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PH.D.

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DIMET

DIVERGENT ROLES OF DENDRITIC CELLS IN THE INDUCTION AND MAINTENANCE OF T CELL TOLERANCE IN DIFFERENT SECONDARY LYMPHOID ORGANS

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Chapter 1: Introduction

1. The immune response to non-self

The immune system represents a dynamic system that has evolved to protect the host from a variety of invading pathogenic microorganisms (including virus, bacteria, fungi and parasites) and cancer. A complex network of cells and molecules is involved and its fine tuning is fundamental to efficiently mount a response to "non-self" while preserving "self" from being attacked and destroyed. Inappropriate immune responses are responsible for a number of dysfunctions like immunodeficiency, autoimmune disease, graft rejection, allergy and asthma.

When a pathogen invades a tissue, the immune system must sense the pathogen and delivers an appropriate immune response which is based on a concerted action of both antigen– (Ag) nonspecific innate immunity and Ag-specific adaptive immunity.

1.1 Innate immunity

Epithelia and mucosal surfaces are normally effective in preventing microbe entry. However, in some circumstances these physical and physiologic barriers are not sufficient and infection occurs. In those cases innate immunity is the first line of defence after pathogenic entrance. Key features of the mammalian innate immune system include the ability to rapidly recognize microbes or tissue injury in a non-specific manner and to initiate adaptive immunity [1]. The innate system comprise phagocytic cells, natural killer (NK) cells, complement, and interferons (IFNs). Most cellular components of innate immunity, including dendritic cells (DCs), macrophages, leukocytes, mast cells and natural killer (NK) cells, are already present in the tissues before the onset of the infection and are able to rapidly recognize conserved specific exclusive microbial components, known as Pathogen Associated Molecular Patterns (PAMPs) via a limited number of germline encoded pattern recognition receptors (PRRs) [2]. Exposure to PAMPs activates multiple signalling pathways resulting in an inflammatory response with the recruitment of leukocytes to the site of infection, activation of antimicrobial effector mechanisms (secretion of cytokines, phagocytosis) and, eventually, initiation of the adaptive immune response.

Innate immunity also includes soluble, non-cell based systems like the complement proteins that normally circulate in an inactive state; once converted to the active state, they have the ability to damage the

membranes of pathogenic organisms, thus destroying the microbes or facilitating their clearance.

1.2 Adaptive immunity

Innate immunity efficiently limits the spread and sometimes is also sufficient to eliminate invading microbes. However, if infection or tissue injury persists, adaptive immunity is triggered.

Adaptive immunity is a potent and sophisticated system based upon a wide variety of unique B and T cell clones capable of recognizing and selectively eliminating foreign microorganisms and molecules (i.e., foreign antigens). Adaptive immunity displays four characteristic attributes: antigenic specificity, diversity, immunologic memory and self/nonself recognition. T and B lymphocytes express unique, monoclonal T and B cell receptors (TCRs and BCRs) that recognize specific antigens and distinguish subtle differences among them. Each T and B cell typically exhibits a restricted antigen specificity; therefore, a tremendous number of different lymphocytes, each carrying a unique receptor, develops to allow efficient recognition of virtually every antigen.

B lymphocytes mature within the bone marrow; when they leave it as naïve B cells, they express a specific BCR which is essentially a membrane-bound antibody molecule (fig. 1a). Upon triggering of the BCR, B cells divide rapidly and differentiate into plasma cells which secrete antibodies, thus driving humoral response; a small proportion of

these cells differentiate also into memory B cells.

T cell precursors also arise in the bone marrow but then migrate to the thymus to mature. During thymic differentiation, TCR is rearranged and expressed. Then, T cells exit the thymus and circulate as naive T cells. Unlike membrane-bound antibodies on B cells, which recognize whole molecules, T-cell receptors bind only antigens in the form of peptides loaded on cell-membrane proteins called major histocompatibility complex (MHC) molecules (pMHC).

Naive T cells are activated by a specific pMHC presented by an antigen presenting cell (APC) which provide also co-stimulatory signals like CD80/CD86; in other words, TCR and CD28 triggering (By pMHC and co-stimulatory molecules) drives T cell activation, proliferation and differentiation into various effector T cells which mediate the so-called cell-based response.

There are two well-defined subpopulations of T cells: T helper (Th) and cytotoxic T lymphocytes (CTL) cells which can be distinguished by the presence of either CD4 or CD8 membrane glycoproteins on their surfaces, respectively (Figure 1b, c).

After the activation, $CD8^+$ cells differentiate into short-lived cytotoxic T lymphocytes (CTL) that exhibit cell-killing activity through cytotoxic molecules like perforin and granzymes [3]. Instead activated $CD4^+$ T cells differentiate into a variety of effector subsets, including classical Th1 and Th2 cells, and the more recently defined Th17, Treg and Th9 cells.

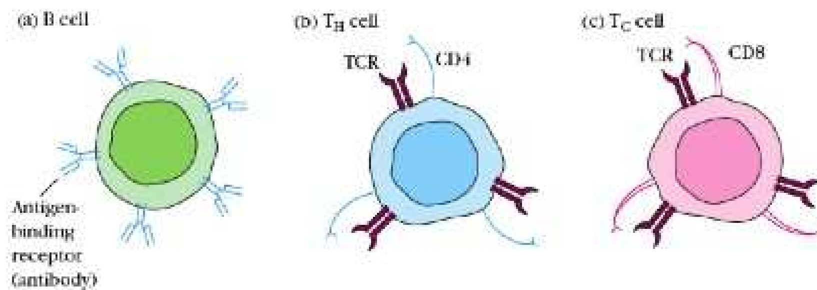


Figure 1: Different classes of lymphocytes and their receptors. (a) B cells have about 10^5 molecules of membrane-bound antibodies (BCR) per cell. All the antibody molecules on a given B cell share the same antigenic specificity and can interact directly with the antigen. (b) T cells bearing CD4 ($CD4^+$ cells) recognize only antigens bound to class II MHC molecules. (c) T cells bearing CD8 ($CD8^+$ cells) recognize only peptide loaded on class I MHC molecules. In general, $CD4^+$ cells act as helper cells and $CD8^+$ cells act as cytotoxic cells. Both types of T cells express about 10^5 identical molecules of T-cell receptor (TCR) per cell. (From Goldsby R. A. et al., Immunology, 5th edition, 2003).

The differentiation any of the T cell subsets is governed predominantly by the cytokines present in the microenvironment in which the activation occurs and, to some extent, by the strength of the interaction of the TCR with antigen. Once differentiated, Th1 cells mainly secrete IFN-γ which activates macrophages and CTL. Th2 cells promote B cell activation and consequent humoral response through IL-3, IL-4, IL-5, IL-10 and IL-13. Th17 cells produce IL-17A, IL-17F, IL-22 and IL-26 and play an important role in the clearance of extracellular bacteria and fungi at the mucosal surfaces [4]; Treg cells play an essential role in the maintenance of immune homeostasis and will be treated in greater details later on.

Finally Th9 subset have been defined recently as an IL-9 producing T cell lineage which is distinct from the other Th subsets; IL-9 is involved in the immune response to helmints as well as allergy.

These effector T helper cell subsets does not represent terminally differentiated lineages: on the contrary, flexibility and high plasticity between the lineages, particularly between Th17 and Treg, is a hallmark of T helper subsets [5, 6].

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. Two major types of memory T cells remain: central memory T cells (TCM) which reside in lymphoid organs and represent a reservoir of memory cells, and effector memory T cells (TEM) that act as sentinels in peripheral tissues such as the skin and the gut [7] and provide a systemic immune surveillance in peripheral non-lymphoid tissues which promptly reacts in case of re-attack.

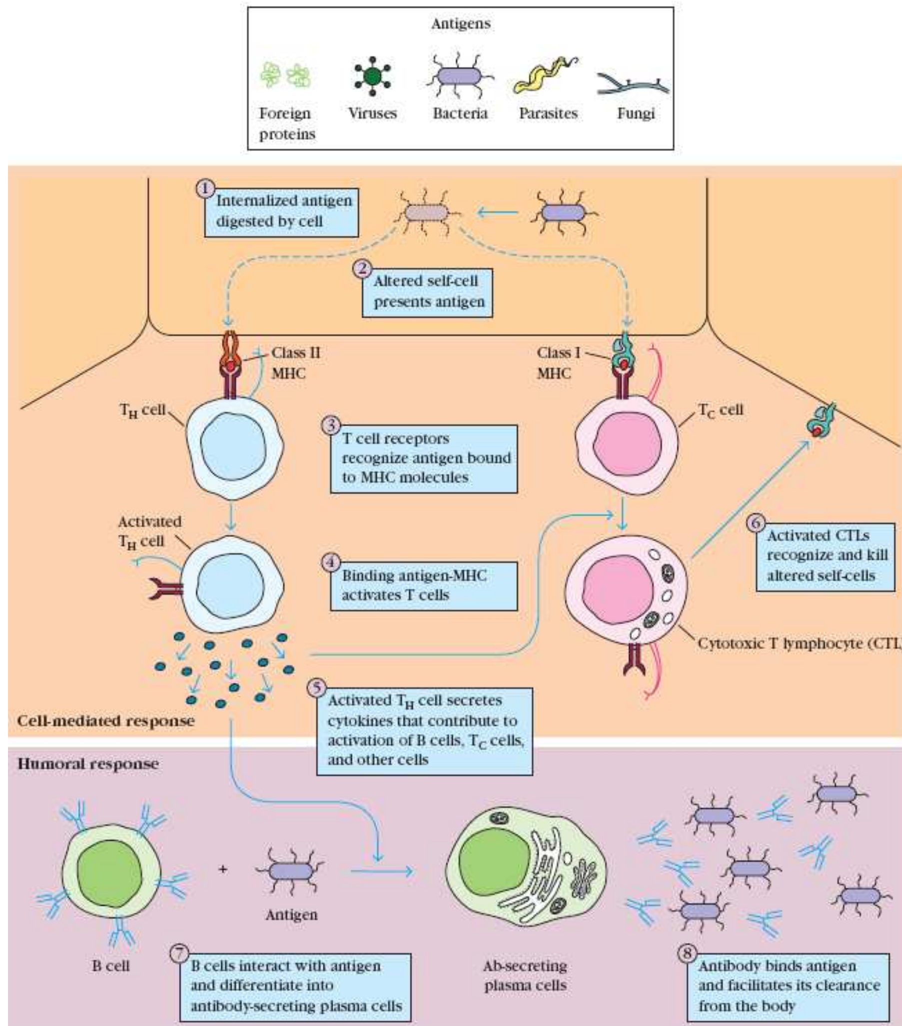


Figure 2: Basic model of the adaptive immunity. DCs that have sensed a pathogen activate T cells which display specific effector functions. CTL have cytotoxic ability, while Th cells secrete cytokines that promote the activation of B cells, macrophages or the same CTL effector response (From Goldsby R. A. et al., Immunology, 5th edition, 2003).

2. Dendritic cells between innate and adaptive immunity

Adaptive immunity is not independent from innate immunity. B and T lymphocytes recognize antigens with high specificity but they do not initiate an immune response, nor do they decide its type. Antigen Presenting Cells (APCs), in particular dendritic cells (DCs) are necessary to efficiently stimulate B and T cells and to determine the type of adaptive immunity [8-10]. DCs contribute also to mediate innate immune responses as well as T cell tolerance.

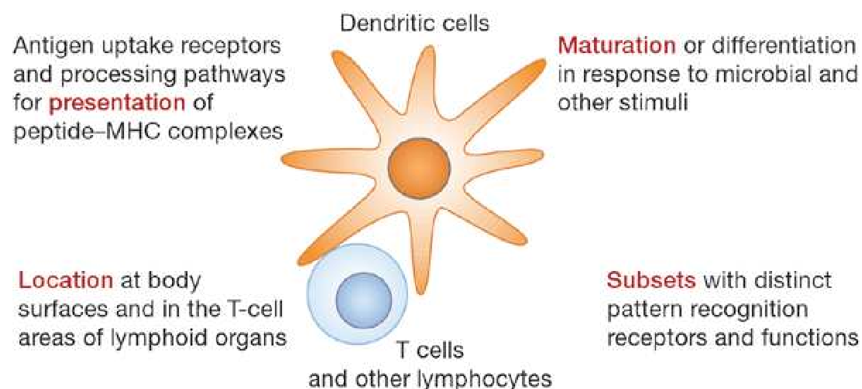


Figure 3: The four main features of DCs. These features render DCs specialized in activating and controlling T cell responses, thus modulating both tolerance and immunity (From Steinman R.M. et al., Nature 2007).

DCs act as "sentinels" perfectly equipped for immune surveillance and for the control of immune responses. These unique abilities depend on

four main features which are DC-specific (Fig. 3). First, they possess a unique distribution and thus are perfectly located to capture both self and external antigens: in fact, they can be found in the blood and lymphoid tissues but also in the peripheral sites like epithelia and mucosal surfaces. Second, they are efficient in antigen capture, processing and presentation to T cells. Third, they rapidly maturate upon stimulation. Fourth, different subsets exist, each specialized for some specific functions.

2.1 Dendritic cell subsets

DCs comprise many subsets characterized by distinct location, life cycle and roles in the initiation of immunity to specific stimuli as well as in the control of tolerance.

There are two main categories of DCs in the mouse: plasmacytoid DCs and conventional DCs [11,12].

Plasmacytoid DCs (pDCs) are characterized by B220/CD45RB expression and moderate levels of CD11c. They normally circulate through the blood and lymphoid tissue and, upon activation, produce type I interferons (IFN I) and have a role in anti-viral immunity [13]. For this peculiarity, they are also defined "natural interferon-producing cell".

cDCs can be divided into migratory DCs and lymphoid-tissue-resident DCs. Migratory DCs [14] are the typical antigen-sampling sentinels; these cells develop from earlier precursors directly in peripheral tissues

where they will reside under steady state consitions: upon stimulation, they travel through the afferent lymphatics to draining lymph nodes; in particular, $CD11b^{+}CD4^{-}CD8^{-}DEC205^{low}$ dermal DCs (or interstitial DCs) migrate from the dermis and can be found in all lymph nodes, while Langherans cells are located in skin epidermis and the epithelia of the intestinal, respiratory and reproductive tracts and are identified for the expression of the characteristic marker langerin.

By contrast, lymphoid-tissue resident DCs capture and present antigens in the lymphoid organ itself. Lymphoid tissue-resident DCs include most of the DCs in the thymus and spleen and half of the lymph-node DCs at the steady state [14, 15]. Lymphoid tissue-resident DCs can be further divided into 3 subsets characterized by different combinations of the surface markers CD4 and CD8: $CD4^{-}CD8^{-}$, $CD4^{-}CD8^{+}$ and $CD4^{+}CD8^{-}$ DCs.

All the DC subsets share the capacity of antigen presentation to T cells, but they have functional specialization and play different roles in immunity: $CD8^{+}$ DCs, for example, generally promote Th1 response, while $CD8^{-}$ DCs, which include $CD4^{-}CD8^{-}$ and $CD4^{+}CD8^{-}$ tend to induce Th2 response. Moreover, it has been suggested the $CD8^{+}$ DCs are by far the most efficient in phagocytosing dead cells and presenting the cellular antigens on the surface of MHC II and MHC I (the latter through cross-presentation): this specialized capacity seems to play a role in the $CD8^{+}$ T cell tolerance induction toward self antigens [16].

2.2 Classical model of DC maturation: the Langherans cell paradigm and beyond

DC life cycle is usually described according to the seminal studies performed in the 1980s on the main DC type found in the epidermis, the Langherans cell. According to this model, usually referred to as "Langherans cell paradigm", tissue-resident, migratory DCs sit in an immature state and interact with microbes through PRRs [17], a large superfamily which include the C-type lectin family receptors, the nucleotide binding and oligomerization domain (NOD)-like receptors (NLR), the retinoid acid-inducible gene I (RIG)-like receptors (RLRs), scavenger receptors (e.g. MARCO) and Toll like receptors (TLRs).

Once they have sensed a microbe, immature DCs undergo a profound functional and phenotypic differentiation process called maturation and reach the mature state (Fig. 4).

In the meanwhile, they migrate from the peripheral tissue to the draining lymph nodes, where they arrive as phenotypically mature DCs. Maturation changes include the expression of co-stimulatory molecules (e.g. CD40, CD80, CD86), adhesion molecules (e.g. CD54, CD58) and chemokine receptors (e.g. CCR7) and the secretion of cytokines (e.g. TNF-α, IL-2 and IL-12). Moreover, DCs lose the antigen uptake abilities and become very efficient in antigen processing and presentation. Processed peptides are then loaded on MHC I and II and are presented to lymphocytes together with co-stimulatory molecules. Naïve T cells can undergo activation and primary adaptive immune response is launched.

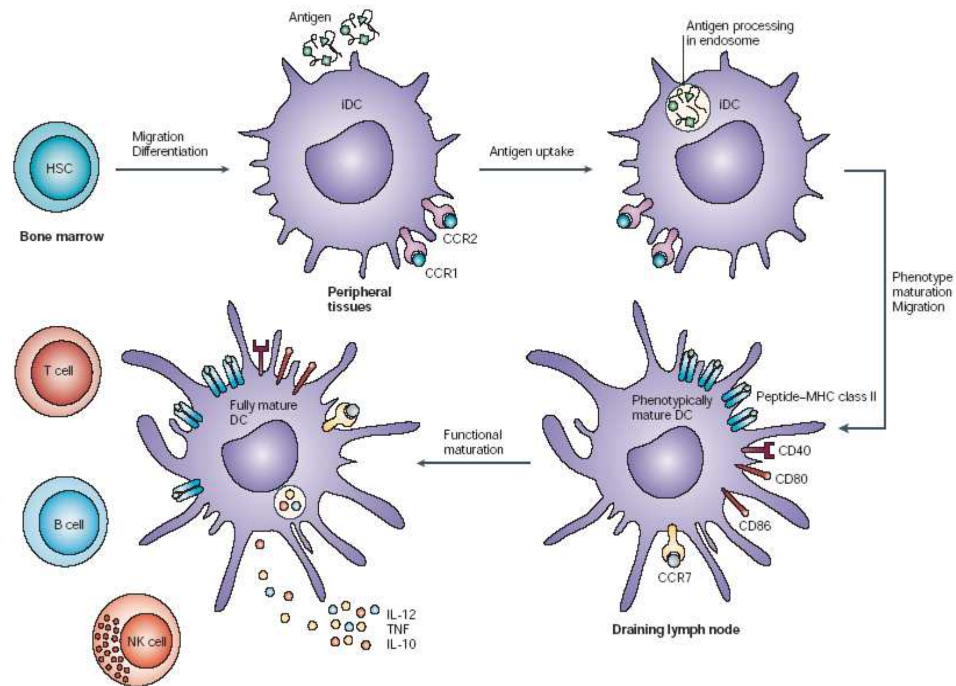


Figure 4: Classical DC maturation process. Once differentiated from hematopoietic stem cells (HSC), DCs are recruited to peripheral tissues as immature DCs. When they sense a microbe, they capture and process the antigen, undergo a maturation process and migrate to secondary lymphoid organs. Mature DCs induce NK cell activation in the first hours after the infection and, later on, prime naive T cells. (From Hackstein et al., Nat. Immunol., 2004).

