

THE ROLE OF ANTIGEN PRESENTING CELLS IN IMMUNE REGULATION MEDIATED BY REGULATORY T CELLS

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Em memória de minha avó Fernanda

Abstract

Foxp3⁺ regulatory T cells are an important component of the immune system. They exert a powerful down-modulation of T-cell mediated immune responses and are essential to maintain immune tolerance.

β -catenin have a central role in the Wnt signaling pathway. We showed that stabilization of β -catenin enhances regulatory T cell survival. The β -catenin-transduced regulatory T cells are anergic and suppressive *in vitro* and *in vivo*. Using the Inflammatory Bowel Disease model (IBD), we determined that fewer β -catenin-transduced regulatory T cells were necessary to control IBD than the number usually used. Moreover, we showed that the stabilization of β -catenin in CD4⁺CD25⁻ non-regulatory T cells induced anergy of these cells, resulting in a similar anti-inflammatory effect. These findings open the possibility of using this pathway for control of pathologic inflammatory responses.

CD4⁺CD25⁻ cells have been shown to have regulatory T cell activity in several experimental systems. We shed light into this question by demonstrating that upon adoptive transfer of CD4⁺CD25⁻ T cells, some of these cells become CD4⁺CD25⁺ regulatory T cells by homeostatic proliferation. These later cells display markers of freshly isolated regulatory T cells and are anergic and suppressive *in vitro*. Moreover, we showed that the maintenance of CD25 expression by transfer CD4⁺CD25⁺ cells is dependent on IL-2 secreted by other cells.

The mechanism(s) of regulatory T cell action is still not fully understood. We used intravital two-photon laser scanning microscopy to visualize an *in vivo* immune response. We found that in presence of regulatory T cells, T cells and antigen-loaded Dendritic Cells (DCs) establish encounters of shorter duration than when regulatory T cells are absent. This result indicates that regulatory T cells exert an effect during priming of naïve T cells by dendritic cells.

One of the possible targets of regulatory T cell action are antigen presenting dendritic cells. We noticed that the number of DCs recovered after adoptive transfer was always lower in recipient animals harboring Foxp3⁺ regulatory T cells than in animals that lack them. While in steady state the number of DCs in mice with or without regulatory T cells was approximately the same, BrdU-labeling experiments indicated that DC turnover was faster in mice that harbored regulatory T cells. Furthermore, there were more apoptotic CD11c⁺ DCs in animals harboring regulatory T cells. We also found that regulatory T cells induced apoptosis of DCs loaded with OVA, but not of DCs loaded with an irrelevant antigen, in presence of OVA-specific T cells. We propose that one of the mechanisms for

regulatory T cell dependent peripheral tolerance is the induction of apoptosis of antigen-loaded DCs.

Sumário

As células T reguladoras, expressam o factor de transcrição Foxp3, são importantes componentes do sistema imunitário. Estas células têm um papel na inibição de respostas imunes mediadas por células T, e são essenciais para manter a tolerância ao próprio.

β -catenina é uma molécula central na via de sinalização Wnt. Nós mostrámos que a estabilização da β -catenina aumenta a sobrevivência das células T reguladoras. Células T reguladoras transfectadas com β -catenina não proliferam e são supressoras *in vitro* e *in vivo*. Utilizando o modelo de IBD (Inflammatory Bowel Disease), determinámos que eram necessárias menos células transfectadas com β -catenina para controlar a doença do que o número que normalmente é utilizado. Para além disso, mostrámos que a estabilização da β -catenina em células não reguladoras $CD4^+CD25^-$ induz nestas um estado não proliferativo, resultando daqui um similar efeito anti-inflamatório. Estas descobertas abrem a possibilidade de utilização desta via de sinalização para controlar respostas inflamatórias patológicas.

Em vários sistemas experimentais, foram identificadas propriedades reguladoras às células não reguladoras $CD4^+CD25^-$. Contribuímos para o esclarecimento desta questão através da demonstração de que após transferência as células não reguladoras $CD4^+CD25^-$ se transformam em células reguladoras $CD4^+CD25^+$ por proliferação homeostática. As células reguladoras $CD4^+CD25^+$ resultantes deste processo expressam marcadores de células reguladoras recentemente purificadas, não proliferam e são supressoras *in vitro*. Mostrámos ainda que a expressão de CD25 em células $CD4^+CD25^+$ após transferência é dependente de IL-2 secretada por outras células.

O(s) mecanismo(s) de acção de células T reguladoras não são completamente conhecidos. Utilizamos microscopia intravital de dois fotões para visualizar uma resposta immune *in vivo*. Descobrimos que na presença de células T reguladoras, os contactos celulares que se estabelecem entre as células T efectoras e células dendriticas carregadas de antígeno são temporalmente mais curtos do que os que se estabelecem entre as mesmas células aquando da ausência de células T reguladoras. Este resultado indica que as células T reguladoras exercem um efeito na primeira fase de activação das células T naïves pelas células dendriticas.

Um dos possíveis alvos de acção das células T reguladoras são as células dendríticas. Notámos que o número de células dendríticas que era possível recuperar após transferência era sempre menor quando os animais recipientes possuíam células T reguladoras do que quando os animais recipientes não as possuíam. Enquanto em estado estacionário, o número de células dendríticas em animais com ou sem células T reguladoras era aproximadamente igual, experiências de incorporação de “BrdU”

indicaram que as células dendríticas tinham um tempo de vida menor em animais portadores de células T reguladoras do que em murganhos que não as possuíam. Para além disso, existiam mais células dendríticas CD11c⁺ em apoptose em animais possuídores de células T reguladoras. Descobrimos ainda que as células T reguladoras induzem apoptose de células dendríticas carregadas com OVA, mas não de células dendríticas carregadas com outros antígenos, quando em presença de células T específicas para OVA. Propomos então que um dos mecanismos de tolerância periférica mediada por células T reguladoras é justamente a indução de apoptose de células dendríticas carregadas com antígeno.

Résumé

Les cellules T régulatrices exprimant le facteur de transcription Foxp3⁺ sont une importante composante du système immunitaire. Elles jouent un rôle dans l'inhibition des réponses immunes sous médiation des cellules T et sont essentielles au maintien de la tolérance immune.

La β -caténine est un acteur-clé dans la voie de signalisation Wnt. Nous avons montré que la stabilisation de la β -caténine augmente la survie des cellules T régulatrices. Les cellules T régulatrices transfectées avec la β -caténine sont non proliférantes et suppressives *in vitro* et *in vivo*. En utilisant le modèle IBD, nous avons déterminé qu'un nombre inférieur de cellules transfectées avec la β -caténine était nécessaire pour contrôler la maladie, par rapport à celui normalement utilisé. Par ailleurs, nous avons montré que la stabilisation de la β -caténine dans des cellules non régulatrices CD4⁺CD25⁻ induisait une anergie dans ces cellules, menant à un effet anti-inflammatoire similaire. Ces découvertes permettent d'envisager une utilisation de cette voie de signalisation pour contrôler des réponses inflammatoires pathologiques.

Des propriétés régulatrices chez les cellules non régulatrices CD4⁺CD25⁻ ont été identifiées dans plusieurs systèmes expérimentaux. Nous avons contribué à éclairer cette question en démontrant qu'après transfert adoptif de cellules T CD4⁺CD25⁻, certaines d'entre elles se transforment en cellules régulatrices CD4⁺CD25⁺ par prolifération homéostatique. Ces dernières expriment des marqueurs de cellules T régulatrices fraîchement purifiées, sont non proliférantes et suppressives *in vitro*. Nous avons par ailleurs démontré que l'expression de CD25 dans des cellules CD4⁺CD25⁺ après transfert dépend de l'IL-2 sécrétée par d'autres cellules.

Le(s) mécanisme(s) d'action des cellules T régulatrices ne sont pas encore entièrement connu(s). Nous avons eu recours à la microscopie intravitale à deux photons pour visualiser une réponse immune *in vivo*. Nous avons découvert qu'en présence de cellules T régulatrices, les contacts entre cellules T effectrices et cellules dendritiques chargées en antigène sont de plus courte durée qu'en absence de cellules T régulatrices. Ce résultat indique que les cellules T régulatrices exercent un effet lors de la première phase d'activation des cellules T naïves par les cellules dendritiques.

Les cellules dendritiques constituent l'une des cibles d'action possible des cellules T régulatrices. Nous avons noté que le nombre de cellules dendritiques récupérées après transfert adoptif était toujours inférieur chez les animaux receveurs possédant des cellules T régulatrices que chez ceux n'en possédant pas. Alors qu'à l'état stationnaire le nombre de cellules dendritiques chez des souris avec ou sans cellules T régulatrices était

approximativement le même, des expériences d'incorporation de BrdU ont indiqué que la durée de vie des cellules dendritiques était inférieure chez les souris possédant des cellules T régulatrices. De plus, les cellules dendritiques CD11c⁺ apoptotiques se sont avérées en nombre supérieur chez les animaux possédant des cellules T régulatrices. Nous avons également découvert que les cellules T régulatrices induisent l'apoptose de cellules dendritiques chargées d'OVA, mais non pas celle de cellules dendritiques chargées d'autres antigènes, en présence de cellules T spécifiques pour l'OVA. Nous proposons donc que l'un des mécanismes de tolérance périphérique sous médiation des cellules T régulatrices est justement l'induction d'apoptose de cellules dendritiques chargées d'antigène.

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Abbreviations

α - anti

Ab – Antibody

Ac - *N*-acetylated

APC – Antigen Presenting Cell

BrdU - Bromodeoxyuridine (5-bromo-2-deoxyuridine)

CFSE – Carboxyfluorescein Succinimidyl Ester

CTLA-4 - cytotoxic T-lymphocyte-associated antigen-4

DC – Dendritic Cell

EAE – Experimental Autoimmune Encephalomyelitis

FCS – Fetal Calf Serum

Foxp3 - Forkhead box P3

GITR – Glucocorticoid-Induced TNF-Receptor family-related gene/protein

HA – Hemagglutinin

IBD – Inflammatory Bowel Disease

IL - Interleukin

ip - intraperitoneal

iv – intravenous

LN – lymph node

LPS – Lipopolysaccharide

MBP – Myelin Basic Protein

MHC - Major Histocompatibility Complex

NOD – Non-obese diabetic

OVA – Ovalbumin

PBS – Phosphate Buffered Saline

Rag – Recombination Activating Gene

SPF - specific pathogen-free

TCR – T Cell Receptor

TGF- β - Tumor Growth Factor

TLR – Toll Like Receptor

Treg – Regulatory T Cell

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Chapter I

General Introduction

The immune system is a collection of defense mechanisms that work together to protect the integrity of the organism. An important feature of the immune system is to distinguish between self and non-self. The immune system protects the organism from a wide variety of pathogens and tumor cells and at the same time spares organism own functional cells and the beneficial non-self, like commensal bacteria.

The immune response of jawed vertebrates can be broken down into two major types of response: innate and adaptive immunity. First, the innate immune response is based on the recognition and binding of highly conserved constituents of many microorganisms by pattern recognition receptors. Second, the adaptive immunity. Its basic feature is the possibility to generate specific receptors for, in theory, every possible antigen. The cells of the adaptive immune system are T, generated in the thymus, and B, generated in the bone marrow, lymphocytes. Each B and T cell bears an antigen receptor of a unique specificity. The diversity of lymphocyte antigen receptors is generated by somatic gene rearrangements that allows the generation of an infinite number of receptor specificities encoded in a finite number of genes. This process is mediated by RAG-1 and RAG-2 proteins, products of Rag genes (Recombination-activating genes), expressed only in developing lymphocytes.

T cells recognize a non-self target only after antigens have been processed and presented to them combined to a self cell-surface molecule encoded by the MHC (Major Histocompatibility Complex). Due to the random way T cell receptors specificities are generated, it would be conceptually possible that receptors that recognize self-antigens are raised, ultimately resulting in autoimmunity. To ensure T cell self-MHC restriction and self-tolerance, T cells undergo positive and negative selection, respectively, during their maturation in the thymus. When a developing thymocyte encounters its corresponding antigen, it dies by apoptosis. This is the basis of T cell negative selection [1-5].

Although negative selection in the thymus does enable the deletion of T cells expressing receptors that are specific for self-peptides, this process is not 100% effective. Not all of the self-peptides that T cells will encounter during their adult life are presented during their development. Variables like antigen concentration, MHC density, TCR affinity (T Cell Receptor), among others, dictate that for some self-reactive T cells the requirements for clonal deletion will not happen intrathymically (reviewed in [2]). This will result in emigration to the periphery of T cell that are potentially harmful. This being true, together with the fact that normal organisms do not develop autoimmunity, implies the existence of other mechanisms to ensure peripheral tolerance. A dominant mechanism is the existence of a

subset of T cells that down-regulate the activation and proliferation of self-reactive lymphocytes (reviewed in [6-8]). These cells are called regulatory T cells.

Regulatory T cells: the first findings of dominant mechanism of self-tolerance

Controversial is the word that better describe the scientific history of regulatory T cell, once called suppressor T cells [9]. If it's true that almost since the discovery of lymphocytes there was the perception of the existence of some T cells with suppression activity, it's also true that upon the discovery of Th1/Th2 cells the concept of suppressor T cells became a not very welcome concept among immunologists.

The first finding that sustanciate the idea of a subset of T cells with suppressor activity dates from 1969 when Nishizuka & Sakakura reported that neonatal thymectomy (NTx) of female mice led to oophoritis [10]. Some years later, Penhale and associates reported autoimmune thyroiditis in adult rats as result of thymectomy followed by sublethal X-irradiation [11]. Both groups reported some years later that both autoimmune diseases could be prevented by reconstituting animals with normal lymphocytes, special CD4⁺ T cells [12-14]. These reports are the first to contain the idea of a subset of T cells with thymus origin, that function as suppressors of auto-reactive cells preventing the development of autoimmunity.

Part of the controversy around regulatory T cells is due to the lack of reliable markers.

Regulatory T cells: cell markers

Naturally-occurring Treg cells are a subset of CD4⁺ T cells generated in the thymus that express the transcription factor Foxp3 (Forkhead box P3), regulatory T cell major regulator, [15-21]; the majority of Foxp3⁺ T cells also express the surface marker CD25 (the IL-2 receptor α chain) [22, 23]. Although there are some Foxp3⁺ within CD4⁺CD25⁻ cells, pretty much all CD4⁺CD25⁺ express Foxp3⁺. Because it's a cell surface marker, CD25 has been extensively used to characterize and purify regulatory T cells.

Many other cell markers were associated with T cell suppressor activity (Figure 1). In 1985, Sakaguchi *et al.* depleted Lyt-1^{high} (CD5^{high}) from CD4⁺ T cells of normal BALB/c mice spleens and transferred the remaining cells to T-cell deficient mice and observe that these

mice spontaneous developed multiple-organ autoimmunity. Co-transfer of total CD4⁺ together with CD4⁺CD5^{low} T cells inhibited the development of autoimmunity [13].

Powrie *et al* and Morrissey *et al* independently showed suppressor activity within CD4⁺CD45RB^{low} cells. They also contributed to the regulatory T cell field with a highly used model of autoimmunity – inflammatory bowel disease (IBD). When CD4⁺CD45RB^{high} cells are transferred into T/B cell-deficient mice they develop IBD. IBD development can be prevented by co-transferring CD4⁺CD45RB^{low} with the pathogenic CD4⁺CD45RB^{high} cells [24, 25]. This mouse model has been extensively used to prove regulatory T cell activity *in vivo*.

The search for a better regulatory T cell marker continued, and the next significant advance was made by Sakaguchi and collaborators that defined CD25 molecule (IL-2R α chain) as a better marker for regulatory T cell marker [22]. CD4⁺CD25⁺ cells constitute 5-10% (depending on the mouse strain) of the peripheral CD4⁺ T cells in normal mice, and are present in the CD45RB^{low} and CD5^{hi} populations. Proving that CD25⁺ is a good marker for regulatory T cells is the fact that transfers of splenic cell suspensions depleted of CD4⁺CD25⁺ cells to BALB/c athymic nude mice results in autoimmunity in a wider spectrum of organs (stomach, thyroid, ovaries, adrenal glands and Langerhans' islets). It was also shown that the removal of CD4⁺CD25⁺ cells enhance immune responses to non-self antigens and allografts [22]. CD25 is a very useful marker for regulatory T cells, but it is also expressed in all T cells upon activation. This fact raised several questions about the role of CD25 in regulatory T cell biology, concerning their generation and maintenance. It also questions the role of T cell activation in regulatory T cell biology. Nevertheless, CD25 is an indispensable molecule in regulatory T cell biology. This issue will be further addressed below, in the role of IL-2 for regulatory T cell biology section of this general introduction.

After the establishment of CD25 as a good cell surface marker for regulatory T cells. In 1998, Takahashi *et al* and Thornton *et al* established an *in vitro* functional assay for regulatory T cell activity. They showed that in presence of antigen-presenting cells (APCs) and TCR stimulation, CD4⁺CD25⁺ co-cultured with CD4⁺CD25⁻ or CD8⁺ suppressed the proliferation of the later T cells. They also showed that CD4⁺CD25⁺ were anergic *in vitro* [26, 27]. Although they do not fully recreate the *in vivo* action of regulatory T cells, the *in vitro* suppression assays become an easy and fast way to test and study certain aspects of regulatory T cell activity.

Other cell markers have been associated with regulatory T cells (Figure 1). For some, the high expression of GITR (glucocorticoid-induced TNF-receptor family-related gene/protein) is the factor that better correlates with Foxp3 expression in thymus and periphery [21, 28].

Regulatory T cells ($CD4^+CD25^+$ cells) constitutively express CTLA-4 (cytotoxic T-lymphocyte-associated antigen-4) [29]. Abnormal CTLA-4 (CD152) expression affects regulatory T cell function and generates an unbalance towards autoimmunity [30, 31]. Attempting to defined better markers of regulatory T cells, it has been reported that $CD103^+$ cells are better suppressors *in vitro* than $CD103^-$ cells [32]. And $CD62L^{high}CD25^+CD4^+$ cells were better in preventing T1D (Type 1 Diabetes mellitus) in NOD (non-obese diabetic) mice than the ones expressing low levels of CD62L [33].

Regulatory T cells were reported to selectively express Toll Like Receptors (TLR) -4, -5, -7 and -8 [34]. Lipopolysaccharide (LPS) treatment in vitro of regulatory T cells induce the expression of activation markers like MHC class I, CD69, B7.1, CD44 and CD38 [34].

In 2003, three independent groups reported the last major finding in regulatory T cell field, the transcription factor Foxp3 as the major regulator of regulatory T cells [15-17].

