Functional features in the steady state						
Maturity	Immature	Immature	Immature	Mature	Mature	N/A
Co-stimulatory	+	+	+	++	++	N/A
Antigen processing and presentation	+++	+++	+++	+/–	+/–	N/A
MHC class II	++	++	++	+++	+++	N/A

Table1: DCs: location, functional features and surface marker expression (Villadangos and Schnorrer, 2007)

MIGRATORY DCS IN IMMUNITY AND TOLERANCE

Recent data have demonstrated an essential role for migratory DCs in the presentation of antigens contained in peripheral tissues. Using an influenza lung infection model, it was shown that $CD11b^-$ migratory DCs can efficiently cross-present antigen in vivo, a property usually attributed to resident $CD8^+$ DCs alones (GeurtsvanKessel et al., 2008). Other studies have demonstrated similar features in migratory dendritic cells from the dermis, also in homeostatic conditions (Henri et al., 2010). Moreover migratory Dendritic Cells have been shown to be essential for viral clearance and activation of $CD8^+$ antigen specific

CTL in the influenza lung infection model (GeurtsvanKessel et al., 2008). Along with their role in protective immunity in these infections settings, migratory DCs are also known to play a major role in tolerance, since they have the unique capability to sample tissue antigens (fig 1). Recent studies have confirmed their strong tolerogenic capacity, especially for the $CD103^+$ subset of dendritic cells, that resides in the lamina propria and migrate from the gut to mesenteric lymph nodes (Agace and Persson, 2011a). Different groups have shown that these cells are able to induce tolerance to orally administered antigens. Murine $CD103^+$ DCs tolerogenic potential can be linked to the fact that they express high amounts of Aldh1a2 mRNA, have increased aldehyde dehydrogenase (RALDH) activity and therefore produce large amounts of retinoic acid (RA) which is known to enhance regulatory T (Treg) cell conversion *in vitro* (Nolting et al., 2009a). These cells are indeed able to mediate Treg Conversion in the mLN in a retinoic acid and TGF-β dependent manner after antigen feeding (Coombes et al., 2007a; Sun et al., 2007a). Retinoic acid produced by $CD103^+$ intestinal dendritic cells has also an important role in the dualism between Treg and Th17 cells. TH17 cells differentiate in presence of TGF-β and IL-6, while the presence of RA inhibit Th17 and enhances Treg Differentiation. *In vivo* studies conducted by (Mucida et al., 2007a) with a *Listeria monocytogen* infection model demonstrated that RA production by $CD103^+$ DCs in the gut, enhance Treg differentiation while blocking the Th17 response. Recent studies also report that migratory dendritic cells from various other tissues are able to produce retinoic

acid (Guilliams et al., 2010a) while absent or very little RALDH activity can be seen in the spleen or from lymph node resident DCs. Sorting out DCs that are active in producing Retinoic Acid from the cutaneous LN the authors have been able to differentiate *in vitro* regulatory T cells. The high tolerogenic potential of migratory DCs from cutaneous LN has been reported also *in vivo* (Azukizawa et al., 2011).

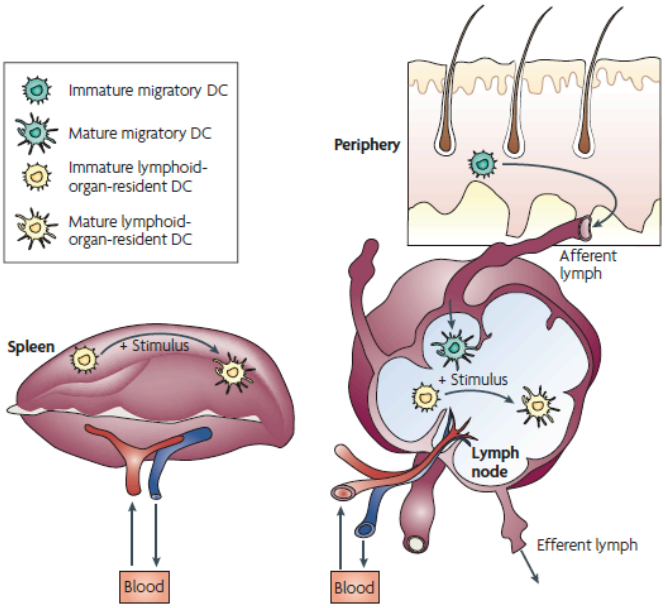

Fig 1: Dendritic cells in different lymphoid organs: Migratory DCs migrate from the Tissues to the LN via the afferent lymph acquiring a semi-mature phenotype. Tissue resident DCs are present both in the spleen and in the LN and activates in situ after pathogen encounter. (Villadangos and Schnorrer, 2007)

TISSUE RESIDENT DENDRITIC CELLS IN IMMUNITY AND TOLERANCE.

Tissue resident DCs reside in lymphoid organs without previous transit through peripheral tissues. In homeostatic conditions, in the absence of inflammatory resident DCs display an immature phenotype. Therefore, almost all splenic DCs and approximately half of the lymph node DCs are resident DCs and can be recognized by their immature phenotype. The role of the DC subsets that are contained in the secondary lymphoid organs is determined by whether they have access only to antigens carried in the blood, only to those contained in peripheral tissues, or both. Unlike the skin, the gut or the respiratory mucosa, the blood is rarely referred to as a peripheral tissue that is subject to DCs surveillance, but the blood represents a major pathway for the dissemination of self-proteins, which might be captured by lymphoid-organ-resident DCs to induce tolerance. Also a variety of pathogens could reach the blood upon inoculation in the skin by arthropods, while other pathogens could enter the blood after colonizing the gut (Villadangos and Schnorrer, 2007). The DCs that reside in the lymph nodes and the spleen are ideally located to monitor the blood, detect these infections and undergo maturation in situ to initiate immunity. In mice, tissue resident DCs can be divided in two main subtypes based on the expression of CD8: "$CD8^+$" DCs and "$CD4^+$ $CD8^-$" DCs (Villadangos and Schnorrer, 2007). These two subsets have also some specialized function. For instance, among the lymphoid-organ-resident DCs, the

CD8 $^{+}$ DCs are the most efficient at phagocytizing dead cells and, consequently, at MHC class II presentation and MHC class I cross-presentation of cellular antigens (den Haan et al., 2000; Iyoda et al., 2002). Among the lymphoid organ resident DCs, $CD8^{+}$ DCs are by far the most efficient at cross-presenting cellular, soluble or latex-beads-associated antigens, or antigens captured by C-type lectin receptors. Comparisons of Langerhans cells with $CD8^{+}$ DCs also showed that although Langerhans cells can cross-present, they are approximately tenfold less efficient than $CD8^{+}$ DCs(Villadangos and Schnorrer, 2007). $CD8^{-}$ DCs are inefficient at cross-presenting antigens that are equally captured by the two subsets (such as pinocytosed soluble antigens or antigens associated with latex beads) or that only they can capture. By contrast, $CD8^{-}$ DCs seem to be more efficient than CD8 $^{+}$ DCs at presenting exogenous antigens by MHC class II molecules(Villadangos and Schnorrer, 2007).

THE HUMAN EQUIVALENTS OF MOUSE DC SUBSETS.

The DC network, as we saw is extremely complex and the possibility to use different mouse models has been crucial to study de roles of each different subset. But how much of this detailed information is applicable to the human immune system? Until recently, the clinical relevance of the various DC subsets had not been apparent. The subtleties of the murine DC system seemed “Lost in Translation”.

Some aspects of the human and mouse DC systems already appeared to be well aligned. The major division into plasmacytoid and conventional DCs, for example, is accepted for both species. Both species also have at least one subset of migratory, conventional DCs in the dermis and a separate subset, known as Langerhans cells, in the epidermis. A notable discrepancy between the two systems has been at the level of the resident DC populations in lymphoid tissues. Four recent papers have shed some light in this field (Bachem et al., 2010; Crozat et al., 2010; Jongbloed et al., 2010; Poulin et al., 2010).

In the mouse, two functionally distinct populations have been recognized, one with high surface expression of CD8, the other lacking this marker. As it turns out, however, CD8 is a poor marker of the eponymous mouse DC subset, as this molecule has no known role in DC development or function. Fortunately, $CD8^+$ DCs possess additional features that are not strictly unique to this population, but together provide an accurate description of this subset. The authors

have indeed found that human CD141^{+}DCs can be the counterpart of mouse CD8^{+} DCs. First CD141 $^{+}$ DCs are capable of phagocytizing dead cells and cross-presenting cell-associated and soluble antigens (Jongbloed et al., 2010), as in mice they express XCR1 (Crozat et al., 2010) they also express transcription factors Batf3 and IRF-8 (Jongbloed et al., 2010; Poulin et al., 2010) which are also expressed in CD8^{+} mice DCs.

TCR DIVERSITY: GREAT OPPORTUNITY AND POTENTIAL DANGER FOR THE IMMUNE SYSTEM

The adaptive immune system of vertebrates has evolved to generate large numbers of T cells, each expressing receptors specific for a single antigen. In order to be able to respond to every kind of pathogenic attack, up to 10^{15} different TCRs are expressed. This great variability is determined by a highly sophisticated random process called “somatic recombination” which takes place in the thymus during T cell ontogeny (Nikolich-Zugich et al., 2004; Turner et al., 2006). TCR is a heterodimer composed of α- and β-chains; these molecules are encoded by genes (*tcr a* and *tcr b*, respectively) composed of a variable and a constant region. The variable region of the β-chain is encoded by variable (V), diversity (D) and junctional (J) gene segments, whereas the same region of the α-chain is encoded by V and J gene segments. The human TCRβ locus has 42 Vβ, 2 Dβ and 12 Jβ gene segments, whereas the TCRα locus has 43 Vα and 58 Jα gene segments. Moreover, regions of hypervariability, known as

complementarity-determining regions (CDRs), are contained within the V gene segments. During thymic development, the random rearrangement of a V, (D) and J gene segment generates the final variable region of each chain (Turner et al., 2006). TCR diversification, thus, depends on the combinatorial variation of the process. The diversity of the naive TCR repertoire is increased further by both a lack of precision during V(D)J genes rearrangement and the addition of non-template encoded nucleotides at V(D)J junctions. The wide repertoire that is generated is virtually sufficient to recognize every non-self antigen. However, the price that is paid for an evolved, effective immune system includes the potential danger of generating autoreactive T cells: it has been estimated that between 20 and 50% of generated receptors can recognize self antigens with a potentially dangerous affinity. Fortunately, however, only a small fraction of humans (3-8%) develop an autoimmune disease: in fact, the immune system has in parallel evolved a number of mechanisms, globally termed tolerance, to control self reactivity, thus preventing dangerous autoimmune reactions (Nikolich-Zugich et al., 2004).

TCELL TOLERANCE

CENTRAL TOLERANCE

T cell tolerance is a fundamental feature of the immune system and is exerted at two levels. It begins during the ontogeny of T cells in a process called “Central Tolerance” and continues later on in the periphery when circulating T cells encounter peripheral antigens with “peripheral tolerance”.

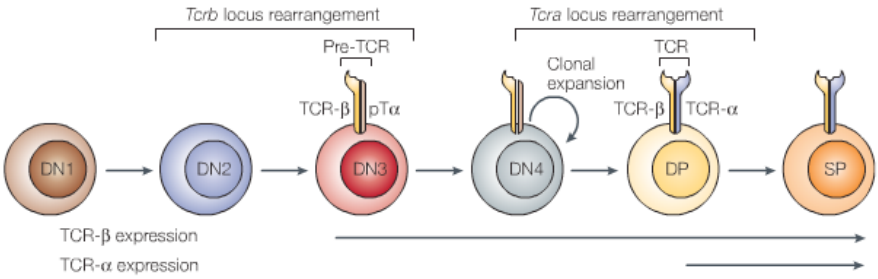

Fig2: Different stages of thymocyte life cycle. (Hogquist et al., 2005)

T cells develop in the thymus and pass through different stages of development. First thymocyte rearrange their *tcr* β *and tcr α* genes, then they express the co-receptor CD4 and CD8. At this point, they pass through different steps of selection with three different possible fates: death by neglect, tolerization and selection with subsequent differentiation into naïve T cells. The commitment to each of the different fate depends on the affinity of the TCR with self-peptides

(fig 3). If the TCR fails to recognize the Peptide MHC complex, the cell die by neglect in a process called “positive selection” in which only the T cells that recognize the pMHC complex receive a survival signal via their TCR. Subsequently, those cells are tested for their affinity to self-peptides and only T cell with an intermediate or low affinity for self-antigens can maturate and exit the thymus. Autoreactive T cells, instead, are tolerized in different ways. The most important one is the negative selection, where cells with a very high affinity are clonally deleted. Another important way of tolerization is the differentiation of these cells in regulatory cells that can be able to control the autoreactive response in the periphery afterwards (natural Tregs). Autoreactive T cells could also undergo re-editing of the TCR with a novel rearrangement of the TCR α chain.

These different processes take part in different district of the thymus: thymocytes enter the cortex where they rearrange the TCR and express both co-receptors CD4 and CD8(fig 2). These double positive thymocytes encounter cortical Thymic Epithelial Cells. Those cells are able to express MHC class I and class II molecules and mediate positive selection triggering survival signals through the TCR. After positive selection and CD4 or CD8 lineage commitment, single-positive (SP) thymocytes rapidly relocate to the medulla, where they scan medullary antigen-presenting cells (mostly dendritic cells (DCs) and medullary TECs (mTECs)), presumably for their entire 4–5 day residency. Here they can encounter not only antigens of thymic origin but also Tissue Restricted Antigens, ectopically expressed by

medullary thymic epithelial cells (mTEC). We know that the ectopic transcription of hundreds if not thousands of tissue-restricted antigens (TRAs) in the thymus is controlled by the autoimmune regulator gene *aire. Aire* gene is expressed in mTEC but TRA presentation is not restricted to this population. For instance among the thymic DCs only the $CD8^+$ population is of thymic origin, whereas the $CD8^-$ $CD11b^+$ $CD172a^+$ population immigrates from peripheral sites (Donskoy and Goldschneider, 2003). These cells are very important for the import of antigens present in the serum acquired in the periphery (Li et al., 2009). Autochthonous DCs instead are very efficient in cross presenting antigen derived from apoptotic cells, including dying mTECs while migratory DCs are much less capable of doing that (Proietto et al., 2008). In the Medulla Single Positive Thymocytes are either selected to differentiate to naïve T cells, in case of an intermediate affinity to the pMHC complexes or, if they recognize pMHC with high affinity, deleted or differentiated to regulatory T cells (nTregs). The forces that drive the choice between the two fates are not clear yet. At the cellular level, both medullary thymic epithelial cells (mTECs) and dendritic cells in the thymus contribute to Treg generation as well as negative selection of naive T cells. Deficiency in the tumour necrosis factor receptor-associated factor 6 (TRAF6) or NF-κB-inducing kinase results in the absence of mature mTECs, hampering the development of Tregs (Akiyama et al., 2005; Kajiura et al., 2004). In humans, the cytokine TSLP (thymic stromal lymphopoietin) secreted by Hassal's corpuscles, which are derived from mTECs, appears to act on thymic dendritic cells to

promote the differentiation of thymocytes to Tregs (Watanabe et al., 2005). Dendritic cells in some cases, seems to be needed for $CD4^+$ Thymocites deletion (Gallegos and Bevan, 2006) and lack of DCs impairs severely central tolerance causing widespread autoimmune reactions (Ohnmacht et al., 2009).