This model of activation fits well to migratory DCs, which are resident in peripheral tissues and migrate upon activation. Lymphoid tissue-resident DCs do not conform to the Langherans cell paradigm [11]; they develop from bone marrow precursors directly within the lymphoid

organs and, in the absence of infections, maintain an immature phenotype throughout their lifespan; in fact, almost all the splenic DCs and half of the lymph node resident DCs are immature in the steady state (Fig. 5).

In addition to the ability of activating adaptive immune response, mature DCs play also a pivotal role in the activation of innate immunity. In the first hours after the interaction with microbes, DCs release membrane-bound molecules and cytokines, such as type I IFN, IL-12, IFN-γ, IL-15 and IL-18, which affect NK cell proliferation, migration, IFN-γ production and cytotoxic activity; more precisely, DCs and NK cells reciprocally influence their maturation: DCs act during the priming phase of NK-cell activation and NK promote further DC maturation and cytokine production [18, 19].

There is now substantial evidence that DCs play also a role in the maintenance of peripheral tolerance to self antigens [18, 20, 21]. Impaired DC homeostasis has been implicated in various human autoimmune diseases including systemic lupus erythematosus (SLE) [22], type I diabetes [23] and multiple sclerosis [24]. Tolerance induction mediated by DCs will be discussed in details in the next sessions.

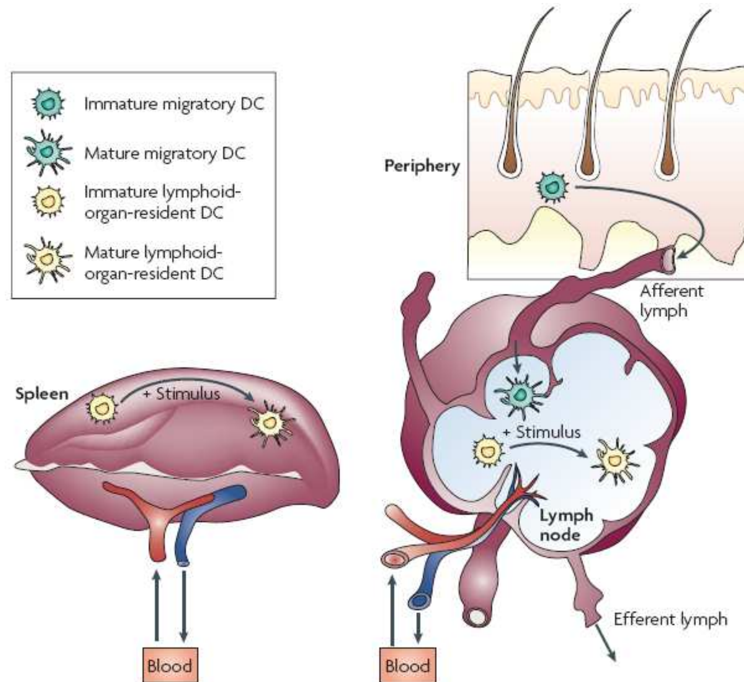


Figure 5: Maturation of migratory and resident DCs. Immature migratory T cells reside in peripheral tissues and migrate to tissue-draining lymph nodes after the activation. The lymph nodes also contain immature resident DCs which activate *in situ* upon pathogen encounter. In the spleen, only resident DCs are present. (From Villadangos J.A. et al., Nat. Rev. Immunol., 2007).

3. 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 take place in the thymus during T cell ontogeny [25,26].

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 [25]. TCR diversification, thus, depends on the combinatorial variation of the process (Fig. 6). The diversity of the naive TCR repertoire is increased further by both a lack of precision during V(D)J gene rearrangement and the addition of non-template encoded nucleotides at V(D)J junctions.

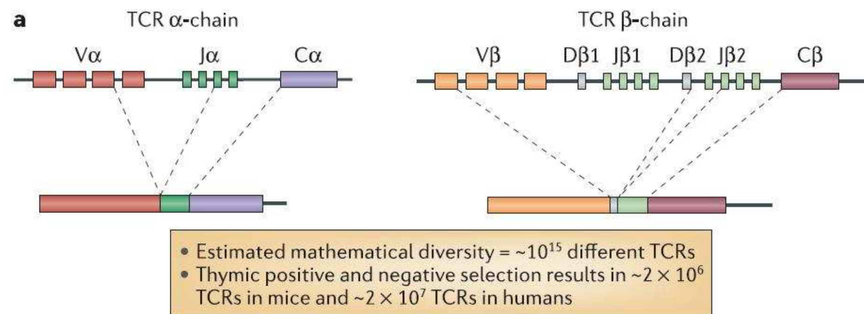


Figure 6: Generation of TCR diversity by somatic recombination of TCR gene segments. Functional TCRs are heterodimers consisting of α- and β-chains that are generated by somatic recombination of variable (V), diversity (D) and junctional (J) gene segments for the β-chain, and V and J gene segments for the α-chain. During T-cell ontogeny, gene segments recombine and are spliced together with the constant region (C) to form the functional αβ TCR. Mathematical estimates of potential TCR diversity are in the range of 1012-1015 different TCRs1. (From Turner S. J et al., Nat. Rev. Immunol. 2006).

The wide repertoire that is generated is virtually sufficient to recognize every non-self antigen [25]. 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. Tolerance markedly limit the diversity of the generated repertoire: in fact, although theoretically there could be

10^{15} TCRs, only $2x10^{8}$ are expressed in a mouse on fully differentiated T cells [27].

4. Tolerance toward self antigens

4.1 Central tolerance

T cell tolerance is a fundamental feature of our immune system and is exerted at two levels: in the thymus, during the ontogeny of lymphocytes (central tolerance) an in the periphery on circulating T cells (peripheral tolerance).

Central tolerance is part of the thymic selection process [28, 29]: during this phase, thymocytes rearrange *tcr a* and *tcr b* loci and express the neo-rearranged TCR on the cell membrane. Then, they undergo a selection process with three possible fates [29, 30]: death by neglect, tolerization or selection with subsequent differentiation into naive T cells which exit the thymus (Fig. 7).

Death by neglect occurs when the TCR fails to interact with pMHC [31]; this condition reflects a useless specificity, i.e., the lack of self-MHC restriction, and results in the deletion of the thymocyte [29, 30].

Instead, if the TCR has sufficient affinity for self pMHC, the cell survives and is positively selected. Then, if the TCR engages pMHC with low affinity, the T cell precursor completes the development and exits the thymus as a naive T cell expressing a specific TCR and the CD4 or CD8 co-receptor.

Negative selection (or central tolerance), on the contrary, occurs when the TCR of the thymocyte binds pMHC with high affinity. Tolerance can be achieved through many strategies: first, the elimination of the T cell

precursor by apoptosis; second, the induction of a state of non-resposiveness (anergy); third, TCR editing, i.e., a second α-chain is expressed as a consequence of the further recombination of *tcr a* locus [32]; fourth, differentiation into a regulatory T cell (Treg) which exits the thymus and controls the immune response in the periphery.

The impact of central tolerance has been estimated by different authors: while it has been calculated that the minimum frequency of positively selected thymocytes that undergo negative selection is 5%[33], the extimated real frequency should be around 50%–70% of positively selected thymocytes [34-36].

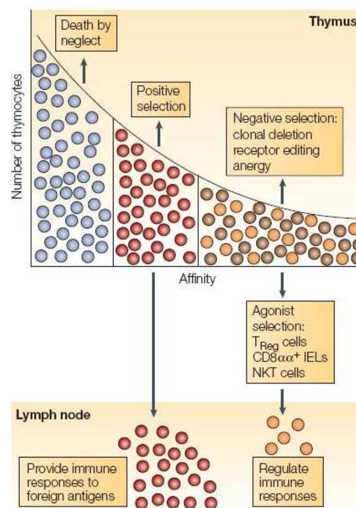


Figure 7: Thymic selection. The affinity of the TCR for self-pMHC is the crucial parameter that drives developmental outcome in the thymus. Thymocytes with no affinity or very low affinity die by neglect. If the TCR has low affinity for self-pMHC the cell survives and mature to naive T cell. When the affinity for self pMHC is high, the cell is tolerized through deletion, anergy, receptor editing or Treg differentiation. (From Hogquist K.A. et. Al, Nat Rev. Immunol. 2005).

All the events which characterize T cell maturation are spatially organized within the thymus. Positive selection occurs in the thymic cortex, where thymocytes interact with cortical thymic epithelial cells (cTEC). Selected T cell precursors then translocate to the medulla, where they interact with medullary antigen-presenting cells, mostly dendritic cells (DCs) [37] and medullary TECs (mTECs). These cells express co-stimulatory molecules, such as CD40, CD80, CD86 and are important for central tolerance toward a number of systemic as well as tissue-specific self antigens (TSA)[28-30, 38-39].

Ectopic or promiscuous TSA expression is an exclusive feature of mTEC. These cells express the transcription factor autoimmune regulator (AIRE) which regulates the ectopic transcription of a number of TSA encoding genes [28, 39]. AIRE plays a fundamental role in the control of self reactivity: in fact, its deficiency in humans results in the severe autoimmune disease APECED; in mice, defective AIRE is associated with impaired clonal selection. TSA expression and presentation in the thymus is an enormous opportunity for widening the repertoire of self antigen presented to T cells in the thymus. Thank to this fine system, T cells which are specific for a TSA will be tolerized directly by central tolerance, thus eliminating the risk of autoreactivity soon after TCR rearrangement.

Medullary DCs also contribute to clonal deletion: they do not express TSA directly but cross-present those expressed by mTEC [29]. Moreover, some circulating DCs carrying antigens captured in the periphery home to the thymus and contribute to TSA presentation [40].

4.2 Mechanisms of peripheral tolerance induction

Central tolerance is fundamental for the prevention of a number of autoimmune diseases [341], but is incomplete [38]. Low affinity self reactive T cells escape negative selection but can potentially be activated in the periphery [42]; moreover, a number of innocuous antigens, such as environmental, food-derived or some tissue-specific antigens are not expressed in the thymus. Peripheral tolerance is therefore necessary to provide supplementary protection.

A number of mechanisms prevent the activation of self-reactive T cell in the periphery. These can be divided into recessive or intrinsic, which regulate the responding state of T cell (i.e. ignorance, apoptosis, anergy, and immune deviation) (Fig. 8) and dominant or extrinsic, which depend on the supply of external signals (i.e. active suppression, the limitation of survival factors, pro-inflammatory mediators and co-stimulatory signals)[43-46].

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. Alternatively, ignorance can be observed when the amount of antigen does not reach the threshold required to trigger a T-cell response.

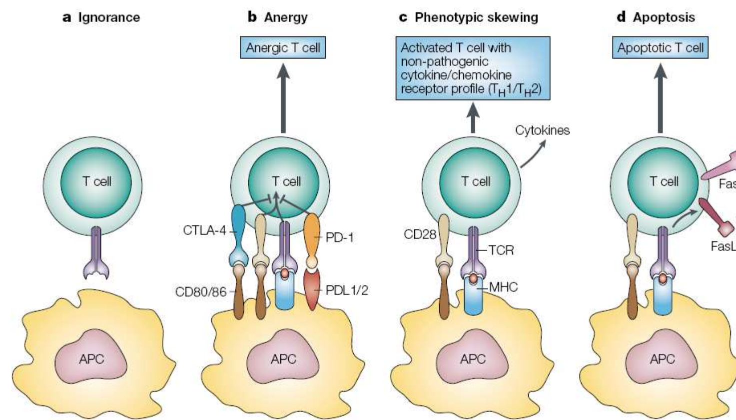


Figure 8: Cell intrinsic mechanisms. a) Self-reactive T cells may never encounter the cognate self-protein and therefore exist in a state of ignorance. b) Encounter with self-protein may induce T-cell anergy, possibly involving interaction of the inhibitory molecules CTLA-4 or PD-1 with their ligands (CD80/86, PDL1/2). c) T cells interacting with self-protein may also undergo full activation, but then develop a non-pathogenic phenotype. d) Self-reactive T cells may be deleted following contact with self-protein by apoptosis. (From Walker S.K. et al., Nat. Rev. Immunol., 2002).

Lymphocytes that encounter self antigens may undergo clonal deletion. On a molecular basis, apoptosis may be mediated by two main convergent pathways: the cascade which 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 [44, 47], 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. 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, [47-49] 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 [49], 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.

Immune deviation, known also as split tolerance or phenotype skewing, is another minor intrinsic mechanism for peripheral T cell tolerance: in this case, T cells become fully activated, but the pathogenic effects are avoided thanks to the so-called T-cell cytokine deviation; in other words, cytokine milieu cause a shift from a potentially dangerous Th1 response to an innocuous Th2 response, therefore controlling autoreactivity and allowing effective tolerance.

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: even if it has been demonstrated that, in the presence of a systemic self antigen, anergy is sufficient to preserve $CD4^+$ T cell tolerance [50], 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.

4.3 Self-nonself discrimination: models of peripheral tolerance

The mechanisms responsible for the induction of peripheral T cell tolerance are well described in the literature with general agreement among the authors. On the contrary, the parameters which can lead to the choice between T cell tolerance or response are still debated. Understanding the factors that govern the distinction between self and nonself is also fundamental for manipulating the response in the clinical practice.

A number of models have been formulated. The first model by Bretscher and Cohn dates back to 1970 and is referred to as the original "two signal model" [51]. According to this, the activation of all resting antigen-specific lymphocytes (both B and T cells) requires the interaction with the antigen and the presence of an antigen-specific T helper cell; in the absence of Th cell, the lymphocyte would be deleted. Actually everyone agrees that this model applies to B cell and CTL. Therefore, the issue of the debate is the activation/tolerization of $CD4^+$ T cells. This issue is strictly dependent upon the definition of the basis of self/nonself (S/NS) discrimination.

Many years after "the two signal model", the same authors have proposed a very basic hypothesis for S/NS distinction called the "minimal model" [52,53]: according to this, the discrimination is set by the timing of the antigen exposure: early in the ontogeny, under maternal protection, the environment is free of nonself and in case of antigen encounter, T cell shall be tolerized. Later in the ontogeny, the outcome of the antigen encounter is immunity. Although attractive, this model requires additional assumptions to explain some phenomena such as the lack of rejection of the fetus, the need of adjuvants for immune responses and many others.

Janeway and colleagues proposed a newer S/NS model based on the reformulated hypothesis of the two signal step for the full activation of naive T cells [54-56]. According to his hypothesis, $CD4^+$ T cells require two distinct signals to activate: the first signal is delivered by TCR; the second signal is costimulatory; in the absence of costimulation, T cells are

directed to die or become anergic [54]. The activation of the innate immune response is mandatory for the delivery of the two signals: the innate immune cells have the ability to discriminate between "non-infectious self" and "infectious nonself" by recognition of PAMPs and, as a consequence, trigger the expression of costimulatory molecules [55, 56]. Infectious nonself is therefore a sort of third signal required for full $CD4^+$ T activation.

Janeway's view was then elaborated by other authors. Matzinger and colleagues elaborated the "danger model" in which the third signal is "danger" [57, 58]: APC can in fact be activated by exogenous microbial PAMPs (as Janeway's model assumes), but also by endogenous alarm signals from stressed or damaged cells. The two class of signals are both categorized as danger signals able to activate APC and consequently induce immune response. The term DAMP (danger associated molecular pattern) has then been formulated to include also the endogenous alarming stimuli. Although the "infectious non self" (INS) and the Danger models have some common features, their basic assumptions about what initiates immunity are fundamentally different. For dangerous foreign pathogens or harmless self, the two models make the same predictions. However, for foreign but harmless (e.g., fetuses) or self but harmful entities (e.g., some mutations), the two models make different predictions.

Instead, Zinkernagel and colleagues have formulated a model starting from a totally distinct point of view [59]: the antigen parameters (the dose, time and particularly the localization -inside or outside the

lymphoid organs-) govern the decision between self and nonself. More specifically, they propose that T cell response may be induced when the antigen is presented in the secondary lymphoid organs in sufficient -non excessive- dose for a sufficient time interval. Lower doses or time periods result in antigen ignorance, as well as in the case in which the antigen never reaches secondary lymphoid organs; instead, excessive dose or permanence in the lymphoid organs induces deletion.

In conclusion, many model have been proposed on this issue, each able to explain some observed phenomena (Table 1), but none has been totally able to give a proper prediction for every situation encountered during the lifespan of an individual. Probably, the truth is a combination of all the described models which interplay in different scenarios; trying to reason in very general terms, we can propose that the choice between T cell tolerance induction or immunity depends on the activation state of the APC (i.e. the presence of one or two signals) and the context (dose, timing, localization) in which the antigen is presented (Fig. 9).