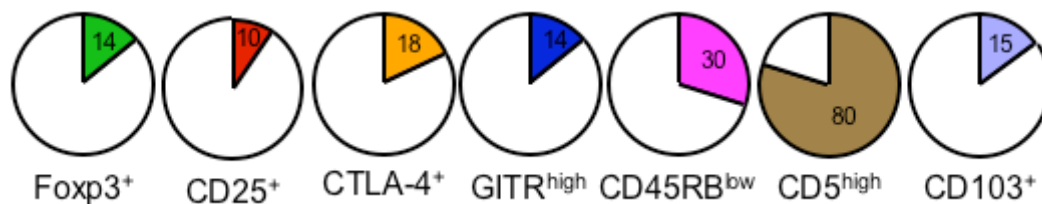


Figure 1: Cell surface markers for regulatory T cells. The colored area and the number represent the fraction of $CD4^+$ T cells that is positive for each cell marker. The most specific and reliable marker for $CD4^+$ regulatory T cells is the transcription factor Foxp3. The percentages represented are reviewed in [35] and for CD103 are reported in [36].

Key role of Foxp3 in regulatory T cell biology

IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome in humans and an analogous lymphoproliferative disease observed in the spontaneous mouse mutant scurfy is the result of a defect in the *Foxp3* gene [37-40].

In mice, the autoimmunity resulting from the scurfy mutation can be rescued by a transgene encoding a wild-type *Foxp3* allele. The scurfy is a X-linked mutation. The hemizygous male mice carrying the scurfy mutation exhibit hyperactivation of $CD4^+$ T cells and overproduction of cytokines and die by four weeks of age [38]. The heterozygous scurfy females remain healthy through out life. This observation implies that the scurfy syndrome is T cell mediated. In heterozygous females, due to random X-chromosome inactivation in T cells, about half of

T cells carry the wild-type *Foxp3* allele, and these last ones are enough to keep in check other T cells harboring the mutant allele. A defective dominant tolerance observed in mice carrying only the mutant *Foxp3* gene suggested some relationship between this gene and CD4⁺CD25⁺ regulatory T cells.

Foxp3 plays a specific role in the development and function of natural regulatory T cells [15-17]. *Foxp3*-deficient mice show similar phenotype than scurfy mutant mice, exhibit hyperactivation of T cells as well as overproduction of cytokines [17]. *Foxp3* overexpressing mice have twice as many CD4⁺CD25⁺ regulatory T cells than the wild-type littermates [16]. Moreover, these mice have fewer CD4⁺ T cells than the non-transgenic mice [41].

In contrast with other molecular markers, like CTLA-4, CD25 or GITR, used to identify regulatory T cells, *Foxp3* is not upregulated upon T cell activation [15-17]. Ectopic expression of *Foxp3* on naïve T cells convert them to Treg like cells [15, 17]. *Foxp3*-transduced cells are able to suppress *in vitro* T cell proliferation [15]. *In vivo*, these cells suppress the development of wasting disease, colitis, and IBD [15, 17] as well as diabetes and other autoimmune diseases in animal models [42, 43] and induce transplantation tolerance [44].

Foxp3 is indispensable in the development of regulatory T cells. Chimeric mice with a mixture of bone marrow (BM) cells from wildtype and *Foxp3*-deficient mice revealed that only the wildtype bone marrow was able to generate CD4⁺CD25⁺ regulatory T cells [17]. The recently available *Foxp3*-reporter mice allowed the establishment of a correlation between *Foxp3* protein and the ontogeny of regulatory T cells [45, 46]. These studies finally solved the issue raised by the first experiments in the field of regulatory T cells. Neonatal thymectomy results into development of autoimmunity because regulatory T cells only become detectable in the periphery of normal mice few days after birth. Thymectomy by day 3 after birth abrogates thymic production of regulatory T cells from the beginning of their ontogeny, but allows some self-reactive T cells to migrate to the periphery before thymectomy. These cells are left unchecked in the periphery and cause autoimmunity [45]. One question that remains to be addressed is why is regulatory T cell ontogeny later than a regular T cell.

Foxp3 is crucial not only during the neonatal period but throughout the lifespan of mice. Mice harboring regulatory T cells that could be deleted at any given age of the mice were generated inserting cDNA encoding a fusion protein of green fluorescent protein (GFP) and the human diphtheria toxin receptor (DTR) into the 3' untranslated region (UTR) of the *Foxp3* locus (*Foxp3*^{DTR} mice) [47]. In these mice diphtheria toxin (DT) treatment results in rapid and complete elimination of *Foxp3* regulatory T cells. When 3 month-old mice were treated with DT, they developed severe lymphadenopathy and splenomegaly, wasting disease, failure to

thrive and reduce mobility. Furthermore, adult mice treated with DT developed a more rapid terminal autoimmune disease than neonates equally treated [47]. Examination of lymphoid and myeloid compartments of *Foxp3*^{DTR} mice treated with DT showed massive self-specific T cell proliferation and activation and expansion of dendritic cells and granulocytes [47, 48]. These studies showed the essential role of Foxp3 in the generation and maintenance of regulatory T cells.

Foxp3 is at this point the most reliable and specific molecular marker for regulatory T cells, whether they are generated in the thymus as functional mature regulatory T cells, these cells are usually called natural occurring regulatory T cells, or they are generated in the periphery from naïve T cells under certain conditions [49-52].

Crucial role of IL-2 in regulatory T cell biology

Interleukin-2 (IL-2) has a well-established role as T cell growth factor. Recent data suggested that IL-2 has also an indispensable role in generation and maintenance of regulatory T cell *in vivo* [53-58].

As mentioned above, CD25 (IL-2 receptor α chain) is a useful marker for regulatory T cells and is component of the high-affinity IL-2 receptor. It is now well established that CD25 is a functionally essential molecule for regulatory T cell biology. Being IL-2 an indispensable cytokine for regulatory T cell existence *in vivo*. IL-2 deficient mice harbor few CD4⁺CD25⁺ T cells and develop spontaneous autoimmunity. The number of other T cells in these mice is not affected and they show normal composition of CD4/CD8 T cells [59, 60]. A recent study using Foxp3-reporter mice has unequivocally shown that CD4⁺CD25⁺ T cells are reduced but not completely absent in IL-2-deficient mice. In contrast, common γ -chain deficiency completely abrogates the generation of Foxp3⁺CD4⁺CD25⁺ T cells [58]. CD25 and CD122 (the IL-2R β chain) deficiency generate fatal lymphoproliferative inflammatory disease similar to the one observed for IL-2 deficiency, and can be prevented by wild-type CD4⁺CD25⁺ T cells transfer [59, 61-63].

Foxp3 suppresses IL-2 transcription and upregulates CD25 expression in Tregs [15]. Meaning that Tregs are unable to produce IL-2 but enable to respond to exogenous IL-2 that must be supplied by other T cells. This together with IL-2 deficiency syndrome described above, is fair to say that regulatory T cells are highly dependent on exogenous IL-2 for their maintenance in the immune system. Several studies were conducted to investigate the role

of circulating IL-2 to regulatory T cells physiology. Anti-IL-2 monoclonal antibodies (mab) were used to neutralize IL-2, that resulted in a substantial reduction of CD4⁺CD25⁺ T cells in the periphery and consequent development of autoimmunity [55, 64].

Thus, the IL-2/IL-2R pathway is a mediator of negative feedback control. Using this pathway, activated effector T cells contribute to regulatory T cells maintenance and activation, while these curb the proliferation of the first ones. This mechanism can resolve immune response at the site of inflammation where both T effectors and Tregs are recruited by antigen presenting cells (APCs) presenting self- or nonself-antigens.

Only in the common γ -chain deficient mice Foxp3⁺ regulatory T cells are totally absent, while a reduced number are present in IL-2R α chain or IL-2 deficient mice suggesting that other cytokines that bind the common γ -chain (like IL-4, IL-7, IL-9 and IL-15) are also involved in the maintenance of regulatory T cells *in vivo* [21]. Tregs express low levels of IL-7R α and need self-antigen recognition to be generated [65]. IL-7 is a key cytokine for thymocyte survival during T cell development [66, 67] and it is possible that down-modulation of IL-7R α during Treg development occurs naturally once they start receiving IL-2-survival signals. In fact, a new study has shown that some Treg precursors were found inside the CD25⁺ Foxp3⁻ CD4⁺ Single-Positive (SP) thymocytes and these cells showed decreased expression of IL-7R α but maintain CD25 expression [68].

The IL-2 neutralization treatment results in reduction of numbers of CD25⁺CD4⁺ thymocytes that indicates that IL-2 is also important for regulatory T cell thymic generation [21, 69].

Regulatory T cell thymic development and TCR repertoire

Lately, thymic generated regulatory T cells are usually designated as natural occurring Tregs or nTregs. This designation is used for T cells that leave the thymus to the periphery as Foxp3 expressing T cells. In the periphery, under certain circumstances, some naïve T cells become Foxp3⁺ T cells, and gain regulatory T cell properties. These cells are usually called peripheral generated Tregs or peripheral induced Tregs (iTregs). Regulatory T cells can also be induced *in vitro* from naïve T cells, using TCR stimulation, in presence the of TGF- β [70-74]. These cells are also called iTregs. The *in vivo* generation of regulatory T cells from CD25⁻ T cells will be the focus of one of the results chapters of this thesis and will be further commented in the Discussion section.

Thymus is required for the maturation of haematopoietic precursors derived from bone marrow into T cells. Its critical role is well demonstrated by the fact that mutant *nude* mice, in which thymic epithelium fails to differentiate, lack mature T cells. Haematopoietic precursors derived from bone marrow enter the thymus at the cortico-medullary junction. Within the thymus they migrate through different microenvironments that support the successive stages of T cell development that are marked by changes in cell surface molecules, in the status of T-cell receptor genes and in the expression of the TCR, as depicted in figure 2. The first T cell developmental checkpoint is positive selection. Double-positive (DP) thymocytes, expressing random generated TCR, that receive relatively weak signals when interacting with self-peptide-MHC complexes on cortical thymic epithelial cells (cTEC) differentiate further. The second major checkpoint is negative selection, which eliminates thymocytes that show high-affinity interactions with self-peptides-MHC complexes presented by dendritic cells (DC) and TECs. Nevertheless, thymic central tolerance is unable to exclude all T cells specific for self-antigen. Some lower self-affinity T cells can be found in the periphery and elude peripheral mechanisms of deletion and anergy induction. These cells are kept in check by regulatory T cells.

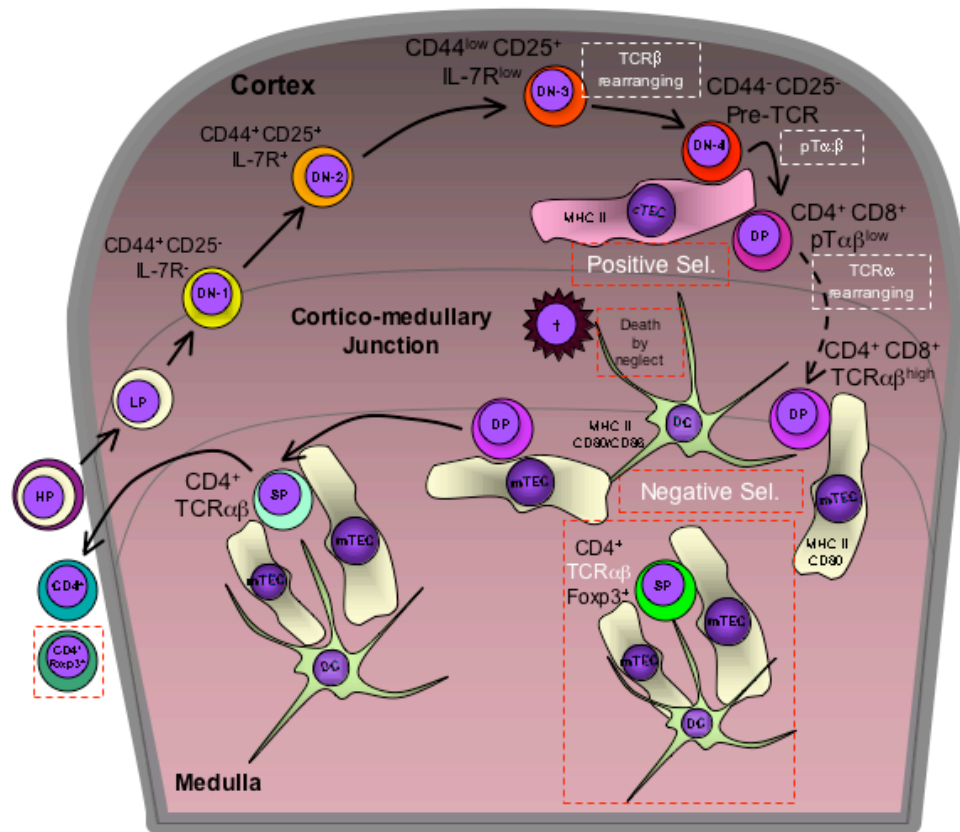


Figure 2: CD4 TCR $\alpha\beta$ T cell development in the thymus. Haematopoietic precursors (HP) enter the thymus at the cortico-medullary junction. Lymphoid progenitor (LP) lack most of the surface molecules that are present in mature T cells. Interactions with the thymic stroma trigger the expression of T cell specific surface molecules like Thy-1, in mice. At this point, thymocytes do not express any of the cell surface markers that define a mature T cell. Due to the absence of CD4 and CD8 they are named, at this moment, double-negative thymocytes (DN). DN cells are subdivided according to CD25 and CD44 expression. DN1 (CD44⁺CD25⁻); DN2 (CD44⁺CD25⁺); DN3 (CD44^{low}CD25⁺); DN4 (CD44⁻CD25⁻). The IL-7 receptor (IL-7R) is essential for early T cell development. Rearrangement of the TCR β -chain gene occurs in DN3. The β chains pair with a surrogate α chain (pT α) to assemble a pre-TCR. DN4 cells proliferate and continue their development, that results in expression of both CD4 and CD8. These cells are called double-positive thymocytes (DP). Small DP cells rearrange α -chain genes, producing thymocytes with a complete $\alpha\beta$ antigen receptor. DP cells are tested for self-MHC restriction during positive selection. Contact between MHC molecules on cortical thymic epithelial cells (cTEC) and the receptors of developing T cells is crucial for positive selection. Thymocytes evolve to express either CD4 or CD8 becoming single-positive (SP) thymocytes. During and after DP stage, thymocytes undergo negative selection to eliminate cells capable of responding to self-antigens. Some TCR $\alpha\beta$ cells that respond to self-antigen are not eliminated but become CD4⁺Foxp3⁺ regulatory T cells. Negative selection is carried out by medullary thymic epithelial cells (mTEC) and dendritic cells (DC) very numerous in the cortico-medullary junction.

Natural occurring regulatory T cells are selected in the thymus, they still express TCRs that show increased affinity for self-peptides-MHC complexes [6]. In Tregs TCR affinities are immediately below deletion threshold or even within the same magnitude of affinity as some negatively selected self-reactive thymocytes. Regulatory T cells are selected in the thymus through a unique differentiative pathway due to selective expression of tissue-specific antigens by TECs (reviewed in [20]).

Studies using double transgenic mice, in which the peptide recognized by the transgenic TCR is expressed in the thymus as a transgene, showed substantial thymic depletion of Tg cells and the remain peripheral T cells exhibited properties of regulatory T cells [75-79]. Many other studies have provided data indicating that increase in TCR signal intensity or signal duration during T cell development promotes regulatory T cell differentiation. It is well accepted that regulatory T cells express TCRs with increased affinities towards self-peptide-MHC when compared to conventional T cells.

It would be fair to think that regulatory T cells have a restricted TCR repertoire. But that does not seem to be the case. Analyses of V α and V β usages by CD25⁺ and CD25⁻ showed overlap between the two cell types [26]. Lately, regulatory T cell TCR usage is being the focus of intense study. TCR repertoire of CD25⁺ and CD25⁻ T cells was analyzed using an experimental system in which TCR β chain was fixed by transgenic expression, and paired with an endogenously rearranged α chain. By cloning and sequencing of TCR α genes from CD25⁺ and CD25⁻CD4⁺ Tg cells the authors showed that CD25⁺ and CD25⁻ display overlapping TCR repertoires [80]. Pacholczyk *et al.* using a somehow similar strategy to limit TCR diversity and enable single-cell analysis of regulatory T cells TCRs reported similar results [81].

Foxp3^{GFP} mice [23] availability allowed a clear identification of regulatory T cells among the thymocytes. Foxp3⁺ thymocytes are in its majority CD4⁺ SP cells, but some were found among the DP cells and a very small percentage were CD8⁺ SP cells [45]. It also allowed a detailed analysis of regulatory T cell repertoire. A recent study showed that the same TCR could be expressed by a conventional T cell as well as by a regulatory T cell [82]. It's also true that TCR transgenic mice in Rag^{-/-} background devoided of regulatory T cells is a reoccurring observation and it was formally demonstrated by Sakaguchi and collaborators [83]. These and many other results show that the existence of a specific regulatory T cell TCR repertoire is still an open question.

Certainly, regulatory T cell function and development are strictly connected with their TCR specificity, but it is now clear that a determined TCR does not make a T cell a regulatory T cell.

Use of TCR transgenic mice in the study of regulatory T cells

TCR transgenic mice have been extensively used in the study of the immune system. T-cell clones of a known TCR antigen specificity were used to make the first few TCR transgenic mice. These mice allowed for elegant set-ups, with antigen either present or absent, to study T-cell development and selection. Lately, TCR Transgenic mice recognizing self-antigens are being used to study autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis in humans; type I diabetes and rheumatoid arthritis. These diseases have a strong T cell component and in many of them regulatory T cell compartment is defective (reviewed in [84]).