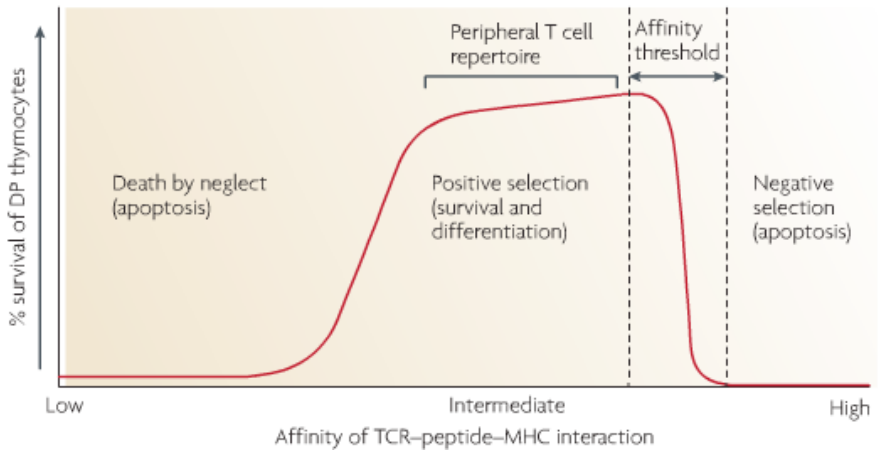

Fig 3: According to this model, the affinity of the T cell receptor (TCR)–peptide–MHC interaction is the key determinant of T cell selection. Double-positive (DP) thymocytes expressing TCRs with no or too low an affinity for self-peptide–MHC complexes die by neglect. Thymocytes with intermediate affinity for self-peptide–MHC complexes receive a survival signal (in a process termed positive selection), commit to the CD4 or CD8 T cell lineage and subsequently pass through the thymus medulla to become part of the peripheral T cell pool. High-affinity binding of the TCR to self-peptide–MHC complexes induces cell death by apoptosis, a process that is known as negative selection (or clonal deletion)

PERIPHERAL TOLERANCE

Central tolerance is fundamental to prevent autoimmune diseases but it is incomplete (Gallegos and Bevan, 2006). Once in the periphery T cells can encounter many tissue-restricted antigens that do not have access to the thymus, moreover a number of antigens can reach the intestine and the circulation after ingestion.

A number of different mechanisms have therefore developed to establish peripheral tolerance. These can be divided into recessive or intrinsic, which regulate the responding state of T cell (i.e. ignorance, apoptosis and anergy) and dominant or extrinsic, which depend on the supply of external signals (i.e. active suppression, limitation of survival or inflammatory signals). The simplest scenario involves T-cell ignorance of self-antigens. Naive T cells have restricted trafficking patterns, circulating from blood to secondary lymphoid organs to efferent lymph and then to blood again; thus, a lot of parenchymal cells expressing tissutal self-antigens are physically separated from potentially autoreactive lymphocytes. Lymphocytes that encounter self-antigens may undergo clonal deletion. On a molecular basis, apoptosis may be mediated by two main convergent pathways: the cascade that depends on Fas receptor engagement by FasL and the mithocondrial Bim-dependent triggering of Bcl-2 and Bcl-xL. Alternatively, autoreactive T cells can become anergic. Anergy is a state of hyporesponsiveness that is induced both *in vitro* and *in vivo* when TCR is triggered in suboptimal conditions, for example in the

absence of CD80/CD86 co-stimulation or in the presence of inhibitory receptors such as CD5, CTLA-4 or PD-1. It is defined as a tolerance mechanism in which the lymphocyte is intrinsically functionally inactivated following an antigen encounter, but remains alive for an extended period of time in the hyporesponsive state. Biologically, clonal anergy represents a growth arrest state in which the production of some cytokines (IL-2, TNF-α and IFN-γ) is significantly impaired. On a molecular basis, T cell anergy is a combined result of a number of factors that negatively regulate proximal TCR- and CD28-coupled signal transduction, such as defective LAT activation or Ras dependent MAPK cascade. Moreover, a selected alternative transcriptional program is activated in anergic T cells compared to activated cells: overactivation of calcium/NFAT signalling in the absence of AP-1, for example, drives the proximal TCR signalling defects but induces also the expression of repressive transcription factors Ikaros, Egr2, Egr3, which actively switch off the expression of a number of genes involved in T cell activation, such as il2 gene (Nolting et al., 2009a; Wells, 2009). The second group of mechanisms are referred to as “extrinsic” and comprise the active suppression exerted by Treg cells (this topic will be discussed in details in the next session) as well as the modulation of the activation and survival of T cells. The survival of circulating T cells at the steady state depends on continuous signalling of TCR with self pMHC as well as exposure to IL-7. Normally, IL-7 levels are low and contribute to maintain T cell homeostasis and limit excessive proliferation. Moreover, the lack of costimulation or inflammation during TCR triggering maintains T cell

tolerance. All the aforementioned modulations rest upon the action of DCs at the steady state. In conclusion, the relative contribution of these strategies, in particular of apoptosis, Treg suppression and anergy, is greatly debated, the most widespread view is that different mechanisms are both overlapping and distinct in response to different forms of self antigens (tissue-restricted or circulating), thus providing multiple possibilities to gain tolerance in different contexts.

While the phenotypes of the tolerized T cells have been thoroughly studied, the forces that drive tolerization are still not completely understood. A lot of work has been done to assess the role of DCs in tolerization of $CD8^+$ T cells. The maturation state of the APC as long as the capacity to cross present auto antigens, detained mainly by $CD8^+$ resident Dendritic cells, seem to be the main characteristic for tolerization of $CD8^+$ T cells that either become anergic or get deleted by DCs (Belz et al., 2002; Kurts et al., 1999; Probst et al., 2003; Probst et al., 2005). The situation is not that clear for CD4 T cells, the maturation state seems not to be the main discriminant, with partially mature dendritic cells with a tolerogenic phenotype and autoreactive T cells activated by immature DCs. Understanding the factors that govern the distinction between self and non-self is fundamental for manipulating the response in the clinical practice.

REGULATORY T CELLS

In the dominant or cell-extrinsic mechanism, certain T cells actively keep in check the activation and expansion of aberrant or overreactive lymphocytes, in particular other types of T cells. Until recently, the physiological significance, and even the existence of T cell-mediated immune suppression, has been highly contentious. Yet there is now firm evidence that the normal immune system produces a population of T cells, called regulatory T cells (Tregs), which are specialized for immune suppression. Disruption in the development or function of Tregs is a primary cause of autoimmune and inflammatory diseases in humans and animals. T cells with a regulatory activity have been noticed the first time in the late 1970s (Gershon and Kondo, 1971). The authors used the term "infectious tolerance" to indicate that normal lymphocytes do not respond to antigen in the presence of thymus-derived lymphocytes transferred from an antigen pre-treated mouse. Only in the nineties these lymphocytes came back to light with the seminal work of Sakaguchi and colleagues (Sakaguchi et al., 1995). He identified regulatory T lymphocytes in a population of $CD4^+$ T cells constitutively expressing the IL2R α subunit (CD25). These cells represented about 10% of all $CD4^+$ T cells and their importance in maintaining peripheral tolerance has been established in a T cell transfer experiment. Nude mice have been replenished either with CD4 T cells depleted of the $CD4^+$ $CD25^+$ Tregs or with the un-depleted population. When $CD25^+$ cells where

depleted all recipients spontaneously developed histologically and serologically evident autoimmune diseases. Reconstitution of $CD4^+CD25^+$ cells within a limited period after transfer of $CD4^+CD25^-$ cells prevented these autoimmune developments in a dose-dependent fashion. These experiments demonstrated the fundamental importance of regulatory T cells in maintaining the homeostasis of the immune system and their key role in establishing and maintaining peripheral tolerance. $Foxp3^+$ Treg cells differentiate in the thymus during T cell ontogeny and are defined "natural" Treg cells (nTreg). In addition, conventional T cells can also acquire Treg phenotype in the periphery under specific conditions and are called "adaptive" or " induced" Treg cells (iTreg).

FUNCTIONAL AND PHENOTYPICAL CARACHTERIZATION OF $CD4^+CD25^+$ $FOXP3^+$ REGULATORY T CELLS

$CD4^+CD25^+Foxp3^+$ Treg cells express a broad TCR repertoire characterized by high affinity for self pMHC II. These cells, then, continuously proliferate in the periphery of healthy mice and Humans. Regulatory T cells strictly depend on IL-2 for survival and homeostatic proliferation (Setoguchi et al., 2005; Suffner et al., 2010). Since Tregs are not able to produce IL-2 they depend on an exogenous source for the cytokine that could be represented by other $CD4^+$ T cells or by Dendritic Cells. Strikingly, IL-2 deficient mice exhibit a severe phenotype characterized by an abnormal expansion

of CD4 T cells and the consequent development of autoimmune diseases. Evidence suggests that the syndrome is due to deficiency or dysfunction of $Foxp3^+$ Tregs. First, the number of $Foxp3^+$ Tregs is reduced in mice lacking either CD25 or IL-2 (Antony et al., 2006), and autoimmunity in CD25-deficient mice can be prevented by inoculating them with wild-type $CD25^+CD4^+$ T cells (Sakaguchi et al., 2008).

Naturally occurring Tregs specifically express the transcription factor Foxp3 that is a master regulator of Treg development and function. This gene was first identified as the defective gene in mouse strain Scurfy. Scurfy mice are characterized by an X linked disease that is lethal in hemizygous male within a month after. This syndrome is characterized by hyperactivation of CD4+ T cells and overproduction of pro-inflammatory cytokines that leads to development of many autoimmune reactions (Brunkow et al., 2001). In human, mutation on the gene *FOXP3* causes a syndrome called IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) that is the human counterpart of mouse scurfy syndrome. Immunological and clinical similarities between IPEX in humans and autoimmunity/infammation produced in rodents by Treg depletion prompted researchers to determine the possible role of Foxp3 in Treg development and function(Fontenot et al., 2003; Hori et al., 2003). $Foxp3^+$ ectopic expression in $CD4^+$ conventional T cells confers to these cells a regulatory phenotype, conferring them the ability to suppress T cell proliferation *in vitro* and the ability to inhibit the

development of autoimmune diseases in vivo (Hori et al., 2003). Foxp3 also regulates a number of genes involved in Treg function and the expression of CD25 and other Treg-associated cell-surface molecules, such as cytotoxic T cell- associated antigen-4 (CTLA-4) and glucocorticoid-induced TNF receptor family-related gene/protein (GITR), whereas it represses the production of IL-2, IFN-γ, and IL-4 (Fontenot et al., 2003). Recent searches for Foxp3 target genes have shown that Foxp3 directly or indirectly controls hundreds (~700) of genes and binds directly to ~10% of them (Zheng et al., 2007).

Many different mechanisms by which regulatory T cells exert their suppressive activity have been described and include the secretion of anti-inflammatory cytokines, direct cytolysis, metabolic disruption, consumption of essential cytokines and targeting of APCs.

Regulatory T cells are known for secreting two of the potent anti inflammatory cytokines, IL-10 and TGF β. Il 10. IL-10 is a homodimeric cytokine produced by a variety of cells, including monocytes (DCs and macrophages) and lymphocytes (B, Th2, CTL, Th1 and Treg cells). It has a wide range of effects on both myeloid and lymphoid cells; it exerts regulatory activity by inhibiting APC functions (maturation, costimulatory molecule expression and proinflammatory cytokines production). IL-10 action is extremely important in controlling a number of situations including allergic reactions and the control of IBD and colitis (Kühn et al., 1993). Recently in different models of IBD, has been shown that IL10 produced by Tregs was required for the suppression of spontaneous Th17 cell-driven colitis and controls

directly Th17 cells but not Th1 cells (Chaudhry et al., 2011; Huber et al., 2011). IL-10 produced by Tregs has also an autocrine effect and amplifies the regulatory response both signalling back to tTregs enhancing their function, and favouring the differentiation of Tr1 cells from naïve T cells (Chaudhry et al., 2011). The transforming growth factor-b (TGF-β) family is composed by a large group of pleiotropic cytokines (the most abundant is by far TGF-β1) with strong suppressive ability affecting most immune cell types. TGF-β - dependent signalling pathway involves the activation of SMAD proteins and other minor cascades that ultimately transduce their signal to the nucleus. There, they control the expression of many genes, thus regulating a number of cell functions. This confers a strong anti-proliferative activity on T cells and the inhibition of CTL, Th1 and Th2 cell differentiation (Rubtsov and Rudensky, 2007). IL-35 is a recently described heterodimeric cytokine composed of IL-12a (p35) subunit, shared with IL-12, and EBI3 subunit, shared with IL-27 (Collison et al., 2007; Niedbala et al., 2007). Apart from the clear suppressive function in mice, the exact understanding of its activity is still limited. The second suppressive mechanism is target-cell killing. This mechanism shares many common features to NK- or CTL-cytotoxic activities, such as the role of perforin and granzymes in the process. In addition, in Treg cells, some unique cytotoxic mechanisms may also exist. Treg cell mediated cytolysis of target T or NK cells has been demonstrated, while no studies to date have been able to document Treg cell-mediated cytolysis of DCs or B cells in an *in vivo* model (Vignali et al., 2008)

The third group of suppressive mechanisms are collectively referred to as “metabolic disruption” of target T cells. These include IL-2 consumption by $CD4^+CD25^+$ Treg cells, through the stably expressed CD25 component of the high affinity IL-2 receptor. The deprivation of IL-2, in fact, mediates the apoptosis of effector $CD4^+$ T cells (Pandiyan et al., 2007; Vignali et al., 2008). Metabolic disruption is also based upon the pericellular adenosine generation catalysed by CD39 and CD73, which are concomitantly expressed on the surface of $CD4^+CD25^+$ Treg cells. Adenosine binds to adenosine receptor 2A (A2AR) and inhibit T cell effector functions. Finally, Treg cells have been shown to suppress the response directly transferring cyclic AMP (cAMP) into effector T cells through GAP junctions. The fourth group of suppressive mechanisms lead to the modulation of DC function and is part of the complex cross-talk between Treg cells and DCs. DC activation is blocked in many ways. The most prominent molecule involved in this process is CTLA-4, which is constitutively expressed by Treg cells. CTLA-4 exerts its functions both directly and indirectly: by binding to CD80 and CD86, it directly blocks the priming capacity of DCs; in addition, CTLA-4 indirectly impairs costimulatory signalling by preventing the upregulation of CD80/CD86 as well as by activating in DCs the expression of indoleamine 2,3-dioxigenase (IDO), which catalyse transformation of exogenous tryptophan into immunosuppressive kynurenine. Tryptophan starvation and kynurenine production both contribute to immunosuppression (Puccetti and Grohmann, 2007). In conclusion, several mechanisms are documented to contribute to Treg mediated suppression. These

probably operate synergistically and in a complementary manner depending on the scenario. There could also be the possibility that some core mechanisms like CTLA-4 action or IL-2 exist, with all the others being accessory (Vignali et al., 2008)

ORIGIN OF REGULATORY TCELLS. NATURAL TREGS VS ADAPTIVE TREGS

$Foxp3^+$ Tregs can be generated in the thymus during thymic selection. $Foxp3^+$ timocytes are first detected in the $CD4^+CD8^+$ double positive population but the majority is detected in cells that have already undergone positive selection and mainly in $CD4^+$ thymocites. Positive selection of Treg cells requires TCR—major histocompatibility complex (MHC) molecular interaction, as for conventional T cells but with a stronger dependence on costimulatory signals through CD28. The TCR specificity of Tregs is mainly self reactive with a small overlap with the repertoire of conventional Tcells (Feuerer et al., 2009). Tregs generate first as a CD25 hi population among $CD4^+$ SP thymocytes that subsequently acquires Foxp3 expression. Exposure to IL-2 can convert these intermediates to fully differentiated Tregs while TGF β seems to be dispensable for thymic selection of Tregs. (Feuerer et al., 2009; Lio and Hsieh, 2008).