Observation/Experiments	Signal 1 + 2	Localization, dose, time
Effects of adjuvants[37]	'Danger', necrosis[14]	Depot, i.e. (dose + time) sufficient + localization: peripheral, draining to secondary lymphoid organs[15,54]
Puberty 'new antigens'	T cells get anergized in periphery e.g. breast[14]	Milk antigens are ignored since they do not reach lymphoid organs
Transplanted cells, organs[11,55–57]	Fresh graft brings 'danger', cultured grafts anergize	Passenger leukocytes induce immune response, but *only* if they reach secondary lymphoid organs[15,25]
		Antigenic cells of solid tissues usually cannot migrate to secondary lymphatic organs
Peripheral tumour carcinoma, sarcoma	Healthy cell 'no danger' anergizes T cell in periphery	Ignored for too long if no tumour cells emigrate to secondary lymphoid organs[20]
		If tumour cells emigrate early, anti-tumour immunity is induced → no tumour[20]
		Successful T help induction via APC/macrophages only via class II, but antibodies cannot reach periphery
Viruses exclusively in peripheral cells (neurones: Borna or rabies virus; epithelial cells: papilloma viruses; some tumour viruses)	Cross-processing obligatory for CTL—induction and cross-processing happens as a rule[58,59]	Ignored for too long[15,19,35]
		Induction of CTL as a rule only if infected (or antigen expressing) cells or replicating virus reach secondary lymphoid organs
Lymphocyte migration in neonates (or irradiation chimaeras) permits T cells to reach periphery! Liver disease in antigen tg+TCR tg mice	T cells are deleted and deletion is maintained by DC cross-presenting peripheral self-determinants via class I[60]	Is a tg TCR observation that does not apply to low-frequency T cells
	Micro-environment of tumour/organ is key to effectiveness of auto-aggression[61]	Permanent regeneration of T cell repertoire from thymus cannot be explained with a mechanism operative only a few days after birth
		Dose response: window is minimal
Anti-CD40 or −/− Anti-CD40 L or −/−	Absence of signal 2 angergizes/deletes[53,62,63]	Change of dose-time-localization parameters[64]
B7 positive tumour cells are more imunogenic	Second signal enhances T induction, prevents peripheral deletion[53,65]	Identical T cell induction directly by $B7^{+}$ and $B7^{-}$ fibro-sarcoma tumours (Ochsenbein, unpublished)
Fibroblasts expressing new antigen without signal 2 are ignored or immunogenic if they stay outside of or are brought into secondary lymphatic organs respectively, in absence of deletion or cross-priming	Unexplained or must be explained via efficient or obligatory cross-processing[58,59,66,67]	Localization and direct induction in secondary lymphoid organs[20,30]

Table 1: Interpretation of some observation according to different models.(From Zinkernagel R.M., Semin. Immunol, 2000).

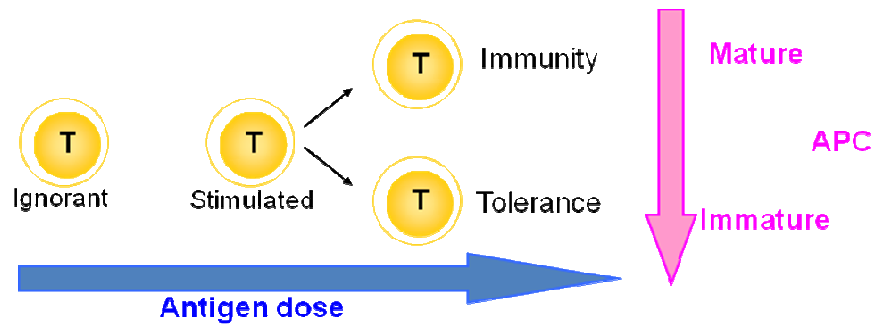


Figure 9: Schematic view of the fundamental parameters which can lead to T cell tolerance or immunity. Different models support the hypothesis that either the activation state of the APC or that the context in which antigen is presented are fundamental parameters for the choice. The truth is that the two conditions are both necessary.

5. Regulatory T cells

Regulatory T cells (Treg) are a subset of T cells specialized in the control of T cell self-reactivity as well as of immunological homeostasis, limiting excessive chronic responses against pathogens. In both cases, they prevent deleterious effects of the activation of the immune system. In the meanwhile, the suppression exerted by these cells can also limit beneficial responses against, for instance, tumors. Moreover, a lot of Foxp3^{+} Treg cell-based therapeutic approaches have been exploited for the treatment of immunological pathologies, including autoimmune diseases and allergy [60, 61].

The description of the phenomenon of T cell-mediated suppression dates back to 1970s [62]: Gershon and Kondo 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. To explain such observation, they postulate that thymus derived lymphocytes could make a suppressive substance. However, no marker existed at that time for the identification of the suppressor cells and the complex experimental models were not easy to replicate. Thus, in few years the idea that these cells could exist did not convinced anymore.

Apart from some hypothesis of few illuminated authors in the 1980s, the concept of the existence of a population of suppressor cells renewed only in 1995, with the seminal paper of Sakaguchi and colleagues [63]: in that study, a population of CD25-expressing CD4^{+} cells able to suppress a

number of autoimmune responses were identified and re-defined "regulatory T cells". From that initial observation, a big number of studies were performed on the topic and now it is known that the original $CD4^{+}CD25^{+}Foxp3^{+}$ Treg population described by Sakaguchi is only one of the documented subsets of regulatory T cells.

$Foxp3^{+}$ 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). These include $CD4^{+}CD25^{+}Foxp3^{+}$ Treg cells (which are phenotypically and functionally similar to nTreg cells), IL-10 secreting Tr1 cells and TGF-β secreting Th3 cells.

In the next paragraphs we will focus on the biology of $CD4^{+}CD25^{+}Foxp3^{+}$ Treg cells and only a dedicate paragraph will be referred to the other iTreg cell subsets.

5.1 Functional and phenotypic characterization of $CD4^{+}CD25^{+}Foxp3^{+}$ Treg 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. Their proliferation and survival is highly dependent on IL-2 and, since Treg cells have an anergic phenotype and do not produce effector cytokines by themselves, the source of IL-2 are probably effector T cells.

$CD4^+CD25^+Foxp3^+$ Treg cells are very efficient in suppressing the activation, proliferation and effector function of a wide panel of target cells including $CD4^+$ and $CD8^+$ T cells, dendritic cells, B cells, macrophages, NK cells, NKT cells, osteoblasts and also mast cells. In this way, they prevent many self reactive responses such as those directed toward allergens [64].

Historically, the identification of specific markers has been a fundamental step for the identification and manipulation of Tregs. Up to 1500 gene products are overexpressed or repressed in Treg with respect with Tconv cells and constitute the "Treg-cell signature" [65-67]: some of these proteins have been ascribed as privileged markers of Tregs.

CD25 molecule (the IL-2 receptor α–chain) was the first surface marker described by Sakaguchi [63]: it is not an exclusive marker as also Tconv cells transiently express it upon stimulation; however, only Treg cells constitutively express CD25. Functionally, the expression of the high affinity IL-2 receptor represents a clear demonstration of the fundamental role of IL-2 for Treg function and development. In fact, mice deficient for IL-2 or CD25 spontaneously develop T cell-mediated fatal lymphoproliferative/ inflammatory autoimmune disease, a phenotype somewhat similar to the one observed in Treg deficient mice.

Apart from CD25, the most important Treg cell marker, at least in the mouse system, is Foxp3. In humans, in fact, Foxp3 is also expressed by some activated Tconv cells.

Foxp3 (forkhead box P3) is a X-linked transcription factor specifically expressed by Treg cells [68]. Foxp3 is considered as a master regulator of

Treg cell biology: Foxp3-deficient mice lack $CD4^{+}CD25^{+}$ Treg cells [65] and, similarly to spontaneous Foxp3-mutant *scurfy* mice, develop fatal systemic lymphoproliferative autoimmune disease with hyperproduction of IgE [68-71]. Similarly, in humans, mutation of the Foxp3 locus is responsible for a severe disorder, IPEX (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked). Moreover, Foxp3 ectopic expression is sufficient to confer to naive T cells a Treg-like phenotype with suppressive capacities [68].

Foxp3 regulates between 700 and 1100 genes [65, 72], either directly or through indirect interaction with other transcription factors, and can function as a transcriptional activator or repressor (Fig. 10). Regulated genes include cytokines (i.e., IL-2, TGF-β, TNF–α, IFN–γ, IL–4), cytokine receptors, chemokines, cell surface or secreted molecules (GITR, CTLA4, CD25) and many other gene categories.

Although Foxp3 is indispensable for Treg cell development and function [72], many studies have demonstrated that the *de novo* lineage commitment and several key features of Treg cells are Foxp3-independent, including anergy and the dependence on paracrine IL-2 [65, 70, 71]. Rather, Foxp3 amplifies and stabilize pre-established molecular features of Treg cells.

Considering Foxp3 as a simple marker to uniformly identify T cells with suppressor ability, has been recently demonstrated to be an oversimplified view of the reality: human Foxp3 expressing cells comprise three phenotypically and functionally distinct subpopulations: $CD45RA^{-}Foxp3^{hi}$ activated Treg cells which rapidly died;

CD45RA^{+}Foxp3^{lo} resting Tregs able to proliferate and switch into activated Treg cells; and nonsuppressive cytokine secreting CD45RA^{-}Foxp3^{lo}. In mice, Foxp3 expressing cells appear to be more homogeneous [73].

Other known markers of Treg cells are CTLA-4 and GITR. CTLA-4 is an inhibitory molecule which belongs to the B7 family. It has multiple roles, e.g. maintaining the Treg anergic phenotype and contributing to the modification of the DC activation state mediated by Treg cells. GITR (glucocorticoid induced TNF receptor family-related gene/protein) is involved in the modulation of Treg mediated suppression.

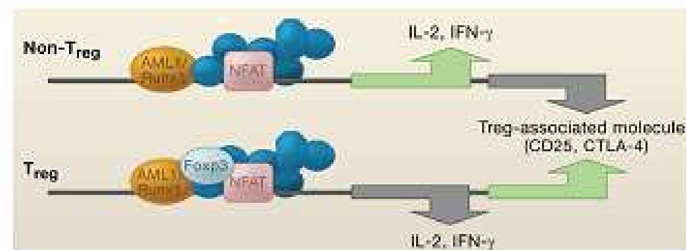


Figure 10: Control of Treg function by Foxp3. Foxp3 interact with estabilished transcriptional complexes and activate or repress the transcription of many genes encoding cytokines or surface molecules. (Modified from Sakaguchi S. et al., Immunity 2009).

5.2 The origin of CD4^{+}CD25^{+}Foxp3^{+} Treg cells

Foxp3^{+} Treg cells can be generated in the thymus during thymic selection: Foxp3^{+} thymocytes are detectable from the late CD4^{+}CD8^{+} double positive stage to the single positive stage, constituting ~5% of

mature $CD4^+CD8^-$ thymocytes and less than 1% of $CD4^-CD8^+$. Thymic Treg cells (also known as natural Treg, nTreg) then migrate to peripheral tissues where other Treg cells (called adaptive or induced Treg, iTregs) are converted from Tconv cells in the periphery (Fig. 11a) [74, 75]. The peripheral population of Treg cells, therefore, comprise both nTreg and iTreg cells and represents 10-15% of circulating $CD4^+$ T cell pool (Fig. 11b).

Evidences [67, 74] suggest that nTreg TCRs display high affinity for thymic pMHC. Indeed, Treg development requires a strong signal via the TCR. Besides, other molecules play critical roles in the process, such as CD28, CD40L and LFA-1 on T cells with their respective partners CD80 and CD86, CD40 and ICAM-1 on thymic stromal cells. Finally some common γ-chain cytokines (i.e. IL-2, IL-15 and to a lesser degree IL-7) are required for thymic development of Treg cells, as well as for their survival in the thymic medulla and in the periphery (fig. 12a) [76]. At the cellular level, both medullary thymic epithelial cells (mTECs) and dendritic cells contribute to Treg generation in the thymus.

While nTreg develop in a highly controlled thymic environment, iTreg cells differentiate under more varied conditions. Induction of Foxp3 expression in naive T cells occurs under suboptimal TCR signalling or by a combination of strong TCR engagement together with TGF-β. Moreover, Treg generation is observed during the induction of oral tolerance in the mesenteric lymph nodes or in the gut lamina propria or after tissue transplantation.

Our understanding of iTreg conversion is still incomplete, but it is

known that the minimal differentiation program for mouse T cells requires TCR stimulation, TGF-β and IL-2 (Fig. 12b)[74, 77]. At the molecular level, TGF-β promote Foxp3 expression and CTLA-4 is required [78]. Stimulation of human T cells in similar conditions also results in Foxp3 expression, but the cells lack regulatory cell function; notably, in humans Foxp3 is a poor marker of suppressor ability.

The role of TGF-β and IL-2 in the murine iTreg conversion is in contrast to the generation of nTreg for which both TGF-β and IL-2, although important, are not so fundamental: lack of TGF-β signalling is rapidly counterbalanced by higher IL-2 production [79], while IL-2 deficiency is supplemented by IL-15.

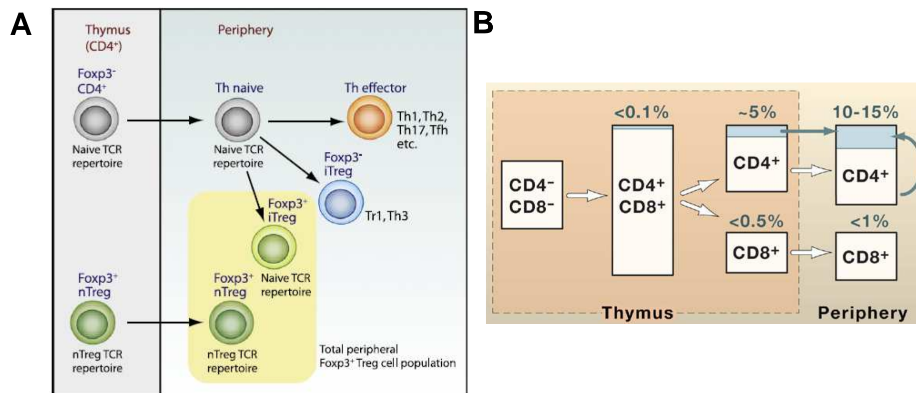


Figure 11: Thymic and peripheral generation of Foxp3+ Treg cells. a) nTreg cells differentiate in the thymus and migrate to peripheral tissues, while iTreg cells differentiate in the secondary lymphoid organs. b) The composition of Foxp3+ cells in each thymocyte subpopulation is shown as a percentage. In the periphery, $CD4^+$ non-Treg cells can differentiate to $Foxp3^+$ Treg cells under certain conditions (Modified from Curotto de Lafaille M.A. et al., Immunity 2009 and Sakaguchi S. et al., Immunity 2009).

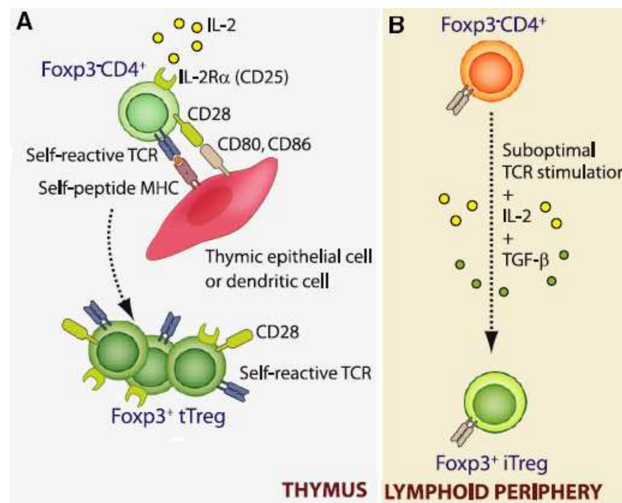


Figure 12: Signals for thymic and peripheral generation of Tregs. a) Treg differentiation in the thymus requires high affinity TCR engagement, CD28 binding and cytokine signalling. b) In the periphery, conditions like chronic low-dose antigen stimulation, the presence of cytokines like TGF–β and IL-2 or other metabolites favor Treg generation. (modified from Josefowicz S.Z. et al., Immunity, 2009).

Apart from the minimal requirements for Treg induction, other factors can promote the process in the periphery. Retinoic acid (RA), a metabolite of vitamin A, have been shown *in vitro* and *in vivo* to induce the differentiation of mouse [80, 81] or human [82] conventional T cells into $CD4^{+}CD25^{+}Foxp3^{+}$ cells with potent suppressor ability while repressing differentiation toward Th17. RA could influence Foxp3 induction through several, nonmutually exclusive mechanisms. First, RA inhibits the synthesis of cytokines (IFN-γ, IL-4, and IL-21), which interfere with the conversion [80, 81]. Second, there is a direct effect on the activation of the Foxp3 locus (e.g., by direct transactivation of the

Foxp3 locus or by potentiating TGF-β signalling). Third, RA interferes with the negative effect of co-stimulation on naive T cells [83]. *In vivo*, RA is strongly produced, together with TGF-β, by a specialized subset of DCs distributed in intestinal lamina propria and mesenteric lymph nodes, CD103$^+$ DCs: orally administered antigens presented by these DCs can efficiently mediate the conversion of Foxp3$^+$ Treg cells, thus contributing to oral tolerance toward food antigens and intestinal flora [66, 74, 84, 85].

Different organs or tissues are more or less permissive to Treg differentiation because of their cellular and molecular composition. Mucosal sites as intestine containing the CD103$^+$ DCs are just one example. In the spleen, CD8$^+$CD205$^+$ DCs have been shown to promote Treg conversion [86]. CNS, on the contrary, does not favor Treg generation in part due to IL-6 production by Tconv cells, which drive Th17 differentiation instead of Treg cells in the presence of TGF-β.

Another important issue regarding Treg biology is the homeostatic mechanism that controls the size of Treg pool in the periphery. Peripheral Treg cells seem to have a minor dependence on homeostatic TCR triggering than conventional naive T cells. Rather, three factors are implicated in this control: IL-2, CD-28 and TGF-β. IL-2 also increases the metabolic fitness and proliferative capacity of Treg cells [87].

5.3 Other induced Treg cell subsets: Th1 and Tr3

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) and Th3 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 [76, 88]. Moreover, they can also be produced *in vitro* by antigenic stimulation of naive T cells in the presence of IL-10 or a combination of IL-4 and IL-10 [76], and can be induced from human and mouse T cells cultured with immature DCs and immunosuppressive drugs [89]; human blood CD4^{+} T cells can also differentiate to Tr1 after repetitive priming with monocyte-derived DC [90].

Tr1 cells do not express Foxp3 [91], express low levels of IL-2 and IL-4 and are hypoproliferative upon restimulation. After TCR engagement, Tr1 cells produce high levels of IL-10 and variable levels of IFN-γ, TGF-β, and IL-5 [92]. More importantly, IL-10-induced Tr1 cells suppress the proliferation of antigen-specific naive T cells *in vitro* and *in vivo* [91]. Although the main suppressive mechanism is the secretion of immunosuppressive cytokines (IL-10 and TGF-β), it is likely that Tr1 cells share some suppressive mechanisms with natural Foxp3^{+} Treg cells. *In vivo*, Tr1 cells play an essential role in many circumstances, often

collaborating with $CD4^{+}CD25^{+}$ Treg cells [92-94]: Tr1 are involved in maintaining transplantation tolerance (avoiding acute GVHD), suppress allergen-specific Th2 reactions in healthy individuals, control responses to self-antigen and have a role in gut tolerance toward food-derived antigens and microflora [95]. Their impairment is associated with autoimmune diseases like rheumatoid arthritis (RA) or experimental autoimmune encephalomyelitis (EAE).