This section will focus mainly in the description of two TCR transgenic mice and its variants used in the following chapters. First, a myelin basic protein (MBP) specific TCR Tg mouse [85]. In these mice, encephalitogenic T cells are not deleted in the H-2^u thymi and can be found in the periphery in a naïve stage [85]. Lafaille *et al.* reported that 100% of these TCR Tg mice when crossed with RAG^{-/-} mice (T/R⁻) developed spontaneous EAE, whereas their Tg RAG⁺ (T/R⁺) littermates did not. EAE is an inflammatory disease of the central nervous system (CNS) provoked by autoreactive T cells that recognize autoantigens in the CNS, in this particular case MBP. Clinically, EAE manifests by an ascending impairment of the tail and limb paralysis. Sub-clinically, spontaneous EAE is preceded of a significant drop in body weight of the mice (Lafaille unpublished observation). Development of spontaneous EAE by T/R⁻ mice but not by T/R⁺ mice is due to the lack of regulatory T cells expressing endogenous TCRs in T/R⁻ mice [85-87]. A restricted endogenous TCR repertoire does not support the generation of regulatory T cells, leading to disease. Monoclonality of the $\alpha\beta$ T cell repertoire can be achieved by genetically removing the non-transgenic $\alpha\beta$ T cells. Mice that lack regulatory T cells can be obtained either by crossing the TCR Tg mice with Rag^{-/-} mice (T/R⁻, mentioned above) or TCR $\alpha^{-/-}$ $\beta^{-/-}$ double knockout mice [87]. Another option is to cross TCR Tg mice with themselves generating homozygous TCR Tg RAG⁺ α^{+} β^{+} mice (TgTg mice) [84, 88, 89]. Development of spontaneous EAE can be prevented by a single injection of regulatory T cells before mice are 4 weeks old [86, 87]. In a 4 to 6 weeks old T/R⁺ mouse

around 95-98% of the CD4⁺ T cells express only the MBP-specific TCR. The remaining CD4⁺ T cells express an endogenous TCR, alone or together with the MBP-specific TCR. This population is absent in T/R⁻ or TgTg mice, and is required for protection against EAE [84, 85, 87, 90]. Moreover, it has been shown that transfer of CD4⁺ T cells purified from T/R⁺ mice and depleted of MBP-specific T cells into T/R⁻ mice do not protect them from spontaneous EAE, and therefore the protective lymphocytes are the double TCR expressing cells [90]. Flow cytometry analysis of lymph node (LN) cells showed that clonotype negative lymphocytes were present in the CD4⁺CD25⁺ fraction of T/R⁺ mice, but not among CD4⁺CD25⁻ cells [90]. The TCR specificity of the cells that prevent EAE remains unknown. Data reported in the past few years provide possible reasons why the use of CD25 molecule as a regulatory T cell marker in this Tg mouse model was sometimes controversial. It was reported that CD25-depleted CD4⁺ cells purified from wild-type mice protected T/R⁻ mice from EAE [88]. These results can now be explained since we now know that CD25⁻ T cells generate CD4⁺CD25⁺Foxp3⁺ T cells by peripheral expansion [49-52]. TgTg mice, unlike T/R⁻ mice, display lymphoid organs with normal architectures and this is mainly due to the presence of B cells [89]. T/R⁺ and TgTg mice are the variants of this TCR Tg mouse strain that have or lack regulatory T cells, respectively, that are used in the following chapters.

The second TCR transgenic mouse strain, from now on referred to as T/B monoclonal mice, was generated by crossing the influenza HA-specific immunoglobulin heavy-and-light-chain Knockin mice [91] and the DO11.10 OVA-specific TCR transgenic mice [92], these mice were extensively backcrossed onto BALB/c background. When crossed with Rag-1 knockout mice, these mice are devoided of regulatory T cells and carry naïve and monoclonal populations of T and B cells. When immunized with a cross-linked antigen OVA-HA develop exacerbated Th2/IgE responses [91]. Likewise in the previously described TCR Tg mice, is the monoclonality of the T cell compartment that determines the hyper IgE response. The IgE in total serum 14 days after a single immunization is 200-fold lower Rag competent mice than RAG deficient, that is due to the presence of regulatory T cells in the previous mice but not in the later ones [91]. Hyper IgE response can be prevented by single transfer of regulatory T cells before immunization [91]. In what regulatory T cell biology is concerned this TCR Tg system is very similar to the one described before. The main advantage of the T/B monoclonal system to the study of regulatory T cells is the fact that OVA is not auto-antigen like MBP, allowing control on antigen availability. Moreover, oral administration of antigen induce development of OVA-specific CD4⁺CD25⁺Foxp3⁺CD45RB^{low} regulatory T cells, and prevents effector/memory Th2 cell development and hyper IgE responses [51].

Molecular mechanisms of regulatory T cell biology

The role of cytokines for T cell suppression is still controversial. IL-10 and transforming growth factor TGF- β are the cytokines that more often have been implicated in suppression. In the context of the murine IBD model, IL-10 and TGF- β are protective cytokines [93-95]. CD4⁺CD25⁺ T cells purified from IL-10^{-/-} mice when co-transferred with CD4⁺CD25⁻ T cells did not protect mice from IBD, whereas the ones purified from IL-10 competent mice did [95]. Some contribution for IL-10 in regulatory T cell mediated suppression was also shown using other murine models, but in a less clear way [96].

The role of IL-10 in the spontaneous EAE model described above is not clear either. It is certain that IL-10 is not needed for spontaneous EAE induction, since T/R⁻ IL-10^{-/-} mice developed EAE in the same fashion than T/R⁻ IL-10^{+/+} mice do. No T/R⁺ IL-10 competent mice develop EAE but 29% of the T/R⁺ IL-10^{-/-} mice did. Transfer of peripheral CD4⁺ T cells purified from IL-10^{-/-} mice into T/R⁻ IL-10 competent mice led to significant protection [88].

A recent study using a dual reporter system for IL-10 and Foxp3 genes shed some light into the role of IL-10 in regulatory T cell mediated suppression. This report showed a differential expression of IL-10 among Foxp3⁺ regulatory T cells depending of the tissue. While secondary lymphoid tissues, like lung and liver, had Foxp3⁺IL-10⁻ T cells, large and small intestine were enriched in IL-10 expressing cells [97]. These results may explain the crucial role of IL-10 for protection in the IBD model.

TGF- β is also involved in regulatory T cell activity. A transgenic mice expressing dominant negative form of TGF- β type II receptor on T cells develop IBD and have an increased production of IL-4 and IFN- γ [98]. Furthermore, TGF- β is effective in down-regulating Th1 and Th2 responses [94, 99]. TGF- β is essential for *in vitro* generation of regulatory T cells [70-74] and certainly plays a role in the generation of at least some regulatory T cells *in vivo* [97].

Many evidences point out for an *in vivo* role for cytokines in regulatory T cell mediated suppression, while *in vitro* suppression seems to be cell contact dependent. When in an *in vitro* suppression assay CD4⁺CD25⁺ T cells and CD4⁺CD25⁻ T cells are physically separated suppression does not occur [26, 27]. Furthermore, supernatants from activated CD4⁺CD25⁺ cells cultured alone or together with CD4⁺CD25⁻ cells do not retain suppressor activity [26]. The *in vitro* suppression assays may not perfectly mimic regulatory T cells activity *in vivo*, but they have been useful not only to test regulatory activity of a cell population but also to test some properties of regulatory T cell suppression. These assays were used to show that the amount of suppression was dependent of the number as well as the quality of the APCs

used. Suppression of proliferation increased when the number of APCs decrease [100]. Therefore, it is possible that regulatory T cells act by weakening APC function. It was reported that CD4⁺CD25⁺ T cells down-modulated the expression of CD80 and CD86 on dendritic cells [100].

Taking all these results together it is possible that regulatory T cells use several mechanisms to exert suppression, depending on the situation and site of action. This can also be the basis for their several functions. Regulatory T cells have been shown to be beneficial in transplantation tolerance [101-104], in resolving of immune responses to non-self-antigens, microbes or allergens [105-109] and, as already mentioned, in preventing autoimmunity. The molecular basis by which regulatory T cells develop their functions is still controversial and under active study.

Thesis outline

In spite of the fact that regulatory T cells have been the subject of innumerable studies in the past few years, many questions about their biology remain unanswered. A crucial role for Foxp3⁺ regulatory T cells in the maintenance of peripheral tolerance is already well established. Nevertheless, questions concerning their generation, maintenance and mechanism of action still remain. Here, using mainly two TCR transgenic mouse models, we demonstrate that regulatory T cells can be generated in the periphery from CD25⁻ T cells.

We also address the mechanism of Treg action *in vivo*.

The big challenge for scientists that study regulatory T cells is making the use of these cells in human therapy possible. Aiming to contribute in that direction, we show that β -catenin, a central molecule in the Wnt pathway, may be key molecule to extend survival of regulatory T cells.

Chapter 1 is the present general introduction that aims an overview of the regulatory T cells features that are important to understand the following chapters:

In chapter 2, *in vivo* and *in vitro* evidences are presented that show the Beta-catenin stabilization extends regulatory T cell survival and induces anergy in CD4⁺CD25⁻ naïve T cells.

In chapter 3, we show the generation of CD25⁺Foxp3⁺ regulatory T cells upon homeostatic proliferation from donor polyclonal CD4⁺CD25⁻ cells. The converted cells are equivalent to natural occurring regulatory T cells.

In chapter 4, we use intra-vital two-photon laser-scanning microscopy to visualize the behavior of MBP-specific T cells in presence or absence of regulatory T cells. We show that regulatory T cells inhibit stable contacts between MBP-specific T cells and dendritic cells in vivo.

In chapter 5, we show that the number of DC recovered after adoptive transfer was always lower in recipient animals harboring Foxp3⁺ Treg cells than in animals that lack Foxp3⁺ Treg cells. Furthermore, there were more apoptotic CD11c⁺ cells in animals harboring Treg cells. We propose that one of the mechanisms for Treg-dependent peripheral tolerance is the induction of apoptosis of antigen-loaded DC.

Chapter 6 is a general discussion of the results presented.

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Chapter II

Beta-catenin stabilization extends regulatory T cells survival and induces anergy in nonregulatory T cells

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Beta-catenin stabilization extends regulatory T cell survival and induces anergy in nonregulatory T cells

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β-catenin is a central molecule in the Wnt pathway. Expression of a stable form of β-catenin on CD4⁺CD25⁺ regulatory T (T_{reg}) cells resulted in a marked enhancement of survival of these cells *in vitro*. Furthermore, stable β-catenin-expressing CD4⁺CD25⁺ T_{reg} cells outcompeted control T_{reg} cells *in vivo*, and the number of T_{reg} cells necessary for protection against inflammatory bowel disease could be substantially reduced when stable β-catenin-expressing CD4⁺CD25⁺ T_{reg} cells were used instead of control T_{reg} cells. Expression of stable β-catenin on potentially pathogenic CD4⁺CD25⁺ T cells rendered these cells anergic, and the β-catenin-mediated induction of anergy occurred even in Foxp3-deficient T cells. Thus, through enhanced survival of existing regulatory T cells, and through induction of unresponsiveness in precursors of T effector cells, β-catenin stabilization has a powerful effect on the prevention of inflammatory disease.

β-catenin is an essential component of the Wnt signaling pathway, which is evolutionarily conserved and is involved in a large variety of developmental processes including the specification of cell fate and the maintenance of stem cell pluripotency^{1–3}. Not surprisingly, β-catenin concentration and its localization within the cell are tightly controlled. β-catenin is usually regulated through sequential phosphorylation by casein kinase I-α and glycogen synthase kinase-3β, which results in β-catenin recognition by β-trCP, polyubiquitinylation and degradation^{4–7}. Casein kinase I-α and glycogen synthase kinase-3β phosphorylate four serine and threonine residues (Ser33, Ser37, Thr41, Ser45) located near the N terminus of β-catenin. β-catenin molecules containing alanine in place of the four serine or threonine residues are not degraded by this pathway, and are hereafter referred to as ‘stable’ β-catenin. These mutations increase the half-life of β-catenin, resulting in higher concentration and localization in the nucleus, where β-catenin associates with transcription factors of the Tcf/Lef family. Only nonphosphorylated β-catenin seems to have transcriptional activity⁸.

T_{reg} cells are a very powerful component of the immune system, as they have the capacity to downmodulate all T cell-mediated immune responses^{9–12}; and absence or abnormality of the T_{reg} compartment results in autoimmunity, atopy and a series of hyper-responses. Most thymus-derived naturally occurring T_{reg} cells express CD4, CD25, glucocorticoid-induced tumor necrosis factor receptor, cytotoxic T lymphocyte antigen-4 and Foxp3 (ref. 11). Naturally occurring T_{reg} cells are referred to as being anergic because they proliferate poorly in response to *in vitro* stimulation with monoclonal antibody to CD3. A number of approaches have been tried to expand naturally occurring T_{reg} cells or to generate Foxp3⁺ T_{reg} cells from naive CD4⁺ T cells^{13–18}, but it remains challenging to obtain large numbers of

Foxp3⁺ T_{reg} cells for therapeutic purposes. In fact, the pathways that could be exploited pharmacologically to enhance the survival of T_{reg} cells are unknown.

Here, we show that stabilized β-catenin enhances the survival of CD4⁺CD25⁺ T_{reg} cells *in vitro* without changing their anergic state or suppressive function. In T_{reg} cells, Bcl-X_L expression (but not Bcl-2 expression) is upregulated by β-catenin, and reduction of mRNA encoding Bcl-X_L neutralizes the β-catenin-enhanced survival of T_{reg} cells. *In vivo* experiments showed that stable β-catenin-expressing CD4⁺CD25⁺ T_{reg} cells outcompete control T_{reg} cells and, most notably, that fewer stable β-catenin-expressing CD4⁺CD25⁺ T_{reg} cells than vector only-transduced T_{reg} cells or freshly isolated T_{reg} cells are needed to achieve similar protection against inflammatory bowel disease (IBD). We also show that stable β-catenin expression in CD4⁺CD25⁺ naive T cells induces anergy on these pathogenic cells. Notably, the induction of anergy by stable β-catenin occurs even in Foxp3-deficient cells. Although the effect of β-catenin is different in T_{reg} and non-T_{reg} cells, the effect of stable β-catenin expression is anti-inflammatory in both cases, thus opening the possibility of using this pathway for control of pathological inflammatory responses.

RESULTS

β-catenin-transduced T_{reg} cells show enhanced survival

We determined the effects of stable β-catenin on CD4⁺CD25⁺ T_{reg} cells by using retroviral constructs that encoded the stable β-catenin molecule, an internal ribosomal entry site (IRES), and GFP¹⁹. GFP expression was detected in 60–85% of transduced CD4⁺ cells (Supplementary Fig. 1a online), and the expression persisted for the entire *in vitro* culture period. Western blot analysis

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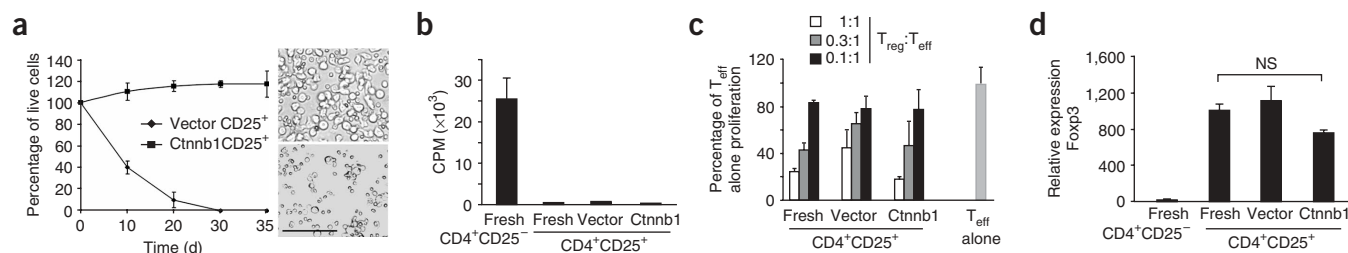


Figure 1 CD4⁺CD25⁺ T cells expressing stable β-catenin show enhanced survival *in vitro*. Splenic CD4⁺CD25⁺ T cells were infected with stable β-catenin (Ctnnb1) or empty vector (Vector), sorted and rested for 1 week. **(a)** At the end of this period, the number of viable cells in each culture was established as 100%, and cells were counted weekly thereafter. Microphotographs of representative cultures (2 weeks of culture) can be found at the right end of the survival curves. Scale bar, 60 μm. **(b)** Proliferation assay. The sorted GFP⁺ retroviral-transduced T cells were used in proliferation assay alongside fresh CD4⁺CD25⁻ T cells and CD4⁺CD25⁺ T cells. **(c)** Suppression assay. Fresh CD4⁺CD25⁻ T cells (T_{eff}) were cocultured with the sorted GFP⁺ retroviral-transduced T cells at the indicated proportions. **(d)** Real-time RT-PCR analysis of Foxp3 expression, normalized to β-actin, from cultures as described above at 3 weeks.

(Supplementary Fig. 1b), confirmed the expression of β-catenin at the protein level.

We examined whether stable β-catenin expression increased long-term growth of T lymphocytes. Empty vector-transduced CD4⁺CD25⁺ T_{reg} cells began to die quickly in medium containing limiting amounts of interleukin (IL)-2 (20 U/ml). Notably, under the same conditions, stable β-catenin-transduced CD4⁺CD25⁺ T_{reg} cells survived for at least 5 weeks (Fig. 1a). During this period, the number of β-catenin-transduced CD4⁺CD25⁺ T_{reg} cells increased slightly and then stabilized, indicating that the cells expressing a stable β-catenin could either survive longer or undergo a slow and limited self-renewal. Thymidine-labeling experiments and ablative morphology indicated a low level of proliferation, which must be offset by equivalent cell death (Fig. 1a and data not shown). Thus, as a population, β-catenin-transduced T_{reg} cells show enhanced survival.

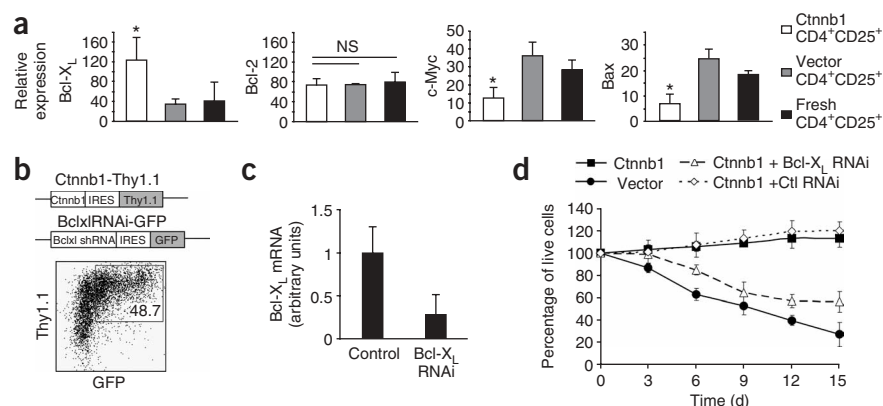
To determine whether expression of a stable form of β-catenin alters CD4⁺CD25⁺ T_{reg} cell function, the proliferative response and *in vitro* suppressive ability of β-catenin⁺ (GFP⁺) CD4⁺CD25⁺ T_{reg} cells were assessed. Stable β-catenin-transduced CD4⁺CD25⁺ T_{reg} cells were as anergic and suppressive as empty vector-transduced or freshly isolated CD4⁺CD25⁺ T_{reg} cells (Fig. 1b,c), while maintaining Foxp3 expression (Fig. 1d). We concluded that stable expression of β-catenin in T_{reg} cells promotes their survival *in vitro* without changing their phenotype or function.