Regulatory T cells can also differentiate from naïve T cells that encounter self-antigens in the periphery. Some of the early evidence of peripheral conversion of naïve conventional $CD4^+$ cells into $Foxp3^+$

T cells originated from adoptive transfer experiments in which polyclonal $CD4^+CD25^-$ naïve T cells were injected into lymphopenic mice or mice containing a monoclonal T cell repertoire devoid of Treg cells(Curotto de Lafaille et al., 2008; Curotto de Lafaille et al., 2004; Sun et al., 2007a). In these experiments, part of the transferred T cells acquired CD25 and Foxp3 expression and exhibit regulatory activity. Other groups studied Treg conversion injecting antigen-specific naive $CD4^+$ T cells where transferred into lymphocyte-deficient RAG- deficient mice that expressed the antigen as a systemic secreted protein. In these mice T cells expanded, causing a violent GvH like reaction and eventually part of these cells differentiated in $Foxp3^+$ Tregs. ITreg cell generation did not require the thymus but was dependent on IL-2 (Knoechel et al., 2005).

Several groups have described iTreg cell induction by foreign antigens. Von Boehmer and colleagues showed the generation of $Foxp3^+$ iTreg cells in mice treated with minute antigen doses by osmotic pump delivery (Apostolou and von Boehmer, 2004), or after delivery of antigen cross-linked to DEC-205 antibody in the absence of costimulatory signals, a process that directs the antigen to DEC-205^+ dendritic cells (Kretschmer et al., 2005a; Yamazaki et al., 2008). The use of the oral route to delivery antigens as a mean to generate $Foxp3^+$ iTreg cells has now become a major method of iTreg cell generation *in vivo* because of its simplicity, the effectiveness of these Treg cells *in vitro* and *in vivo* assays, and the fact that concomitant effector T cell generation is inefficient.

Although nTreg cells develop in a highly controlled thymic micro environment, iTreg can be generated in many different settings; they can be generated in homeostatic conditions in mesenteric lymph nodes during the induction of oral tolerance (Coombes et al., 2007a; Mucida et al., 2007a) and migrate to the lamina propria where they are expanded by "gut-resident macrophages" (Hadis et al., 2011) , or they can differentiate in response to microbiota directly in the intestinal lamina propria (Sun et al., 2007a). Even in inflammatory conditions, such as in chronic infections (Curotto de Lafaille et al., 2008) or tumours (Liu et al., 2007b) we can see generation of iTreg.

Our understanding of the different microenvironments of iTreg cell development *in vivo* is still incomplete. We do know though that Treg cells can be easily obtained from CD4 T cells *in vitro* by addiction of TGF β in cultures of plate-bound anti-CD3 and CD28-stimulated naive T cells (Chen et al., 2003), thus, antigen-presenting cells (APCs) were not required for *in vitro* conversion. The importance of TGF β signalling in generating iTreg has been demonstrated also *in vivo* by Mucida et al. (2005).

In addition to the minimal cytokine conditions for iTreg cell development, other micro-environmental factors impair iTreg cell induction, like cytokines that induce the differentiation of other T helper cell types (Pasare and Medzhitov, 2003; Zhou et al., 2009a) or promote Treg conversion, like retinoic acid. Retinoic acid is a metabolite of vitamin A that is well known to enhance Treg conversion both *in vitro* in an APC free setting (Nolting et al., 2009a)

and in vivo. Many different groups has demonstrated the capacity of Retinoic Acid to enhance iTreg conversion, especially in the gut(Sun et al., 2007a) with $CD103^+$ intestinal DCs as the main RA producers(Coombes et al., 2007a). Moreover Retinoic Acid plays a role in the balance between Th17 or Treg induction, inhibiting the IL-6–driven induction of proinflammatory TH17 cells and promoting anti- inflammatory Treg cell differentiation(Mucida et al., 2007a). Retinoic acid has been proved to enhance Treg conversion also in other district such as the skin(Guilliams et al., 2010a).

A number of studies have provided data that tried to shed light on the differences between nTreg and iTreg cells as well as the differences between iTreg cells generated in different ways. The absence of reliable molecular marker that differentiate natural Tregs and induced Tregs made rather complicated the studies in this field. One of the first identified difference between the two lineages is the methylation of CpG motifs in the Foxp3 locus of natural $Foxp3^+$ Tregs that showed complete demethylation within an evolutionary conserved region upstream of exon 1 which is not observable in iTregs(Floess et al., 2007). Further efforts in finding differences between the two lineages have brought to suggest the transcription factor Helios as an nTreg specific marker. Thornton et al. (2010) have observed that Helios is selectively expressed in a subset representing the 70% of mice and human T regs. Moreover in the first days after birth (when nTregs are likely to be the vast majority of Tregs) $Foxp3^+$ Helios- cells are absent and begin to be detectable 12 days after

birth. A more recent paper used a clever experimental setting to distinguish between natural Tregs and iTregs and, taking advantage of Foxp3-DTR mice have been able to study the different properties of the two subsets. Strikingly Haribhai et al. (2011a) demonstrated that nTreg alone are not sufficient for rescue completely Foxp3 deficient mice. Co-transfer of Foxp3 proficient $CD4^{+}Foxp3^{-}$ T cells lead to the differentiation of those cells in iTreg that completely rescued the Foxp3 KO phenotype. Moreover control $CD4^{+}Foxp3^{-}$ T cells from a Foxp3 deficient animal failed to rescue completely those mice and depletion of newly differentiated iTreg abolished their protective effect. Comparison between nTregs and iTregs isolated by the same mice revealed that Helios is not a reliable marker (While all the nTreg are $Helios^{+}$ only a small fraction of iTreg is Helios-) while demethylation of the CpG domains in the Foxp3 gene can be considered a good marker (almost no demethylation detected in iTreg cells). The gene expression profile of the two populations showed huge similarity between iTreg and nTreg while *in vitro* generated iTreg are very dissimilar from iTreg generated in vivo. TCR repertoire analysis showed a very small overlap between the TCR specificity of nTregs compared to iTregs and this could be consistent with the requirement of both lineages for maintaining peripheral tolerance.

OTHER INDUCED REGULATORY CELL SUBSETS: TR1

Besides Foxp3$^+$ Treg cells, there are other types of Treg cells that can be induced from naive T cells in the periphery, such as Type 1 regulatory (Tr1) cells. Tr1 cells are defined for their capacity to secrete IL-10 and TGF-β. They arise *in vivo* from effector cells which lose the typical cytokine profile and become suppressor cells upon chronic antigenic stimulation, for example by repeated intranasal peptide administration (Wan and Flavell, 2006)

Tr1 cells, upon activation via the TCR, produce high amounts of IL-10 but are distinct from Th2 cells since they do not produce IL-4, and produce very low levels of IL-2, which are both potent T cell growth factors. Tr1 cells are inducible cells and for this reason, similar to Th1 and Th2 cells, they arise from naive precursors and can be differentiated both *ex vivo* and *in vivo*. IL-10 is considered the driving force for Tr1 cell generation, as shown by experiments in which antigen-specific murine Tr1 cells can be induced *ex vivo* by repeated TCR stimulation in the presence of high doses of IL-10. IL-10 is therefore not only responsible for the regulatory function of murine Tr1 cells but also it is also fundamental for their differentiation. However, for human Tr1 cells it is now evident that in many experimental settings IL-10 is necessary but probably not sufficient for their differentiation (Battaglia et al., 2006). Tr1 cells are able to suppress colitis in mice (Huber et al., 2011; Wan and Flavell, 2006)

and they can be exploited to induce tolerance to transplants (Battaglia et al., 2006)

SCOPE OF THE THESIS

Induction and maintenance of T cell tolerance towards self-antigens is vital to preventing autoimmunity. To this purpose, many different overlapping and non-overlapping mechanisms of T cell tolerization exist, both at central and peripheral level. In this thesis we wanted to investigate whether DCs in general are able to induce T cell tolerance or it is a prerogative of a specialized subset.

In Chapter 2 we investigated the question with an experimental system where antigen presentation is not confined to a specific DC subpopulation but it is extended to all conventional DCs.

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CHAPTER 1

MIGRATORY AND NOT LYMPHOID-RESIDENT DENDRITIC CELLS MAINTAIN PERIPHERAL SELF-TOLERANCE AND PREVENT AUTOIMMUNITY VIA INDUCTION OF ITREG CELLS

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Migratory and not lymphoid-resident dendritic cells maintain peripheral self-tolerance and prevent autoimmunity via induction of iTreg cells

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Abstract

There is evidence that dendritic cells (DCs) induce peripheral tolerance. Nevertheless, it is not known whether immature DCs in general are able to tolerize $CD4^+$ T cells or if this is a prerogative of specialized subtypes. Here, we show that, when autoantigen presentation is extended to all conventional mouse DCs, immature lymphoid tissue resident DCs are unable to tolerize $CD4^+$ T cells. In contrast, this is a prerogative of steady state migratory DCs. The way they contribute to tolerance development is via the induction of autoantigen-specific regulatory T (iTreg) cell conversion. Since only lymph nodes host migratory DCs, iTreg cells develop solely in lymph nodes, and not in the spleen, and are retained inside the lymph nodes. Mechanistically, in cutaneous lymph nodes, DC-derived CCL22 contributes to the retention of iTreg cells. The importance of the local generation of iTreg cells is emphasized by their essential role in preventing autoimmunity.

Introduction

Induction and maintenance of T cell tolerance towards self-antigens is vital to preventing autoimmunity. To this purpose, many different overlapping and non-overlapping mechanisms of T cell tolerization exist, both at central and peripheral level(Goodnow et al., 2005; Lutz and Kurts, 2009; Mathis and Benoist, 2004; Steinman et al., 2003; Wing and Sakaguchi, 2010).

A common vision of how dendritic cells (DCs) contribute to the induction and maintenance of peripheral $CD4^+$ T cell tolerance is that, in resting conditions, immature DCs, expressing low levels of signal 1 (specificity) and low or no levels of signal 2 (costimulation), are able to induce T cell unresponsiveness.

However, the effective knowledge concerning the contribution of DCs in inducing and maintaining $CD4^+$ T cell tolerance in peripheral lymphoid organs derives from the selective analysis of specific DC subpopulations. In particular, $CD205^+$ DCs are able to induce $CD4^+$ T cell tolerance in conditions of suboptimal activation(Belkaid and Oldenhove, 2008; Curotto de Lafaille and Lafaille, 2009; Kretschmer et al., 2005b; Yamazaki and Steinman, 2009). Moreover, steady state migratory DC (ssmDC) subpopulations from the gut and the skin, phenotypically $CD103^+$ and $CD103^-$ respectively, mediate the

conversion of naïve T cells into Foxp3^{+} regulatory T cells (iTreg) in a retinoic acid (RA)-dependent manner(Coombes et al., 2007b; Guilliams et al., 2010b; Mucida et al., 2007b; Sun et al., 2007b). Since the lymph constantly carries peptides for loading on both migratory and lymphoid tissue resident immature DCs in a dose range suitable for tolerization(Clement et al.), it cannot be excluded that, in addition to the analyzed populations and in agreement with the common vision, all conventional immature DCs can induce autoantigen specific CD4^{+} T cell tolerance in the periphery. Therefore, it was relevant to know whether DCs in general are able to induce T cell tolerance at the steady state or if this is a prerogative of specialized subsets. We investigated this question by using an experimental system where antigen presentation, in contrast to previous studies, is not *a priori* confined to a specific DC subpopulation but is extended to all conventional DC subtypes. Specifically, we adopted the 2a T transgenic animal model(Granucci et al., 1996). In this experimental system, T cell receptor (TCR) transgenic T cells (2a T cells) recognize a portion of the CH3 region (435-451) of the IgG2a^{b}, the Bpep, in association with the MHC molecule, I-A^{d}. We also generated a mouse model in which the Bpep was presented by CD11c^{+} cells that include all conventional DC subtypes. By performing a systematic study of

the behavior of naïve autoantigen-specific T cells after interaction with all conventional $CD11c^+$ DCs in homeostatic conditions we found that DCs are able to induce $CD4^+$ T cell tolerance exclusively by promoting the conversion of autoantigen-specific naïve T cells into iTreg cells. Among the different DC subtypes, ssmDCs possess unique ability to induce antigen-specific iTreg cells in a RA-dependent manner. Diversely, lymphoid tissue resident DCs are not able to induce T cell tolerance. Therefore, iTreg cells develop solely in lymph nodes and not in the spleen, which does not host the migratory DC subtype. We also show that iTreg cells that are newly generated in lymph nodes, do not re-circulate but are retained inside the lymph nodes. Specifically, in cutaneous lymph nodes (CLNs) CCL22, produced by CLN DCs, contributes to the retention of iTreg cells that all express the CCL22 receptor, CCR4.

Protection from autoimmunity is assured although the exclusive regional nature of the tolerization process controlled by DCs, as assessed using the Herpes Stromal Keratitis (HSK) autoimmunity model.

Material and Methods

Generation of DC-tg mice

The expression cassette used for the generation of the DC-tg mice was generated in our laboratory: a 1000 bp EcoRI-DraIII fragment containing the external domain of I-A^d β chain fused to the Bpep coding sequence was amplified by PCR; Dra III-EcoRI fragment containing intracellular and transmembrane domain of I-A^d β chain was amplified by RT-PCR using RNA obtained from B-blasts as template; the two fragments were digested with DraIII (Promega), ligated and the resulting fragment was cloned into EcoRI site of a previously generated pBS vector containing the β-globin cassette under the control of the CD11c promoter; the resulting vector was used to obtain the DC-tg trasgenic animals.

Transgenic mice were generated as described(Kollias et al., 1986). In brief, CBA x C57/Bl6 oocytes were microinjected with the genetic material, implanted into pseudopregnant females and transgenic founder animals were obtained. To assess for the presence of the transgene, 2mm of tail was digested in 200 μl of digestion buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 0.1 mg/mL gelatin, 0.45% Np40, 0.45% Tween20, 30 μg proteinase K) o.n. at 56 °C followed by incubation at 95 °C for 30 min. The presence of the transgene was verified by PCR using 2 ml of the extract as template and the following primer pairs:

DCtgF (5'-CTCAGAGTACAAAAGAGCACTTGG-3') and

DCtgR (5'-TCCACATGGCAGGTGTAGAC-3') (Primm Srl).

Mice

2a T BALB/c Rag-2–deficient mice (2a T mice) expressing the transgenic TCR recognizing the 435–451 peptide (Bpep) in the CH3 region of $IgG2a^b$ in association with $I\text{-}A^d$ have been generated in our laboratory as described elsewhere(Raimondi et al., 2006). DC-tg mice were backcrossed on the BALB/c Rag-2-deficient background for at least 10 generations. BALB/c and congenic CB-17 mice (expressing the $IgG2a^b$) were obtained from Harlan Italy. All the animals were maintained in specific pathogen-free conditions. All experiments were carried out in accordance with the relevant laws and institutional guidelines.

In vitro Bpep presentation assay

B5HII39 (B5) hybridoma cells(Bartnes et al., 1993) were grown in IMDM supplemented with 2 mM L-Glutamine, 100 U/ml Penicillin, 100 mg/ml Streptomycin, 10% heat inactivated FBS (all EuroClone) and 50 μM 2-Mercaptoethanol (Sigma-Aldrich).

For the Bpep presentation assay, $CD11c^-$ cells, DCs ($CD11c^+$) and macrophages ($F4/80^+$, $CD11b^+$, $CD11c^-$) were sorted from the spleen and peripheral lymph nodes of DC-tg mice using MoFlo cell sorter (purity >98%); graded cell numbers were co-coltured with 10^5 B5 cells in 200 μl of complete medium. After 24 hours, supernatants were collected and IL-2 concentration measured using mouse IL-2 duoset ELISA (R&D systems).