Antigen-specific TGF-β-secreting T cells [96], called Th3 cells, were originally propagated from animals that became tolerant to orally administered protein antigen. Their generation is dependent on the same TGF-β; however, the absence of known markers strongly limits the studies on this cell subset.

5.4 Basic mechanisms of Treg cell function

Treg cells can exert suppressive functions through a number of mechanisms contact-dependent or mediated by soluble factors. The most common view is that Treg cells need TCR triggering for their activation but then suppress the response of T cells independently of their antigen specificity [97]. However, recent findings demonstrate that Treg mediated suppression could also occur in the absence of TCR stimulation [98]. These different conclusions can be ascribed to the diverse experimental setting used.

Suppressive mechanisms exploited by activated Treg cells can be divided into four groups: production of inhibitory cytokines, cytolysis, metabolic

disruption and modulation of DC maturation/function (Fig. 13) [99-101]. Inhibitory cytokines secreted by Treg cells have dual roles: they control conventional T cell activation and promote the induction of other Treg cells, such as Tr1 and Th3 [90, 93, 94, 101]. Inhibitory cytokines include IL-10, TGF-β and IL-35 [102].

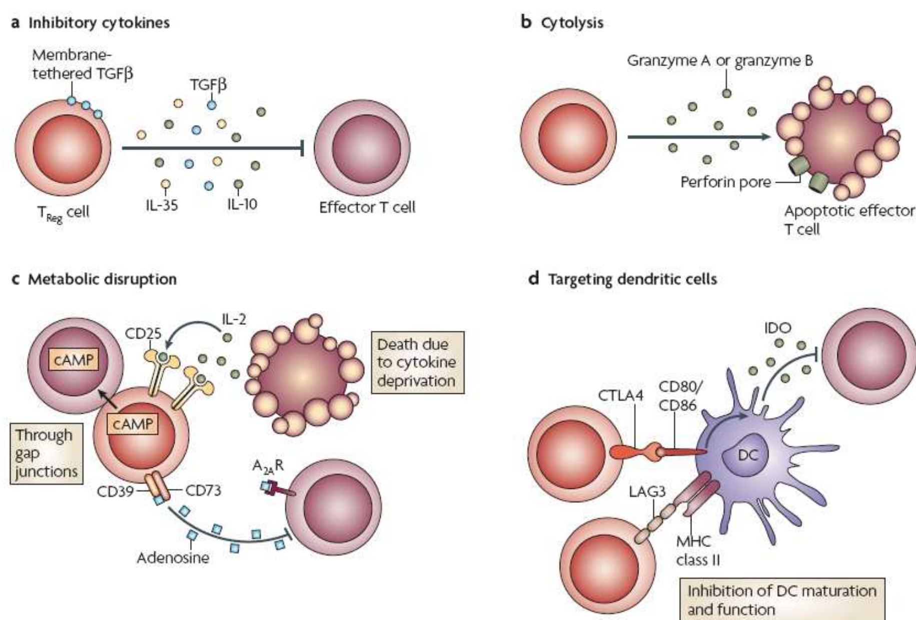


Figure 13: Molecular mechanisms of Treg cell-mediated suppression. a) Inhibitory cytokines include IL-10, IL-35 and TGF-β. b) Cytolysis is mediated by granzymeA, granzymeB and perforin. c) Metabolic disruption includes cytokine deprivation-mediated apoptosis, cyclic AMP (cAMP)-mediated inhibition of T cell functions and CD39- and/or CD73-generated adenosine, which mediate immunosuppression upon ligation to adenosine receptor 2A ($A_{2A}R$), expressed bu T cells. d) DC maturation and/or function are modulated by Treg cells through mechanisms such as LAG3–MHC-class-II mediated suppression of DC maturation and induction of IDO production, an immunosuppressive enzyme, by DCs. (From Vignali D.A.A. et al., Nat. Rev. Immunol., 2008).

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) as well as Th1 cell differentiation [88]. Moreover, IL-10 promote the conversion of naive T cells into Tr1 cells, with subsequent amplification of IL-10 effects [86, 88-90]. IL-10 action is important in controlling a number of situations including allergic reactions [92] and intestinal homeostasis [95].

The transforming growth factor-β (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 which ultimately transduce their signal to the nucleus. There, they control the expression of many genes, thus regulating a number of cell functions. [103]. This confers a strong anti-proliferative activity on T cells and the inhibition of CTL, Th1 and Th2 cell differentiation.

IL-35 is a recently described heterodimeric cytokine composed of IL-12α (p35) subunit, shared with IL-12, and EBI3 subunit, shared with IL-27. Apart from the clear suppressive function in mice, the exact understanding of its activity is still limited.

Although the general suppressive activity of these three cytokines as well as their role in iTreg cell functions are undisputed, their

contribution to the function of nTreg cells and the extent to which they are employed in specific biological settings are still debated. In addition, TGF-β produced by Foxp3+ Treg cells could be bound to the cell surface by an as yet uncharacterized receptor and would mediate suppression in a cell contact-dependent fashion rather than in a soluble fashion [78, 99, 100].

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 [99, 100].

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 [104]. Metabolic disruption is also based upon the pericellular adenosine generation catalyzed by CD39 and CD73 which are concomitantly expressed on the surface of CD4+CD25+ Treg cells [105]. Adenosine binds to adenosine receptor 2A (A^2AR) 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 catalyze transformation of exogenous tryptophan into immunosuppressive kynurenine. Tryptophan starvation and kynurenine production both contribute to immunosuppression [106].

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 [99, 100].

6. Tolerogenic dendritic cells

A common view in the past was that DCs exist in two functional states: mature DCs, efficient in eliciting immunity, and immature DCs that induced T cell tolerance in the steady state [37, 105]. This paradigm has been challenged by a number of studies showing that also fully mature DCs can induce differentiation of Treg cells [107]. Thus, DC maturation *per se* cannot longer be considered a distinguishing feature of immunogenic or tolerogenic DC. Rather, the integration of different signals by the DC, including antigen dose, cytokine milieu and maturation state will define a DC as "tolerogenic" [21, 108, 109].

Immature DCs in the steady state indeed efficiently induce T cell deletion, anergy or conversion into Treg cells, but some cytokines can strongly modulate tolerogenic functions of DCs: in the presence of TGF-β, for example, DCs can induce Treg differentiation *in vitro*, even when stimulated with LPS. IL-10 is another cytokine which can induce tolerogenic DCs: IL-10 interferes with maturation of DCs and promote tolerance induction in a number of models.

DCs are also target of Treg cell-regulation: these cells specifically inhibit DC function in multiple ways, even in the presence of maturating stimuli and the consequence is the induction of "infectious tolerance", which is believed to allow the expansion of the regulatory environment in a bystander manner.

In conclusion, the distinction between immature and mature DCs does not fit very well with the definition of tolerogenic vs. immunogenic DCs;

given these premises, it is fundamental to investigate the conditions which can render a DC able to induce tolerance *in vivo*. The results of these studies will provide new tools for the design of therapeutic strategies for many pathologic conditions including autoimmune diseases, allergy, allograft acceptance and cancer.

Chapter 2: Scope of the thesis

The study of T cell tolerization dynamics has been historically conducted using both *in vitro* and *in vivo* approaches. *In vitro* approaches have been employed for mechanistic studies of processes which are easily reproduced *ex vivo*; a typical example of such processes is T cell conversion into Treg cells: cell culture experiments have been useful for demonstrating the role of RA or TGF-β and for evaluating the ability of a specific DC subset [78-86] in the process of Treg cell conversion. Moreover, i*n vitro* experiments aimed to find the best condition for therapeutic *ex vivo* expansion of Treg cells have been performed [110].
Despite this, *in vitro* models are not sufficient for the study of a complex phenomenon like tolerance and *in vivo* models are necessary for such investigation. Two main experimental settings are commonly exploited. First, the generation of mice expressing transgenic TCRs specific for an antigen which is usually administered at a given dose in a controlled way: this approach is particularly useful to study tolerance toward circulating, airway- or food-derived antigens administered endovenously, intranasally or orally, respectively [111, 84]; second, the adoptive transfer of T cells (often transgenic) into recipients expressing the cognate antigen in many forms, soluble [112-114] or membrane-

bound [115], systemic or tissue-restricted [112], under steady state or inflammatory conditions and/or presented by one [116,117] or more APC types. Also, T cells can be transferred into recipient mice which also receive the antigen: this approach was used, for example, to deliver Ovalbumin to a specific APC subpopulation, DEC205^{+} DCs, by the use of an engineered anti-DEC205-OVA antibody [86].

Our laboratory has historically used a peptide of the IgG2a^{b} molecule (Bpep) as a model antigen for tolerance-related studies. 2a T cells specific for Bpep presented in the context of class II MHC molecule I-A^{d} have been generated. These anti-IgG2a^{b} 2a T cells are not deleted in the thymus of IgG2a^{b+} mice and are responsible for a complete and chronic suppression of IgG2a^{b} production [113,114]. *In vivo,* in the IgG2a^{b+} mice, B cells demonstrated to be the only APCs able to present the Bpep to 2a T cells [118]. This peculiar presentation pattern was exploited to study the fine parameters which promote peripheral tolerance in antigen-specific T cells when the exclusive APCs are B cell [116,117]: 2a T cell fate was evaluated upon transient or chronic antigen exposure in the presence or absence of APC activation. Surprisingly, it was shown that the fate of transferred naïve T cells depended on the antigen dose and persistency and not on the activation status of the APC: in particular, transient antigen presentation in the absence of inflammation and in a self-context induces CD4^{+} T cell activation and memory formation. In contrast, CD4^{+} T cells that re-encounter the antigen for a prolonged period, presented either by resting or activated B cells, become nonfunctional and lose any autoimmune reactivity. Therefore, when the exclusive APCs are B cells

the presence/absence of a danger signal appears to be a dispensable parameter for T cell immunity/tolerance in the periphery; instead, antigen persistency is the fundamental parameter.

Anyway, the most important physiologic APCs are DCs. Although the role of DCs in inducing and maintaining T cell tolerance in a confined environment like the gut and mesenteric lymph nodes have been described quite well [78-86], less is known about their role *in vivo* in the tolerization toward endogenous self-antigens in peripheral lymph nodes (PLN) and spleen. Therefore we decided to generate a new model which exploited the same antigen (Bpep) and the same $CD4^+$ Bpep-specific T cells (2a T cells) used for B cell-induced tolerance studies to dissect the dynamics of peripheral T cell tolerization process within distinct secondary lymphoid organs when the exclusive APCs are DCs under steady state.

We set up a new transgenic model (DC-tg) in which Bpep is expressed by $CD11c^+$ cells as a transgene covalently linked to the class II $I\text{-}A^d$ β-chain molecule. Two founders (DC-tg and DC-tg^{high}) were generated whose DCs constitutively present the Bpep with lower and higher efficiency, respectively. In these mice, we could follow the fate of naive 2a T cells transferred in the absence of DC activation and some unexpected results emerged. Among secondary lymphoid organs, lymph nodes were privileged sites for T cell tolerization and distinct molecular mechanisms (anergy or Treg conversion) were employed in the presence of DCs presenting the same antigen with different efficiency. Spleens, on the contrary, did not appear to be site of tolerance induction

Chapter 3: Materials and methods

Generation of the 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 microinjected into CBA x C57/Bl6 oocytes which were implanted into pseudopregnant females to obtain transgenic founder animals. All the procedure for the generation of transgenic mice were performed by Dr. G Kollias laboratory.

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. Digested material was analyzed by PCR using 2 µl 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 Rag2-deficient -/- mice (2a T mice) expressing transgenic TCR that recognize 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 elsewere [113]. DC-Tg mice were backcrossed on the BALB/c Rag2-deficient background for at least 10 geerations. BALB/c and congenic CB-17 mice (expressing the $IgG2a^b$) were bred in our animal facility. 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 cell line was generated by Hannestad e Bartnes using a $CD4^+$ Th1 clone obtained from a BALB/c mouse immunized with an allogenic $IgG2a^b$ molecule [119]. They 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, DCs ($CD11c^+$) and macrophages

(F4/80$^+$, CD11b$^+$, CD11c$^-$) were sorted from the spleen, peripheral lymph nodes and mesenteric lymph nodes of BALB/c mice using MoFlo cell sorter (purity >98%); the indicated numbers were co-cultured with 10^5 B5 cells in 200 µl of complete medium. After 24 hours, surnatants were collected and IL-2 concentration was 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 and mesenteric lymph nodes were collected from of 2a T mice. Single cell suspension 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, Vβ14$^-$ T cells were purified from the spleen of CB-17 mice by negative selection. In brief, after spleen processing and red blood cell lysis, the cells were incubated 20 min. on ice 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), washed in PBS and incubated with streptavidin MicroBeads (Miltenyi Biotec). Labelled 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 µl PBS. Two weeks later, blood samples were collected and the presence of $CD4^+V\beta14^-$ 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. If necessary, a secondary 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-CD8a-PE (53-6.7), anti-CD25-PE or –biotin (7D4), anti-Vβ14-FITC or –biotin (14-2), all from BD Bioscience; anti-CD25-APC (7D4, Southern Biotech); anti-CCR4-PE or -APC (2G12, Biolegend). Intracellular detection of Foxp3 was performed using PE-anti mouse/Rat Foxp3 staining set (FJK16s, eBioscience). CFSE cell labeling (Invitrogen) was performed following manifacturer's instructions. Data were acquired using a BD FACScalibur and analyzed with CellQuest software (BD Biosciences).

Reagents

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

Suppression assay

Responder 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 upon depletion of T cells using biotinilated anti-CD3ε and anti-TCRβ-chain antibodies (BD), streptavidin microbeads and MACS LS columns (Miltenyi Biotec).

Cells assessed for their suppressive capacity were recovered from the spleen or the 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 separating labeled cells with 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 and putative Tregs at a 2:1 ratio were co-cultured with splenocytes in 96-well round bottom plate in complete

medium supplemented with 0,6 μg/ml of purified anti-CD3ε mAb (BD). 72 hrs later, cells were collected, and proliferation of CFSE labelled cells was assessed by flow cytometry.

In vitro 2a T restimulation

To assess the functionality of 2aT cells after the primary response, T cells recovered from the spleen or lymph nodes of transferred mice were re-purified as illustrated in the previous paragraph and co-cultured with splenocytes depleted of T cells (as described above) in the presence or not of 1 μg/ml Bpep. After 48 or 72 hr, surnatants were collected and IFNγ production was evaluated by ELISA using BD OptiEIA mouse-IFNγ kit (BD).

In vivo proliferation assay

Mice were i.v. injected with 1 mg of purified anti-CD25 antibody (clone PC61) (Bio X Cell) or PBS. After 5 days, $5x10^5$ purified 2a T cells were labeled with 1 μM CFSE and i.v. injected in the animals. After 48 or 72 hr spleen, mLNs and pLNs of injected animals were collected and proliferation of CFSE labelled cells was assessed by flow cytometry, gating the analysis on $CD4^+V\beta14^+$ cells.

In vitro conversion assay

2a T cells were enriched using MoFlo from a pool of spleens and lymph nodes of 2a T Rag2-/- mice by negative selection of $CD11c^+$, $B220^+$, $DX5^+$, $CD11b^+$ cells (purity 70%). For the purification of $CD11c^{high}$ and $CD11c^{low}$

cells, spleen or lymph nodes of BALB/c mice were pooled; T and B cells were depleted using biotinilated anti-CD19 and anti-CD3ε Abs (BD bioscience), streptavidin microbeads and MACS LS columns (Miltenyi Biotec); then, the negative fraction was sorted using MoFlo (purity >98%). Propidium Iodide positive cells were excluded. 10^5 2a T cells and $2*10^4$ CD11c$^{high/low}$ cells were cultured in a 96-well plate in final volume of 200 µl of complete medium supplemented, where indicated, with 3 ng/ml rhTGF-β1 and 3 ng/ml rhTGF-β2 (R&D systems), 1 or 10 µM RA (Sigma-Aldrich) and 1 µM LE540 (Wako). After 5 days, cells were collected and Foxp3 ICS was performed.

Microarray and quantitative rt-PCR

CD11c$^+$ cells were sorted from the spleen or LNs 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. Total RNA integrity was assessed with a Agilent Bioanalyser and the RNA Integrity Number (RIN) was calculated. Only high-quality RNA preparations, with RIN greater than 8.5, were used for microarray analysis.

cRNA was prepared from 3 µg of total RNA according to the Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix) using the one cycle target labelling kit and according to the manufacturer's instructions. 10 µg of biotinylated cRNA was hybridized to the Affymetryx GeneChip Mouse Genome 430A 2.0 arrays. Bioconductor32

was used for most data handling. The Guanine Cytosine Robust Multi-array Analysis GCRMA33 method was used to calculate probe set intensity. The normalization method applied was the quantile.

For microarray data validation, $CD11c^+$ cells were sorted from the spleen, mesenteric or other peripheral lymph nodes of BALB/c mice using MoFlo (purity >98%) and resuspended in 500 µl TRIzol® reagent (Gibco-BRL). Total RNA was extracted using chlorophorm separation followed by purification using RNeasy Micro kit (QIAGEN) and quantified using RiboGreen RNA quantitation reagent (Invitrogen). qRT-PCR reaction was performed on 1 ng RNA using Power SYBR® Green RNA-to-CT™ 1-Step Kit (Applied Biosystems) Each reaction was performed in duplicate on a Applied Biosystem 7500 PCR system using the following primers: . Data are represented as Aldh1a2 Ct normalized on Hprt1 Ct.

Statistical analysis

Results are expressed as the means ± SEM. Data were analyzed using a nonpaired Student's *t* test.