β-catenin-transduced T_{reg} cells show enhanced Bcl-X_L expression

Death of lymphocytes that lack growth signals, as occurs in the vector only-transduced T_{reg} cells in Figure 1a, is likely to involve the mitochondrial pathway. This pathway is regulated by the Bcl-2 family, the two main antiapoptotic members being Bcl-2 and Bcl-X_L. We therefore tested whether transduction of stable β-catenin on T_{reg} cells would result in increased expression of Bcl-2 or Bcl-X_L. Our real-time PCR data showed that Bcl-X_L expression but not Bcl-2 expression was significantly upregulated upon stable β-catenin transduction of T_{reg} cells (Fig. 2a). Although discovered as an oncogene, it has been known for a long time that, depending on the metabolic state of the cell, Myc (also known as c-Myc) can also induce apoptosis²⁰. It has also been shown that c-Myc augments apoptosis by suppressing Bcl-X_L expression²¹. It was thus possible that stable β-catenin expression resulted in the downmodulation of c-Myc and concomitant Bcl-X_L upregulation. Indeed, Myc mRNA was downmodulated in stable β-catenin-transduced T_{reg} cells compared to vector-only controls (Fig. 2a). Regarding death effectors, c-Myc induces apoptosis *in vivo* exclusively through Bax but not Bak^{22–24}. It has also been proposed that Bax is a transcriptional target of c-Myc²⁵. We thus assessed by real-time PCR whether Bax expression in T_{reg} cells was affected by stable β-catenin transduction, and observed a significant decrease in Bax expression compared to T_{reg} cells transduced with vector-only or freshly isolated T_{reg} cells (Fig. 2a). To

Figure 2 β-catenin-transduced T_{reg} cells show enhanced Bcl-X_L and decreased Bax expression.

(a) β-catenin-transduced T_{reg} cells express more mRNA encoding Bcl-X_L and less mRNA encoding c-Myc and Bax than control T_{reg} cells. Mean expression of the genes encoding Bcl-X_L, Bcl-2, c-Myc and Bax determined by real-time RT-PCR, using RNA derived from sorted GFP⁺ cells from either Ctnnb1- or vector-transduced samples, results were normalized to β-actin. **P* < 0.05 for Ctnnb1- versus Vector-transduced or fresh CD4⁺CD25⁺ T cells. NS, not significant. Value indicates the mean ± s.e.m. of three separate experiments. **(b)** Splenic CD4⁺CD25⁺ T cells were coinfect with stable β-catenin (Ctnnb1-Thy1.1) and Bcl-X_L shRNA (BclxIRNAi-GFP). Cells coexpressing Thy1.1 and GFP were sorted. **(c)** Knockdown of Bcl-X_L results in repression of mRNA encoding Bcl-X_L in mouse primary CD4⁺CD25⁺Thy1.1⁺GFP⁺ T_{reg} cells. Relative expression of Bcl-X_L was quantified by real-time RT-PCR, using β-actin as an internal control. **(d)** The cell survival was monitored as described in Figure 1a.



determine whether Bcl-X_L upregulation accounted for the enhanced survival of stable β -catenin–transduced T_{reg} cells, we used a short hairpin RNA (shRNA) knockdown approach. We cotransduced T_{reg} cells with retroviral vectors expressing stable β -catenin (selectable by Thy1.1 expression) and Bcl-X_L shRNA (selectable by GFP expression; Fig. 2b and Supplementary Fig. 2 online). The expression of mRNA encoding Bcl-X_L was reduced by about 60–80% in β -catenin–transduced T_{reg} cells cotransduced with Bcl-X_L shRNA (Fig. 2c). Notably, the reduction of levels of mRNA encoding Bcl-X_L considerably counteracted the survival effect of stable β -catenin expression on T_{reg} cell survival (Fig. 2d), even though other antiapoptotic molecules were not affected. Thus, in β -catenin–transduced T_{reg} cells one

observes downmodulation of mRNA for c-Myc and Bax, as well as an upregulation of Bcl-X_L mRNA that is responsible for the enhanced survival of these cells.

β -catenin–transduced T_{reg} cells have a competitive advantage

We sought to determine whether the substantial survival advantage of stable β -catenin–expressing T_{reg} cells *in vitro* would also be observed *in vivo*. We thus carried out competition experiments in which equal numbers of allelically distinguishable stable β -catenin–transduced and vector only–transduced T_{reg} cells were coinjected into TCR $\alpha\beta$ -deficient mice, and the proportion of each subset was monitored over time. Likewise, we also set up competition experiments in which stable

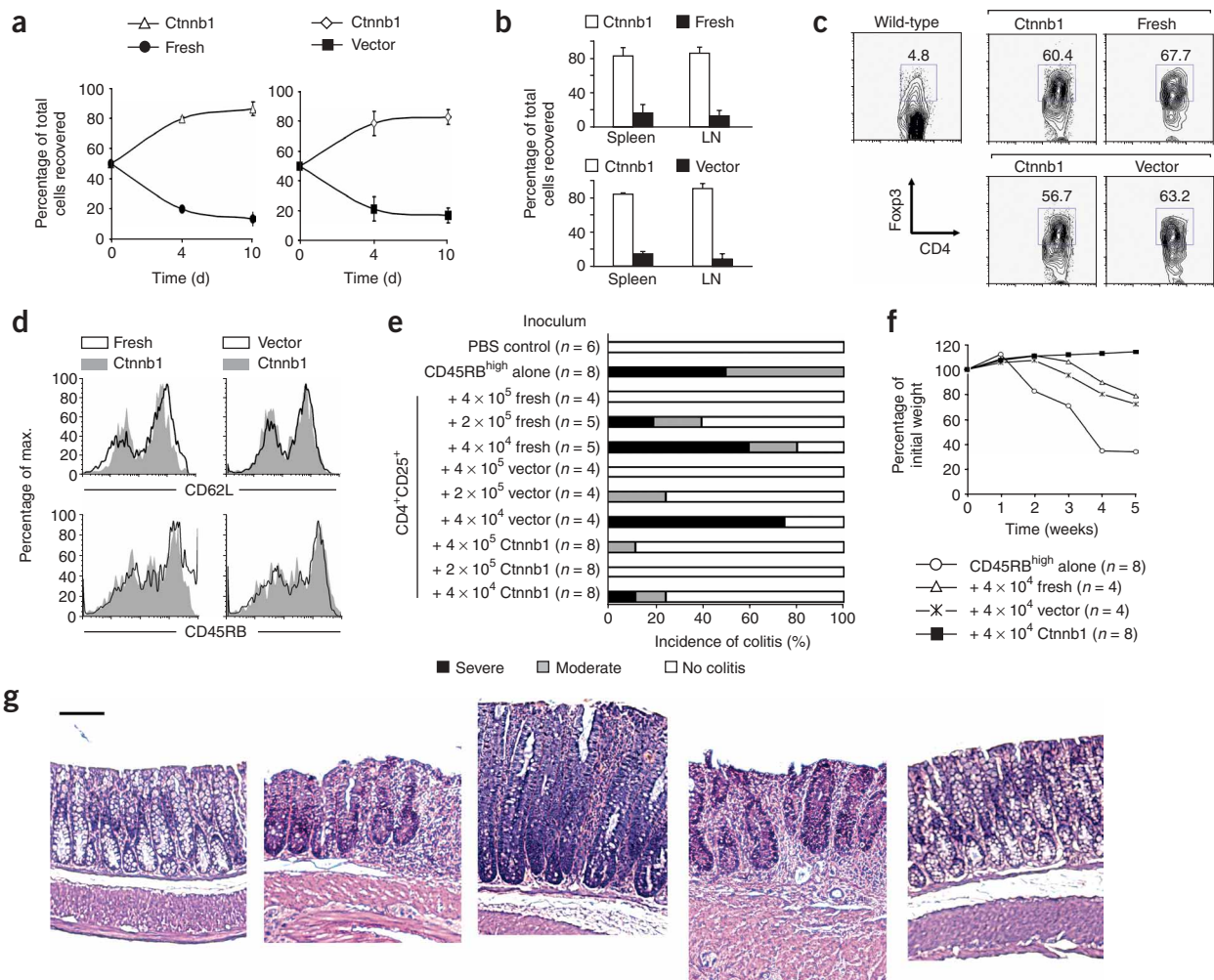


Figure 3 Stable β -catenin–expressing T_{reg} cells have a competitive *in vivo* advantage. (**a–g**) Ctnnb1–transduced CD4⁺CD25⁺ T cells (1×10^6 per recipient, Thy1.2⁺) were cotransferred with empty vector–transduced (1×10^6 per recipient, Thy1.1⁺) or freshly isolated CD4⁺CD25⁺ (1×10^6 per recipient, Thy1.1⁺) into *Tcr α ^{-/-}Tcr β ^{-/-}* hosts. (**a**) Peripheral blood analysis 4 and 10 d after transfer, showing the proportion of Ctnnb1 transduced T_{reg} cells and control T_{reg} cells after exclusion of host $\gamma\delta$ T cells. (**b**) Spleen and lymph node (LN) cell analysis indicating the proportion of CD4⁺ gated T cells derived from Ctnnb1–transduced T_{reg} cells and control T_{reg} cells 10 d after coinjection. (**c**) Analysis of Fopx3 expression 10 d after coinjection in lymph node. (**d**) Analysis of CD62L and CD45RB expression 10 d after coinjection in lymph node. (**e–g**) Colitis was induced in *Tcr α ^{-/-}Tcr β ^{-/-}* mice by transfer of 4×10^5 CD4⁺CD25⁺–CD45RB^{high} cells. (**e**) Different numbers of fresh CD4⁺CD25⁺ cells, empty vector–transduced CD4⁺CD25⁺ cells or Ctnnb1–transduced CD4⁺CD25⁺ cells were coinjected, as indicated in each line. Disease progression was monitored by body weight loss, diarrhea and histology analysis, as detailed in Methods. A control group received PBS intravenously instead of CD4⁺CD25⁺–CD45RB^{high} cells. (**f**) Data show the mean weights of mice for each group. Numbers in parentheses indicate number of mice. (**g**) Representative photomicrographs of distal colon of *Tcr α ^{-/-}Tcr β ^{-/-}* mice after transfer of CD4⁺CD25⁺–CD45RB^{high} T cells. Scale bar, 100 μm. Original magnification, 100× (H&E). From left to right: PBS control; 4×10^5 CD4⁺CD25⁺–CD45RB^{high} cells alone; 4×10^5 CD4⁺CD25⁺–CD45RB^{high} cells cotransferred with 4×10^4 freshly isolated CD4⁺CD25⁺ T cells; cotransferred with 4×10^4 vector–transduced CD4⁺CD25⁺ T cells; or cotransferred with 4×10^4 Ctnnb1–transduced CD4⁺CD25⁺ T cells.

Table 1 T cell recovery in competition experiments^a

Coinjected CD4 ⁺ subpopulation	Number of recovered CD4 ⁺ cells ($\times 10^4$) ^b		
	Spleen	Lymph nodes ^c	Blood ^d
Cttnb1 CD25 ⁺ (Thy1.2)	272.06 ($\pm$ 1.05)	46.43 ($\pm$ 8.17)	21.53 ($\pm$ 1.15)
Fresh CD25 ⁺ (Thy1.1)	54.94 ($\pm$ 7.63)	7.00 ($\pm$ 1.63)	2.20 ($\pm$ 0.46)
Cttnb1 CD25 ⁺ (Thy1.2)	137.88 ($\pm$ 17.09)	48.70 ($\pm$ 14.34)	14.87 ($\pm$ 7.79)
Vector CD25 ⁺ (Thy1.1)	25.22 ($\pm$ 3.75)	4.95 ($\pm$ 1.23)	2.16 ($\pm$ 3.56)

^a*Tcr α ^{-/-}Tcr β ^{-/-}* recipient mice ($n = 4$ per group) were coinjected with 1×10^6 CD4⁺ T cells, either Cttnb1-transduced CD25⁺ T_{reg} cells or control T_{reg} cells. CD4⁺ T cells of each origin were quantified in the indicated organs after 10 d. ^bMean $\pm$ s.e.m. ^cInguinal, cervical and mesenteric lymph nodes. ^dData are expressed as cell number per milliliter of blood.

β -catenin–transduced T_{reg} cells were coinjected with equal number of freshly isolated T_{reg} cells. In both cases, stable β -catenin–transduced T_{reg} cells outcompeted the control T_{reg} populations, in peripheral blood (Fig. 3a), spleen or lymph nodes (Fig. 3b). The number of recovered Thy1.1⁺ and Thy1.2⁺ CD4⁺ T cells in spleen and lymph nodes is shown in Table 1. It is thus clear that β -catenin–transduced T_{reg} cells are better fit to survive and expand *in vivo*.

Next we investigated the phenotype of the transferred T_{reg} cells *ex vivo* 10 d after injection into T cell–deficient hosts. Foxp3 expression was maintained by the majority of T_{reg} cells, with no difference between the T_{reg} population derived from stable β -catenin–transduced T_{reg} cells and either of the control T_{reg} groups (Fig. 3c). It is notable that in all experimental groups, irrespective of β -catenin expression, there was some loss in Foxp3 expression upon expansion in lymphopenic hosts. Indeed, in Foxp3-GFP reporter mice it has been shown that 13% of Foxp3⁺ cells recovered from lymphopenic hosts had lost Foxp3 expression²⁶. We also assessed whether β -catenin–transduced T_{reg} cells had an altered phenotype *in vivo* by determining the expression of memory T cell markers and effector cytokine production. After 10 d *in vivo*, there was no difference between stable β -catenin–transduced T_{reg} cells and either group of control T_{reg} cells regarding the expression of CD62L or CD45RB (Fig. 3d). Finally, neither β -catenin transduction nor control treatment resulted in the secretion of effector cytokines by T_{reg} cells (Supplementary Fig. 3 online). We therefore conclude that stable β -catenin–transduced T_{reg} cells have a competitive *in vivo* advantage over control T_{reg} cells without any noticeable phenotypical change.

Fewer β -catenin–transduced T_{reg} cells protect against IBD

We speculated that the improved survival of β -catenin–transduced CD4⁺CD25⁺ T cells *in vitro* and fitness *in vivo* could translate into an enhanced regulatory potency as a population. To test this hypothesis, we compared the ability of different numbers of freshly isolated or stable β -catenin–transduced CD4⁺CD25⁺ cells to suppress colitis induced by wild-type CD4⁺CD25⁺CD45RB^{high} cells²⁷. At a 1:10 ratio of CD4⁺CD25⁺ to pathogenic CD4⁺CD45RB^{high} cells, β -catenin–transduced

CD4⁺CD25⁺ cells protected 75% of the recipient mice from colitis (Fig. 3e). This protection was far superior to that afforded by either freshly isolated wild-type CD4⁺CD25⁺ cell group (20% protection) or vector-transduced CD4⁺CD25⁺ cells (25% protection). The weight curves of the groups treated with the lowest dose of T_{reg} cells are shown in Figure 3f, and representative histology pictures are shown in Figure 3g. A complete set of data including all T_{reg} doses is shown in Supplementary Figure 4 online. It is noteworthy that none of the mice transferred with stable β -catenin–expressing T cells developed malignancies throughout the period of observation (4 months); this is compatible with the reduction in c-Myc expression shown in Figure 2a. Thus, a reduced number of T_{reg} cells can be used to confer the same protection when β -catenin is stabilized, an important finding given the difficulty of isolating large quantities of T_{reg} cells.

Stable β -catenin expression induces anergy in CD4⁺CD25⁺ T cells

We next turned our attention to the effect of stable β -catenin expression on CD4⁺CD25⁺ T cells. When we transduced CD4⁺CD25⁺ T cells, we observed that stable β -catenin–expressing cells proliferated poorly, in contrast to the vigorous proliferation of T cells from control vector-transduced cells or freshly isolated CD4⁺CD25⁺ cells (Fig. 4a). To compare the proliferation of stable β -catenin–transduced CD4⁺CD25⁺ T cells with the cells in the same cultures that did not harbor the stable β -catenin–expressing retrovirus, we monitored the carboxyfluorescein succinimidyl ester (CFSE) dilution of CD4⁺CD25⁺ T cells stimulated with antibody to CD3. To distinguish transduced from nontransduced T cells while analyzing CFSE dilution, the same stable β -catenin construct was subcloned into Thy1.1 reporter retroviral vector (Supplementary Fig. 2). We noted that proliferation was markedly reduced in the cells that expressed stable β -catenin (Thy1.1⁺) compared to untransduced T cells (Fig. 4b).

Given the reduced proliferation by stable β -catenin–expressing CD4⁺CD25⁺ T cells, we sought to determine whether β -catenin stabilization resulted in the upregulation of genes associated

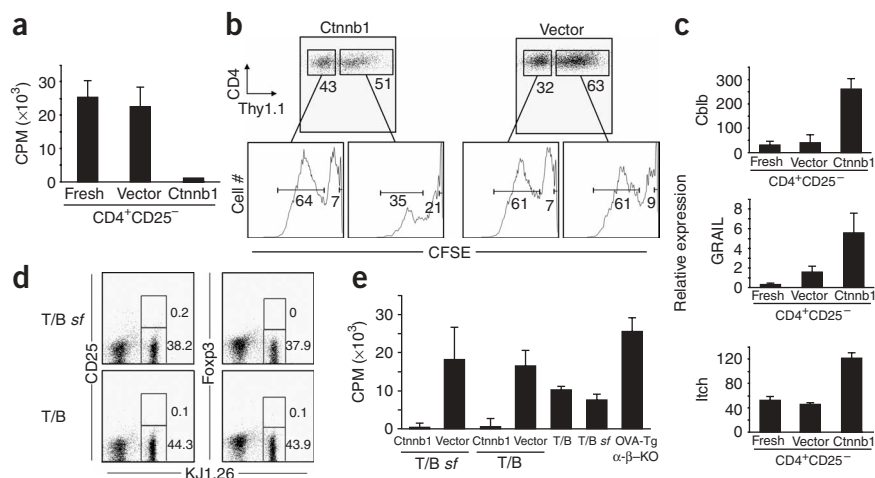


Figure 4 Stable β -catenin expression induces anergy in CD4⁺CD25⁺ T cells. Splenic CD4⁺CD25⁺ T cells were infected with stable β -catenin or empty vector. (a) Proliferation assay. The sorted retroviral-transduced GFP⁺ T cells were used in a proliferation assay alongside with fresh CD4⁺CD25⁺ T cells. (b) CFSE dilution was analyzed in the Cttnb1- and empty vector-transduced CD4⁺CD25⁺ T cells upon stimulation. (c) Real-time RT-PCR analysis of expression of genes encoding Cblb, GRAIL(Rnf128) and Itch, normalized to β -actin. (d) CD4⁺ T cell from T/B monoclonal and T/B monoclonal sf mice are stained for CD25 and Foxp3. (e) Proliferation assay. The sorted GFP⁺ retroviral transduced T cells were used in proliferation assay alongside with fresh OVA-Tg *Tcr α ^{-/-}Tcr β ^{-/-}* (α - β -KO) T cells.

with the anergic state. Indeed, real-time quantitative PCR analysis showed that stable β -catenin–transduced $CD4^+CD25^-$ T cells had significantly higher levels of mRNA encoding *Cblb* ($P = 0.0228$), *GRAIL* (*Rnf128*; $P = 0.0231$) and *Itch* ($P = 0.0258$) than freshly isolated $CD4^+CD25^-$ T cells or $CD4^+CD25^-$ T cells transduced with empty vector (Fig. 4c). Thus, expression of stable β -catenin on mature $CD4^+CD25^-$ T cells results in the induction of a typical anergic state.