Cell preparation and adoptive transfer

To obtain naive TCR-Tg anti-IgG2a^b T cells (2a T cells) the spleen and inguinal, axillary, maxillary, brachial, mesenteric lymph nodes were collected from of 2a T mice. Single cell suspensions were prepared and red blood cells were lysed incubating spleen cells in 5 ml of RBC lysis buffer (8.29 g/l NH_4Cl, 0.037 g/l tetrasodic EDTA, 1 g/l $KHCO_3$, pH 7.3) for 5 minutes on ice. Cell preparations were resuspended in IMDM supplemented with 10% FBS and plated in cell culture treated plates. After 1 hour, cells in suspension were collected, washed extensively in PBS and purity of 2a T cell preparation was evaluated by flow cytomery. DC-tg mice were i.v. injected with 10^5 2a T cells/200 µl PBS.

To obtain non-lymphopenic recipients, total Vβ14$^-$ T cells were purified from the spleen of CB-17 mice. Single cell suspensions were prepared and red blood cells were lysed. The cells were then incubated with biotinylated anti-Vβ14, anti-CD11c, anti-B220, anti-CD19, anti DX5 anti-CD11b, and anti-GR1 Abs (20 µg/ml, all from BD biosciences) 20 min. on ice, washed in PBS and incubated with streptavidin MicroBeads (Miltenyi Biotec). Labeled cells were negatively selected on LS MACS separation columns according to manufacturer's instructions. DC-tg mice were i.v. injected with 10^7 CD4$^+$ cells/200 ml PBS. Two weeks later, blood samples were collected and the presence of CD4$^+$Vβ14$^-$ in the blood was evaluated by flow cytometry: animals were used as non-lymphopenic recipients when the percentage of CD4$^+$ cells was >5%. 2a T cells were adoptively transferred in these mice as described above.

Flow cytometry

Blood samples (50 µl) and single-cell suspensions of 1 x 10^6 splenocytes or lymph node cells were pelleted and resuspended with the appropriate amount of Ab in 200 µl of PBS, and incubated for 20 min on ice in the dark. The cells were than washed once with 1 ml of PBS. When required, secondary reagent incubation in 100 µl of PerCP-Cy5.5-conjugated streptavidin (diluted 1/500; Sigma-Aldrich) was performed for 15 min on ice in the dark. For FACS analysis, the following Abs were used: anti -CD4-FITC, -PerCP, -Cy5.5 or -PE (RM4-5), anti-CD8α-PE (53-6.7), anti-CD25-PE or -biotin (7D4), anti-Vβ14-FITC or -biotin (14-2) were from BD Bioscience; anti-CD25-APC (7D4) was from Southern Biotech; anti-CCR4-PE (2G12) was from eBioscience. Intracellular detection of Foxp3 was performed using PE- anti mouse/Rat Foxp3 staining set (clone FJK16s, eBioscience). CFSE cell labeling (Invitrogen) was performed following manifacturer's instructions. Data were acquired using a BD FACScalibur and analyzed with CellQuest or Flowjo softwares (BD Biosciences).

Reagents

LE540 (Wako Chemicals) was resuspended in DMSO and stored -20°C; aliquots were diluted in PBS and 100µg/200 µl PBS 5%DMSO were i.p. injected every two days for 20 days. Concerning the CCR4 inhibitor (AF399/42018025), 1,5 µg/200 µl PBS were i.v. injected three times every second day starting 18 days after the adoptive transfer. For all of the compounds, analysis was performed 24 hours after the last treatment.

Suppression assay and in vitro restimulation

Responder naive polyclonal T cells were positively selected from the spleen of BALB/c mice using anti-CD4 microbeads and MACS LS columns (Miltenyi Biotec). Cells were labeled with 1 μM CFSE. Splenocytes were obtained from the spleen of BALB/c mice and were depleted of T cells using biotinilated anti-CD3ε and anti-TCRβ-chain antibodies (BD), streptavidin-microbeads and MACS LS columns (Miltenyi Biotec).

To analyze the suppressive capacity, cells were recovered from the spleen or lymph nodes of transferred animals and were split in two samples: one of these was depleted of $CD25^+$ cells using biotinilated anti-CD25 antibody (BD) and magnetic streptavidin microbeads (Dynal). Then, $CD4^+$ T cells were positively purified using anti-CD4 microbeads and MACS MS columns (Miltenyi Biotec).

Responder CFSE labeled T cells were co-coltured with putative Tregs at a 1:1 ratio and splenocytes in 96-well round bottom plate in complete medium supplemented with 0,6 μg/ml of purified anti-CD3ε (145.2C11). Proliferation of CFSE-labeled cells was assessed by flow cytometry after 72 hr of colture.

In vitro 2a T restimulation

To assess the functionality of 2a T cells after the primary response, T cells recovered from the spleen or lymph nodes of transferred mice were purified as illustrated in the previous paragraph and co-coltured with splenocytes depleted of T cells as described above either loaded or not with 1 μg/ml of Bpep. After 48 or 72 hr, supernatants were

collected and IFNγ production was evaluated by ELISA using BD OptiEIA mouse-IFNγ kit.

In vivo proliferation assay

Purified 2a T cells ($5x10^5$) were CFSE labeled and i.v. injected in the animals. After 48 or 72 hr spleen and CLNs of injected animals were collected and proliferation of CFSE-labeled cells was assessed by flow cytometry, on gated $CD4^+V\beta14^+$ cells.

In vitro conversion assay

2a T cells were sorted by negative selection of $CD11c^+$, $B220^+$, $DX5^+$, $CD11b^+$ cells from a pool of spleen and lymph node single cell suspensions. For the purification of CLN resident DCs (MHC class $II^{low}CD11c^+$), ssmDCs (MHC class $II^{high}CD11c^+$), and spleen resident DCs (MHC class $II^{low/int}CD11c^+$) from BALB/c mice, spleens and lymph nodes were pooled, T and B cells were depleted using biotinilated anti-CD19 and anti CD3ε monoclonal antibodies (BD bioscience), streptavidin microbeads and MACS LS columns (Miltenyi Biotec). The negative fraction was then sorted with the MoFlo high speed sorting (purity > 98%). Propidium iodide positive cells were excluded. 2a T cells (10^5) and MHC class $II^{low/high}CD11c^+$ cells ($2x10^4$) were cocultured in 96 well plates in a final volume of 200 µl of complete medium in presence of Bpep (1 µg/ml). In some experiments 1µg LE540 (Wako) and 3 ng/ml rhTGF-β1 and 3 ng/ml

TGF-β2 were added. After 5 days, cells were collected and analyzed for the expression of Foxp3 and CD25.

Microarray

$CD11c^+$ cells were sorted from the spleen or lymph nodes of BALB/c mice using MoFlo (purity> 98%). Total RNA was extracted by the double extraction protocol: RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction (TRIzol Invitrogen) followed by a Qiagen RNeasy clean-up procedure

Because of the small amount of the cells, 20 ng of Qiagen RNA carrier was used in every extraction. Total RNA concentration was calculated by Quant-IT RiboGreen (Invitrogen). Total RNA integrity was assessed by Agilent Bioanalyzer (Pico kit) and the RNA Integrity Number (RIN) was calculated. Only high-quality RNA preparations, with RIN greater than 8.4, were used for microarray analysis.

Biotinylated cDNA was prepared from 2ng of total RNA and fragmented according to the Nugen protocol (WT-Ovation Pico RNA Amplification System). Five micrograms of biotinylated cRNA were hybridized to the Affymetryx GeneChip Mouse Genome 430A 2.0 arrays. Data handling was mainly done using Bioconductor 2.6 (Gentleman et al., 2004; Pelizzola et al., 2006). The Robust Multichip Average (RMA) (Irizarry et al., 2003), method was employed to calculate probe set intensity. To filter out noisy data before the selection of differentially expressed genes a filter was applied based on a Inter Quantile Range (IQR) greater than 0.25. The identification of differentially expressed genes was addressed using Linear Models

for Microarray Data (LIMMA) (Smyth, 2004) and a discovery rate correction of the p-value (Benjamini-Hochberg). The corrected p-value selected in the analysis is p.value less or equal to 0.01.

Ocular infection and scoring of HSK.

Corneas of mice were scarified using a sterile gauge needle before infection with HSV-1 (5x104 PFU) in the right eye and disease severity was scored on different days after infection as described(Panoutsakopoulou et al., 2001) based on the degree of corneal opacity: ≤25% of cornea, 1; ≤50%, 2; ≤75%, 3; ≤100%, 4.

Statistical analysis

Means were compared by paired or unpaired t- tests. Data are expressed and plotted as means ± SE values. Statistical significance was defined as: *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$. Sample sizes for each experimental condition are provided in the figures and the respective legends.

Results

Experimental model

In order to obtain an animal model in which all of the DC subpopulations present the selected class II restricted self-peptide, a transgenic mouse expressing the Bpep covalently linked to the β chain of the I-A^d molecule under the control of the CD11c promoter was produced, (DC-tg animals, Figure S1). In agreement with previous results(Brocker, 1997; Brocker et al., 1997), CD11c regulatory region limited Bpep presentation to conventional DCs (Figure S2A).

Bpep presentation occurred efficiently *in vivo* in secondary lymphoid organs. Initially, we focused our analysis on two secondary lymphoid organs clearly described to be populated by different DC subtypes. Namely the spleen, exclusively populated by lymphoid tissue resident DC subsets which arrive there as precursors by a hematogenous route, and cutaneous lymph nodes (CLNs), in which, diversely from the spleen, ssmDCs represent an abundant population(Jakubzick et al., 2008; Lutz and Kurts, 2009). Subsequently, the analysis was extended to other districts. Adoptive transfer of CFSE labeled naïve anti-Bpep TCR transgenic T cells (2a T cells) into DC-tg mice showed a comparable antigen-driven expansion between spleen and CLNs

(brachial, inguinal and axillary), (Figure 1A). Homeostatic proliferation in Rag2-deficient recipients was negligible (Figure 1A).

Conversion of naïve T cells into iTreg cells occurs in the lymph nodes and not in the spleen

To investigate whether $CD11c^+$ DCs presenting a self-peptide, in homeostatic conditions, were able to tolerize naïve antigen-specific $CD4^+$ T cells in lymph node and spleen, the fate of 2a T cells after the encounter of antigen presenting DCs was investigated. Naïve ($CD44^{low}$, data not shown) $CD4^+V\beta14^+CD25^-$ 2a T cells sorted from Rag2-deficient TCR transgenic mice were transferred into DC-tg or non-transgenic mice as a control. As shown in Figure 1B all the naïve transferred cells were also Foxp3-negative. Cell behavior was followed over time (Figure 1C). We initially used DC-tg mice on Rag2-deficient background as recipients to formally exclude some predictable complications of the system: i) the possibility that anti-Bpep Treg cells, originated in the thymus (data not shown) due to the forced presentation of the Bpep by DCs(Atibalentja et al., 2009), could suppress 2a T cell responses immediately after transfer; ii) the possibility that the endogenous $IgG2a^a$, whose 435-451 sequence

(Apep) is very close to the Bpep and could be presented by B cells, could bias the results by leading to a suboptimal activation or to the inactivation of 2a T cells.

During the first two weeks after transfer, a robust expansion of 2a T cells was observed in spleen, lymph nodes and blood of DC-tg mice. Following a slight decrease, the frequency of 2a T cells reached a plateau in the blood and both types of peripheral lymphoid organs (Figure 1C). Moreover, IFNγ serum levels could be detected starting from 4 days after the transfer with a peak at Day 7 (Figure 1C). The expression of surface markers, including CD44 and CD25, indicated a robust antigen-specific T cell response (Figure 1D). CD25 was transiently upregulated on 2a T cells in CLNs and spleen very early after transfer (3-5 days). Subsequently, it was completely downregulated in the spleen, while a discrete population of 2a T cells maintained CD25 expression over time in CLNs (Figure 1D). Interestingly, a significant percentage of these cells also expressed the Treg cell marker, Foxp3 (Figure 1D). We then analyzed different districts. $CD25^{+}Foxp3^{+}$ cell differentiation was not limited to CLNs but was apparent in all the different types of lymph node analyzed. In mesenteric lymph nodes, the efficiency of $Foxp3^{+}$ cell generation was

comparable with previous data (Coombes et al., 2007b; Sun et al., 2007b) (Figure S3). To determine whether $CD25^{+}Foxp3^{+}$ 2a T cells present in CLNs had, indeed, regulatory properties, their suppressive activity was investigated. *In vitro*, 2a T cells recovered from CLNs of DC-tg mice 4 weeks after transfer were able to suppress anti-CD3 induced polyclonal T cell proliferation and antigen-induced IFNγ production (Figure 2A, B). This activity was entirely due to the $CD25^{+}$ cells, since it was completely abolished upon $CD25^{+}$ cell depletion. In contrast, 2a T cells recovered from the spleen did not display any regulatory activity (Figure 2A) and fully responded to restimulation (Figure 2B). Similar results were obtained after the transfer of naïve polyclonal BALB/c $CD4^{+}$ T cells: only T cells from spleen and not T cell from lymph nodes could be restimulated in vitro in response to the Bpep (Figure 2C). This indicated that tolerance was not achieved in the spleen but only in the lymph nodes.

In a kinetic analysis, we determined that $CD25^{+}Foxp3^{+}$ 2a T cells first appeared in CLNs around 5 days after transfer and eventually persisted (Figure 2D). Diversely, in the spleen we could only document the transient appearance of a $CD25^{int}Foxp3^{low}$ 2a T cell population (Figure 2D), which did not show suppressive activity.

Indeed when the spleen cells were re-challenged *in vitro* with the Bpep, 10 days after transfer, the presence of $CD25^+$ T lymphocytes did not influence the production of IFNγ by 2a T cells. In contrast, $CD25^+FoxP3^+$ cells of CLNs showed suppressive activity since their first appearance (Figure 2E).

These *in vitro* results suggested that induction of Treg cells specifically occurred in CLNs and not in the spleen. An *in vivo* analysis confirmed this split situation. Indeed, CFSE labelled 2a T cells, injected into DC-tg animals four weeks after the first 2a T cell transfer, proliferated in the spleen but not in CLNs (Figure 2F). The CLN inhibitory activity could be removed by inhibiting $CD25^+$ cells, indicating that these cells were responsible for the suppression. Thus, in agreement with Sun et al.(Sun et al., 2007b), we found that splenic DCs were clearly inefficient in inducing iTreg cell conversion, in contrast to what happens in the CLNs.

The lack of iTreg cell generation in the spleen was not due to the lymphopenic environment. iTreg cells did not differentiate in the spleen under non-lymphopenic conditions as well (Figure S4). On the other hand, a more efficient generation of iTreg cells was observed in

CLNs of non-lymphopenic hosts (Figure S4), suggesting that T cells can influence the efficiency of DCs to induce iTreg cell conversion.

ssmDCs have the exclusive capacity to induce iTreg cell conversion via RA production

The observed results posed the questions why iTreg cells differentiate only in lymph nodes.

To investigate the first question, we reasoned that the major difference between the spleen and lymph nodes could be represented by ssmDCs. This DC subpopulation could, therefore, be responsible for the conversion of iTreg cells. To explore this possibility, migratory and resident DCs(Dakic et al., 2004; Kissenpfennig et al., 2005) from CLNs and spleen DCs all purified from wild type BALB/c mice were co-cultured with naïve 2a T cells in the presence of the Bpep. iTreg cell conversion was then evaluated. Only when the antigen was presented by CLN migratory DCs, a fraction of 2a T cells converted into iTreg cells (Figure 3A). As control, the addition of TGFβ rendered spleen DCs and CLN resident DCs capable of inducing iTreg cells (Figure 3A).

It has been shown that migratory CLN DCs express the aldehyde

dehydrogenase, RALDH2(Guilliams et al.), which catalyzes the synthesis of RA from retinaldehyde. RA, the active derivative of vitamin A (retinol), has been extensively shown to be one of the active molecules responsible, either directly(Nolting et al., 2009b) or indirectly(Hill et al., 2008), for the conversion of iTreg cells in mesenteric and CLNs(Coombes et al., 2007b; Iwata et al., 2004; Sun et al., 2007b). Therefore, we predicted that migratory DCs could induce iTreg conversion in a RA-dependent manner. Accordingly, we observed that treatment with the RA receptor (RAR) inhibitor, LE540,(Li et al., 1999) significantly abrogated the iTreg cell conversion (Figure 3A).