Chapter 4: Results

1. Experimental model

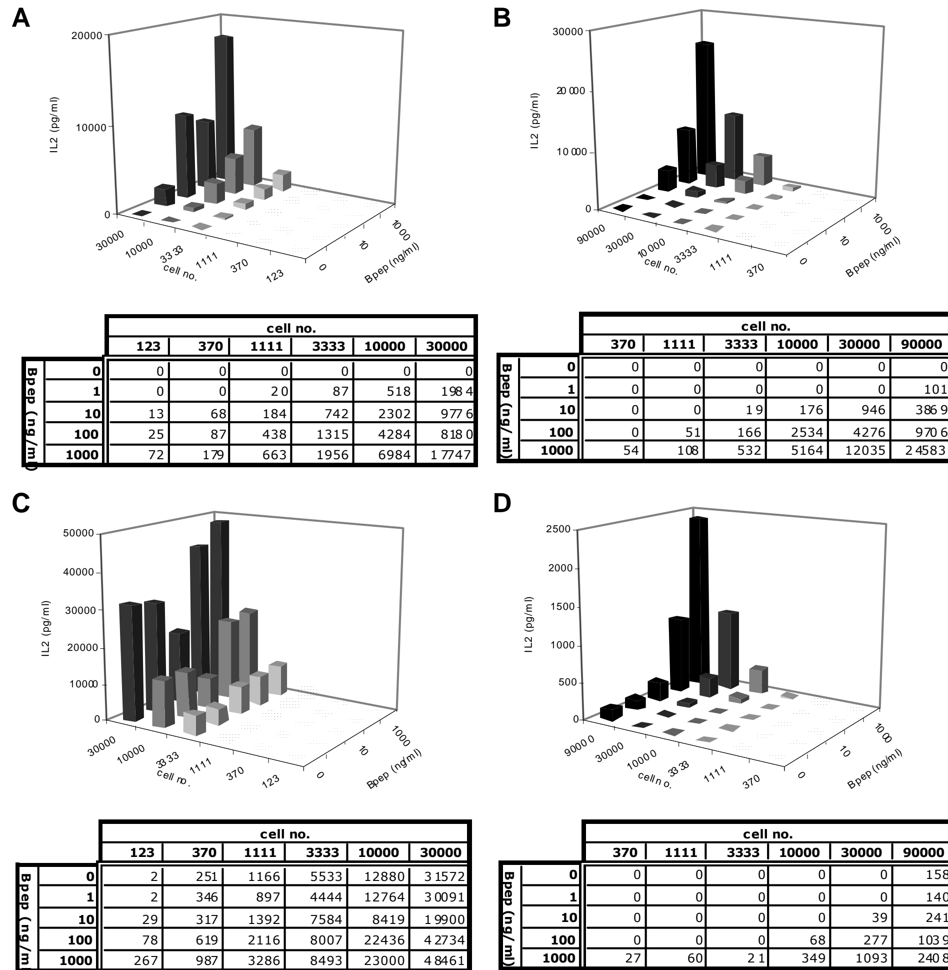
To investigate the outcome of DC-mediated antigen presentation to $CD4^+$ T cells *in vivo* under steady state conditions, we generated an experimental transgenic mouse model (called DC-tg) in which an antigenic peptide (the 435-451 epitope of $IgG2a^b$, Bpep) is exclusively presented by $CD11c^+$ cells. More specifically, the Bpep coding sequence was fused to the I-A^d β-chain coding sequence and the expression of the construct was restricted to $CD11c^+$ cells using the CD11c promoter (Fig. 1). As a result, in the DC-tg mice $CD11c^+$ cells should express, other than endogenous I-A^d molecules, a "chimeric" I-A^d composed by the endogenous α–chain and the transgenic Bpep-β-chain.

Figure 1: Transgene construct and Bpep sequence. The unique Bpep form expressed in the DC-tg mice is the transgene, which is composed by the β-chain of the I-A^d molecule fused to the Bpep. The expression of the transgene is controlled by the CD11c promoter.

Transgenic animals were obtained on BALB/c Rag2-deficient background (Igh a) in order to avoid the possibility that endogenous Bpep could be derived from the processing of the $IgG2a^b$ molecule.

To verify which cell type was able to present the transgenic peptide in the DC-tg and to establish the efficiency of antigen presentation, we sorted different cell populations from the spleen and lymph nodes and tested their ability to activate B5 T cell hybridoma. These cells express a TCR which shows high affinity for the $I\text{-}A^d$+Bpep complex and, once activated, efficiently secrete IL-2 [119]. This system is very sensitive: IL-2 can be secreted in measurable amounts (by ELISA) when up to a minimum number of 370 splenic DCs or 3330 macrophages from BALB/c mice loaded with as low as 10 ng/ml of Bpep are used as APCs (a complete panel of IL-2 amounts produced in response to different combinations of decreasing DC or macrophage cell numbers and Bpep concentrations is shown in fig. 2a and 2b).

Using this assay, Bpep presentation could be detected when as few as 370 $CD11c^+$ cells from the spleen of DC-tg mice were used as APCs, while at least $9x10^4$ macrophages ($CD11b^+F4/80^+CD11c^-$) were required to obtain a minimal IL-2 production by the same cell number of B5 hybridoma (Fig. 2c and 2d). Moreover, measurable Bpep presentation occurred using total lymph node- and spleen-cells from the DC-tg animals and was completely abolished when the same cell preparations were deprived of $CD11c^+$ cells, thus confirming that the major Bpep presenting cells in DC-tg are $CD11c^+$ cells (Fig. 3a).



A

Bpep (ng/ml)	cell no. 123	370	1111	3333	10000	30000
0	0	0	0	0	0	0
1	0	0	20	87	518	1984
10	13	68	184	742	2302	9776
100	25	87	438	1315	4284	8180
1000	72	179	663	1956	6984	17747

B

Bpep (ng/ml)	cell no. 370	1111	3333	10000	30000	90000
0	0	0	0	0	0	0
1	0	0	0	0	0	101
10	0	0	19	176	946	3869
100	0	51	166	2534	4276	9706
1000	54	108	532	5164	12035	24583

C

Bpep (ng/ml)	cell no. 123	370	1111	3333	10000	30000
0	2	251	1166	5533	12880	31572
1	2	346	897	4444	12764	30091
10	29	317	1392	7584	8419	19900
100	78	619	2116	8007	22436	42734
1000	267	987	3286	8493	23000	48461

D

Bpep (ng/ml)	cell no. 370	1111	3333	10000	30000	90000
0	0	0	0	0	0	158
1	0	0	0	0	0	140
10	0	0	0	0	39	241
100	0	0	0	68	277	1039
1000	27	60	21	349	1093	2408

Figure 2: Bpep presentation in the spleen of DC-tg mice is driven by $CD11c^{+}$ cells. DCs (a) or macrophages (b) sorted from the spleen of BALB/c animals or DCs (c) and macrophages (d) from the spleen of DC-tg animals were co-coltured in different numbers with B5 hybridoma cells in the presence of different concentrations of Bpep. 24 hours later, surnatants were collected and IL-2 were measured by ELISA (The results are representative of three independent experiments).

Bpep presentation in these animals was also investigated *in vivo*. CFSE labelled naïve anti-Bpep TCR transgenic T cells (2aT cells) were adoptively transferred in the DC-tg recipients and their division profile was analyzed at different time points after the injection. Indeed, 2aT cell divisions could be observed 48 hours after the transfer and, strikingly, the division profile was very similar between spleen and peripheral lymph nodes (PLN)(Fig. 3b).
As the trangene expression is controlled by the CD11c promoter, it was fundamental to define if Bpep presentation *in vivo* was mediated solely by $CD11c^{high}$ or also by $CD11c^{low/medium}$ cells. For this purpose, we generated DC-tg DTR double-transgenic mice, in which $CD11c^{high}$ cells can be depleted *in vivo* upon diphtheria toxin (DT) treatment. CFSE labelled naïve 2aT cells were injected in the DC-tg DTR animals and the division profile in treated and non-treated animals was compared (Fig. 3c). Bpep presentation in $CD11c^{high}$-depleted animals was still observed and was only slightly retarded demonstrating that Bpep presentation in DC-tg mice could be carried out both by $CD11c^{high}$ and $CD11c^{low/medium}$ cells.

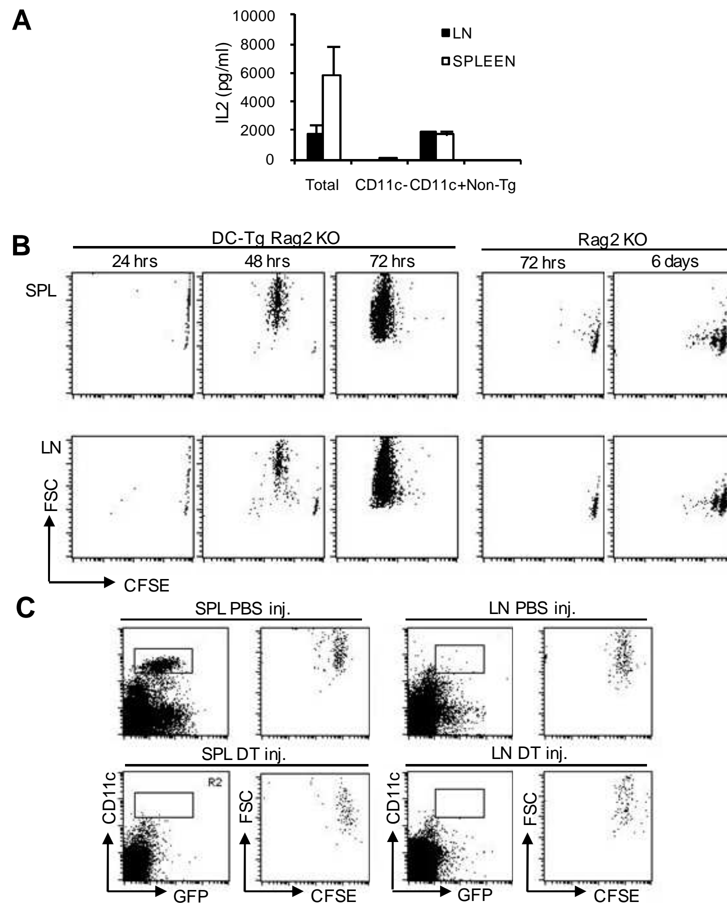


Figure 3: Bpep presentation in DC-tg animals is driven by CD11c⁺ cells. a) B5 hybridoma cells were co-coltured with different spleen or lymph nodes cell populations ; 24 hrs later, surnatants were collected and IL-2 was measured by ELISA. b) CFSE-labelled 2a T cell were transferred into DC-tg or Rag2-deficient control animals; 24, 48, 72 hours or 6 days later, spleen and pooled PLNs were collected and the 2a T cell division profile was analyzed by flow cytometry. c) 2aT cell division profile was analyzed 48 hours after the transfer into DC-tg DTR mice either treated or not with difetria toxin. All the results are representative of at least two independent experiments.

2. Conversion of naive T cells into Treg cells in lymphopenic hosts occurs in the lymph nodes but 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 the periphery, the fate of 2aT cells after the encounter of antigen presenting DCs was investigated. Thus, truly naïve ($CD44^{low}$, data not shown) $CD4^+V\beta14^+CD25^-Foxp3^-$ 2aT cells (Fig. 4a) purified from Rag2-deficient TCR transgenic mice were transferred into DC-tg or Rag2-deficient mice as controls and their behaviour was followed over time (Fig. 4b). We initially used Rag2-deficient mice as recipients to formally exclude some predictable complications of the system: i) the possibility that endogenous anti-Bpep Treg cells could suppress 2a T cell response immediately after transfer; ii) the possibility that B cell-produced $IgG2a^a$, whose 435-451 sequence (Apep) is very close to that of $IgG2a^b$ (Bpep), could lead to some nonspecific activation of the 2aT TCR.

In the DC-tg mice, a very robust primary response was observed after the adoptive transfer. 2a T cells strongly proliferated during the first two weeks and represented increasing percentages among spleen, lymph nodes (Fig. 4c) and blood (Fig. 4d) cell populations. Then, following a slight decrease, 2a T cell fractions reached a plateau in both peripheral lymphoid organs (spleen and pooled LNs). In contrast, in non-tg control mice, limited homeostatic proliferation of 2a T cells, probably due to the lymphopenic environment, occurred (Fig. 4d and data not shown).

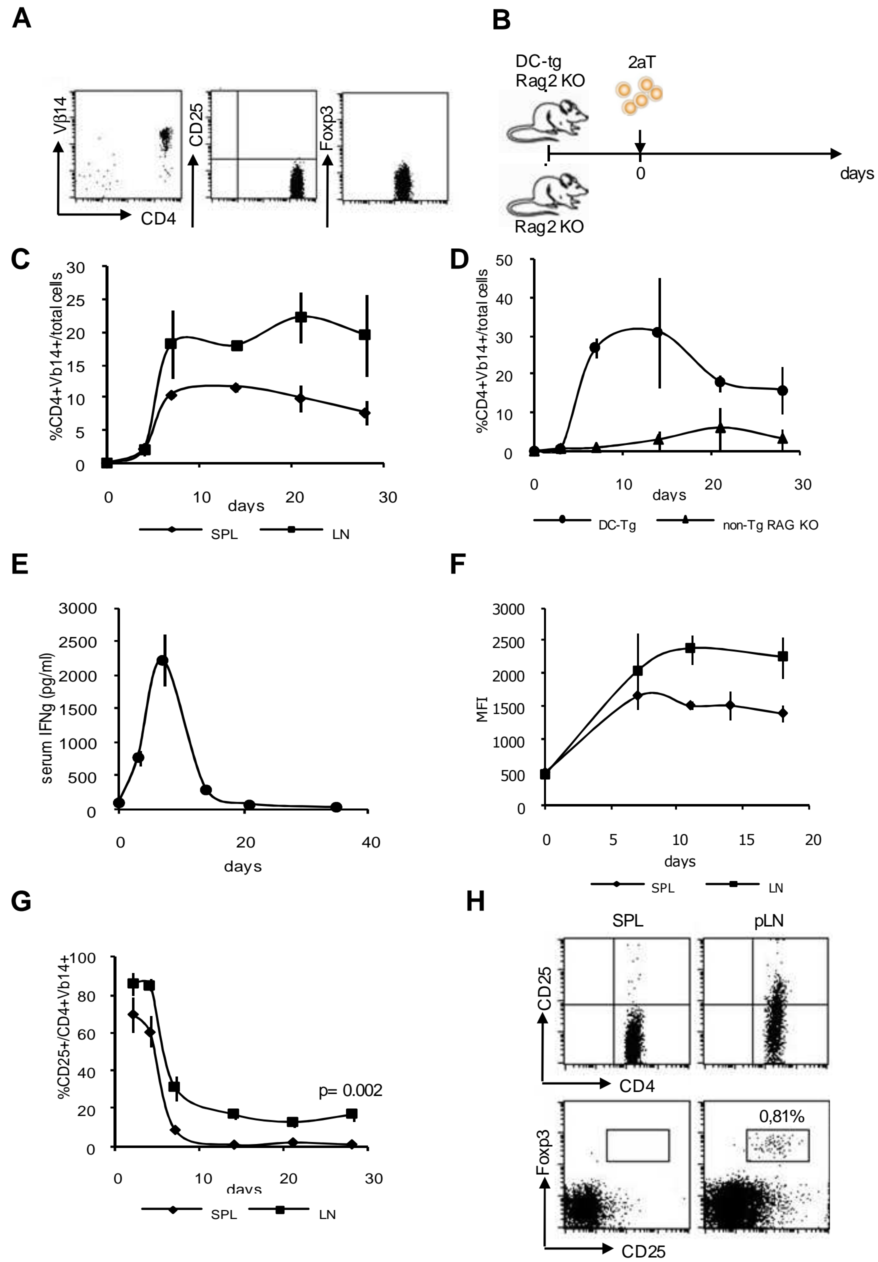

A
Vβ14
CD4
CD25
Foxp3
B
DC-tg
Rag2 KO
2aT
0
days
Rag2 KO
C
%CD4+Vb14+/total cells
days
SPL
LN
D
%CD4+Vb14+/total cells
days
DC-Tg
non-Tg RAG KO
E
serum IFNg (pg/ml)
days
F
MFI
days
SPL
LN
G
%CD25+/CD4+Vb14+
p= 0.002
days
SPL
LN
H
SPL
pLN
CD25
CD4
0,81%
Foxp3
CD25

Figure 4: 2aT cells transferred into lymphopenic hosts strongly respond to the antigen and can convert to Treg cells in the PLN but not in the spleen. a) Cytofluorimetric analysis of naive 2a T cells before the transfer. b) schematic representation of adoptive transfer experiments. c) and d) expansion of 2a T cells after the transfer was evaluated as percentage of $CD4^+V\beta14^+$ cells on total cell population. e) transient production of IFN-γ as measured by ELISA on blood samples collected at different time points. f) CD44 MFI was calculated over time as indication of the activation-induced CD44 upregulation. g) CD25 expressing 2aT cells ($CD4^+V\beta14^+$) kinetic analysis clearly shows initial transient upregulation in both the secondary lymphoid organs and subsequent sustained expesion in some of lymph node 2a T cells at later time points. h)Dot plots of Foxp3 and CD25 expression on gated $CD4^+V\beta14^+$ cells 3 weeks after the transfer; $CD25^+Foxp3^+$ cells can be identified exclusively in the PLN. Data are representative of at least 3 indipendent experiments using at least 3 mice per group.

Moreover, high serum IFN-γ was detectable starting at day 4 after the transfer, with a peak at day 7 in DC-tg (Fig. 4e) but not in Rag2-deficient mice (data not shown). Then it returned to basal level within day 18 (Fig. 4e). The expression of markers indicative of antigen recognition further indicated that 2a T cells were responding to the antigen. CD44 upregulation was already detected 7 days after the transfer and reached a peak at day 11 in both the lymph nodes and spleen (Fig. 4f). Moreover, the early activation marker CD25 was transiently upregulated in both the two secondary lymphoid organs very early after transfer (3-5 days). Subsequently, its expression was downregulated over time in the spleen, while some 2a T cells in PLN maintained CD25 at later time points (Fig. 4g). Interestingly, a significant percentage of these $CD25^+$ 2a T cells expressed also the Treg cell marker Foxp3 (Fig. 4h) while $CD25^+Foxp3^+$

2a T cells were not documented in the spleen.

To verify that $CD25^+Foxp3^+$ cells were truly Treg cells with regulatory properties, their suppressive activity was investigated *in vitro*. 2a T cells recovered from the lymph nodes of DC-tg mice 5 weeks after transfer were able to suppress anti-CD3ε induced polyclonal T cell proliferation and this activity was entirely due to $CD25^+$ cells, since it was completely abolished upon $CD25^+$ cell depletion. In contrast, splenic 2a T cells did not display any regulatory activity (Figure 5a).