As we showed above that $CD4^+CD25^+$ T_{reg} cells transduced with β -catenin showed a markedly enhanced survival, it was possible that our results with $CD4^+CD25^-$ T cells were clouded by a small number of T_{reg} contaminants that would influence the growth of $CD4^+CD25^-$ T cells. Furthermore, it was clear that there is a small population of $Foxp3^+CD4^+CD25^-$ T cells. In order to ensure that we were analyzing the effect of stable β -catenin expression on nonregulatory $CD4^+$ T cells, we analyzed the effect of stable β -catenin expression on the proliferation of T cells purified from TCR transgenic RAG-deficient mice, which lack *Foxp3*-expressing T cells²⁸. To validate the system, we showed that ovalbumin (OVA)-specific DO11.10 *Rag1*^{-/-} T cells from T/B monoclonal mice²⁹ express neither CD25 nor *Foxp3* (Fig. 4d). The few *Foxp3*-positive dots represent background, as they also occur in *Foxp3*-deficient (scurlfy) T/B monoclonal mice (Fig. 4d). We then evaluated the proliferative status of stable β -catenin–transduced and control $CD4^+$ T cells in this experimental context, in which the contamination of $CD4^+CD25^-$ T cells with T_{reg} cells was clearly excluded. As we had observed with wild-type polyclonal $CD4^+CD25^-$ T cells, stable β -catenin expression also induced anergy in $CD4^+CD25^-$ T cells from T/B monoclonal mice (Fig. 4e). This β -catenin–mediated induction of anergy occurred without the induction of *in vitro* suppressive capacity (data not shown), confirming the notion that anergy and the capacity to exert suppression are properties that can be separated. Notably, the induction of anergy by stable β -catenin expression occurred even in T cells from *Foxp3*-deficient mice, indicating that anergy can be induced in T cells independently of *Foxp3* expression (Fig. 4e).

It is possible that lack of proliferation in response to CD3-specific antibody *in vitro* does not correspond to an anergic state *in vivo*. To examine the *in vivo* activity of $CD4^+CD25^-$ cells transduced with stable β -catenin, we used the IBD model mentioned above. As anticipated, all of the mice that received fresh $CD4^+CD25^-CD45RB^{high}$ cells or vector only–transduced $CD4^+CD25^-$ cells developed IBD. Notably, only one of eight mice transferred with stable β -catenin transduced $CD4^+CD25^-$ cells did (Fig. 5a). Histological analysis showed that 25% of the mice that received control empty vector–transduced cells had severe pathological changes in the colon and 75% had moderate pathology, whereas the one mouse that developed IBD in the group that received stable β -catenin–transduced cells showed moderate histological findings, and seven of eight mice had no findings (Fig. 5a). Mice transferred with stable β -catenin–transduced T cells did not lose weight or lost little weight, whereas all mice in the

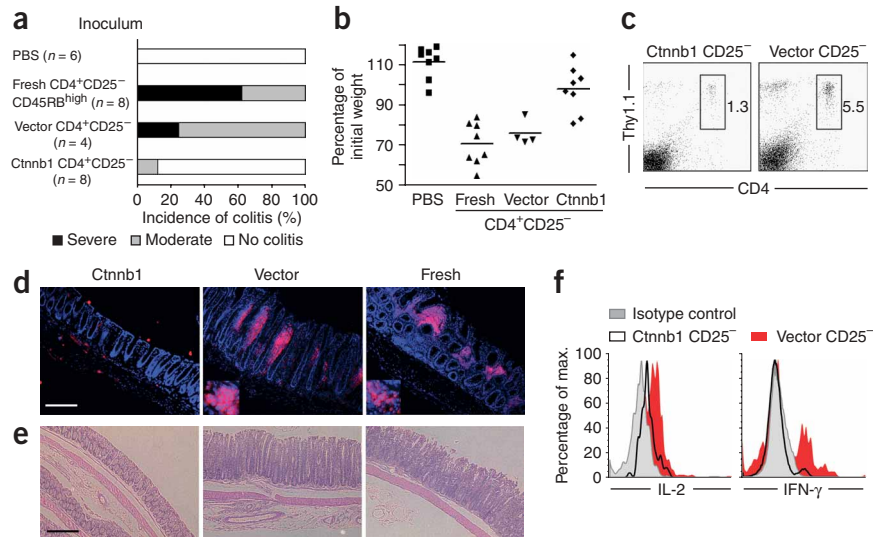


Figure 5 $CD4^+CD25^-$ T cells expressing stable β -catenin do not induce IBD *in vivo*. *Tcr α* ^{-/-}*Tcr β* ^{-/-} mice (Thy1.2) were transferred with 4×10^5 Vector-Thy1.1–transduced $CD4^+CD25^-$ T cells, Ctnnb1-Thy1.1–transduced $CD4^+CD25^-$ T cells or fresh $CD4^+CD25^-CD45RB^{high}$ cells (Thy1.1). PBS was injected as control. (a) Disease progression was monitored by body weight loss, diarrhea and histology analysis, as detailed in Methods. (b) Data represent body weight as a percentage of the initial weight of individual mice. (c) Staining of $CD4^+Thy1.1^+$ cells recovered from spleen 2 weeks after transfer. (d,e) Histological sections of colon. (d) Thy1.1 staining (red) and DAPI counterstaining (blue). Microphotographs were taken at $\times 100$ magnification (insets, $\times 400$ magnification). Scale bar, 100 μ m. (e) H&E staining. Insets show Thy1.1 staining of donor $CD4^+CD25^-$ cells transduced with Ctnnb1-Thy1.1 (left), vector-Thy1.1 (middle) or freshly sorted $CD45RB^{high}$ cells (from Thy1.1 donor) (right). Microphotographs were taken at $\times 100$ magnification. Scale bar, 100 μ m. (f) Intracellular staining of IL-2 and IFN- γ in $CD4^+Thy1.1^+$ cells recovered from spleen 2 weeks after transfer.

control group lost weight (Fig. 5b). Thus, stable β -catenin–transduced $CD4^+CD25^-$ T cells are anergic *in vitro* and show greatly reduced capacity to induce inflammatory disease.

We monitored the fate of the transferred β -catenin–transduced $CD4^+CD25^-$ T cells in the lymphopenic recipient mice by cell counting, FACS and immunohistology. In accordance to the *in vitro* anergic phenotype, we consistently recovered 60–80% fewer stable β -catenin–transduced $CD4^+CD25^-$ T cells than we did control $CD4^+CD25^-$ T cells (Fig. 5c and Supplementary Table 1 online). Within the gut, there was a clear reduction in the number of transferred β -catenin–expressing T cells compared to control T cells (Fig. 5d), a reduction associated with lowered gut inflammation (Fig. 5e). Finally, amongst the recovered T cells that had been transduced with stable β -catenin, there was a decrease in IL-2 and IFN- γ production compared to that in control T cells (Fig. 5f). Thus, it is apparent that stable β -catenin expression on $CD4^+CD25^-$ T cells induces an anergic phenotype *in vitro* with loss of pathogenicity *in vivo*.

DISCUSSION

We report here the strong effect of β -catenin stabilization on mature $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. β -catenin stabilization in both cell types was beneficial with regards to their effect on inflammation. $CD4^+CD25^+$ T_{reg} cells transduced with stable β -catenin survived longer *in vitro* and outcompeted control T_{reg} cells *in vivo*, making possible the utilization of substantially fewer T_{reg} cells in the control of inflammatory disease.

$CD4^+CD25^-$ T cells transduced with stable β -catenin became anergic. The effect of β -catenin stabilization may be similar on mature $CD4^+CD25^-$ T cells and developing thymocytes. In the latter cells, it

was reported that conditional early stabilization of β -catenin or conditional early deletion of the APC gene prolonged the mitotic metaphase-to-anaphase checkpoint resulting in impaired cell division and a very small thymus³⁰. Thus, our results are consistent with a cell context-specific effect of β -catenin. Fortunately, stabilization of β -catenin in CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells resulted, in both cell types, in phenotypes that are beneficial in inflammatory conditions such as IBD.

This effect of stable β -catenin on CD4⁺CD25⁺ T_{reg} cells was dependent on Bcl-X_L upregulation and correlated with the decrease in c-Myc mRNA levels. The latter fact may explain the absence of malignancy in mice that received stable β -catenin-expressing T cells. Our results with CD4⁺CD25⁺ T_{reg} cells are consistent with the observation that the Wnt pathway has an important role in self-renewal of hematopoietic stem cells¹⁹. Furthermore, the survival of CD4⁺CD8⁺ double-positive thymocytes was shown to be dependent on β -catenin-Tcf-1 interactions³¹. In addition, enhanced survival of CD8⁺ T cells or antigen-presenting cells could explain the enhanced proliferation of CD8⁺ T cells at late, but not early, time points in lithium-treated *in vitro* cultures³².

Numerous reports described the importance of β -catenin on T cell development^{30,33,34}. To bypass the effects of β -catenin on T cell development, we used transduction of stable β -catenin into mature T cells in this study.

Mechanistically, it was important to determine that the β -catenin-mediated induction of anergy in CD4⁺CD25⁻ T cells was independent of Foxp3 expression, as anergy could be induced in Foxp3-deficient naive T cells. Naturally occurring regulatory T cells are anergic and suppressive. It has been shown that induction of Foxp3 expression in naive CD4⁺CD25⁻ T cells results in the acquisition of the anergic phenotype as well as the capacity to suppress proliferation of naive T cells *in vitro*^{35,36}. Human T cell clones rendered anergic could suppress the proliferation of responder T cells³⁷. T cells that reported Foxp3 expression but had no functional Foxp3 gene were less anergic than Foxp3⁺ cells in the presence of IL-2 or CD28-specific antibody costimulation^{26,38}; however, they were anergic in the classical CD3-specific antibody stimulation assay²⁶. It is thus probable that the full induction of regulatory T cell function is a multistep process in which anergy induction is an early step. This early step can be accomplished experimentally by stable β -catenin expression, although our data do not address whether this is the physiological pathway. Multistep T_{reg} cell differentiation could be mediated by sequential change of NFAT partners, as has been described for NFAT-Foxp2 complexes³⁹.

It has been shown that Foxp3⁺ T_{reg} cells can be generated *in vitro* by stimulation of CD4⁺CD25⁻ T cells with TCR-specific antibodies and TGF- β ¹³. In order to generate a large number of polyclonal regulatory T cells (for instance when the antigen recognized by effector cells is not known, as is the case in IBD) repeated cycles of TCR-specific antibody stimulation would be necessary, which is difficult as the antibody treatment results in the downmodulation of TCR expression. Additional methods to enhance polyclonal T_{reg} cell numbers or potency are therefore important. Our findings with stable β -catenin transduction open the possibility of enhancing T_{reg} cell activity in clinical settings by modifying the Wnt- β -catenin pathway pharmacologically or using a gene therapy approach, with the addition of suicide genes.

The question of whether an intact β -catenin pathway is involved in the biology of regulatory T cells remains a major unresolved issue and is the subject of ongoing studies. Regardless of this role, our report shows that the system is amenable to manipulation in a manner that is advantageous to the host.

METHODS

Mice. We housed all mice under specific pathogen-free conditions at the Skirball Institute Central Animal Facility, New York University School of Medicine (NYUSM). We used the C57BL/10.PL (Thy1.2), Thy1.1 congenic C57BL/10.PL Thy1.1 and C57BL/10.PL TCR $\alpha^{-/-}$ $\beta^{-/-}$ mice (Thy1.2) at 6–12 weeks of age. We obtained T/B monoclonal mice²⁸ by crossing the 17/9 immunoglobulin knock-in mice with DO11.10 TCR transgenic mice and with Rag1^{-/-} mice. We obtained T/B monoclonal Foxp3-deficient mice by crossing Scurfy (sf) mice (Jax) with T/B monoclonal mice, and backcrossed them to the BALB/c genetic background.

FACS analysis. We purchased the following reagents from PharMingen: monoclonal antibodies to CD3 ϵ (145-2C11), CD28 (37.51); CD4 (RM4-5); CD4 (H129.19), CD8 (53-6.7), CD45RB (16A), CD62L (MEL-14), Thy1.1 (OX-7), Thy1.2 (53-2.1), IL-17 (TC-11-18H10.1), IFN- γ (25723.11) and TCR $\gamma\delta$ (GL3). Monoclonal antibody to CD25 (7D4) was from Caltag (RM-6004). We purchased monoclonal antibody to Foxp3 (FJK-16s) from eBioscience (72-5775-40).

For surface staining, we incubated single-cell suspensions for 40 min at 4 °C with the antibody cocktails. We analyzed samples in a FACSCalibur machine (Becton Dickinson).

For intracellular cytokine staining, we stimulated cells with phorbol 12-myristate 13-acetate (100 ng/ml) and ionomycin (250 ng/ml) for a total of 4 h at a concentration of 1×10^6 cells/ml. In the last 2 h, we added monensin (3 μ M), we fixed cells and permeabilized them with Cytofix/Cytoperm (BD, 554722), and stained them with monoclonal antibodies to IL-17 (phycoerythrin, PE) and IFN- γ (allophycocyanin, APC).

We performed Foxp3 staining as previously described²⁸, in brief, after surface staining, we permeabilized samples with Cytofix/Cytoperm for 20 min, and then with 1% paraformaldehyde plus 0.5% Tween-20 in PBS for 30 min. After permeabilization, we stained samples with monoclonal antibody to Foxp3-PE antibody at 25 °C for 45 min.

Cell purification. We purified CD4⁺CD25⁺ and CD4⁺CD25⁻ lymphocytes from spleen of wild-type C57BL/10.PL mice by magnetic cell sorting using Miltenyi reagents and a Vario MACS apparatus. The purity of the CD25⁺ population was 85–95%. We purified CD4⁺CD25⁻CD45RB^{high} fractions used in the experiments of colitis induction by cell sorting in a MoFlo cytometer (DakoCytomation) at NYUSM. The purity of MoFlo sorted fraction was $\geq 95\%$.

Retroviral transduction. For retroviral infection, we cultured T cells at 37 °C for 24 h in complete RPMI medium⁹ with 100 U/ml rhIL-2 at a density of 2×10^6 cells per well in 24-well plates precoated with 5 μ g/ml monoclonal antibodies to CD3 and CD28.

The retroviral constructs containing bicistronic GFP expression and stable β -catenin (Ctnnb1), and GFP-only vector (vector) were transfected into the Phoenix packaging cells.

***In vitro* proliferation and suppression assays.** We set up proliferation assays in 96-well plates that contained, per well, 2×10^4 responder cells, 4×10^4 mitomycin C-treated spleen cells from TCR $\alpha^{-/-}$ $\beta^{-/-}$ mice, and antibody to CD3 at 0.5 μ g/ml. We cocultured putative suppressor cells at suppressor: responder ratios of 1:1, 0.3:1 and 0.1:1. We determined proliferation by adding [³H] thymidine (1 μ Ci/well; Du Pont/NEN) on the third day of culture and determining incorporation 6 h later. We performed cultures in triplicate, and the s.d. was within 15% of the mean for each experiment. Background counts in the wells with antigen-presenting cells alone were always $< 2,000$ cpm.

RNAi. A shRNA sequence targeting the mouse Bcl-X_L (*Bcl2l1*) 3' untranslated region sequence (5'-GCTCTCTGCTGTACATATTCG-3') was picked by Invitrogen Block-iT. The corresponding hairpin RNAi sequence were 5'-GATC CCCGCTCTCTGCTGTACATATTCGTTCAAGAGACGAATATGTACAGCAGAGAGCTTTTGGAAA-3' and 5'-TCGATTTCACAAAAGCTCTCTGCTGTACATATTCGTTCTTTGAACGAATATGTACAGCAGAGAGCGGG-3'. We annealed these oligonucleotides and subcloned them downstream of the H1 promoter in pQCXIP by using *Xho*I and *Bgl*II to generate BclxlRNAi-GFP, which we then stably transfected into CD4⁺CD25⁺ T_{reg} cells.

Real-time PCR analysis. We extracted total cellular RNA from 5×10^5 sorted cells using TRIzol. We reverse transcribed the total amount of RNA using Superscript II reverse-transcriptase and oligo(dT)₁₂₋₁₈ primer (Invitrogen). We treated RNA samples with RNase-free DNase I (Invitrogen) before reverse transcription to eliminate contaminating genomic DNA. We determined the expression of the mRNAs for Bcl-X_L, Bcl-2, c-Myc, Bax, Foxp3, Cblb, Itch, GAIL and β -actin by real-time PCR using the primers below. Expression was normalized to the level of β -actin in each sample: Bcl-X_L, 5'-GTCGCCGGAG ATAGATTGA-3', 5'-GTCCAAACACCTGCTCAC-3'; Bcl-2, 5'-AGTACCTG AACCGCATCTG-3', 5'-CATGCTGGGGCCATATAGTT-3'; c-Myc, 5'-GCTG GAGATGATGACCGAGT-3', 5'-AACCGCTCCACATACAGTCC-3'; Bax, 5'-TG GAGATGAAGTGGACGCA-3', 5'-TGAAGTTGCCATCAGCAAA-3'; Foxp3, 5'-ACTGGGGTCTTCTCCCTCAA-3', 5'-GTGGGAAGGTGCAGAGTAG-3'; Cblb, 5'-CTTAAATGGGAGGCACAGTAGAAT-3', 5'-CAGTACACTTTATGC TTGGGAGAA-3'; Itch, 5'-GTGTGGAGTACCAGACCCT-3', 5'-GCTTCTAC TTGCAGCCCATC-3'; GAIL (Rnf128), 5'-GTAACCGGCACACCAATTC-3', 5'-TGAGACATGGGGATGACCT-3'; β -actin, 5'-TGACAGGATGCAGAAGGA GA-3', 5'-GTAATGCGCTCAGGAGGAG-3'.