Based on these *in vitro* results, we investigated whether RA was involved also *in vivo* in the conversion of antigen-specific $FoxP3^{+}CD25^{+}$ T cells. DC-tg mice were transferred with naive 2a T cells and systemically treated for three weeks with LE540. As shown in Figure 3B a significant reduction in the percentage of iTreg cells was observed in CLNs of treated recipients. Since RALDH2 expression is restricted to migratory DCs(Grainger et al.; Guilliams et al.), together these results indicate that migratory and not lymphoid tissue resident DCs are able to induce iTreg cell conversion under steady state conditions with a mechanism dependent on RA.

CCL22 contributes to the retention of iTreg cells in CLNs

A second question to answer was why newly generated iTregs persist only within lymph nodes. As matter of fact the absence of differentiated iTregs in the spleen and circulation (data not shown) suggested that these iTregs do not re-circulate among different lymphoid organs.

To investigate this question, we analyzed the differences between $CD11c^+$ cells of CLNs and spleen from resting wild type BALB/c animals by performing a comparative global gene expression analysis. Genes showing a fold change of at least three in the level of expression in lymph node versus spleen DCs were considered differentially expressed. The total list of differentially expressed genes is shown in supplementary Table 1. Strikingly, *Aldh1a2* (coding RALDH2) resulted on the top of the list of genes specifically expressed in lymph nodes DCs, thus substantiating our finding that exclusively migratory DCs expressing RALDH2 were involved in the conversion of antigen-specific iTreg cells.

The other gene that was far more expressed in lymph nodes compared to spleen was *Ccl22*. We, thus, focused our attention on CCL22, a chemokine whose receptor, CCR4, is particularly highly expressed in Treg cells(Iellem et al., 2001), and we hypothesized that CCL22 was

involved in the homing of iTreg cells in CLNs.

We first confirmed CCR4 expression by $CD4^+V\beta14^+CD25^+Foxp3^+$ 2a T cells (Figure 3C). Secondly, we tested the effect exerted by a CCR4 antagonist on Treg cell homing. DC-tg mice adoptively transferred with 2a T cells were systemically treated with the potent CCR4 antagonist, AF399/42018025(Bayry et al., 2008), to breakdown the possible CCL22-mediated mechanism of retention, and Treg cells enumerated three days later. As shown in Figure 3D, a strong reduction in the percentage of $Foxp3^+CD25^+$ among $CD4^+V\beta14^+$ cells was observed in CLNs. Notably, ssmDCs from CLNs were able to induce CCR4 expression on the converted iTreg 2a T cells *in vitro* (Figure 3E). The converted iTreg cells obtained *in vitro* after DC exposure were the only T cell population that was expressing CCR4, indicating that ssmDCs have not only the exclusive capacity to induce iTreg cell conversion but also the exclusive capacity to induce CCR4 expression. This is a further indication of the homing function of this receptor. Overall these results indicate that DC-derived CCL22 contributes to the retention of iTreg cells in CLNs.

Newly generated iTreg cells protect from autoimmunity

To investigate whether newly generated iTreg cells in DC-tg mice could protect from autoimmunity, we took advantage of the HSK model. HSK is a $CD4^+$ T cell-mediated autoimmune disease of the eye that can be induced in susceptible mice by ocular infection with HSV-1(Streilein et al., 1997). It has been proven that $CD4^+$ T cells responsible for HSK cross-recognize a corneal antigen, the $IgG2a^b$ Bpep and a peptide of the viral UL6 protein(Zhao et al., 1998). Ighb animals, such as CB-17 mice, are resistant to HSK and their resistance has been attributed to the tolerant state of T cells versus the Bpep. Conversely, BALB/c (Igha) mice are susceptible to the disease since keratogenic T anti-$IgG2a^b$ T cells are not tolerized due to the absence of the Bpep in this strain. We reasoned that DC-tg mice, once reconstituted with polyclonal naive BALB/c $CD4^+$ T cells containing keratogenic T cells, should develop resistance to HSK few weeks after T cell transfer, concomitantly with iTreg cell conversion. Therefore, we transferred polyclonal naive BALB/c $CD4^+$ T cells into DC-tg *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$ mice. We then infected the recipient mice with HSV-1 (strain KOS) at two different time points: three days after transfer, at a time when iTreg cells had not yet generated, and three weeks after transfer, thus after iTreg cell conversion. HSK incidence

and severity were scored 7, 10 and 14 days after ocular infection. As shown in Figure 3F, in agreement with the prediction, DC-tg mice were almost totally protected form HSK if ocularly infected with HSV three weeks after T cell transfer. In contrast, recipient mice infected with HSV three days after T cell transfer showed a higher HSK susceptibility compared to *Rag2*$^{-/-}$ recipients, indicating a hyper-reactivity of anti-Bpep T cells, consistent with a priming effect. These results indicate that tolerance induced locally by migratory DCs confers protection from autoimmunity.

Discussion

By using an experimental system in which antigen presentation is not confined to a particular DC subset but is extended to all $CD11c^+$ DCs, we have demonstrated that lymphoid tissue resident DCs are not able to tolerize T cells. Migratory DCs have, instead, the privileged capacity to induce self-antigen-specific iTreg cell conversion and autoimmunity protection. This ssmDC function is RA-mediated.

Since the only DCs able to convert naïve T cells into iTreg cells come from non-lymphoid tissues we can hypothesize that immature DCs are not generally able to tolerize T cells by default (simply because of the absence of signal-2, the co-stimulation) but need to receive a specific conditioning. At the steady state the appropriate signals that render DC tolerogenic (or alternatively activated(Pulendran et al., 2010)) may be delivered by the organs/tissues where migratory DCs reside. For instance tissue-specific stimuli could induce the expression of RALDH2 essential for RA synthesis. In the intestine it has been proposed that GM-CSF, presumably produced by lamina propria macrophages or eosinophil-like cells, and RA, produced by $ALDH1A1^+$ intestinal epithelial cells from dietary vitamin A or blood-derived retinol, may represent the tissue-derived stimuli that elicit Aldh1a2 expression by migratory DCs(Guilliams et al., 2010b;

Yokota et al., 2009b). The same environmental factors may be also responsible for DC conditioning in the skin.

In addition to endogenous signals, exogenous signals can alternatively activate DCs. A well-characterized exogenous signal is the anti-DEC205(Daniel et al., 2011; Yamazaki et al., 2008), that leads to a suboptimal DC activation(Kretschmer et al., 2005b). Targeting the antigens to DCs via the CD205 receptor is a very efficient system for antigen-specific iTreg cell induction and autoimmunity protection(Daniel et al., 2011; Steinman et al., 2003). Other exogenous signals may be some Toll like receptor (TLR) stimuli, such as TLR2 agonists(Manicassamy et al., 2009).

Given that among the conventional DCs only ssmDCs are able to induce tolerance in the periphery *via* iTreg cell generation, and that ssmDCs constantly deliver tissue-sequestered antigens to draining lymph nodes, we can predict that DCs are specialized to induce tolerance to tissue-sequestered antigens. This hypothesis is substantiated by the recent finding that iTreg cells have a T cell receptor (TCR) repertoire that only partially overlaps with the repertoire of nTreg cells(Haribhai et al., 2011b). Moreover, the two Treg cell subsets have very similar effector mechanisms but are functionally complementary and non-redundant(Haribhai et al.,

2011b), indicating that their major difference resides in their TCR diversity. Therefore, the multiple peptides that drive the differentiation of nTreg cells in the thymus should partially differ from the peptides that drive the generation of iTreg cells in the periphery. The peptides that most likely never reach the thymus are exactly tissue-sequestered antigens. ssmDCs maybe the cells that contribute to the diversification of the antigen-specificity of iTreg cells compared to nTreg cells since they are the cells that present tissue-sequestered antigens to the adaptive immune system to prevent autoimmune reactions. Nevertheless, there is also a certain overlapping in the specificities of the two Treg cell subtypes. Therefore, we can hypothesize that other cells, in addition to conventional DCs, contribute to the conversion of Treg cells in peripheral lymphoid organs. The repertoire of peptide presented by these additional cells, including for instance plasmacytoid DCs, may not differ from the repertoire of peptide presented in the thymus. We predict that, if iTreg cells differentiate in the spleen (thanks to cells different from conventional DCs), their TCR repertoire could in large part overlap with the repertoire of nTreg cells.

In this view, it is important that the iTreg cells induced locally by DCs do not recirculate but remain localized in the lymph nodes. Since their

specificity may be directed to tissue-sequestered antigens, their regulatory activity may be locally required. The possibility that the newly generated iTreg cells not only home in the lymph node but also in the organ/tissue where ssmDCs originate should be also taken into account. A clear drop in the percent of iTreg cells in the lymph nodes is, indeed, observable very soon after generation (Figure 2D). As DC-induced iTreg cells are not found in circulation, this observation is compatible with a re-localization of these cells into the non-lymphoid tissue. Leukocyte trafficking is critically regulated by chemokines and Treg cells have been previously shown to express CCR4. We have found that ssmDCs from CLNs specifically induce CCR4 expression by iTreg cells. Therefore their localization in CLNs and possibly the skin is regulated by the abundant CCL22 (CCR4 ligand) production by lymph node DCs(Vulcano et al., 2001) and keratinocytes(Rot and von Andrian, 2004b).

Interestingly the small number of iTreg cells generated in the lymph nodes is sufficient to protect from autoimmunity. In the HSK experimental system, mice are totally protected from disease if the induction is performed when Treg cells have fully differentiated. In contrast, DC-tg recipient mice are strongly susceptible to the disease if viral infection is performed early after T cell transfer, when Treg cells

have not differentiated yet but keratogenic T cells have been primed by Bpep-presenting DCs.

In conclusion, some tissue-sequestered antigens may escape the penalties of thymus exclusion for specific tolerance induction, by using RALDH2^{+} migratory DCs. These cells function as antigen shuttles to tissue draining lymph nodes that become, in this way, additional and exclusive sites of iTreg cell conversion in non-inflammatory conditions. In this scenario, the retention of ssmDC-induced iTreg cells at the site of conversion is consistent with their function to keep at bay autoreactive T cells where the cognate antigen is preferentially transported.

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Figure legends

Figure 1 Generation of $CD25^+Foxp3^+$ 2a T cells in CLNs and not in the spleen. (A) Expansion of 2a T cells in the spleen and CLNs of DC-tg Rag2-deficient or Rag2-deficient mice at the indicated time points after transfer. The percentage of divided cells is shown. (B) Flow cytometry of CD25 and FoxP3 expression on 2a T cells before transfer. (C) Kinetic of 2a T cell expansion in spleen, CLNs (left panel) and blood (middle panel) of DC-tg Rag2-deficient or control mice at the indicated time points after transfer. Right panel, IFNγ serum levels measured at the indicated days after transfer in DC-tg Rag2-deficient or control mice. (D) Upper panels, kinetic of CD44 and CD25 expression by 2a T cells in spleen and CLNs of DC-tg Rag2-deficient recipient mice at the indicated time points after transfer; lower panels, CD25 and Foxp3 expression by 2a T cells ($CD4^+V\beta14^+$ cells) in CLNs and spleen three weeks after transfer in DC-tg Rag2-deficient mice. Data shown in C and D are representative of at least three independent experiments performed using at least three mice per group.

Figure 2 $CD25^+Foxp3^+$ 2a T cells generated in CLNs have suppressing activity *in vitro* and *in vivo*. (A) $CD25^+$ 2a T cells recovered from CLNs of DC-tg mice inhibit mitogen-induced proliferation of polyclonal T cells. CFSE-labeled polyclonal T cells were stimulated with an anti-CD3ε monoclonal antibody and syngeneic splenocytes in presence or not of 2a T cells recovered from spleen or CLNs of DC-tg mice four weeks after transfer. Polyclonal T

cell proliferation was assessed by flow cytometry 72 hours later. Where indicated, recovered 2a T cells were depleted of the $CD25^+$ population ($CD25^-$ 2a T cells, left panels; $CD25^-$, right panel). Left panels, histograms of CFSE labeled polyclonal T cell proliferation. Right panel, quantitative analysis of the frequency of undivided cells calculated on three independent experiments. Data represent means and SDs. (B) IFNγ production by 2a T cells, recovered from CLNs or spleen of DC-tg Rag2-deficient mice three weeks after transfer, upon *in vitro* re-stimulation in the presence of splenocytes loaded or not with the Bpep. Where indicated, 2a T cells recovered from CLNs were depleted of the $CD25^+$ population ($CD25^-$CLN). The activation of naïve 2a T cells is also shown. (C) IFNγ production by polyclonal BALB/c T cells, recovered from CLNs or spleen of Rag2-deficient and DC-tg Rag2-deficient mice three weeks after transfer, upon *in vitro* re-stimulation in the presence of splenocytes loaded or not with the Bpep. (D) Kinetic of $CD25^+Foxp3^+$ 2a T cells generation in CLNs and spleen of DC-tg Rag2-deficient mice. (E) IFNγ production by 2a T cells, recovered from CLNs or spleen of DC-tg Rag2-deficient mice one week after transfer, upon *in vitro* re-stimulation in presence of splenocytes loaded with the Bpep. Where indicated, 2a T cells recovered from CLNs and spleen were depleted of the $CD25^+$ population ($CD25^-$). All the experiments were repeated three times with similar results. (F) Upper diagram, schematic representation of the adoptive transfer experiment. DC-tg RAG2-deficient mice were first transferred with naïve 2a T cells; three weeks later reconstituted mice were either non-treated (NT) or treated with the anti-CD25 blocking antibody, PC61 (labeled as αCD25), and one week later

transferred with CFSE-labeled 2a T cells. Lower left panels, *in vivo* proliferation of CFSE-labeled 2a T cells measured 48 hours after transfer. Lower right panel, quantitative analysis of the frequency of undivided cells. Data represent means and SDs of four mice analyzed in two independent experiments.

Figure 3 Migratory DCs are responsible for iTreg cell conversion. (A) *In vitro* differentiation of $CD25^{+}Foxp3^{+}$ 2a T cells induced by migratory (MHC class $II^{high}CD11c^{+}$, ssmDCs), CLN resident (MHC class $II^{low}CD11c^{+}$, CLN res DCs) or spleen resident (MHC class $II^{low/int}CD11c^{+}$, spleen DCs) DCs from wild type BALB/c mice loaded with the Bpep. Where indicated the RAR inhibitor, LE540, and TGFβ have been added to the co-cultures. The experiment has been repeated three times with similar results. (B) Inhibition of $CD25^{+}Foxp3^{+}$ 2a T cell generation *in vivo* by the LE540. DC-tg Rag2-deficient animals were adoptively transferred with naïve 2a T cells and treated every second day with the inhibitors for 3 weeks. The percentage of $CD25^{+}Foxp3^{+}$ 2a T cells on the total 2a T cells population was determined by cytofluorimetric analysis three weeks after transfer. (C) Flow cytometry of the CCR4 expression by the indicated populations of 2a T cells two weeks after transfer in DC-tg Rag2-deficient mice in lymph nodes. (D) Reduction in the frequency of $CD25^{+}Foxp3^{+}$ 2a T cells in the lymph nodes of transferred mice after treatment with the CCR4 inhibitor, AF399/42018025. DC-tg Rag2-deficient mice were transferred with naïve 2a T cells and treated with the CCR4 inhibitor. The frequency of $CD25^{+}Foxp3^{+}$ 2a T cells on total 2a T cells was then assessed by flow cytometry. NT, non-treated mice. (E) Flow

cytometric analysis of CCR4 expression by $CD25^{+}Foxp3^{+}$ 2a T cells generated *in vitro* as described in A. CCR4 mean fluorescence intensity (MFI) on $CD25^{+}Foxp3^{+}$ and $CD25^{-}Foxp3^{-}$ 2a T cells after co-culture with ssmDCs from BALB/c mice in presence of the Bpep in shown. The experiment was repeated twice with similar results. (F) HSK susceptibility of DC-tg Rag2-/- mice at Day 3 and 21 after polyclonal T cell transfer. DC-tg Rag2-/- and Rag2-/- mice were transferred with 106 polyclonal naïve CD4+ T cells from BALB/c animals. At the indicated days after T cell transfer recipient mice were infected with HSV-1 and incidence and severity of the disease scored on days 7, 10 and 14 after treatment. Data represent means and standard deviations of 5 mice per group.