Moreover, 2a T cells from the lymph nodes failed to produce IFNγ if re-stimulated *in vitro* with Bpep-loaded T cell-depleted splenocytes. This effect was specifically due to Treg-mediated suppression and was not dependent on a general defective functionality of 2a T cells: in fact, the same cells deprived of $CD25^+$ fraction returned to secrete the cytokine. In contrast, 2a T cells recovered from the spleen were not tolerant; rather, they were totally responsive to re-challenge *in vitro* and produced large amount of IFN-γ (Figure 5b).

In summary, *in vitro* analysis revealed that a state of peripheral 2a T cell-tolerance was instaurated in the PLN but not in the spleen and that this was dependent on Treg conversion. To confirm this split situation *in vivo*, we evaluated the capacity of CFSE-labelled naive 2a T cells (CFSE 2a T) to mount a secondary response and proliferate in the same recipient animals. According to *in vitro* results, 2a T cell proliferation was suppressed in the lymph nodes while full proliferation was observed in the spleen; moreover, the suppression of CFSE 2a T cell response was dependent on $CD25^+$ lymph node-2a T cells: in fact, when the mice were

pre-treated with the depleting/neutralizing anti-CD25 antibody (PC61), the proliferation of CFSE 2a T cells in the lymph nodes was restored (Figure 5c and 5d).

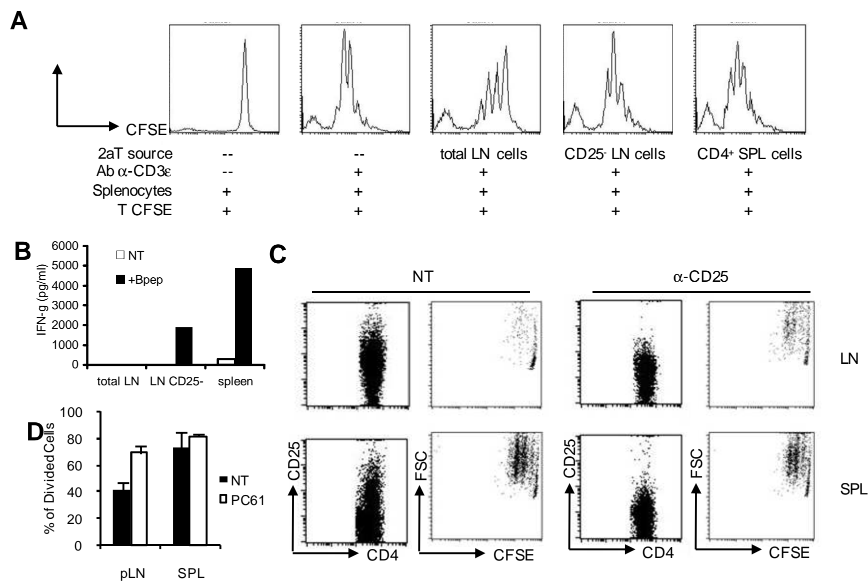


Figure 5: 2a Treg cells convert exclusively in the lymph nodes of the DC-tg mice and are sufficient to locally suppress 2a T cell responsiveness. a) CFSE-labelled polyclonal T cells were stimulated with splenocytes and α-CD3ε antibody in the presence of 2a T cells recovered from the spleen or pLN of the DC-tg mice 5 weeks after the transfer. 72 hours later, proliferation was assessed by flow cytometry. b) 2a T cells recovered from DC-tg mice 3 weeks after the transfer were restimulated *in vitro* with splenocytes loaded or not with 1 μg/ml of Bpep; 72 hours later, surnatants were collected and IFNγ was measured by ELISA. Lymph nodes cells were sometimes pooled from 2-3 animals. One representative experiment of three is shown. c) CFSE labelled naive 2a T cells were injected in DC-tg mice 3 weeks after the first adoptive transfer. 48 hours later spleen and pLNs were collected and analyzed by flow cytometry. Dot plots are gated on $CD4^+V\beta14^+$ cells. d) The percentage of divided cells of the experiment c) is shown in the bar graph. Representative of two experiments with two mice per group.

These results reveal an unpredicted different capacity of pLN and spleen-DCs in converting self-antigen-specific Treg cells in the periphery of a lymphopenic host at the steady state. Lymph nodes DCs appear to be particularly efficient in this process. Treg cells induced in the lymph nodes are sufficient to locally maintain tolerance but do not control the responsiveness of 2a T cells isolated from the spleen.

3. PLN- but not spleen-DCs are able to induce conversion of naive T cells into Treg cells in non-lymphopenic hosts

To clarify whether the lymphopenic environment could influence peripheral Treg generation observed in our system, DC-tg Rag2-deficient mice were reconstituted with polyclonal T cells before the adoptive transfer in order to simulate a more physiologic lymphoid environment. Polyclonal T cells were obtained from the CB.17 mice, congenic to BALB/c except for the immunoglobulin haplotype (Igh b). In this way, purified CB.17-derived T cells should be naturally tolerant to the IgG2a^{b}/Bpep. The cells were depleted of the Vβ14^{+} population to be able to track 2a T cells once transferred. At day 14 after reconstitution with CB.17 Vb14^{-} total T cells (Fig. 6a), 2a T cells were transferred and the phenotype of the recipient mice was analyzed three weeks later (Fig. 6b).

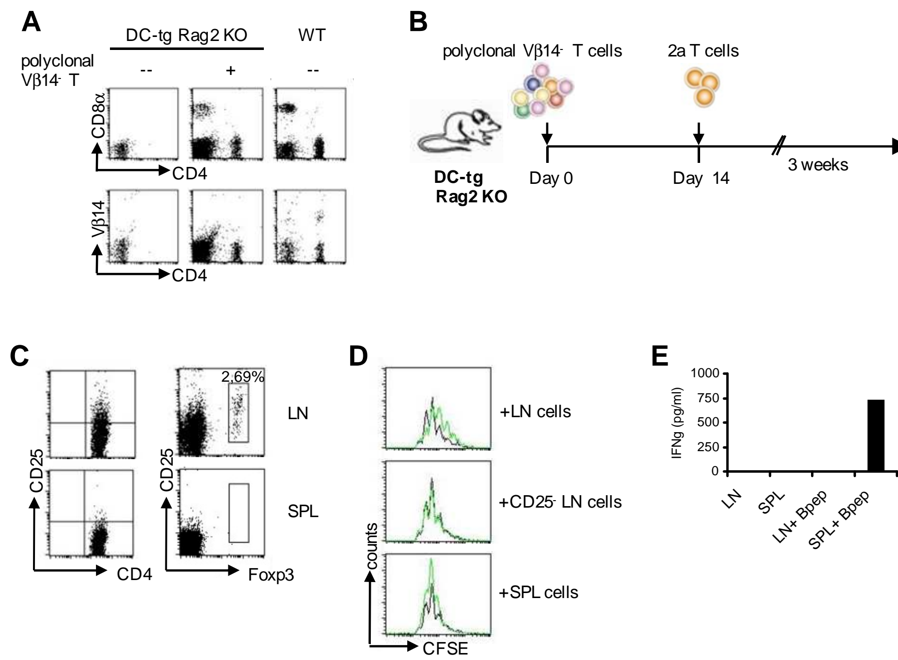


Figure 6: 2a T cells convert to Treg cells in the PLN of non-lymphopenic hosts. a) Blood samples were collected 14 days after the injection with polyclonal Vβ14$^-$ T cells and the reconstitution was verified by flow cytometry. b) Experimental design of the experiment. c) 3 weeks after 2a T transfer, spleen and PLN of the recipients were collected and Foxp3 expression was assessed by flow cytometry. Dot plots are gated on CD4$^+$Vβ14$^+$ cells. Representative of at least three experiments with two distinct mice per group. d) *In vitro* suppression assay was performed as in fig. 5a. The proliferation of CFSE 2a T cells in the presence of the population indicated at the right side of each panel (green) is compared to the proliferation of the same CFSE 2a T cells in the absence of those populations (black). Representative of two independent experiments, each performed with at least two pooled animals. e) Cytokine production was assesed as described in fig. 5b. Representative of at least two independent experiments.

According to the results obtained in non-reconstituted Rag2-deficient recipients, conversion of antigen-specific naive 2a T cells into $CD25^+Foxp3^+$ Treg cells (Fig. 6c) able to suppress polyclonal T cell proliferation (Fig. 6d) and antigen-specific T cell effector functions, such as cytokine production (Fig. 6e), was observable only in the PLN versus spleen. The conversion efficiency was even higher in non-lymphopenic compared to lymphopenic hosts, suggesting that "endogenous" non-antigen specific T cells could positively influence this particular functional activity of lymph node-DCs.

The fate of 2a T cells was indeed very similar between lymphopenic and non-lymphopenic recipients. However, an interesting difference exists between the two groups of animals in the mesenteric lymph nodes (MLN): $CD25^+Foxp3^+$ Treg cells, in fact, was never documented in the lymphopenic hosts, while they can be converted in MLN of non-lymphopenic recipients (data not shown). One possible explanation for this divergence between MLN and other PLN may rest upon the different DC subsets which populate gut-draining and skin-draining lymph nodes. However, further experiments are necessary to clarify this point.

4. Molecular characterization of the differences between spleen and lymph node CD11c^{high} and/or CD11c^{low} DCs/1: non-redundant role of retinoic acid in the conversion of Treg cells

The results obtained with this experimental system pose two types of questions: i) why iTreg cells differentiate only in lymph nodes and ii) why they do not re-circulate once differentiated: accordingly, Foxp3^{+}CD4^{+}CD25^{+} cells were never documented in the blood of the hosts (data not shown). If the first problem is certainly due to differences in the DC composition of lymph node and spleen, the second problem may or may not be due to such DC divergences.

To explore the possible molecular differences between spleen- and lymph node-DCs that could explain their divergent capacity in converting Treg cells and possibly in Treg cell homing, a global gene expression analysis was performed on CD11c^{high} and CD11c^{low} DCs sorted from these two secondary lymphoid organs in resting BALB/c mice. Genes showing an absolute $\log_2$ ratio of a least three in the level of transcript expression in spleen- compared to lymph node-DCs were considered differentially expressed. The lists of differentially expressed genes (DEG) of CD11c^{high} and CD11c^{low} DCs include genes involved in a number of cellular functions both linked to immunity and to unrelated processes (for the full list, see Table 1 and 2).

Probe Set ID	Gene Symbol	Gene Title	Entrez Gene ID	LogRatio	P.Value
1417925_at	Ccl22	chemokine (C-C motif) ligand 22	20299	-6,5144196	0,0002739
1422789_at	Aldh1a2	aldehyde dehydrogenase family 1, subfamily	19378	-6,2137691	0,0015012
1449757_x_at	Dntt	deoxynucleotidyltransferase, terminal	21673	-5,8304812	0,0002308
1420166_at	Dntt	Deoxynucleotidyltransferase, terminal	21673	-5,5941649	0,0003781
1439036_a_at	Atp1b1	ATPase, Na+/K+ transporting, beta 1 polypep	11931	-5,3567957	0,0007254
1439368_a_at	Slc9a3r2	solute carrier family 9 (sodium/hydrogen exc	65962	-5,3107614	0,000681
1439369_x_at	Slc9a3r2	solute carrier family 9 (sodium/hydrogen exc	65962	-5,2197974	0,0002211
1415837_at	Klk1	kallikrein 1	16612	-5,0969958	0,004241
1418726_a_at	Tnnt2	troponin T2, cardiac	21956	-5,0355362	0,0001541
1438365_x_at	Laptm4b	lysosomal-associated protein transmembran	114128	-4,9007888	0,0007419
1417185_at	Ly6a	lymphocyte antigen 6 complex, locus A	110454	-4,8230112	0,0025591
1436915_x_at	Laptm4b	lysosomal-associated protein transmembran	114128	-4,7833105	0,0005109
1448107_x_at	Klk1	kallikrein 1	16612	-4,6906074	0,0048798
1421321_a_at	Net1	neuroepithelial cell transforming gene 1	56349	-4,6273943	0,0025591
1428492_at	Glipr2	GLI pathogenesis-related 2	384009	-4,5311065	0,0022403
1427419_x_at	Ccr9	chemokine (C-C motif) receptor 9	12769	-4,5212107	0,0014777
1421256_at	Gzmc	granzyme C	14940	-4,4591969	0,0042997
1448261_at	Cdh1	cadherin 1	12550	-4,2728093	0,0014693
1419907_s_at	Fcrla	Fc receptor-like A	98752	-4,2289691	0,0008288
1422706_at	Pmepa1	prostate transmembrane protein, androgen i	65112	-4,1695453	0,0001541
1428077_at	LOC100047091 //	hypothetical protein LOC100047091 /// transr	100047091 /// 721	-4,1654637	0,0018315
1448576_at	Il7r	interleukin 7 receptor	16197	-4,0976072	0,0020187
1419908_at	Fcrla	Fc receptor-like A	98752	-4,0778448	0,0044418
1419605_at	Mgl1	macrophage galactose N-acetyl-galactosamin	17312	-4,0602345	0,0023106
1438975_x_at	Zdhhc14	zinc finger, DHHC domain containing 14	224454	-4,0152411	0,0003428
1427562_a_at	Prkca	protein kinase C, alpha	18750	-3,957173	0,0049173
1418346_at	Insl6	insulin-like 6	27356	-3,9306202	0,004008
1451780_at	Blnk	B-cell linker	17060	-3,879941	0,0012738
1424967_x_at	Tnnt2	troponin T2, cardiac	21956	-3,8323496	0,0028745
1449300_at	Cttnbp2nl	CTTNBP2 N-terminal like	80281	-3,8129487	0,0025284
1425506_at	Mylk	myosin, light polypeptide kinase	107589	-3,8118428	0,0009471
1437614_x_at	Zdhhc14	zinc finger, DHHC domain containing 14	224454	-3,7814739	0,0005821
1434062_at	Rabgap1l	RAB GTPase activating protein 1-like	29809	-3,6832337	0,0020187
1448575_at	Il7r	interleukin 7 receptor	16197	-3,5465107	0,0025591
1438619_x_at	Zdhhc14	zinc finger, DHHC domain containing 14	224454	-3,5463914	0,000553
1422259_a_at	Ccr5	chemokine (C-C motif) receptor 5	12774	-3,5441182	0,000462
1452358_at	Rai2	retinoic acid induced 2	24004	-3,5394119	0,0048798
1452445_at	Slc41a2	Solute carrier family 41, member 2	338365	-3,4642754	0,0041482
1420286_at	---	---	---	-3,4135662	0,0009771
1422601_at	Serpinb9	serine (or cysteine) peptidase inhibitor, clade	20723	-3,3696746	0,0020187
1438151_x_at	Zdhhc14	zinc finger, DHHC domain containing 14	224454	-3,3268207	0,0008288
1424727_at	Ccr5	chemokine (C-C motif) receptor 5	12774	-3,1939085	0,000462
1424733_at	P2ry14	purinergic receptor P2Y, G-protein coupled, 1	140795	-3,1677195	0,0020187
1422804_at	Serpinb6b	serine (or cysteine) peptidase inhibitor, clade	20708	-3,1626046	0,0016685
1425182_x_at	Klk1b22 /// Klk1l	kallikrein 1-related peptidase b22 /// kallikre	13646 /// 13648	-3,1561777	0,0041482
1453004_at	Slc22a23	solute carrier family 22, member 23	73102	-3,1379881	0,0033188
1423668_at	Zdhhc14	zinc finger, DHHC domain containing 14	224454	-3,0630588	0,0020205
1416022_at	Fabp5	fatty acid binding protein 5, epidermal	16592	-3,0426254	0,001025

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1456174_x_at	Ndrg1	N-myc downstream regulated gene 1	17988	-3,0061492	0,0020187
1420693_at	Myom1	myomesin 1	17929	-3,0016452	0,0046271
1448788_at	Cd200	CD200 antigen	17470	-3,000619	0,0006829
1425215_at	Ffar2	free fatty acid receptor 2	233079	3,17888886	0,0005109
1419758_at	Abcb1a	ATP-binding cassette, sub-family B (MDR/TAI	18671	3,22669214	0,0026257
1422837_at	Scel	sciellin	64929	3,33351512	0,0040723
1419759_at	Abcb1a	ATP-binding cassette, sub-family B (MDR/TAI	18671	3,45162602	5,49E-05
1421463_at	Siglece	sialic acid binding Ig-like lectin E	83382	3,494678	0,0004448
1452561_at	---	---	---	3,56847639	5,63E-07
1417622_at	Slc12a2	solute carrier family 12, member 2	20496	3,58460619	0,0025591
1425251_at	Ptger3	prostaglandin E receptor 3 (subtype EP3)	19218	3,84855812	0,0002211
1448005_at	Sash1	SAM and SH3 domain containing 1	70097	3,88247874	0,0016685
1426004_a_at	Tgm2	transglutaminase 2, C polypeptide	21817	3,90041394	0,0029496
1448780_at	Slc12a2	solute carrier family 12, member 2	20496	4,13441253	0,0024707
1425822_a_at	Dtx1	deltex 1 homolog (Drosophila)	14357	4,46858611	0,0004448
1427809_at	---	---	---	4,48879489	1,31E-05

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Table 1: List of differentially expressed genes of spleen- vs. lymph nodes-CD11c^{high} cells. Genes with an absolute log_2 ratio > 3 are depicted. Negative values refer to genes whose expression is more abundant in the lymph nodes-CD11c^{high} cells compared to the spleen-CD11c^{high} cells. Positive values refer to genes whose expression is more abundant in the spleen-CD11c^{high} cells compared to the lymph node counterparts.