Western blot. We lysed cells in RIPA buffer supplemented with protease and phosphatase inhibitors. We loaded equal amounts of protein on a 7.5% SDS-PAGE, blotted on nitrocellulose. We probed blots with antibody to β -catenin (clone 7D11, Upstate) and antibody to α -tubulin (clone DM 1A, Sigma) and visualized them using ECL system (Amersham).

In vivo competition assay. We intravenously injected C57BL/10.PL *Tcr α ^{-/-}Tcr β ^{-/-}* mice with equal numbers (1×10^6 cells/mouse) of stable β -catenin-transduced (Thy1.2) and empty vector-transduced (Thy1.1) CD4⁺CD25⁺ cells or, alternatively, injected them with equal numbers of stable β -catenin-transduced (Thy1.2) and freshly isolated (Thy1.1) CD4⁺CD25⁺ cells. We collected blood, spleen and lymph node samples from the recipient mice at different time points after injection. To determine equal number of input cells, we carried out hemocytometer cell counts in combination with monoclonal antibodies to Thy1.1 and Thy1.2 staining of the mixed cells. We carried out a final validation by Thy1.1 and Thy1.2 staining of the material injected in the mice.

Carboxyfluorescein succinimidyl ester labeling. We added CFSE to the cell suspensions (1×10^7 cells/ml) at a final concentration of 3 μ M, 37 °C, for 10 min; we then stopped the reaction with FCS at a final concentration of 10%, and we washed cells twice with PBS and resuspended them with complete RPMI media.

T cell transfer model of colitis. We intravenously injected C57BL/10.PL *Tcr α ^{-/-}Tcr β ^{-/-}* mice with 4×10^5 sorted CD4⁺CD25⁺CD45RB^{high} T cells alone or in combination with the indicated numbers of CD4⁺ T cell subpopulations. We weighed mice twice a week and inspected them for clinical signs of colitis such as diarrhea, rectal prolapse and ruffled fur. We carried out scoring by histological examination, as previously described²⁷.

Histochemistry and immunohistochemistry. We histochemically stained paraffin-embedded sections with H&E. For immunohistochemical analysis, we immunolabeled the sections with primary antibodies to FITC-B220 (clone RA3-6B2, Pharmingen), Thy1.1-PE (clone OX-7, Pharmingen), CD4-APC (clone S6.5, Caltag) and CD3-APC (Clone 145-2C11, Pharmingen) at 4 °C overnight. We performed DAPI staining for 2 min at 25 °C then mounted the slides with Fluoromount-G (Fisher). We acquired images with an Axioplan 2 fluorescent microscope (Zeiss).

Statistical analysis. We determined *P* values by a two-tailed *t*-test, assuming equal variance. We considered differences between groups significant at *P* < 0.05.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

Y.D. was involved in the execution of all experiments and the preparation of the manuscript. S.S. contributed to some of the *in vivo* experiments, A.C.L. contributed to some of the *in vitro* experiments and M.A.C.L. contributed to the experiments with the T/B monoclonal and T/B monoclonal Scurfy mouse lines. J.J.L. supervised the project and prepared the manuscript.

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Supplementary Information

Beta-catenin stabilization extends regulatory T cell survival and induces anergy in non-regulatory T cells

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and Juan J. Lafaille^{1,2*}**

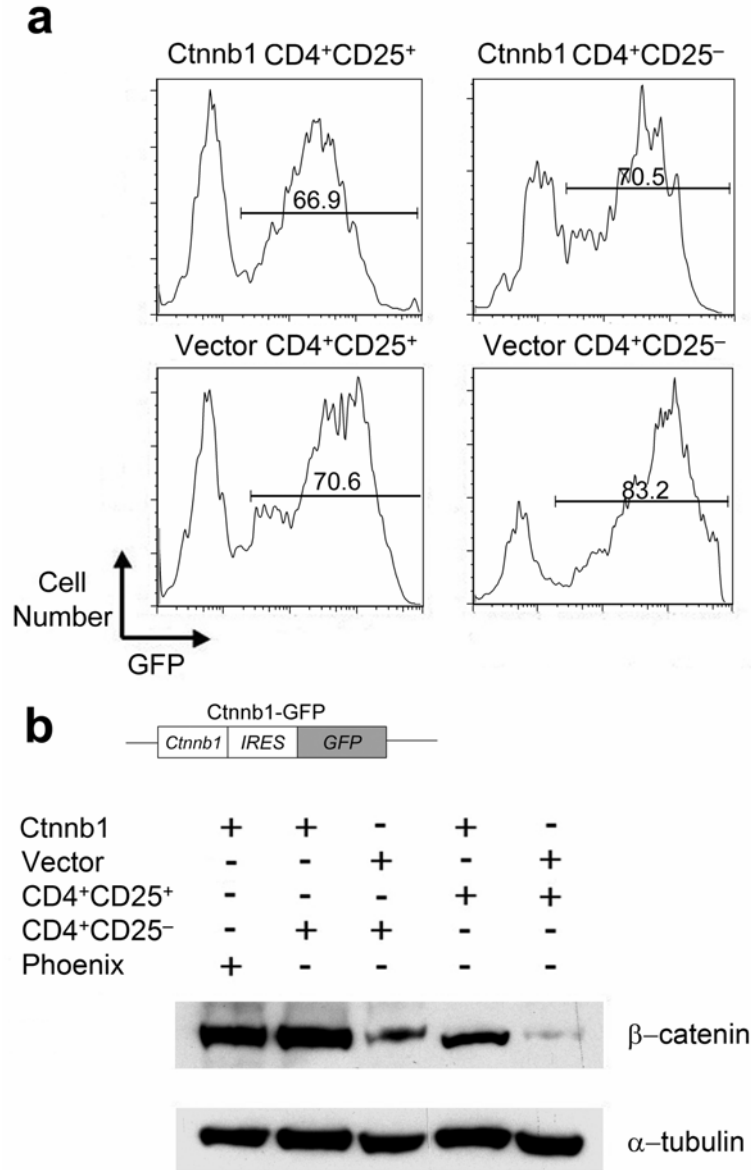


Figure S1. Expression of stable β -catenin in CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells.

CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells from spleen of wild-type C57BL/10.PL mice were stimulated with antibodies to CD3, CD28 and rhIL-2. The activated T cells were then infected with pMIGR retrovirus expressing either stable β -catenin (Ctnnb1) or GFP-only vector (Vector). **(a)** Detection of GFP expression by flow cytometry. **(b)** Western blot analysis of the Ctnnb1 or vector transduced CD4⁺ cells using monoclonal antibody specific for β -catenin. Blotting with antibody to α -Tubulin (lower panels) is used as loading control. The leftmost lane show β -catenin transfected Phoenix cells, used as positive control.

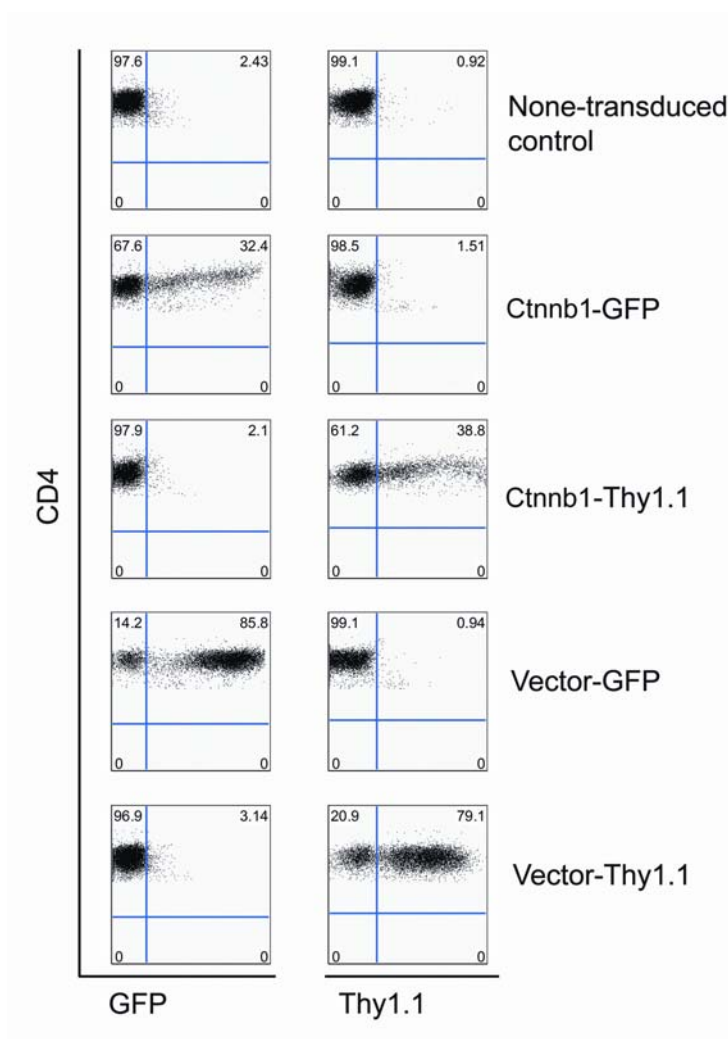


Figure S2. Vectors with selection marker GFP and Thy1.1 have similar transduction efficiency.

Splenic CD4⁺ T cells were infected with stable β -catenin (Ctnnb1) or empty vector (Vector) with different selection marker GFP or Thy1.1 and transduction efficiency was examined by flow cytometry.

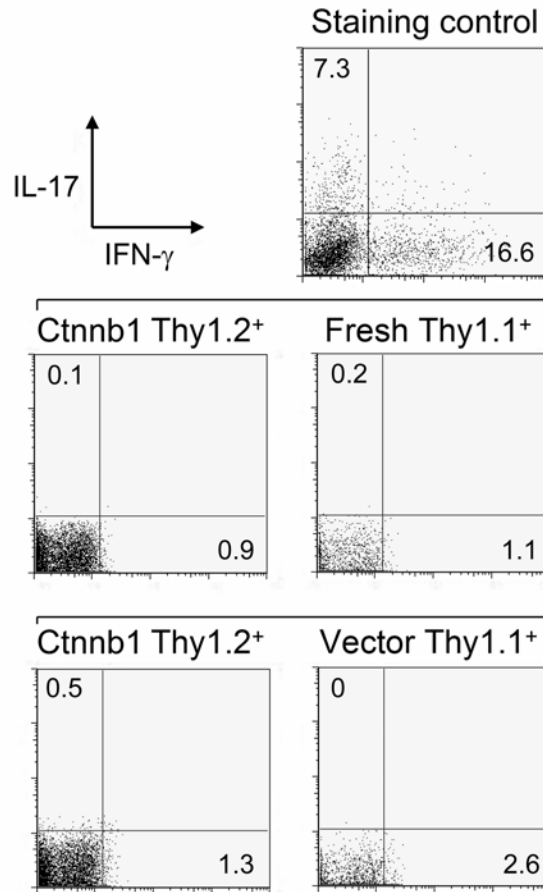


Figure S3. β -catenin-transduced Tregs do not produce effector cytokines.

Ctnnb1 transduced CD4⁺CD25⁺ (1×10^6 per recipient) were co-transferred with empty vector transduced (1×10^6 per recipient) or freshly isolated CD4⁺CD25⁺ (1×10^6 per recipient) into TCR $\alpha^{-/-}$ $\beta^{-/-}$ hosts. Ten days after transfer, spleen and lymph nodes cells were analyzed by flow cytometry for IFN- γ and IL-17 expression. In vitro cultured T cells are used as staining control. There are four mice in each group.

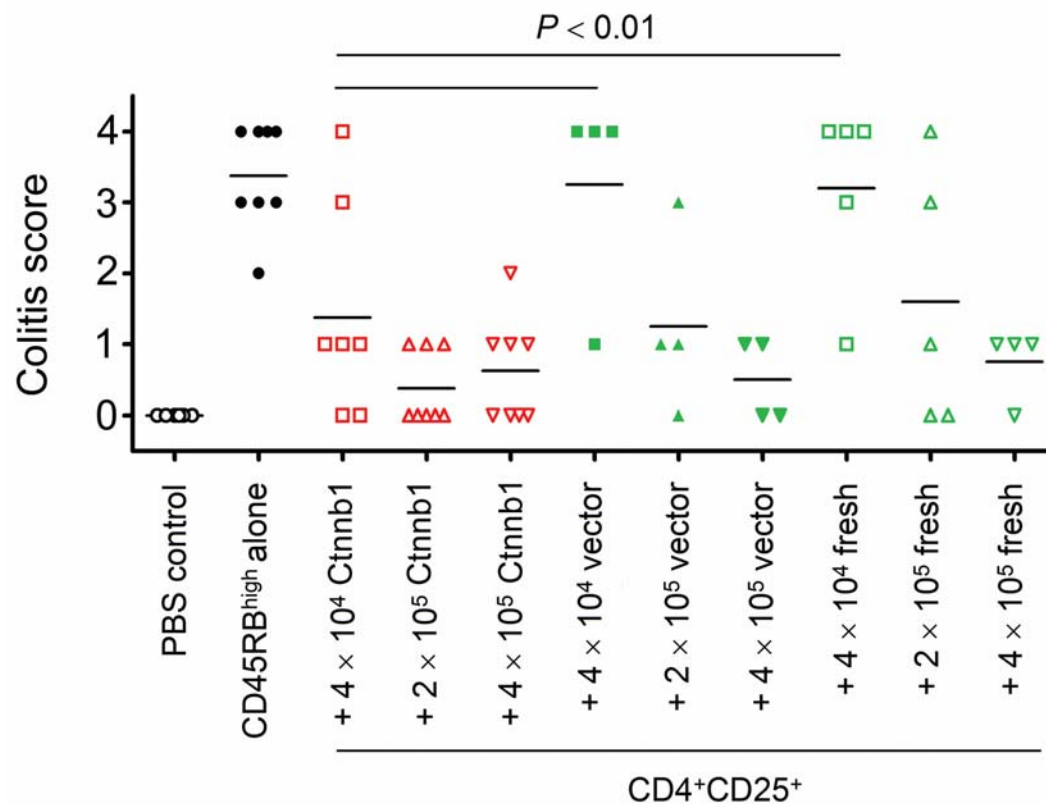


Figure S4. Fewer CD4⁺CD25⁺ T cells expressing stable β -catenin than fresh CD4⁺CD25⁺ T cells are needed for protection against IBD.

These data supplements **Fig. 3e**. Colitis was induced in TCR $\alpha^{-/-}\beta^{-/-}$ mice by transfer of 4×10^5 CD4⁺CD25⁻CD45RB^{high} cells. In addition, some mice received the indicated number of freshly isolated CD4⁺CD25⁺ T cells, empty vector transduced CD4⁺CD25⁺ T cells, or Ctnnb1 transduced CD4⁺CD25⁺ T cells. Data show colitis scores for individual mice taken from three independent experiments.

S. Table 1. Organ distribution of CD4⁺ cells in TCR $\alpha^{-/-}\beta^{-/-}$ mice Reconstitute with different CD4 subpopulations^a.

CD4 ⁺ subpopulation	No. of Recovered CD4 ⁺ Cells (x 10 ⁴) ^b			
	Blood ^c	Spleen	mLN ^d	Colon
Fresh CD4 ⁺ CD25 ⁻	9.35±1.72	46.35±10.2	7.3±1.64	12.2±2.66
Vector CD4 ⁺ CD25 ⁻	11.35±1.23	57.6±10.97	12.25±2.86	10.92±3.07
Ctnnb1 CD4 ⁺ CD25 ⁻	3.45±0.72	16.45±2.17	2.57±0.8	2.73±0.76

a. TCR $\alpha^{-/-}\beta^{-/-}$ recipient mice (n=4/group) were transferred *i.v.* with 3 x 10⁵ T cells either freshly isolated or Ctnnb1, Vector transduced CD4⁺CD25⁻ cells. CD4⁺ T cells of each origin were quantified in the indicated organs after 3 weeks.

b. Average ± SEM.

c. Data are expressed as cell number per milliliter of blood.

d. Mesenteric lymph nodes.

Chapter III

CD25⁻ T cells Generate CD25⁺Foxp3⁺ Regulatory T cells by Peripheral Expansion

By: Maria A. Curotto de Lafaille, Andreia C. Lino, Nino Kutchukhidze &
Juan J. Lafaille

CD25⁻ T Cells Generate CD25⁺Foxp3⁺ Regulatory T Cells by Peripheral Expansion¹

Maria A. Curotto de Lafaille,² Andreia C. Lino, Nino Kutchukhidze, and Juan J. Lafaille²

Naturally occurring CD4⁺ regulatory T cells are generally identified through their expression of CD25. However, in several experimental systems considerable T_{reg} activity has been observed in the CD4⁺CD25⁻ fraction. Upon adoptive transfer, the expression of CD25 in donor-derived cells is not stable, with CD4⁺CD25⁺ cells appearing in CD4⁺CD25⁻ T cell-injected animals and vice versa. We show in this study that CD25⁺ cells arising from donor CD25⁻ cells upon homeostatic proliferation in recipient mice express markers of freshly isolated T_{reg} cells, display an anergic state, and suppress the proliferation of other cells in vitro. The maintenance of CD25 expression by CD4⁺CD25⁺ cells depends on IL-2 secreted by cotransferred CD4⁺CD25⁻ or by Ag-stimulated T cells in peripheral lymphoid organs. *The Journal of Immunology*, 2004, 173: 7259–7268.

Regulatory T cells (T_{reg}),³ also called suppressor T cells, negatively modulate immune responses (1–4). The best-characterized, naturally occurring T_{reg} cells are CD4⁺ T cells of thymic origin that constitutively express CD25 and are unresponsive to stimulation in vitro (5–8). CD4⁺CD25⁺ T_{reg} cells constitutively express the transcription factor Foxp3 and the coreceptor CTLA-4 (9–13). Genetic deficiencies in these two genes cause profound immune dysregulation, leading to autoimmune diseases and allergy (14–18). T cells with T_{reg} activity are also contained in the CD4⁺CD25⁻ T cell population of normal animals. The regulatory T cell function of CD4⁺CD25⁻ T cells has been demonstrated in animal models of autoimmune encephalomyelitis (19, 20), diabetes (21–23), allergy (24), and inflammatory bowel disease (25). However, although CD4⁺CD25⁻ cells produced by depletion of CD25⁺ T cells can exert regulatory activity in several disease models, genetically deficient CD25^{-/-} cells are unable to do so (26). Thus, CD25 must be expressed at some point for T cells to exert suppression.