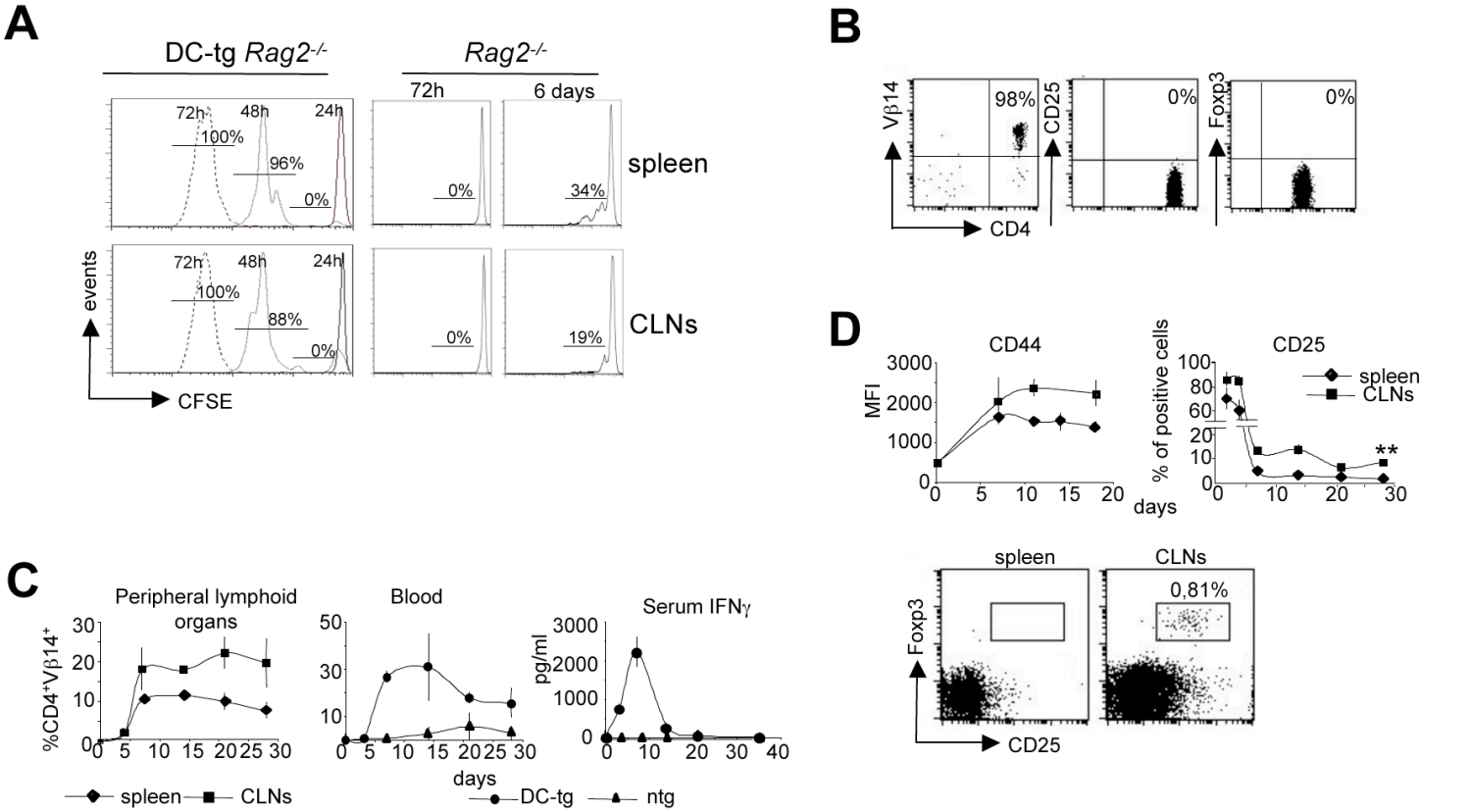

A
DC-tg Rag2-/-
Rag2-/-
72h
6 days
72h
48h
24h
100%
96%
0%
0%
34%
spleen
events
88%
19%
CLNs
CFSE
B
Vβ14
98%
CD25
0%
Foxp3
0%
CD4
C
Peripheral lymphoid organs
Blood
Serum IFNγ
%CD4+Vβ14+
pg/ml
days
spleen
CLNs
DC-tg
ntg
D
CD44
MFI
CD25
% of positive cells
spleen
CLNs
**
days
spleen
CLNs
0,81%
Foxp3
CD25

Figure 1

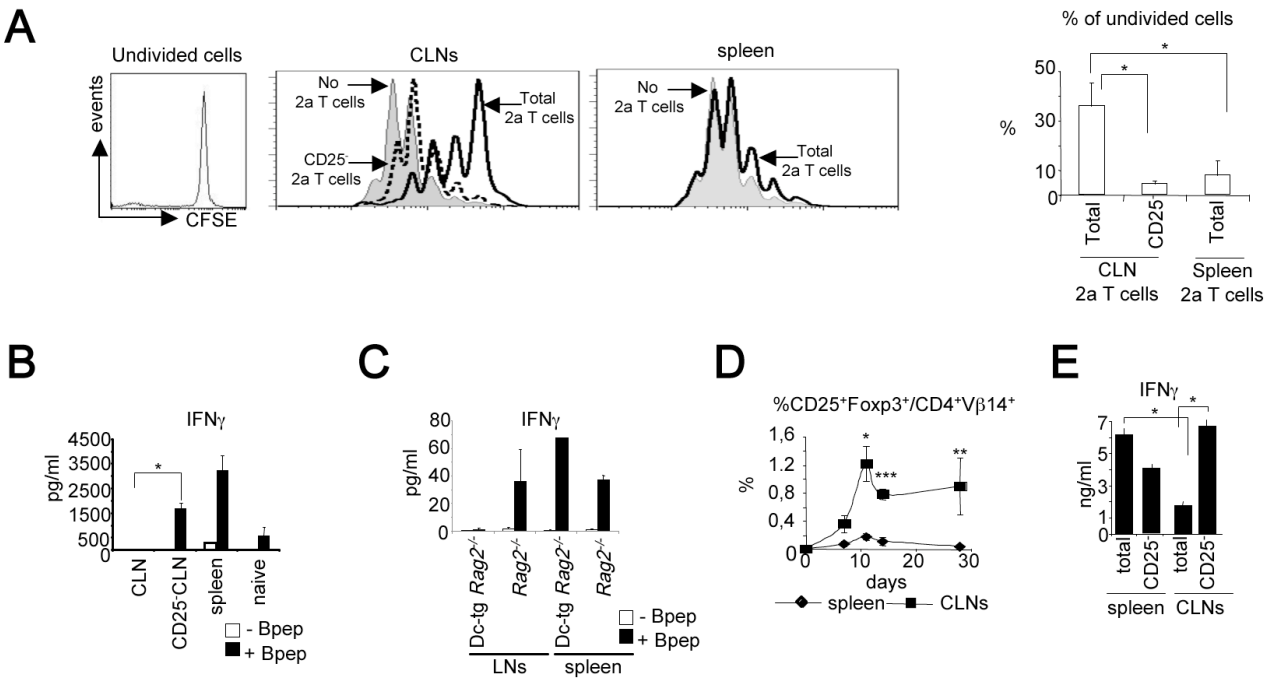
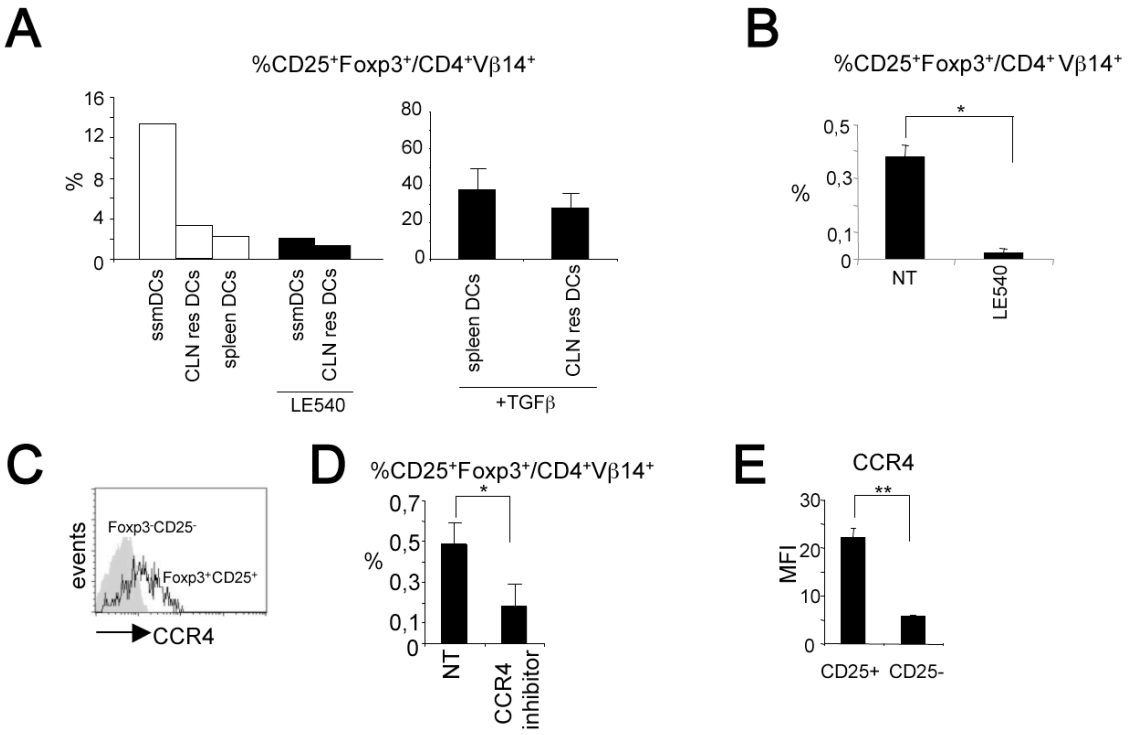

A
Undivided cells
CLNs
spleen
events
CFSE
No 2a T cells
Total 2a T cells
CD25- 2a T cells
% of undivided cells
Total
CD25
CLN 2a T cells
Spleen 2a T cells
B
IFNγ
pg/ml
CLN
CD25-CLN
spleen
naive
- Bpep
+ Bpep
C
IFNγ
pg/ml
Dc-tg Rag2-/-
Rag2-/-
LNs
spleen
D
%CD25+Foxp3+/CD4+Vβ14+
%
days
spleen
CLNs
E
IFNγ
ng/ml
total
CD25
spleen
CLNs
F
2a T cells
αCD25
CFSE 2a T cells
days
0
21
27
CLNs
spleen
events
αCD25
NT
CFSE
% of undivided cells
αCD25
NT
%
CLNs
spleen

Figure 2


A
%CD25+Foxp3+/CD4+Vβ14+
%
ssmDCs
CLN res DCs
spleen DCs
LE540
+TGFβ
B
%CD25+Foxp3+/CD4+ Vβ14+
NT
LE540
C
events
Foxp3-CD25-
Foxp3+CD25+
CCR4
D
%CD25+Foxp3+/CD4+Vβ14+
CCR4 inhibitor
E
CCR4
MFI
CD25+
CD25-

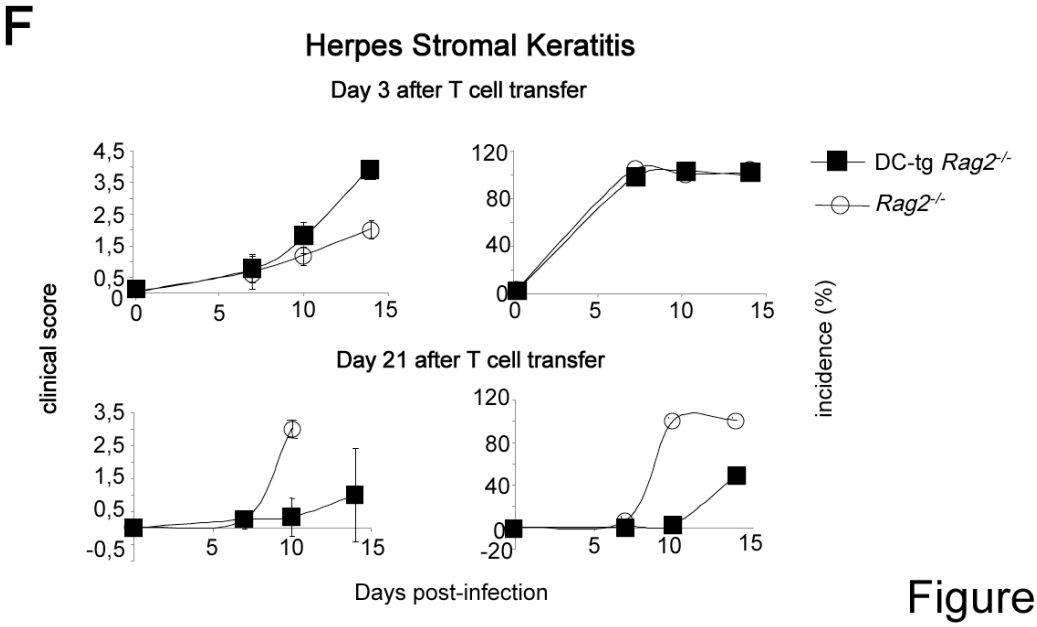

F
Herpes Stromal Keratitis
Day 3 after T cell transfer
Day 21 after T cell transfer
clinical score
incidence (%)
DC-tg Rag2-/-
Rag2-/-
Days post-infection

Figure 3

Supporting Online Material

Figs S1-S4

Table 1

Supporting Online Material

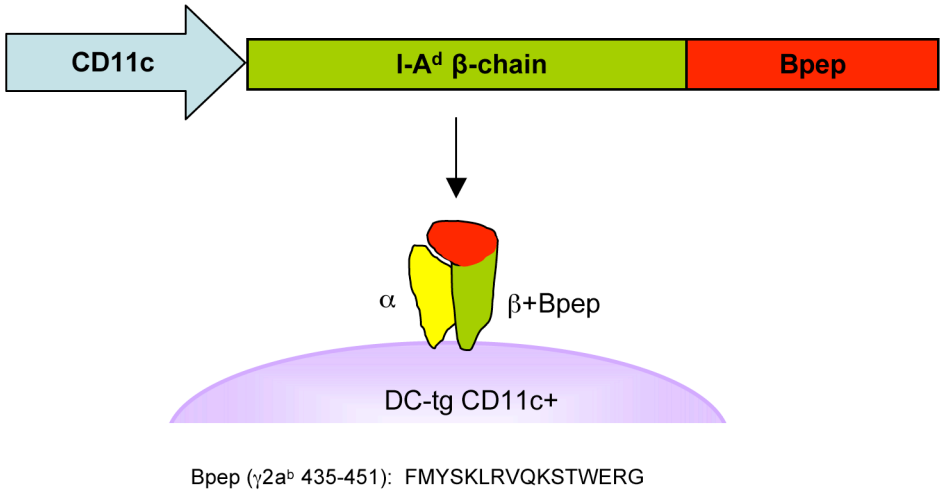


Figure S1. Schematic representation of the DC-tg transgenic model. The 435-451 fragment of the CH3 region of IgG2a^b (Bpep) covalently linked to the β chain of I-A^d class II molecule is presented at the surface of CD11c$^+$ cells.