Probe Set ID	Gene Symbol	Gene Title	Entrez Gene ID	LogRatio	P.Value
1417925_at	Ccl22	chemokine (C-C motif) ligand 22	20299	-5,5175168	0,0012166
1422789_at	Aldh1a2	aldehyde dehydrogenase family 1, subfamily A2	19378	-4,4003087	0,0164821
1421256_at	Gzmc	granzyme C	14940	-4,3655501	0,0099733
1454737_at	Dusp9	dual specificity phosphatase 9	75590	-3,4230904	0,0056586
1420166_at	Dntt	Deoxynucleotidyltransferase, terminal	21673	-3,3266979	0,0099733
1449757_x_at	Dntt	deoxynucleotidyltransferase, terminal	21673	-3,233831	0,0095197
1424229_at	Dyrk3	dual-specificity tyrosine-(Y)-phosphorylation regulatec	226419	-3,2160067	0,0312772
1427839_at	Igh-VJ558	Immunoglobulin heavy chain (J558 family)	16061	-3,1532848	0,0304237
1424842_a_at	Arhgap24	Rho GTPase activating protein 24	231532	-3,1341509	0,0005853
1418726_a_at	Tnnt2	troponin T2, cardiac	21956	-3,0973127	0,0040253
1450545_a_at	Dntt	deoxynucleotidyltransferase, terminal	21673	-3,0919315	0,0061509
1436363_a_at	Nfix	nuclear factor I/X	18032	3,04185665	0,037167
1448620_at	Fcgr3	Fc receptor, IgG, low affinity III	14131	3,11233067	0,047644
1448123_s_at	Tgfbi	transforming growth factor, beta induced	21810	3,14166623	0,028188
1417623_at	Slc12a2	solute carrier family 12, member 2	20496	3,15786171	0,0303406
1423153_x_at	Cfh /// LOC1000	complement component factor h /// similar to complen	100048018 /// 126	3,1626321	0,0001337
1416978_at	Fcgrt	Fc receptor, IgG, alpha chain transporter	14132	3,16485369	0,0035767
1415904_at	Lpl	lipoprotein lipase	16956	3,17794553	0,0177942
1452210_at	Dna2	DNA replication helicase 2 homolog (yeast)	327762	3,19952835	0,0006334
1448655_at	Lrp1	low density lipoprotein receptor-related protein 1	16971	3,20239633	0,0002506
1417622_at	Slc12a2	solute carrier family 12, member 2	20496	3,24745226	0,0095197
1423593_a_at	Csf1r	colony stimulating factor 1 receptor	12978	3,31932282	0,0066182
1420361_at	Slc11a1	solute carrier family 11 (proton-coupled divalent metal	18173	3,36970817	0,0296926
1428018_a_at	AF251705	cDNA sequence AF251705	140497	3,38289518	0,0299094
1423754_at	Ifitm3	interferon induced transmembrane protein 3	66141	3,39454035	0,0022052
1450876_at	Cfh /// LOC1000	complement component factor h /// similar to complen	100048018 /// 126	3,54615392	5,92E-06
1425216_at	Ffar2	free fatty acid receptor 2	233079	3,55209889	0,0147368
1417676_a_at	Ptpro	protein tyrosine phosphatase, receptor type, O	19277	3,58321644	0,0035767
1420249_s_at	Ccl6	chemokine (C-C motif) ligand 6	20305	3,59859211	0,0035767
1421685_at	Clec4b1	C-type lectin domain family 4, member b1	69810	3,65076392	0,0066182
1419872_at	Csf1r	colony stimulating factor 1 receptor	12978	3,65411823	0,0156689
1448780_at	Slc12a2	solute carrier family 12, member 2	20496	3,69697033	0,0095197
1450020_at	Cx3cr1 /// LOC10	chemokine (C-X3-C) receptor 1 /// similar to chemokin	100047704 /// 100	3,70100014	0,0019853
1425546_a_at	Trf	transferrin	22041	3,70585916	0,0094549
1422412_x_at	Ear3	eosinophil-associated, ribonuclease A family, member	53876	3,73932007	0,0296926
1417460_at	Ifitm2	interferon induced transmembrane protein 2	80876	3,78498184	0,0128255
1422645_at	Hfe	hemochromatosis	15216	3,79330799	0,0294658
1450731_s_at	Tnfrsf21	tumor necrosis factor receptor superfamily, member 21	94185	3,8276068	0,0054175
1425407_s_at	Clec4a2 /// Clec	C-type lectin domain family 4, member a2 /// C-type le	26888 /// 69810	3,85848923	0,006923
1422041_at	Pilrb1	paired immunoglobin-like type 2 receptor beta 1	170741	3,92759576	0,02059
1451563_at	Emr4	EGF-like module containing, mucin-like, hormone rece	52614	4,13640246	0,0461683
1417266_at	Ccl6	chemokine (C-C motif) ligand 6	20305	4,49600381	0,0008941
1440865_at	Ifitm6	interferon induced transmembrane protein 6	213002	4,72446163	0,0048832
1422411_s_at	Ear1 /// Ear12 //	eosinophil-associated, ribonuclease A family, member	13586 /// 13587 //	4,90875702	0,0141068
1417936_at	Ccl9	chemokine (C-C motif) ligand 9	20308	5,02433188	0,0007479
1449846_at	Ear2	eosinophil-associated, ribonuclease A family, member	13587	5,06841129	0,02805
1451263_a_at	Fabp4	fatty acid binding protein 4, adipocyte	11770	5,27495987	0,0450292
1448756_at	S100a9	S100 calcium binding protein A9 (calgranulin B)	20202	5,66327324	0,0259886

Table 2: List of differentially expressed genes of spleen- vs. lymph nodes-CD11c^{low} cells. Genes with an absolute log2 ratio > 3 are depicted.

We initially focused our attention on two DEGs, *Aldh1a2* and *Ccl22*, for two reasons. First, because in both comparison they resulted the first in the list with absolute $\log_2$ ratio of -6,21 and -6,51 in the CD11c^{high} cells and of -4,40 and -5,51 in the CD11c^{low} cells, respectively; in other words, they are far more abundant in the lymph nodes-DCs with respect to spleen-DCs and, thus, could indeed mediate one of the observed phenomena. Second, these two genes are already known to be involved in Treg cell biology in some scenarios. *Aldh1a2* encodes retinaldehyde dehydrogenase 2 (RALDH2), an aldehyde dehydrogenase that catalyzes the synthesis of retinoic acid (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 or indirectly, for the conversion of iTreg cells in mesenteric lymph nodes and lamina propria [80-85]. *Ccl22* encodes for the homonymous chemokine CCL22, whose receptor, CCR4, is reported to be highly expressed on the surface of Treg cells [120]. Thus, we hypothesized that, analogously to MLN, RA produced by DCs in PLN but not in the spleen was involved in the peripheral conversion of naïve T cells into Treg cells and that CCL22 was required for the homing of iTreg cells in the lymph nodes. In the next paragraph we will focus our attention on CCL22. Now we will follow the hypothesis of a role of RA in the conversion of Treg cells.

qPCR analysis confirmed that *Aldh1a2* gene was significantly more expressed in the PLN-CD11c^{high} cells compared to the splenic counterparts (Fig. 7a); in these analysis we included MLN-CD11c^{high} cells as positive control expressing very high levels of *Aldh1a2* [84].

To verify if RA produced by DCs in PLN was involved in the conversion of antigen-specific Foxp3$^+$CD25$^+$ T cells we set up both *in vitro* and *in vivo* experiments. *In vivo* DC-tg mice transferred with naive 2a T cells were systemically treated for three weeks with LE540 [121], a Retinoic Acid Receptor (RAR) inhibitor which is able to block Foxp3$^+$ Treg cell conversion mediated by mucosal DCs [84,85]. The presence of Treg cells was then analyzed in the PLN (Fig. 7b): a significant reduction in the percentage of Foxp3$^+$CD25$^+$ cells was observed in PLN of treated recipient mice, indicating that RA was responsible, at least partially, for the different capability of PLN- and spleen-DCs of inducing Treg cell conversion.

Given the pleiotropic effects of RA on a number of physiological functions, both related or not with immunity, there could be the possibility that the contraction of Foxp3$^+$CD25$^+$ Treg conversion was dependent on indirect effects of RA on the process.

To rule out this possibility, naive 2a T cells sorted from 2a T Rag2-deficient mice were co-coltured for 5 days with different subpopulation of CD11c$^+$ cells sorted from the spleen or lymph nodes of the DC-tg mice (purity >98%); then their conversion to CD25$^+$Foxp3$^+$ cells was assessed.

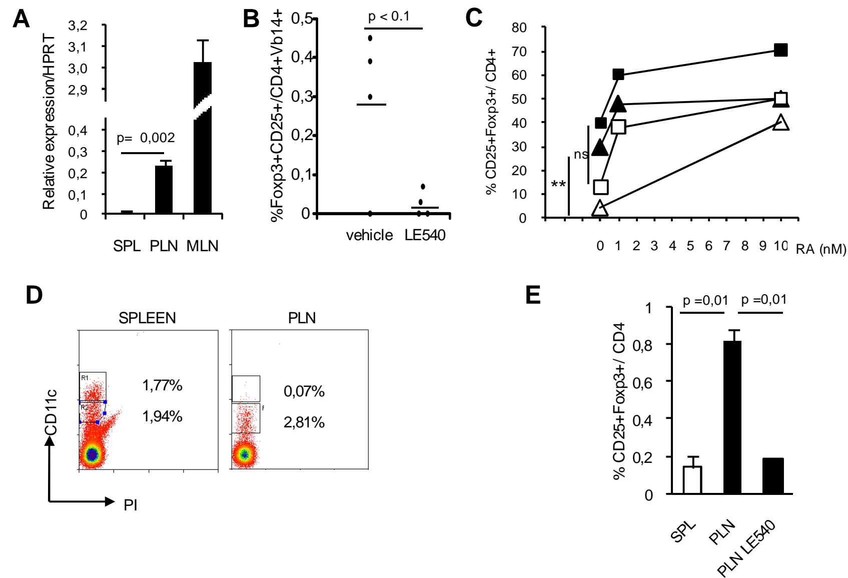

Figure 7: RA produced by PLN- but not spleen-DCs plays a fundamental role in the conversion of 2a T cells into CD25⁺Foxp3⁺ Treg cells. a) Validation of microarray data was performed by Real Time PCR. The bar graph represents *Aldh1a2* expression relative to *Hprt1* transcript. Results are representative of two independent experiments. b) DC-tg Rag2-deficient mice were adoptively transferred with 2a T cells and were treated with LE540 or vehicle every second day for 3 weeks. Then animals were sacrified and the percentage of $CD25^+Foxp3^+$ cells (gated on $CD4^+V\beta14^+$ cells) in the PLN was analyzed by flow cytometry. c) Naive $CD4^+V\beta14^+$ T cells sorted from 2a T Rag2-deficient mice were cultured with $CD11c^{high}$ (white dots) and $CD11c^{low}$ cells (black dots) sorted from spleen (squared dots) or PLN (triangles) of the DC-Tg animals in the presence of TGF-β and increasing doses of RA. 5 days later cells were collected and the percentage of $CD25^+Foxp3^+$ cells was assessed by flow cytometry. (** p= 0,001). d) Spleen or peripheral lymph node cell preparations depleted of T cells were stained with Ab α-CD11c APC and Propidium Iodide to exclude dead cells; $CD11c^{high}$ and $CD11c^{low}$ populations and their respective percentage are indicated in the dot plot. Data are represenative of four experiments. e) The experiment was performed as in c) but in the absence of TGF-β and in the presence of LE540 where indicated.

First, to exclude functional defects of the sorted cells and to confirm the positive effect of RA on Treg cell conversion, co-culture in the presence of TGF-β and increasing doses of RA were set up. In fact, TGF-β strongly promotes the induction of Treg *in vivo* [78,79] and is widely used in many *in vitro* conversion protocols [109]. Indeed (fig.7c), TGF-β strongly boosted Treg conversion in the presence of both splenic and lymph node-$CD11c^{low}$ cells and RA was able to further increase the percentage of $Foxp3^{+}CD25^{+}$ T cells in a dose-dependent manner. $CD11c^{high}$ cells from both the lymphoid organs, instead, was less efficient in the conversion capacity in the presence of TGF-β than $CD11c^{low}$ cells, although the difference was statistically significative only for PLN; however, RA were able to fill the gap between the two cell types, thus excluding an aspecific biological defect in $CD11c^{high}$ cells.
Given the less efficient intrinsic capacity of $CD11c^{high}$ cells in inducing Treg cells even in the presence of TGF-β and the paucity of $CD11c^{high}$ cells in the lymph nodes at the steady state (Fig.7c), we decided to analyze *in vitro* conversion of Treg in the absence of TGF-β using $CD11c^{low}$ cells as APC (Fig. 7e). A minimal (0,79%) but statistically significant fraction of 2a T cells converted to $CD25^{+}Foxp3^{+}$ cells when the antigen was presented by lymph nodes $CD11c^{low}$ cells; on the contrary, splenic $CD11c^{low}$ cells scarcely induced $CD25^{+}Foxp3^{+}$ cells, thus mirroring the divergent behaviour observed *in vivo*. Moreover, LE540 treatment significatively abrogated the conversion, suggesting a role for RA in the process. Indeed, the addition of TGF-β in the culture restored the ability of splenic $CD11c^{low}$ cells to efficiently convert Treg cells (Fig.

7c). These results suggested some conclusions: first, RA produced by PLN- (but not spleen-) CD11c^{low} cells plays a previously unappreciated role for in the peripheral induction of Foxp3^{+} T cells. Second, RA contribution was minor with respect of that of TGF-β *in vitro* but, *in vivo,* TGF-β appeared not to be involved in our experimental setting (data not shown). Third, it was possible that the divergence between PLN- and spleen-CD11c^{low} cells could not be appreciated in many of the experiments performed by others [e.g: 105] because Foxp3^{+} cell conversion was always evaluated in the presence of recombinant TGF-β.

5. Treg retention in the lymph nodes is dependent on CCL22 produced by lymph node (but not spleen) CD11c^{high} and/or CD11c^{low} DCs

CCL22 (or MDC, macrophage derived chemokine) is a chemokine produced by macrophages, activated pro-B cells [118] and DCs. In particular, Langherans cells and lymph nodes-DCs residing in the T cell area but not spleen DCs produce high level of CCL22 and increase the secretion of the chemokine upon DC activation [123-126].

CCL22 is chemotactic for a number of cells which express CCR4, the receptor shared with CCL17 (TARC); CCR4 expressing cells include activated NK cells, Th2 cells [122], skin homing memory T cells and also CD4^{+}CD25^{+}Treg [120].

Acting on these cells, CCL22 drives homing to the site of antigen presentation, i.e. the skin and the lymph nodes, and plays a role in a number of pathological conditions, including Th2 allergic and

inflammatory responses [127], autoimmune diseases such as diabetes and IBD [128,129] and tumor evasion: the recruitment of $CCR4^+$ Treg cells by tumor infiltrating CCL22-producing DCs, in fact, is associated to an adverse clinical outcome [130].

Our microarray analysis demonstrated that, at the steady state, both $CD11c^{high}$ and $CD11c^{low}$ cells resident in the lymph nodes actively transcribed *ccl22* (and presumably produced CCL22) while in the spleen its expression, if present, was indeed far less abundant. This observation, together with the documented expression of CCR4 on Treg cells, could explain why 2a Treg cells in the PLN of DC-tg animals did not recirculate once converted.

To follow this hypothesis, CCR4 expression on $CD4^+V\beta14^+CD25^+Foxp3^+$ 2a T cells was verified (Fig. 8a): 25,3±2,9% of $CD25^+Foxp3^+$ 2a T cells found in the lymph nodes of transferred animals expressed CCR4 compared to 11,9±2,8 % of $CD4^+V\beta14^+Foxp3^-$ cells. Then, the potent CCR4 antagonist AF399/42018025 was systemically administered into DC-tg mice adoptively transferred with 2a T cells 3 weeks before and the effect on Treg frequency was assessed three days later. This inhibitor was able to reduce the percentage of $Foxp3^+CD25^+$ cells among $CD4^+V\beta14^+$ 2a T cells populating PLN (Fig. 8b). Our result suggested that the CCR4 antagonist was able to inhibit CCL22-mediated 2a Treg retention in PLN: as a consequence, the cells were not retained anymore, exited the lymph nodes and this led to reduced Treg cell frequency in the lymphoid organ. The absence of Treg cells in the circulation of treated as well as non treated animals (data not shown) is not necessarily against our theory as

the Treg cell numbers are so small that, once outside the lymph nodes, they cannot be detected any more due to excessive dilution in the blood. Instead, we can speculate that retention within the lymph node is a unique opportunity for our immune system to concentrate very limited numbers of Treg cells optimizing their localization at the site of antigen encounter when the antigen is present.

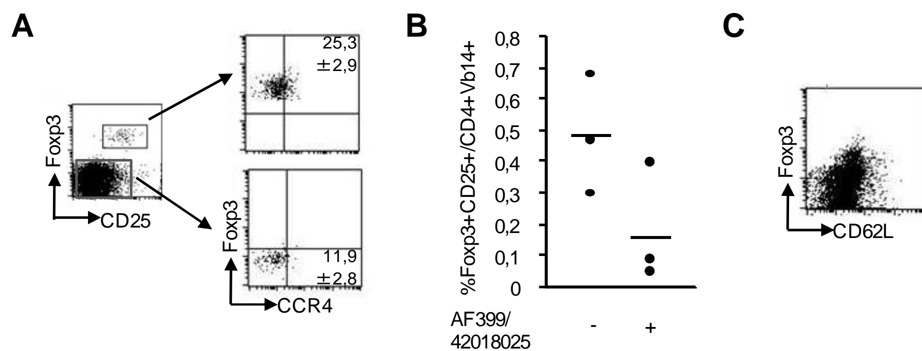

Figure 8: CCR4 signalling contribute to the retention of neo-converted 2a Treg cells within the PLN. a) The expression of CCR4 on Foxp3^{+} and Foxp3^{-} 2a T cells present in the lymph nodes was evaluated 2 weeks after the transfer by flow cytometry. Dot plots are gated on CD4^{+}Vβ14^{+} cells. b) Transferred mice were treated with AF399/42018035 or vehicle for 3 weeks; then, PLN were collected and the frequency of CD25^{+}Foxp3^{+} on total CD4^{+}Vβ14^{+} cells was assessed by flow cytometry. c) CD62L expression in nTreg cells was evaluated by flow cytometry on the lymph node cells prepared from BALB/c animals. Representative of at least three independent experiments.

It is worth noting that our results do not necessarily exclude that other additional molecules other than CCL22-mediated chemotaxis can also contribute to 2a Treg retention in the PLN, such as CCL17, which shares

the CCR4 receptor with CCL22, or CD62L and CCR7, other well known lymphoid homing molecules which are expressed by nTreg cells recirculating through lymph nodes (Fig. 8c and reviewed in ref. 131).

6. The capacity of peripheral lymph node DCs to induce Treg cell differentiation depends on the efficiency of antigen presentation.