Upon transfer of purified CD4⁺CD25⁺ cells into T cell-deficient mice, scurfy mice, or TCR transgenic RAG^{-/-} mice, a substantial fraction of the cells become CD25⁻. Conversely, transfer of purified CD4⁺CD25⁻ cells into T cell-deficient mice generates a population of cells expressing CD25 (10, 25–28). CD25 expression appears to be more stable upon transfer of purified CD4⁺CD25⁺ T cells into wild-type mice (29). Laurie et al. (30) depleted CD4⁺CD25⁺ T cells by in vivo administration of anti-CD25 Abs to thymectomized BALB/c recipients and observed that CD25⁺ T cells returned to pretreatment proportion and function-

ality 48 days after Ab injection. In this and the previous cases it is unclear whether the CD25⁺ cells derived from the outgrowth of a small residual CD25⁺ population or the conversion of CD25⁻ into CD25⁺ T cells. The biological properties of the cells that acquired CD25 expression through homeostatic proliferation are not well known; likewise, the factors that influence the degree of CD25 marker switching remain poorly understood.

To study these issues we used an experimental system, the T/B monoclonal mice, in which we showed that both CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells display in vivo suppressive activity. On the BALB/c background, T/B monoclonal mice produce hyper-Th2 and IgE responses after a single immunization. These hyperresponses can be prevented by adoptive transfer of total CD4⁺, purified CD4⁺CD25⁺, or CD4⁺CD25⁻ T cells from BALB/c mice (24). T/B monoclonal mice harbor an I-A^d-restricted, monoclonal, OVA-specific T cell repertoire that lacks CD4⁺CD25⁺ T cells, and a monoclonal B cell repertoire specific for a linear peptide from influenza virus hemagglutinin (HA). T/B monoclonal mice were derived by crossing DO11.10 OVA-specific TCR transgenic mice, 17/9 HA-specific Ig H and L chain knockin mice, and RAG-1^{-/-} mice (24).

We show in this study that donor polyclonal CD4⁺CD25⁻ cells can convert in vivo to CD4⁺CD25⁺ upon homeostatic proliferation; the newly generated CD4⁺CD25⁺ cells are phenotypically and functionally equivalent to naturally occurring T_{reg} cells. The expression of CD25 in T_{reg} cells, which correlates with their T_{reg} ability, depends on IL-2 secreted by cotransferred CD25⁻ or by Ag-stimulated conventional T cells in peripheral lymphoid organs.

Materials and Methods

Mice

The T/B monoclonal mice (17/9 DO11.10 RAG-1^{-/-}) were previously described (24). T/B monoclonal mice harbor monoclonal populations of T and B lymphocytes that are specific for chicken OVA_{323–339} and HA of influenza virus, respectively. BALB/c Thy1.2 mice and BALB/c IL-2^{-/-} mice were purchased from The Jackson Laboratory (Bar Harbor, ME). BALB/c Thy1.1 mice were obtained from Dr. F. Zavala (New York University, New York, NY). TCRαβ-deficient mice were backcrossed to the BALB/c background in our facility. All mice were bred and housed at the Skirball specific pathogen-free animal facility.

Cell purification and adoptive transfer

BALB/c CD4⁺CD25⁺ and CD4⁺CD25⁻ lymphocytes were purified from spleen and lymph nodes by MACS using Miltenyi reagents and a Vario MACS apparatus (Miltenyi Biotec, Auburn, CA).

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³ Abbreviations used in this paper: T_{reg}, regulatory T cell; HA, hemagglutinin from influenza virus; MFI, mean fluorescence intensity; GITR, glucocorticoid-induced TNFR.

To purify spleen and lymph node CD4⁺CD25⁻ cells by MACS, the cells were first incubated with FITC-labeled Abs to CD8, B220, and CD25 (in some experiments Abs to CD11b, CD11c, and TER119 were also added), followed by incubation with anti-FITC magnetic beads. The cells were then passed through the first depletion column, incubated with more anti-FITC magnetic beads, and passed through a second depletion column. Thymic CD4⁺CD25⁻ cells were purified by depletion of CD8⁺ and CD25⁺ cells using a similar two-step protocol. The unbound fraction typically contained 85–95% CD4⁺CD25⁻ cells, 0.1–0.3% CD25^{low} cells, and no CD25^{high} cells. To purify CD4⁺CD25⁺ cells, spleen and lymph node cell suspensions were first depleted of CD8⁺ and B220⁺ cells by MACS, incubated with PE-anti-CD25 Abs followed by anti-PE magnetic beads, and subsequently purified as the bound fraction from a MACS column. The purity of the CD25⁺ population was >95%.

CD25⁺, CD25⁻CD45RB^{high}, and CD25⁻CD45RB^{low} fractions from BALB/c mice used in the experiments shown in Fig. 5 were purified by cell sorting in a MoFlo cytometer (DakoCytomation, Carpinteria, CA) at New York University School of Medicine with ≥99% purity. Typically, 1–5 × 10⁶ purified cells were injected i.v. into recipient mice. All recipient mice were treated with broad-spectrum antibiotic (Bactrim; Sulfatrim Pediatric Suspension; Alapharma USPD, Baltimore, MD). Immunization of T/B monoclonal mice was performed the day after cell transfer, i.p. with 100 μg of cross-linked OVA-HA in alum (24).

CFSE labeling

Cells purified as described above were labeled with CFSE (Molecular Probes, Eugene, OR) by incubating 10⁷ cells/ml in PBS with 5 μM CFSE resuspended in DMSO. Labeling was stopped with medium containing 10% FCS.

Immunostaining and RNA analysis

Spleen and lymph node cells from recipient mice were analyzed by flow cytometry using fluorochrome-labeled Abs purchased from BD Pharmingen (San Diego, CA), Caltag Laboratories (Palo Alto, CA), and R&D Systems (Minneapolis, MN). The samples were analyzed using a FACSCalibur or an LSR II cytometer (BD Biosciences, Mountain View, CA). For quantitative mRNA expression analysis, donor-derived CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were MoFlo-sorted. RNA was extracted from the samples using TRIzol, and cDNA was synthesized using standard procedures. The expression of Foxp3, CTLA-4, glucocorticoid-induced TNFR (GITR), and β-actin was determined by quantitative real-time PCR using the primers below.

Expression was normalized to the level of β-actin in each sample: Foxp3, ACTGGGGTCTTCTCCCTCAA, CGTGGGAAGGTGCAGAGTAG; CTLA-4, GTTGGGGGCATTTTCACATA, TTTTACAGTTTCCTGGTCTC; and β-actin, TGACAGGATGCAGAAGGAGA, GTACTTGCGCTCAGGAGGAG.

In vitro suppression assays

Donor-derived CD4⁺CD25⁺ and CD4⁺CD25⁻ cells from spleen and lymph nodes of recipient mice were MoFlo-sorted. Proliferation assays were set up in 96-well, round-bottom plates and contained, per well, 1 × 10⁴ responder cells, 2 × 10⁴ APC (mitomycin C-treated spleen cells from TCRαβ-deficient BALB/c mice), and anti-CD3 Ab at a concentration of 0.5 μg/ml. Putative suppressor cells were cocultured at responder:suppressor ratios of 1:1, 1:0.3, and 1:0.1. Proliferation was determined by adding [³H]thymidine on the third day of culture and determining incorporation 6 h later.

Results

Peripheral CD4⁺CD25⁻ T cells generate CD4⁺CD25⁺ cells by peripheral expansion

We and others have observed that in conditions of homeostatic proliferation, CD25 expression is not stably maintained. Expanding CD4⁺CD25⁻ cells generate a population of CD25⁺ cells, although expanding CD25⁺ cells partially lose CD25 expression (25–28). To analyze the phenotypic and functional characteristics of the CD25⁺ cells derived from CD25⁻ cells, we adoptively transferred purified CD25⁻ T cells into host mice in which donor cells could undergo homeostatic expansion, such as RAG-1 knockout mice and T/B monoclonal mice. T/B monoclonal mice are DO11.10 TCR transgenic mice crossed with 17/9 Ig H and L chain knockin mice and RAG-1 knockout mice on the BALB/c genetic

background. T/B monoclonal mice are devoid of T_{reg} cells, and their peripheral T cells have a naive phenotype (24).

To investigate the relationship between homeostatic proliferation and peripheral conversion of CD25⁻ T cells to CD25⁺, we purified CD4⁺CD25⁻ cells from BALB/c mice (Fig. 1A), labeled

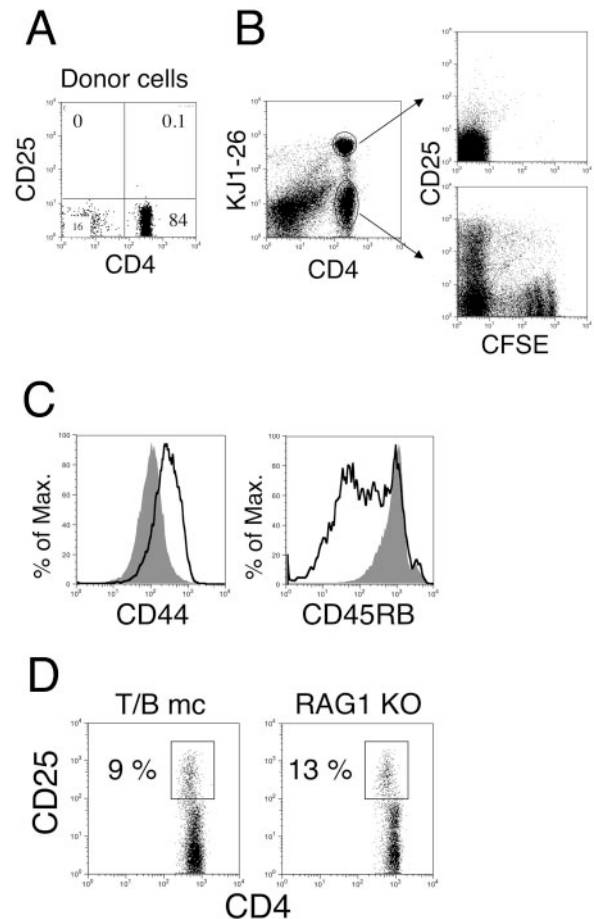


FIGURE 1. Conversion of CD4⁺CD25⁻ cells to CD25⁺ cells in T/B monoclonal and RAG-1^{-/-} recipient mice during peripheral expansion. **A**, CD4⁺CD25⁻ cells were purified from spleen and lymph nodes of BALB/c mice with ≤0.1% contamination by CD25⁺ cells. The cells were labeled with CFSE and transferred to T/B monoclonal mice (5 × 10⁶ cells/mouse; *n* = 3). One month later, recipient mice were killed, and spleen and lymph node cells were analyzed by flow cytometry. Representative analyses are shown in **B** and **C**. **B**, Cells were stained with anti-CD4, anti-CD25, and KJ1-26 Abs. The left plot shows the gates used for the analysis of endogenous cells (CD4⁺KJ1-26⁺) and the donor-derived cells (CD4⁺KJ1-26⁻). The lower right plot shows CFSE dilution and CD25 expression in the donor-derived population. The upper right plot shows endogenous cells (CFSE-negative and naive). **C**, Cells were stained with anti-CD4, anti-CD45RB, anti-CD44, and KJ1-26 Abs. Donor and endogenous populations were gated as described in **B**. The figure shows overlapping histograms of CD44 (left) and CD45RB (right) expression in donor cells (CD4⁺KJ1-26⁻; line) and endogenous cells (CD4⁺KJ1-26⁺; solid gray). **D**, CD4⁺CD25⁻ cells were purified from spleen and lymph nodes of BALB/c (Thy 1.1/1.2) mice and transferred to T/B monoclonal mice (T/B mc; Thy 1.2) and RAG-1^{-/-} mice (2 × 10⁶ cells/mouse; *n* = 3). Two weeks after immunization, the expression of CD25 in donor-derived CD4⁺ cells (Thy1.1⁺) was analyzed in peripheral blood samples stained with Abs to Thy1.1, CD4, and CD25. The plots show CD25 expression in donor-derived cells gated as CD4⁺Thy1.1⁺. Homeostatic proliferation of polyclonal CD4⁺ T cells in T/B monoclonal mice, and conversion of CD25⁻ into CD25⁺ cells in T/B monoclonal and RAG-1^{-/-} mice has been extensively confirmed in numerous experiments using both MACS- or MoFlo-sorted donor cells obtained from peripheral lymphoid organs or thymus.

them with CFSE, and transferred them to T/B monoclonal mice. Homeostatic proliferation of polyclonal CD4⁺ T cells in T/B monoclonal mice was demonstrated by CFSE dilution in the donor-derived population (gated as KJ1-26⁺CD4⁺ in Fig. 1B). Cells that became CD25⁺ were found in the population that underwent extensive proliferation (Fig. 1B). As in T cell-deficient hosts, cells that expanded in T/B monoclonal mice acquired a memory phenotype, expressing high levels of CD44 and low levels of CD45RB (Fig. 1C). In contrast, the endogenous OVA-specific KJ1-26⁺CD4⁺ cells maintained a naive phenotype (CD25[−], CD44^{low}, CD45RB^{high}). Simultaneous transfer of purified CD4⁺CD25[−] BALB/c cells into T/B monoclonal mice and RAG1^{−/−} mice demonstrated similar rates of conversion to CD25⁺ cells in both host strains (Fig. 1D).

It has been known for some time that the level of CD4 on the surface of CD4⁺CD25⁺ T_{reg} cells is lower than the level of CD4 on naive CD4⁺ T cells (for instance, see BALB/c cells in Fig. 2A). Upon injection of CD4⁺CD25[−] cells obtained from secondary lymphoid organs (designated peripheral in Fig. 2A) or CD4⁺CD25[−] single-positive cells from thymus (designated SP in Fig. 2A), we monitored the kinetics of appearance of CD4⁺CD25⁺ cells as well as the levels of CD4 surface expression. FACS analysis was performed on peripheral blood 5, 8, and 11 days after transfer of CD25[−] T cells (Fig. 2A). Although the donor-derived population contained CD25⁺ cells on day 5, only on day 8 was the population expressing high CD25 and low CD4 evident. The kinetics of generation of CD25⁺ cells as well as the down-modulation of CD4 were similar regardless of whether CD25[−] cells were obtained from peripheral lymphoid organs or thymus.

To demonstrate that the peripherally generated CD4⁺CD25⁺ cells derive from acquisition of CD25 expression by CD4⁺CD25[−] T cells, rather than by expansion of contaminant CD25⁺ cells (usually 0.1–0.3% in the donor CD25[−] population), we spiked purified BALB/c CD4⁺CD25[−] Thy1.2 cells with 0.5% contaminant CD4⁺CD25⁺ Thy1.1 cells and transferred the mixed population to T/B monoclonal mice. After 10 days, contaminant CD4⁺Thy1.1⁺ cells constituted ~1% of total donor-derived cells (2-fold increase from initial input), whereas Thy1.2⁺ donor-derived cells contained >7% CD25⁺ cells (49-fold increase from initial input; Fig. 2B). Thus, CD25⁺ cells derived from donor CD25[−] cells did not originate from preferential expansion of a few initial CD25⁺ cells. Instead, a fraction of CD25[−] cells converted to CD25⁺ cells in the periphery of recipient mice. FACS analysis of TCR V α and V β gene usage in peripherally converted CD25⁺ cells showed a broad diversity, similar to the freshly isolated CD4⁺ cells from BALB/c mice, arguing against the conversion-expansion of a few clones of CD25[−] donor cells (data not shown).

CD4⁺CD25⁺ T cells derived from peripherally expanded CD25[−] cells are anergic and suppressive

We subsequently investigated whether the CD4⁺CD25⁺ cells arising from donor CD4⁺CD25[−] cells displayed phenotypic and functional properties ascribed to freshly isolated CD4⁺CD25⁺ T_{reg} cells (9–13, 31–34). Analysis of RNA expression by real-time quantitative PCR in purified donor-derived cells demonstrated high levels of Foxp3 in the donor-derived CD25⁺, but not in the donor-derived CD25[−] fraction (Fig. 3A). It is important to note that unlike other T_{reg} markers, Foxp3 is not expressed by activated conventional mouse CD4⁺ cells (9–11). Flow cytometric analysis showed that donor-derived CD25⁺ cells, but not CD25[−] cells, expressed GITR, CD103, and CTLA-4 (Fig. 3B). Thus, phenotypically, CD4⁺CD25⁺ cells derived from homeostatic expansion of CD4⁺CD25[−] cells are very similar to freshly isolated CD4⁺CD25⁺ T cells.