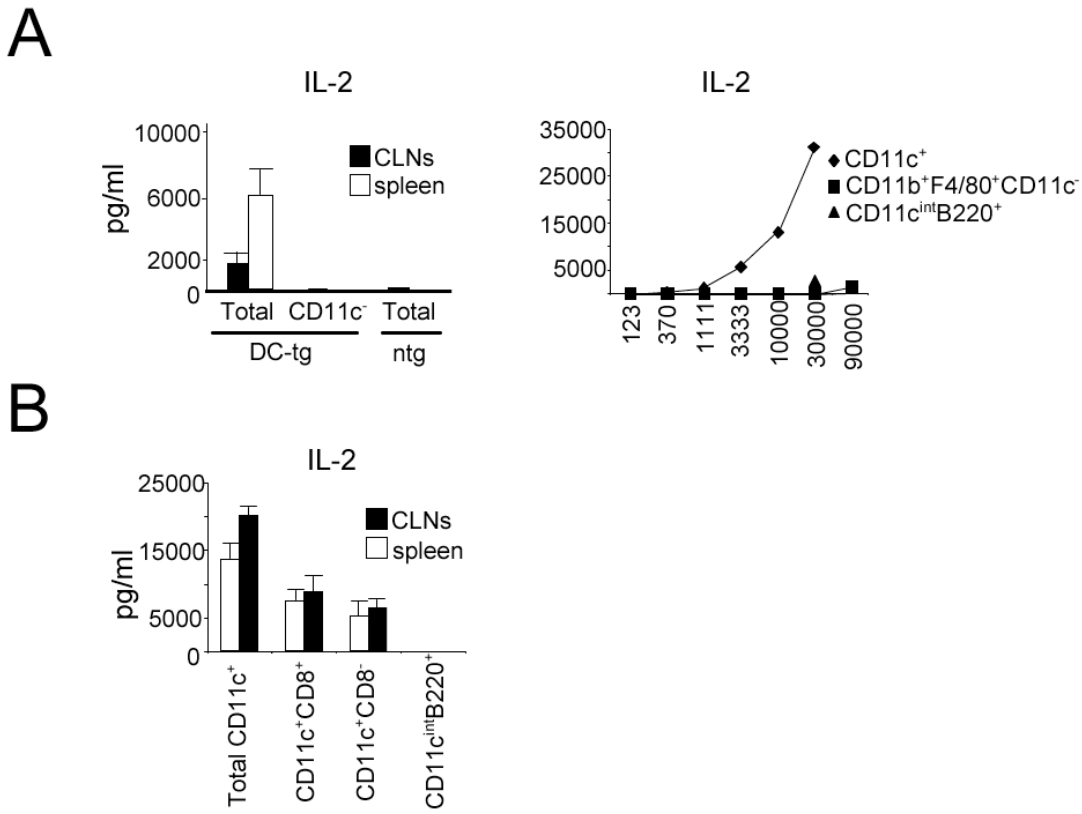


Figure S2. Efficiency of Bpep presentation by different professional APCs from DC-tg Rag-2-deficient mice. (A) Left panel, total or $CD11c^+$cell-depleted ($CD11c^-$) spleen and CLN cells (10000 cells) from DC-tg and wild type BALB/c mice (ntg) were cocultured with the B5HII39 anti-Bpep T hybridoma and the levels of IL-2 in the supernatant measured 24 hours later. The B5HII39 is high sensitive hybridoma, showing high affinity for the $I\text{-}A^d$+Bpep complex (Bartnes et al., 1993). Right panel, graded numbers of DCs ($CD11c^+$),

macrophages ($CD11b^{+}F4/80^{+}CD11c^{-}$) and 30000 plasmacytoid DCs ($CD11c^{int}B220^{+}$) from DC-tg mice were incubated with the B5HII39 hybridoma and the amount of IL-2 secreted in the supernatant measured 24 hours later. (B) Total $CD11c^{+}$ cells and different DCs subtypes, $CD11c^{+}CD8\alpha^{+}$ (lymphoid tissue resident), $CD11c^{+}CD8\alpha^{-}$ and plasmacytoid cells $CD11c^{int}B220^{+}$ (in all cases 10000 cells) were incubated with the B5HII39 hybridoma and the amount of IL-2 measured 24 hours later.

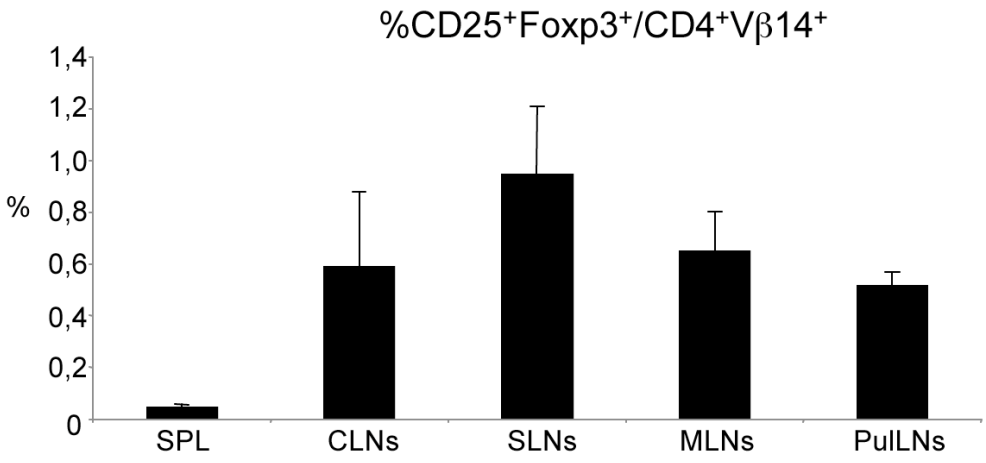


Figure S3. Generation of $CD25^{+}Foxp3^{+}$ 2a T cells in different types of lymph nodes. Naïve 2a T cells were transferred into DC-tg Rag-2-

deficient mice and the percentage of $CD25^{+}Foxp3^{+}$ cells on the total $CD4^{+}V\beta14^{+}$ cells measured three weeks later in spleen, cutaneous (CLNs), sublingual (SLNs), mesenteric (MLNs) and pulmonary (PulLNs) lymph nodes. Data are representative of at least three independent experiments performed using at least three mice per group.

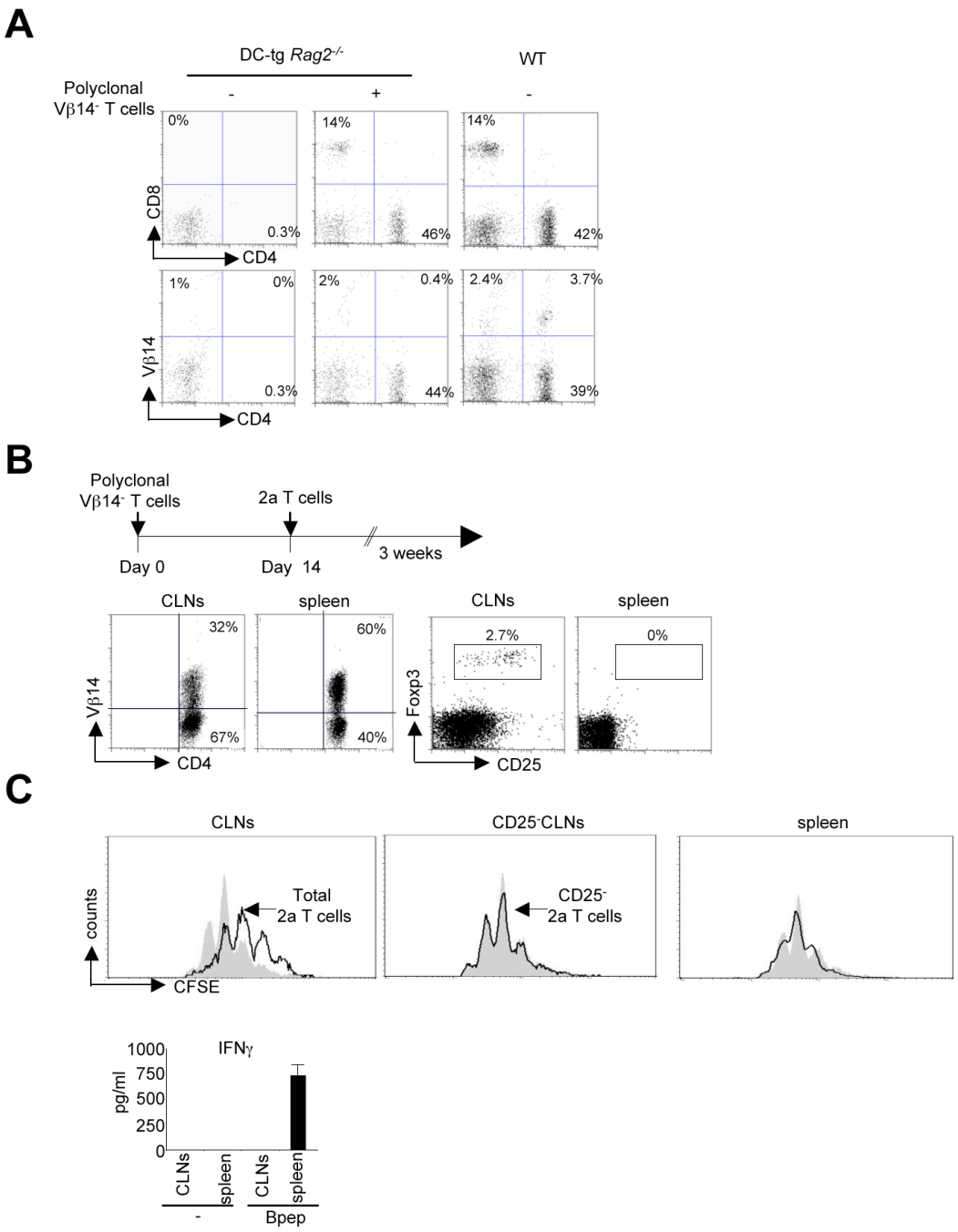

A
DC-tg Rag2-/-
WT
Polyclonal
Vβ14- T cells
-
+
-
0%
14%
14%
CD8
0.3%
46%
42%
CD4
1%
0%
2%
0.4%
2.4%
3.7%
Vβ14
0.3%
44%
39%
CD4
B
Polyclonal
Vβ14- T cells
2a T cells
Day 0
Day 14
3 weeks
CLNs
spleen
CLNs
spleen
32%
60%
2.7%
0%
Vβ14
67%
40%
Foxp3
CD4
CD25
C
CLNs
CD25-CLNs
spleen
counts
Total
2a T cells
CD25-
2a T cells
CFSE
IFNγ
1000
750
500
250
0
pg/ml
CLNs
spleen
CLNs
spleen
-
Bpep

Figure S4. Naïve 2a T cells convert into iTreg cells in CLNs but not in the spleen in non-lymphopenic hosts. To guarantee the absence of naturally occurring anti-Bpep Tregs, DC-tg Rag-2-deficient mice were reconstituted with polyclonal T cells from CB-17 mice depleted of the $V\beta14^+$ population. CB-17 mice are Igh-1b and the endogenous anti-Bpep T cells are in a state of Treg independent, irreversible non-functionality induced by $IgG2a^b$-positive B lymphocytes, representing the exclusive cells able to present the Bpep in natural conditions (Raimondi et al., 2006). Therefore, the polyclonal anti-Bpep CB-17 T cells do not interfere with the response of naïve 2a T cells. Moreover, the depletion of $V\beta14^+$ cells ensures that 2a T cells ($V\beta14^+$) can be easily followed after adoptive transfer. At Day 14 after reconstitution of the polyclonal T cell pool (Figure S4A), 2a T cells were introduced and the generation of $V\beta14^+$ Treg cells was analyzed three weeks after transfer (Figure S4B). According with the results obtained in non-reconstituted Rag-2-deficient recipients, conversion of antigen-specific naïve T cells into iTreg cells was observable only in CLNs and not in the spleen (Figure S4B). The efficiency of conversion was even higher in non-lymphopenic compared to lymphopenic hosts, suggesting that polyclonal T cells can influence this particular functional activity of lymph node DCs. iTreg cells generated in CLNs of non-lymphopenic hosts were able to suppress polyclonal T cell proliferation *in vitro* and antigen-induced cytokine production upon restimulation (Figure S4C).

Figure legend (A) Reconstitution of DC-tg Rag-2-deficient mice with Vβ14$^-$ T cells. DC-Tg Rag-2-deficient mice were injected with 10^7 Vβ14$^-$ T cells and the efficiency of reconstitution evaluated in the blood 14 days later. (B) Upper diagram, schematic representation of the adoptive transfer experiment. At Day 0 DC-tg Rag2-deficient mice received polyclonal Vβ14$^-$ T cells. Two weeks later, naïve 2a T cells were transferred into reconstituted mice and their fate analyzed three weeks later. Lower panel, cyfluorimetric analysis of Vβ14, CD4, CD25 and Foxp3 expression by transferred 2a T cells in CLNs and spleen three weeks after transfer. (C) Upper panels, CD25$^+$ 2a T cells recovered from CLNs of DC-tg mice inhibit mitogen-induced proliferation of polyclonal T cells. CFSE-labeled plyclonal T cells were stimulated with an anti-CD3ε monoclonal antibody and syngeneic splenocytes in presence or not of 2a T cells recovered from spleen or CLNs of DC-tg non-lymphopenic mice three weeks after transfer. Their proliferation was then assessed by flow cytometry 72 hours later. Where indicated, recovered 2a T cells were deprived of the CD25$^+$ population. This experiment is representative of two independent experiments, each performed with at least two pooled animals. Lower panels, IFNγ production by 2a T cells, recovered from spleen and CLNs three weeks after transfer, upon *in vitro* restimulation with splenocytes in presence or absence of the Bpep. This experiment is representative of two independent experiments.