Self-reactive lymphocytes can be rendered unresponsive in the periphery through many tolerization mechanisms, each used, alone or in combination, in specific biological contexts. It has been widely shown that the tolerization modality depends on a number of factors, such as the soluble or membrane-bound form of the self antigen [112], the tissue in which it is expressed [132] and/or its dose [133]. Given these premises, we exploited our experimental system to assess whether 2a Treg induction could be affected by the antigenic levels. Therefore, we selected a second founder of DC-tg Rag2-deficient animals (DC-tg^{high}) whose DCs spontaneously presented the Bpep with much higher efficiency, evaluated in terms of IL2 production by B5 T cell hybridoma; indeed, in the presence of bone marrow-derived DCs (BMDCs) (Figure 9a) as well as splenic DCs (Figure 9b) from the DC-tg^{high} animals, B5 cells secreted 4-5 times more IL2 in the culture surnatants than in the presence of the DC-tg counterparts. The different presentation efficiency of the two strains was specifically restricted to the transgenic Bpep and did not depend on general defective presentation in the DC-tg compared to DC-tg^{high}; in fact, when increasing amounts of exogenous soluble Bpep was

supplemented to the culture, DC-tg- as well as non-tg-derived DCs induced B5 hybridoma cell activation and IL2 production with comparable efficiency than that of the DC-tg^{high}-derived DCs (Figure 9c).
Using DC-tg and DC-tg^{high} mice as recipients, we had the possibility to compare two situations in which the antigen was presented in the same qualitative context but with different levels of presentation. Therefore, we transferred naïve 2a T cells in the two groups of recipients and their fate at the exhaustion of the primary response (evaluated through IFNγ serum measurement, data not shown) was compared. In the DC-tg^{high}, five weeks after transfer, 2a T cells found in the spleen were all CD44^{high} and CD25^{-}, according to what happened in DC-tg. However, in PLN, around 80% of 2a T cells downregulated the TCRs and none converted to Treg cells. TCR downregulation is a well known mechanism for extrathymic tolerance as T cells, in the absence of their TCR, cannot respond anymore. This mechanism of anergy strictly depends on the constant presence of the antigen: it has been documented that TCR can be soon re-expressed upon short activation *in vitro*[132,134]. Indeed, 2a T cells isolated from the lymph nodes of DC-tg^{high} immediately restored TCR expression once resorted and cultured for few hours (data not shown).
Globally, our results indicate that, in conditions of chronic antigen presentation by DCs, tolerance is always specifically induced in the PLN, with the mechanism of T cell tolerization being dictated by the dose of the antigen. Instead, spleen are never site of tolerization.

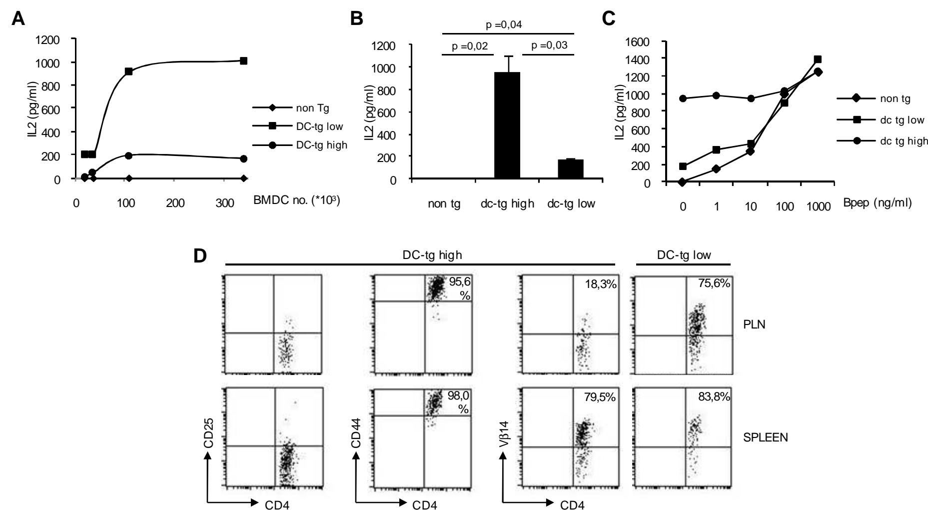

Figure 9: DC-tg^high mice present Bpep more efficiently than DC-tg animals and induce distinct tolerization mechanisms on transferred 2a T cells. a) BMDCs were differentiated *in vitro* from BM precursors in the presence of GM-CSF ($CD11c^+$>90%) and were cultured at the indicated numbers with 10^5 B5 cells; 24 hrs later, surnatants were collected and IL2 was measured by ELISA. b) 10^5 DCs were sorted from the spleen of the indicated mice and co-cultured as in a). c) cultures were performed as in b) with the addition of the indicated amounts of exogenous Bpep. d) 2a T cells were transferred in DC-tg^{high} or DC-tg mice and the phenotype of transferred cells was evaluated by flow cytometry 5 weeks later. Plots are gated on $CD4^+$ cells. Representative of two independent experiments, each with at least three mice per group.

Chapter 5: Summary, conclusions and future perspectives

Defining the rules which drive T cell tolerization in the periphery has been a matter of debate in the immunology community.

On one side, there have been many attempts to define which are the fundamental parameters for self-nonself discrimination. According to the classical view, two functional DC states (immature and mature) strictly linked to T cell outcome (tolerization or activation) exist: under steady state conditions immature DCs capture proteins and dying cells, present the derived self-antigens and silence T cells; in contrast, when DC maturation is induced, antigen presentation results in T cell activation [37, 51-58] and blockade of Treg-mediated suppression [135]. This classical paradigm has been recently challenged by the demonstration that also mature DCs expressing high levels of MHC or co-stimulatory molecules can be tolerogenic in some circumstances [107,109] and can also promote $CD4^{+}CD25^{+}$ Treg expansion [136].

Therefore, it is now well accepted that, beside the APC state, the context

in which the antigen is presented (i.e., the dose, timing, localization and cytokine milieu) is also important for the choice between tolerance or immunity; the expression of the same antigen in various non-lymphoid tissues [132], its dose [59], its soluble/cell-associated, systemic/tissue-restricted form [112], the transient/chronic exposure to this [59,116,117,133,137,138] and the presence of cytokines such as IL10 or TGF-β [108], in fact, deeply condition T cell responsiveness, resulting in activation or tolerance. Moreover, tolerance can be achieved through distinct and overlapping molecular mechanisms in different biological contexts [116,117].

On the other side, many reports have characterized the specific role of a particular APC subset in tolerance induction: for example, ovalbumin (OVA) has been delivered to $CD8^-33D1^+$ and $CD8^+DEC205^+$ DCs (the two major splenic DC subtypes) using chimeric anti-33D1 and anti-DEC205 antibodies, respectively; as a result of both triggering, OT-II OVA-specific $CD4^+$ T cells underwent strong initial proliferation followed by deletion [139].

Other than DCs, also B cells can induce T cell tolerance: Raimondi et al. demonstrated that when the antigen is chronically/persistently presented exclusively by B cells (either activated or not), antigen-specific $CD4^+$ transgenic T cells are tolerized in the periphery without formation of Treg cells [116,117 and unpublished results]. Moreover, Knoechel et al. showed that the transfer of OVA specific $CD4^+$ T cells into OVA-expressing mice which lack endogenous T and B cells leads to severe autoimmune reactions, while the transfer in animals lacking T cells but

not B cells results in TCR downregulation, control of T cell autoreactivity and milder disease [140].

Speaking about the role of different APC subsets, moreover, many studies have dissected the role of distinct APCs in inducing Treg conversion in the periphery, given that the accumulation of these cells has been associated to positive or negative outcome in many pathological conditions like autoimmune diseases [141] or cancer [142], respectively. These studies demonstrated that Treg conversion are mediated by distinct APC subsets in different biological settings: in the spleen, $CD25^+Foxp3^+$ Treg cells are induced only when the antigen is targeted to $DEC205^+$ DCs [86]. Instead, after cardiac transplantation, the only intracardiac APCs able to induce Treg cells and mediate graft acceptance are pDCs with partially mature phenotype. In the gut associated lymphoid tissues (mesenteric lymph nodes and lamina propria), $CD103^+$ DCs induce Treg conversion from naive T cells after antigen exposure[84].

Here, we present a new experimental setting in which the peptide 435-451 of $IgG2a^b$ (Bpep) is chronically presented by DCs under steady state conditions. We have generated a transgenic mouse model (DC-tg) in which the self antigen (Bpep) is expressed exclusively on the surface of $CD11c^+$ cells in a covalently linked form with the β–chain of $I\text{-}A^d$. Naive Bpep-specific $CD4^+$ T cells (2a T cells) have been transferred into the DC-tg mice and their behaviour have been followed over time.

This system represents an original approach for studying peripheral tolerance for many reasons. First, the contribution of DCs to tolerance

induction can be dissected excluding the role of other APCs while including all the DC subsets, without confining *a priori* antigen presentation to a single subset. Second, the antigen is already expressed in the form of peptide-MHC complex; therefore, the variability in uptaking, processing and presentation among different antigen forms (soluble or membrane-bound, systemic or tissue-specific) is bypassed. Third, exploiting the two transgenic lines naturally presenting the Bpep with different efficiency (DC-tg and DC-tg^{high}), we can appreciate the role of antigenic abundance in the tolerization process.

We demonstrated that peripheral skin-draining lymph nodes (PLN) are privileged sites for the induction of 2a T cell tolerance when the exclusive APCs for the self-antigen are CD11c^{+} cells. Once transferred into the DC-tg or DC-tg^{high} mice, 2a T cells underwent an acute clonal expansion and primary response followed by contraction and tolerization, a behaviour already documented by other authors in similar adoptive transfer experiments [112]. However, with our model, we could observe a previously unappreciated divergent behaviour between distinct lymphoid organs: in the spleen, 2a T cells remained functional and responsive to *ex vivo* restimulation, while tolerance was specifically established in the PLN.

To our knowledge, an intrinsic distinct role of spleen- and skin-draining lymph nodes-DCs in peripheral tolerance has never been documented before, despite the huge number of reports on the topic. Indeed, the evaluation of the intrinsic role of splenic-DCs compared to PLN-DCs is impossible when the model antigen is tissue-specific or is locally

administered (subcutaneously, intradermically, intranasally or orally): in these experimental settings, in fact, the self antigens are drained by the local lymphoid organ and does not reach the spleen or other lymph nodes. Instead, this feature could have been appreciated in models based on circulating systemic antigens; however, in those experiments, spleen and lymph nodes were often pooled or, more simply, only spleen was considered, thus losing a piece of information.

Splenic DCs seem to be defective in inducing T cell tolerance. However, we can speculate that this defect may not emerge in a physiologic context and may simply mirror the divergent tolerization "necessity" of the two lymphoid organs. Spleen and PLN receive qualitatively distinct antigens both at the steady state and upon activation. In the spleen, circulating (blood-derived) self antigens are processed and presented on MHC II directly by resident DCs at the steady state. Instead, in PLN, besides resident DCs, many migratory DCs capture the antigen in the periphery and present them on MHC II upon migration. As a consequence of these different routes of antigen entry, most of T cells which encounter the cognate self-antigen in the spleen should have been previously tolerized in the thymus, where circulating antigens surely arrive. Instead, some of the tissutal antigens carried into the lymph nodes by migratory DCs may not be expressed in the thymus via AIRE and are presented for the first time to naive T cells in the lymph nodes. Thus, to avoid autoreactivity, PLN and particularly migratory DCs must be particularly well equipped for being tolerogenic at the steady state. Someone may argue that migratory DCs are minor constituents of PLN-DC pool at the steady state

[143]; however, if these few APCs are sufficient for self antigen presention, then, they could also be sufficient for T cell toleration. The recent observation that migratory $CD103^{-}CD11b^{+}langerin^{-}$ DCs induce Treg conversion via RA at steady state in skin-draining lymph nodes strongly supports our hypothesis [144].

Beneath lymph node tolerizing specificity, another unexpected result was that distinct mechanisms accounted for 2a T cell tolerization depending on the antigen levels: when the antigen presentation was more efficient (i.e., in the DC-tg^{high} mice) most of 2a T cells downregulated the TCR. TCR as well as CD4/CD8 downregulation is commonly considered a valid mechanism for T cell anergy *in vivo* [132-134] probably dependent on the continuous presentation of the antigen: indeed, TCR is quickly re-expressed as soon as T cells are purified and cultured *in vitro* [112 and our observation].

In the presence of lower antigen presentation efficiencies, TCR downregulation is restricted to a limited fraction of 2a T cells, with most of 2a T cells still positive for Vβ14 staining. However, among these, a small but significative percentage in the PLN converts to $Foxp3^{+}CD25^{+}$ Treg cells within 10 days upon the transfer (data not shown); once generated, 2a Treg cells are stably maintained in the lymph nodes (where they can be detected even after 5 weeks, data not shown). These cells are *bona fine* suppressive Treg cells which locally block the restimulation of 2a T cells co-localized in the lymph nodes. Moreover, they can limit the proliferation of other transferred naive 2a T cells.

Our results suggest that the antigenic levels play a role in the tolerization

process. Other authors already demonstrated the relationship between tolerance and antigen doses: when the exclusive APCs are resting B cells, for example, lower antigenic dose (and the consequent transient TCR triggering) leads to T cell activation; tolerance is induced only with a higher dose or repeated/chronic exposure to the antigen [116]. Now we demonstrate that, in the periphery, under steady state consitions, when the exclusive APCs are CD11c^{+} cells, tolerance is induced despite the abundance of the antigen. Simply, distinct tolerization mechanisms are employed in the two situations. Similar results were already described using different doses of fed or injected antigens [145,146]. Now, using an endogenously expressed self-peptide, we provide the ultimate evidence that the antigen level is a universal parameter for establishing T cell tolerance or immunity.

In attempt to define the distinct features of spleen- and lymph node-DCs at the steady state on a molecular level, we performed comparative whole trascriptome analysis on CD11c^{high} or CD11c^{low} cells of the two lymphoid organs. Among DEGs, one of the most relevant was *aldh1a2,* which encodes for RALDH2, an enzyme involved in the synthesis of RA. Further experiments indicated that *de novo* 2a Treg generation in the DC-tg PLN was at least partially dependent on RA selectively produced by CD11c^{+} cells. *In vitro*, the impact of RA alone in the conversion process is minimal to that of TGF-β; indeed, RA is usually considered as a cofactor during TGF-β mediated Treg conversion [80-85 and our observations]. However, RA alone can play a decisive role *in vivo* in PLN, maybe in tolerogenic contexts which are independent on TGF-β.

RA production and Treg conversion are strictly linked. In the gut-draining lymph nodes and lamina propria, the ability of intestinal $CD103^+$ DCs to promote Treg conversion via RA is well documented [84,85]. Here we show that also skin-draining PLN-DCs constitutively express *Aldh1a2* gene transcript at the steady state, although at lower levels than MLN-DCs. Spleen-DCs, instead, do not express *Aldh1a2* and presumably do not produce RA at the steady state. The absence of RA-dependent Treg-inducing DCs therefore, could explain the consequent absence of 2a Treg cells in the spleen of transferred mice. The incapacity of splenic DCs to produce RA is not intrinsic. Indeed, it has been reported that TLR2 signalling induces splenic DCs to express *Aldh1a2,* transform vitamin A to RA and induce $Foxp3^+$ Treg cells [136].
Migratory DCs are normally absent from the spleen while are present in the lymph nodes, even at the steady state; therefore, we hypothesized that the DC population responsible for RA production in the PLN could be migratory DCs. Strikingly, in a very recent report Malissen's group has discovered a skin-draining lymph node DC subset which produce RA at steady state and defined it as a tissue-derived, migratory $CD103^-$ $CD11b^+$langerin$^-$ DC subset [144]. Thus, the RA-producing cells described by Mallissen likely represent at least one of the DCs responsible for Treg induction in our system, which function possibly throught RA.
Another DEG which raised our attention was *ccl22,* which was highly expressed in lymph node-DCs compared to splenic counterparts. *Ccl22* encodes for CCL22, a chemokine never associated to lymph nodes-DCs before. $Foxp3^+$ Treg cells are known to express CCR4, the receptor shared

by CCL17 and CCL22, responsible for the homing of Treg to peripheral tissues, where normally CCL22 is secreted in high amounts. Based on these premises, we formulated the hypothesis that CCL22 could be also produced by DCs directly in the lymph nodes at least in the absence of inflammatory conditions and that, as a consequence, 2a Treg cells could be retained at the site of differentiation via CCR4, probably together with other known lymph node-retaining factors, such as CD62L or CCR7 [131]. Our experiments provide indirect evidence that CCR4 is expressed in 2a Treg cells isolated from the PLN and promotes their retention into PLN via CCL22; indeed, the inhibition of CCR4 signalling diminishes the frequency of these 2a Treg, thus suggesting their egress from the lymph nodes, possibly due to the breakdown of the CCL22-mediated mechanism of retention.

Taken together, our results provide new mechanistic insights into the fine tuning of the tolerization process and opens new questions to be addressed, such as the regulation of the production and the role of CCL22 in the lymph nodes.

Moreover, given the importance of both the antigenic levels and APC activation states in the tolerization process, as suggested by our work and many other reports, it will be fundamental to test the effect of the combination of these two parameters in a unique system: our model is particularly well suited for this purpose; in fact, using anti-CD40 antibody, for example, we will be able to follow the fate of 2a T cells transferred in the DC-tg mice in the presence of activated DCs in comparison with our current results obtained under steady state

conditions. This will eventually provide other helpful information which would also be applied for the design of novel therapeutic approaches aiming at maximizing positive effect while minimizing unwanted responses.

Ultimately, the identification of the RA-producing DC population within the PLN at the steady state together with its probable migratory origin has an enormous therapeutic potential for unwanted responses. In fact, it will be possible to design new approaches which promote migration to PLN of tolerogenic migratory DCs which induce Treg cell conversion. In this way, Treg cells will be amplified *in vivo*, without the need of *in vitro* manipulations of the cells.

On the contrary, strategies aimed to block migratory DCs-mediated Treg conversion will be a reasonable approach for the treatment of some specific tumor classes, such as melanoma, whose tumor-associated antigens (TAA) are specifically carried by migratory skin-resident DCs to the local lymph nodes. Toward these TAA, T cell tolerance is probably induced, with bad prognosis for the patient. Therefore, local breakdown of tolerizing mechanisms, in these case, is auspicable.

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