Table 1. Differentially expressed genes

Gene Symbol	Entrez Gene ID	Pathway	LogRatio
Ccl22	20299	---	-6,51441965
Aldh1a2	19378	---	-6,213769115
Dntt	21673	---	-5,830481197
Dntt	21673	---	-5,594164912
Atp1b1	11931	Calcium_regulation_in_cardiac_cells	-5,356795679
Slc9a3r2	65962	---	-5,310761363
Slc9a3r2	65962	---	-5,219797418
Klk1	16612	---	-5,096995829
Tnnt2	21956	Striated_muscle_contraction	-5,035536189
Laptm4b	114128	---	-4,900788767
Ly6a	110454	---	-4,823011249
Laptm4b	114128	---	-4,783310464
Klk1	16612	---	-4,690607353
Net1	56349	---	-4,627394306
Glipr2	384009	---	-4,531106511
Ccr9	12769	GPCRDB_Class_A_Rhodopsin-like	-4,521210684
Gzmc	14940	---	-4,459196899
Cdh1	12550	Cell_Cycle_KEGG	-4,272809331
Fcrla	98752	---	-4,22896912
Pmepa1	65112	---	-4,169545326
LOC100047091	100047091 ///	mRNA_processing_binding_Reactom	-4,165463714
Il7r	16197	---	-4,097607213
Fcrla	98752	---	-4,077844778
Mgl1	17312	---	-4,060234529
Zdhhc14	224454	---	-4,015241134
Prkca	18750	Calcium_regulation_in_cardiac_cells	-3,957172955
Insl6	27356	---	-3,930620166
Blnk	17060	---	-3,879940985
Tnnt2	21956	Striated_muscle_contraction	-3,832349571
Cttnbp2nl	80281	---	-3,812948706
Mylk	107589	---	-3,81184277
Zdhhc14	224454	---	-3,781473879
Rabgap1l	29809	---	-3,683233681
Il7r	16197	---	-3,546510736
Zdhhc14	224454	---	-3,546391401
Ccr5	12774	GPCRDB_Class_A_Rhodopsin-like	-3,544118244
Rai2	24004	---	-3,539411881
Slc41a2	338365	---	-3,464275443
---	---	---	-3,413566227
Serpinb9	20723	---	-3,369674614
Zdhhc14	224454	---	-3,326820668
Ccr5	12774	GPCRDB_Class_A_Rhodopsin-like	-3,193908539
P2ry14	140795	GPCRDB_Class_A_Rhodopsin-like	-3,167719532
Serpinb6b	20708	---	-3,162604562
Klk1b22 ///	13646 /// 13648	---	-3,156177688
Slc22a23	73102	---	-3,137988114

Zdhhc14		224454	---	-3,06305882
Fabp5		16592	---	-3,042625415
Ndrg1		17988	---	-3,006149156
Myom1		17929	Striated_muscle_contraction	-3,001645232
Cd200		17470	---	-3,000619027
Sec24d		69608	---	-2,9444241
Gbp1		14468	---	-2,941037068
Cd1d2		12480	---	-2,928764261
Gnb4		14696	Calcium_regulation_in_cardiac_cells	-2,910024819
Slc22a4		30805	---	-2,900638411
Gnb4		14696	Calcium_regulation_in_cardiac_cells	-2,845978709
Il2ra		16184	Inflammatory_Response_Pathway	-2,831415265
Chchd6		66098	---	-2,793167515
Ccr5		12774	GPCRDB_Class_A_Rhodopsin-like	-2,756751254
Pvr		52118	---	-2,711019311
Txndc17		52700	---	-2,689011399
Slco3a1		108116	---	-2,676663453
Map4k4		26921	---	-2,671977614
Gca		227960	---	-2,636107273
Il15ra		16169	---	-2,613949502
EG620603	///	16592 /// 620603	---	-2,533439828
Ahnak		66395	---	-2,522834277
Litaf		56722	---	-2,503180149
Rasa3		19414	MAPK_Cascade	-2,496277925
Hrasls3		225845	---	-2,460606372
Slco3a1		108116	---	-2,458242857
Pkig		18769	Calcium_regulation_in_cardiac_cells	-2,408853685
Tmem39a		67846	---	-2,254290283
Map4k4		26921	---	-2,244415169
Fer1l3		226101	---	-2,229628471
Plscr1		22038	---	-2,219340325
Pi4k2b		67073	---	-2,205684184
Plxdc2		67448	---	-2,184878833
Asph		65973	---	-2,169778414
Tcf12		21406	---	-2,161316248
Lgals1		16852	---	-2,149351634
Rnf19a		30945	---	-2,142407481
Idi1		319554	Cholesterol_Biosynthesis ///	-2,07717455
Adk		11534	---	-2,060704082
Txndc17		52700	---	-2,025185404
Dhcr24		74754	---	-2,02417237
Csrp1		13007	---	-1,999997896
Map4k4		26921	---	-1,986796817
Sqle		20775	Cholesterol_Biosynthesis	-1,942930038
---	---		---	-1,912413434
Dnajb2		56812	---	-1,901948039
---	---		---	-1,894400516
Cd44		12505	---	-1,881111601
Galnt7		108150	---	-1,86822688

Idi1	319554	Cholesterol_Biosynthesis ///	-1,863516791
Plcb4	18798	---	-1,83921826
Scpep1	74617	---	-1,835975924
Cd44	12505	---	-1,812751571
Ccng2	12452	G1_to_S_cell_cycle_Reactome	-1,780426365
Hmgn3	94353	---	-1,745163164
Mx1	17857	---	-1,738126004
EG545878 /// 21968 /// 545878		---	-1,68028904
Pam	18484	---	-1,676257181
Map4k5	399510	---	-1,669662332
Lgals1	16852	---	-1,665283558
Acadl	11363	Fatty_Acid_Degradation ///	-1,653859931
Tcf12	21406	---	-1,647394725
Arl5a	75423	---	-1,619010217
Oxct1	67041	---	-1,53587785
Oxct1	67041	---	-1,527204714
Darc	13349	GPCRDB_Other	1,538875782
EG668525	668525	---	1,574457397
Prkd2	101540	---	1,578732336
Map3k3	26406	MAPK_Cascade	1,579994311
B4galnt1	14421	---	1,595872231
Osgin1	71839	---	1,595958602
5730469M10Ri	70564	---	1,621176279
Mapk11	19094	---	1,628091184
Rab8a	17274	---	1,629280096
Ier5	15939	---	1,638192121
Cr2	12902	---	1,64720561
Parvg	64099	---	1,647466633
Lysmd2	70082	---	1,66379516
Sirpa	19261	---	1,694306532
Mta3	116871	---	1,721060273
Arhgap9	216445	---	1,737135705
Ndst1	15531	---	1,757680577
Rufy3	52822	---	1,758802427
Arhgap6	11856	---	1,76961789
Mapk14	26416	MAPK_Cascade	1,783132403
Mapk14	26416	MAPK_Cascade	1,817759766
Mapk14	26416	MAPK_Cascade	1,833474856
Actr1b	226977	---	1,87839594
Npl	74091	---	1,882281176
Oas2	246728	mRNA_processing_binding_Reactom	1,887970209
Il10ra	16154	Inflammatory_Response_Pathway	1,947882307
Rtn1	104001	---	2,031083865
Rasa4	54153	---	2,04121382
Ddx60	234311	---	2,065147159
Tspan2	70747	---	2,076163734
Rnf149	67702	---	2,077624719
Cyp4f16	70101	---	2,102032387
Scn3a	20269	---	2,148323559

Slfn2		20556	---	2,166815165
Arhgap6		11856	---	2,180600885
Matk		17179	---	2,190385779
Frmd5		228564	---	2,230768307
---	---		---	2,237044707
Ggt5		23887	---	2,256142497
Cyb5r3		109754	---	2,266549749
Fcrl1		229499	---	2,276783956
Sirpa		19261	---	2,298591028
Cd244		18106	---	2,307013531
Man2b1		17159	---	2,359229019
Lpar1		14745	---	2,447672763
Bbs9		319845	---	2,480058902
Tspan2		70747	---	2,485077772
Fcrl1		229499	---	2,490439035
Itln1		16429	---	2,50652958
Dzip1		66573	---	2,536385082
Ggt5		23887	---	2,563370431
Limd2		67803	---	2,567981214
Actr1b		226977	---	2,659305419
Myo1b		17912	---	2,714741614
Dym		69190	---	2,7551573
EG666031	/// 15481 /// 624853 ///		Circadian_Exercise	2,763366467
Marveld1		277010	---	2,78749135
Dscam		13508	---	2,821765852
Bex6		328660	---	2,857175423
Arhgap6		11856	---	2,99732982
Ffar2		233079	---	3,178888858
Abcb1a		18671	---	3,226692142
Scel		64929	---	3,333515123
Abcb1a		18671	---	3,451626018
Siglece		83382	---	3,494677997
---	---		---	3,568476386
Slc12a2		20496	---	3,584606189
Ptger3		19218	GPCRDB_Class_A_Rhodopsin-like	3,848558121
Sash1		70097	---	3,882478738
Tgm2		21817	---	3,900413942
Slc12a2		20496	---	4,134412529
Dtx1		14357	---	4,468586112
---	---		---	4,488794894

Supplementary table 1. List of differentially expressed genes in spleen versus lymph node $CD11c^+$ cells. Genes with an absolute log_2 ratio ≥ 3 are shown. Negative values refer to genes whose expression is more abundant in lymph node $CD11c^+$ cells compared to the spleen ones. Viceversa, positive values refer to genes more expressed in $CD11c^+$ spleen cells compared to lymph node cells.

CHAPTER 3: SUMMARY CONCLUSIONS AND FUTURE PERSPECTIVES

A major challenge in immunology and medicine is to determine how tolerance toward self-antigens is established and maintained, to avoid damage to the host. Understanding the mechanisms of immunological self-tolerance will also provide insights into how weak immune responses, such as those against tumour antigens in cancer patients or against microbial antigens in chronic infection, can be augmented, or conversely, how strong immune responses such as graft rejection can be restrained. The huge amount of knowledge we have on peripheral Tolerance in mice and specifically on Tregs render these cells good candidates to exploit in immunotherapy. Cellular therapy based on ex vivo expansion of Treg cells and their transfer to patients is currently the focus of intense research to treat autoimmune diseases and inhibit the occurrence of graft vs host disease after BM transplantation (Miyara et al., 2009). Unfortunately this approach presents many problems. *In vitro* generated or expanded Tregs are much less stable then iTregs generated in vivo, both in mice and in humans (Sakaguchi et al., 2010). Moreover, recent reports demonstrated that *in vitro* generated iTreg are very different from *in vivo* generated iTreg and nTreg, which in contrast are very similar to each other, as shown by a global gene expression analysis (Haribhai et al., 2011a).

In this picture understanding the rules that drive tolerization *in vivo* becomes a topic of great interest. Particularly, since DCs have been a major target for immunotherapy, understanding their role in inducing tolerance and the different behaviour of different DC subsets is fundamental for the development of targeting strategies in order to maximize the tolerogenic potential of the treatments.

The classical view postulates that, in homeostatic conditions, immature dendritic cells expressing low level of signal 1 (specificity) and low or no level of signal 2 (co-stimulation) are able to render T cells tolerant, while upon activation, mature DCs activate a protective response. This classical paradigm has been challenged by the demonstration that also mature DCs expressing high levels of MHC or co-stimulatory molecules can be tolerogenic in some circumstances (Yamazaki et al., 2007). Moreover $CD205^{+}$ DCs in conditions of suboptimal activation (Kretschmer et al., 2005a) and steady state migratory dendritic cells from the gut and the skin are able to mediate induced Treg (iTreg) conversion (Azukizawa et al., 2011; Coombes et al., 2007a; Sun et al., 2007a) despite their partially activated phenotype (Villadangos and Schnorrer, 2007).

Another open question is whether under certain conditions DCs are able to tolerize T cells *per se* or if certain subsets are naturally more tolerogenic than others. The study of this aspect has been limited by experimental settings that analysed the contribution of specific DC subpopulation. Since the lymph constantly carries peptides for loading on both migratory and lymphoid tissue resident immature

DCs in a dose range suitable for tolerization, it cannot be excluded that, in addition to the analysed populations, all conventional immature DCs can induce autoantigen specific $CD4^+$ T cell tolerance in the periphery. We therefore set up an experimental setting in which antigen presentation is not restricted *a priori* to a specific DC subpopulation and is extended to all conventional DC subtypes giving us the possibility to study if DCs in general are able to induce T cell tolerance at the steady state or if this is a prerogative of specialized subsets.

In this scenario we could demonstrate that lymphoid resident DCs are not able to tolerize T cells while steady state migratory DCs have the capacity to induce self antigen specific iTreg conversion and autoimmunity protection in a retinoic acid dependent manner. Since ssmDCs are the only subset able to tolerize T cell we can hypothesize that immature DCs are not tolerogenic *per se* but need a specific conditioning. Steady state migratory DCs continuously drain the tissues where they can be conditioned to become tolerogenic. SsmDCs, indeed, exhibit a semi-mature phenotype, even in homeostatic conditions after the exposure to tissue derived signals since ssmDCs from Myd88 and TRIF defective mice or mice grown in germ free conditions show the same degree of maturation markers than wild type (Villadangos and Schnorrer, 2007). It has been already reported that tissue specific stimuli could induce the expression of RALDH2 essential for RA synthesis. In the intestine it has been proposed that GM-CSF, presumably produced by lamina propria

macrophages or eosinophil-like cells, and RA, produced by ALDH1A1^{+} intestinal epithelial cells from dietary vitamin A or blood-derived retinol, may represent the tissue-derived stimuli that elicit Aldh1a2 expression by migratory DCs (Guilliams et al., 2010a; Yokota et al., 2009a).

Since ssmDCs are the only subset able to induce iTreg differentiation along with their unique ability to drain tissue antigen made us predict that DCs are DCs are specialized to induce tolerance to tissue-sequestered antigens. This picture is consistent with recent findings that revealed that iTreg and nTreg TCR repertoire is very dissimilar. Moreover the two lineages has very similar effector mechanisms but are functionally non redundant (Haribhai et al., 2011a). While circulating peptides could easily recirculate between the thymus and the spleen (Donskoy and Goldschneider, 2003) tissue-sequestered antigens most likely never reach the thymus, so ssmDCs maybe the cells that contribute to the diversification of the antigen-specificity of iTreg cells compared to nTreg. Nonetheless a partial overlap between the two subsets of regulatory T cells exists and if some iTreg differentiate in the spleen, thanks to cells different from conventional DCs, their TCR repertoire could be responsible for the overlap. Other cells in addiction to conventional DCs have been indeed shown to be able to drive iTreg differentiation, such as pDCs (Ochando et al., 2006).

In this view, it is important that the generated iTreg do not recirculate but stay in the lymph nodes or the tissues where the

antigen is. In our model, indeed, Treg cells are retained in the lymph nodes and never migrate to the spleen. However the clear drop in Foxp3^{+} cells percentage in the lymph nodes raises the possibility that Tregs can home also directly in the tissues where ssmDCs generate. Others have indeed already reported for iTreg generated in the gut, a process that involves generation in the mLN with subsequent migration to the lamina propria where they're expanded by intestinal macrophages (Hadis et al., 2011). Another group, using an experimental model in which the antigen is transiently expressed in the skin has demonstrated that iTregs cell can persist in the target tissues and suppress autoimmune responses upon repeated or chronic encounters with tissue autoantigen(Rosenblum et al., 2011). Since trafficking is tightly regulated by chemokines and chemokine receptors and Tregs are known to express CCR4, their localization in CLNs and possibly the skin can be regulated by the abundant CCL22 (CCR4 ligand) production by lymph node DCs and keratinocytes (Rot and von Andrian, 2004a).

The non-redundant function of iTregs in preventing autoimmunity is most likely due to their diverse antigen specificity so, also a small number of antigen specific iTregs could be effective in preventing autoimmunity. Indeed in the HSK experimental system, mice are totally protected from disease if the induction is performed shortly after transfer, when Treg cells have not differentiated yet but keratogenic T cells have been primed by Bpep-presenting DCs.

In conclusion we discovered that ssmDCs are a privileged subpopulation of DCs capable to induce iTreg differentiation, while lymphoid resident DCs are not able to induce Tregs and that the newly generated iTreg can protect the mice from autoimmune diseases. These findings can help clinical investigators to chose the right target for inducing Tregs in vivo, for instance targeting strategies that involve only conventional DCs (such as CD205 targeting) or DCs in general, can be less effective than strategies that target directly migratory DCs. This work also defines a new concept of T cell tolerance induction, demonstrating that immature DCs are not *per se* able to induce peripheral tolerance but there are specialized tolerogenic DC subsets. Moreover there is a striking difference between different lymphoid organs. The spleen, only accessible via the blood, seems not to have a high tolerogenic capacity, and the immune homeostasis could be maintained only by central tolerance mechanisms (nTreg, clonal deletion). This is consistent with the fact that blood derive antigens can easily reach the thymus. On the other hand Lymph nodes continuously drain tissue antigens that aren't present in the thymus and therefore need to have a high tolerogenic potential.

It still remains to be defined whether migratory DCs are intrinsically tolerogenic or if they need to be instructed by the tissues and if so, which is the tissue-derived signal that renders these DCs tolerogenic and if other signals beside retinoic acid are involved in Treg Tolerization. The role of resident DCs in tolerance still remains to be

defined. Even if they do not seem to play a part in Tregs differentiation, they might have a role in favouring survival and the homeostatic proliferation of Tregs *via* IL-2 production.

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