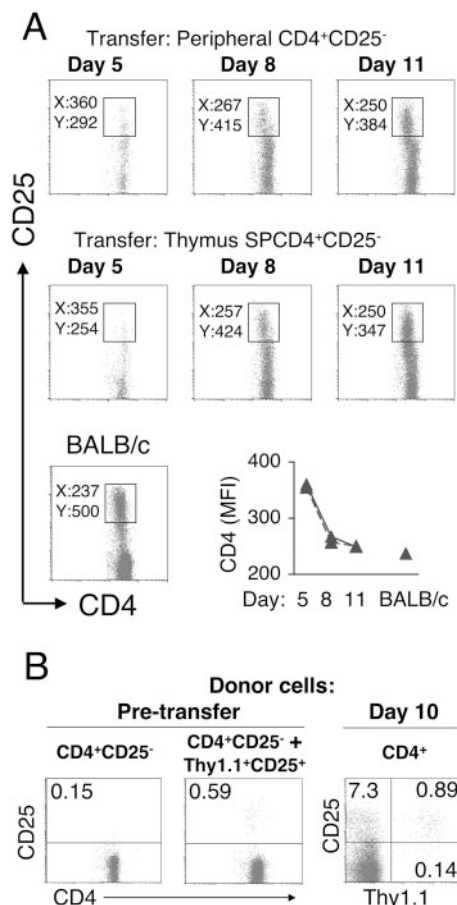


FIGURE 2. A, Kinetics of conversion of CD25[−] cells into CD25^{high}CD4^{low} cells. Purified CD4⁺CD25[−] cells from spleen and lymph nodes (peripheral) or from thymus of BALB/c mice were transferred to T/B monoclonal mice (2×10^6 cells/mouse). The expression of CD25 in donor-derived CD4⁺ cells was analyzed in peripheral blood samples on days 5, 8, and 11 after transfer. MFI values for CD25 (Y) and CD4 (X) in the CD25⁺ gate are indicated. The graphic on the bottom right shows the overlay of CD4 MFI (CD25⁺ cell gated) in donor-derived T cells from periphery (solid line) and thymus (dashed line). Note the overlapping curves, showing, in both cases, the decrease in CD4 MFI over time. The kinetic analysis is from a representative mouse from each group of four mice. Expression of CD4 (CD25⁺ cell gated) in peripheral blood of a BALB/c mouse is shown for comparison. B, Conversion, rather than selective expansion, of contaminant cells explains the appearance of CD25⁺ cells from a population of CD25[−] donor cells. CD4⁺CD25[−] cells were purified from spleen of BALB/c Thy1.2 mice. CD4⁺CD25⁺ cells were purified from spleen of BALB/c Thy1.1 mice. The purified cells were mixed in a ratio of 200:1 (CD25[−]:CD25⁺) and injected into T/B monoclonal mice. A total of 2×10^6 CD4⁺CD25[−] cells was injected per mouse. Pretransfer donor cells (CD4⁺CD25[−]) before and after addition of the contaminant CD25⁺ Thy1.1⁺ cells are shown in the left and middle plots. The right plot shows CD25 expression in CD4⁺Thy1.2⁺ and Thy1.1⁺ donor-derived CD4⁺ cells from spleen of recipient mice (day 10 after transfer; endogenous cells were excluded by staining with the anti-OVA-TCR Ab KJ1-26). The data are representative of two experiments.

The suppressive ability of CD25⁺ cells derived from CD25[−] donor cells was tested in *in vitro* proliferation assays. In these assays, donor-derived CD4⁺CD25⁺ cells were as anergic and suppressive as freshly isolated BALB/c CD4⁺CD25⁺ cells (Fig. 3C). Little or no suppressor activity was detected in the donor-derived CD25[−] population.

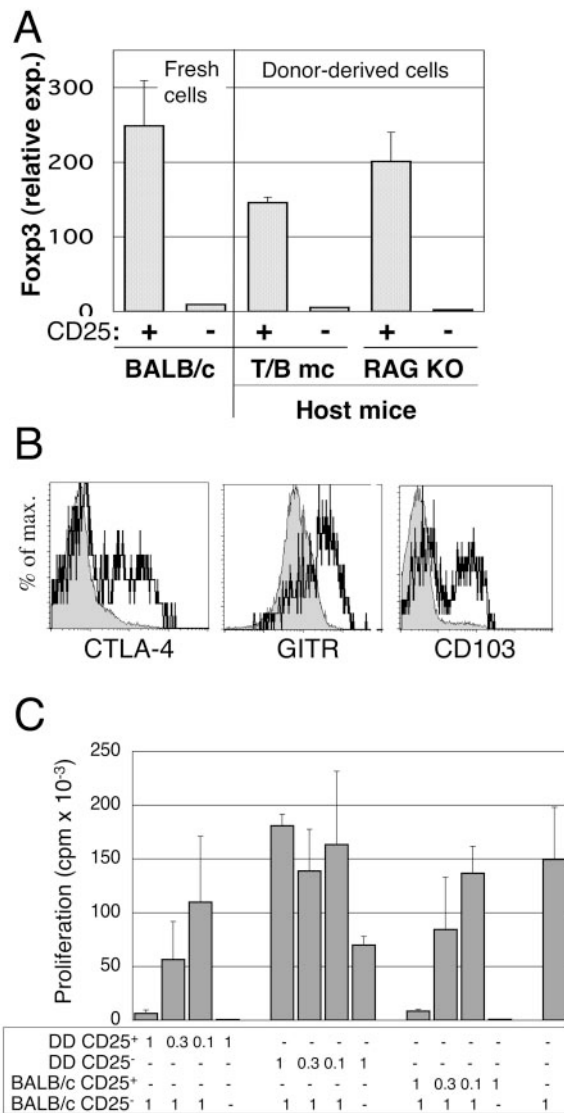


FIGURE 3. CD4⁺CD25⁺ cells derived from CD4⁺CD25⁻ cells have phenotypic and functional characteristics of T_{reg} cells. **A**, CD25⁺ cells derived from CD25⁻ cells express FcγR3 mRNA. CD4⁺CD25⁻ cells were purified from spleen and lymph nodes of BALB/c mice and transferred into T/B monoclonal mice and RAG-1^{-/-} mice. Donor-derived CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were isolated from T/B monoclonal mice or RAG-1^{-/-} mice 11 days after transfer. The expression of FcγR3 in mRNA from donor-derived cell samples as well as from freshly isolated CD4⁺CD25⁺ and CD4⁺CD25⁻ BALB/c cells was determined by real-time PCR. β-Actin amplification was used for normalization. The figure shows the average and SD of triplicate wells. **B**, CD25⁺ cells derived from CD25⁻ cells express GITR, CD103, and CTLA-4. CD4⁺CD25⁻ cells were purified from spleens of BALB/c mice and transferred into T/B monoclonal mice. Two weeks after transfer, the mice were killed, spleen cells were harvested, and the expression of T_{reg} markers (CTLA-4, CD103, and GITR) in the donor-derived population (CD4⁺KJ1-26⁻) was determined by flow cytometry. The figure shows histograms of gated CD4⁺KJ1-26⁻CD25⁺ (line) and CD4⁺KJ1-26⁻CD25⁻ (■) donor-derived cells. **C**, CD25⁺ cells derived from CD25⁻ cells are anergic and suppressive. Donor-derived CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were isolated from T/B monoclonal mice that had been transferred with CD4⁺CD25⁻ cells from BALB/c mice as in **A**. Donor-derived CD4⁺CD25⁺ and CD4⁺CD25⁻ cells as well as freshly isolated CD4⁺CD25⁺ BALB/c cells were cultured alone or were cocultured with 1 × 10⁴ freshly isolated CD4⁺CD25⁻ BALB/c cells (responder cells) in wells containing anti-CD3 Abs and APC. Ratios of responder to suppressor cells were 1:1, 1:0.3, and 1:0.1. On the third day of culture, proliferation was measured by [³H]thymidine incorporation. Results are the average and SD of triplicate wells. DD, donor-derived.

Peripheral conversion of CD4⁺CD25⁻ T cells to CD4⁺CD25⁺ T cells in wild-type newborn mice

As shown above, peripheral conversion of polyclonal CD4⁺CD25⁻ to functional CD25⁺ T_{reg} cells occurs efficiently in mice with no T cells (such as RAG-deficient mice) or mice harboring large numbers of monoclonal T cells (such as T/B monoclonal mice). We investigated whether peripheral generation of T_{reg} cells from CD25⁻ T cells could take place in mice with normal immune systems.

To study the expansion and conversion of CD25⁻ cells in wild-type mice, CD4⁺CD25⁻ cells from BALB/c Thy1.1/1.2 mice were purified, labeled with CFSE (to follow cell division), and transferred into newborn and adult BALB/c Thy1.2 recipient mice.

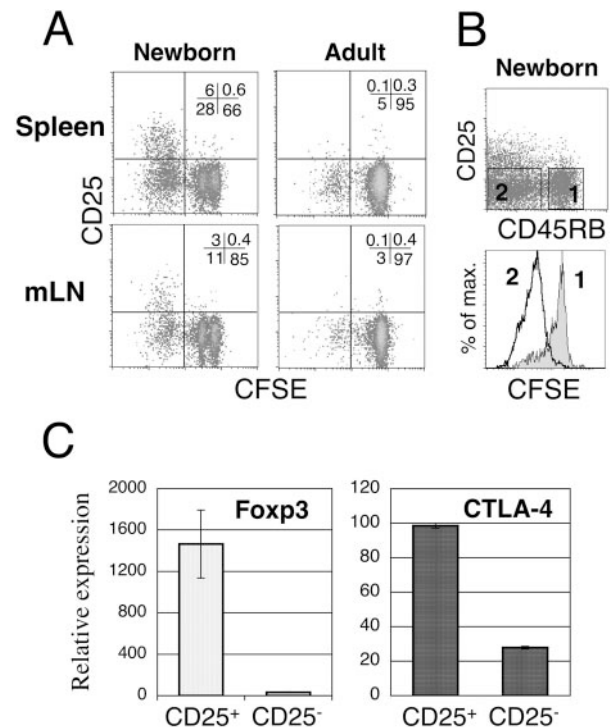


FIGURE 4. CD4⁺CD25⁻ cells transferred into wild-type BALB/c neonates give rise to CD4⁺CD25⁺CD45RB^{low} cells. CD4⁺CD25⁻ cells were purified from spleen and lymph nodes of BALB/c Thy1.1/1.2 mice, labeled with CFSE, and transferred into neonates (0.5–1 × 10⁶/mouse) or adult (5 × 10⁶ cells/mouse) BALB/c Thy1.2 mice. Three weeks later, mice were killed, and spleen and lymph node cells were analyzed by flow cytometry using Abs to CD4, CD25, CD45RB, and Thy1.1. **A**, Representative plots showing CFSE intensity and CD25 expression in gated donor cells (CD4⁺Thy1.1⁺) from spleens and lymph nodes of a neonatally injected mouse and an adult recipient mouse. **B**, Expression of CD45RB and CD25 in gated donor cells from a neonatally injected mouse is shown on the top. Cell divisions of fractions 1 and 2 are shown by histograms of CFSE dilution. CFSE dilution in gated donor-derived CD25⁻CD45RB^{high} (fraction 1) and CD25⁻CD45RB^{low} (fraction 2) cells from the same sample are shown on the right panel. CFSE dilution of CD25⁺CD45RB^{low} cells is shown in **A**, left panels, and is completely overlapping with the fraction 2 shown in **B**. Data are representative of three experiments. **C**, CD4⁺CD25⁻ cells transferred to BALB/c neonates give rise to CD25⁺FcγR3⁺ cells. CD4⁺CD25⁻ cells were purified from spleen and lymph nodes of BALB/c Thy1.1/1.2 mice and transferred into neonatal BALB/c Thy1.2 mice. One month after transfer, donor-derived Thy1.1⁺CD4⁺CD25⁺ and donor-derived Thy1.1⁺CD4⁺CD25⁻ cells were purified from spleen and lymph nodes. The levels of mRNA for FcγR3 and CTLA-4 in the samples were determined by real-time PCR as described. Expression levels (average ± SD) relative to β-actin are shown.

In neonatally injected mice, the donor CD25⁺ population efficiently generated CD25⁺ cells within the proliferating (CFSE^{low}) cells (Fig. 4A). Very low proliferation and conversion occurred in adult mice. Cells that converted to CD25⁺ in neonatally injected mice were found in the CD45RB^{low} fraction of donor-derived CD4⁺ cells, similarly to naturally occurring T_{reg} cells (Fig. 4B). All CD45RB^{low} cells within the donor population had undergone three or more cell divisions at the time of analysis. Similarly to naturally occurring T_{reg} cells, CD25⁺ T cells derived from CD25⁺ T cells in normal newborn mice expressed high levels of Foxp3 and CTLA-4 (Fig. 4C). Thus, peripheral conversion of CD25⁺ into CD25⁺ T_{reg} cells occurs in wild-type mice in conditions of peripheral expansion, such as the neonatal period (35).

Naive T cells give rise to T_{reg} cells in vivo with delayed kinetics

Peripheral CD4⁺CD25⁺ T cells represent a heterogeneous population containing naive and memory cells. Among other markers, this heterogeneity can be visualized by the differential expression of CD45RB. In clean animal facilities, a large fraction of CD4⁺CD25⁺ T cells are naive T cells that express high levels of CD45RB (CD45RB^{high}), and the remaining CD4⁺CD25⁺ peripheral cells express intermediate or low levels of CD45RB (CD45RB^{low}). Peripheral CD4⁺CD25⁺ cells are CD45RB^{low} (12, 25). We sought to determine whether the CD45RB^{high} and CD45RB^{low} fractions of CD4⁺CD25⁺ cells had a different capacity to convert to CD25⁺ T_{reg} cells during homeostatic expansion. CD4⁺CD25⁺CD45RB^{high} and CD45RB^{low} cells from spleen and lymph nodes of BALB/c mice were purified by cell sorting and transferred into T/B monoclonal mice. Although a comparable fraction of CD45RB^{high}- and RB^{low}-derived CD4⁺ T cells expressed CD25 on day 6 after transfer, only CD25⁺ cells derived from CD25⁺CD45RB^{low} T cells had a phenotype similar to that of wild-type T_{reg} cells, such as higher CD25 and lower CD4 expression (Fig. 5A). However, 21 days after transfer, CD25⁺ cells derived from CD25⁺RB^{high} or RB^{low} cells had similar FACS profiles (Fig. 5A). Similarly, CD4⁺CD25⁺ T cells derived from donor CD25⁺CD45RB^{high} cells took a longer time to reach comparable expression levels of T_{reg} genes, Foxp3 and CTLA-4, than the cells derived from the CD25⁺CD45RB^{low} donor population (Fig. 5B). Similar results were obtained when CD4⁺CD25⁺CD45RB^{high} and CD45RB^{low} BALB/c cells were transferred into RAG1^{-/-} recipients (data not shown). We concluded that both CD45RB^{high} and CD45RB^{low} fractions of CD25⁺ cells are able to generate peripheral CD25⁺ cells with phenotype of T_{reg} cells, but the CD45RB^{low} fraction is (at least kinetically) more efficient.

CD25 expression in peripheral lymphoid organs is regulated by IL-2-producing effector-type T cells

It has been reported that upon transfer of CD25⁺ T cells into alymphoid recipients, the expression of CD25 is lost in a large part of the population unless CD4⁺CD25⁺CD45RB^{high} T cells are co-transferred (25). We observed similar down-regulation of CD25 expression when CD25⁺ cells were transferred into T/B monoclonal mice. Given the fact that the T/B monoclonal mice contain a large number of CD4⁺CD25⁺CD45RB^{high} T cells and virtually no CD4⁺CD25⁺ T cells, our finding was unexpected. The difference between the experiments was that the CD4⁺CD25⁺CD45RB^{high} cells in the T/B monoclonal mice were OVA-specific resting cells, whereas polyclonal T cells injected into alymphoid mice were undergoing homeostatic proliferation. Peripheral expansion of the polyclonal T cells would lead to, among other things, IL-2 production. To test whether IL-2 production by CD25⁺ T cells was necessary for the maintenance of CD25 expression by

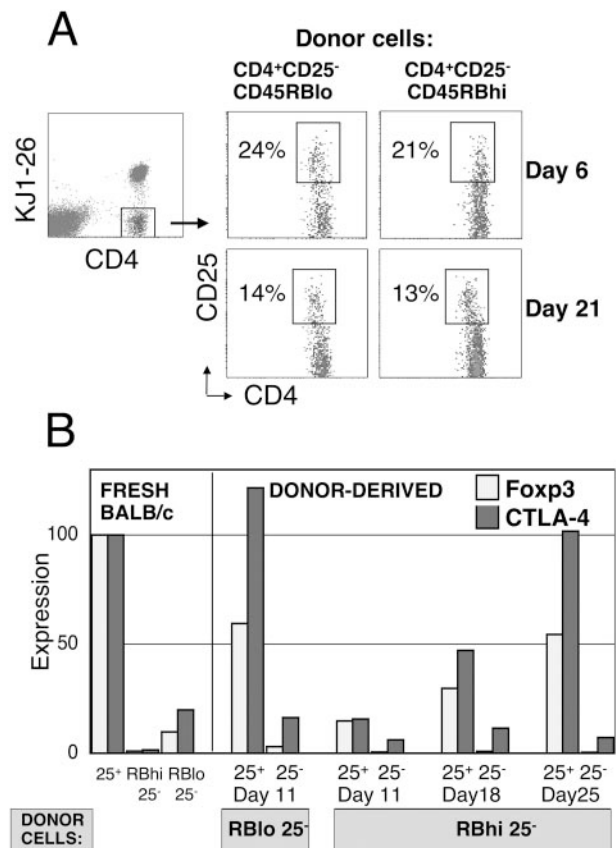


FIGURE 5. CD4⁺CD25⁺CD45RB^{high} naive T cells give rise to T_{reg} cells in vivo with delayed kinetics. **A**, CD4⁺CD25⁺CD45RB^{low} (RB^{lo}) and CD4⁺CD25⁺CD45RB^{high} (RB^{hi}) T cells were purified from BALB/c mice and transferred to T/B monoclonal mice (1×10^6 cells/mouse). The expression of CD25 in donor-derived cells (CD4⁺ KJ1-26⁺) was monitored in peripheral blood of recipient mice. The figure shows representative FACS analysis of samples obtained 6 and 21 days after transfer. **B**, Cells were transferred as indicated in **A**. Donor-derived CD4⁺CD25⁺ and CD4⁺CD25⁺ cells were sorted from spleens of recipient mice 11, 18, and 25 days after transfer. The expression of mRNA for Foxp3 and CTLA-4 in the samples was determined by real-time PCR. Purified CD4⁺CD25⁺, CD4⁺CD25⁺CD45RB^{low}, and CD4⁺CD25⁺CD45RB^{high} cells from spleen and LN cells of BALB/c mice were included in the analysis. Levels of Foxp3 and CTLA-4 are expressed as a percentage of the value obtained for fresh CD4⁺CD25⁺ cells from BALB/c mice. Data are representative of three experiments. Similar results were obtained in two experiments in which RAG-1^{-/-} mice were used as recipients.

CD4⁺CD25⁺ cells, we cotransferred a polyclonal population of CD25⁺ T cells from IL-2^{-/-} BALB/c Thy1.2 mice with purified IL-2⁺ CD4⁺CD25⁺ T cells from BALB/c Thy1.1 mice into T/B monoclonal mice. Although cotransfer of polyclonal CD25⁺ IL-2⁺ T cells allowed the maintenance of CD25 expression on 80% of CD25⁺ cells, IL-2^{-/-} T cells were unable to affect the frequency of CD25⁺ T cells among Thy1.1 cells (Fig. 6A). FACS analysis of the Thy1.1 donor population in spleens of recipient mice revealed that the cotransfer of CD25⁺ IL-2⁺ cells helped sustain Thy1.1 donor cells expressing high levels of CD25 and CTLA-4 (Fig. 6B). We conclude that polyclonal CD25⁺ cells help to maintain peripheral T_{reg} cells through IL-2 production.

Activation of naive T cells in vivo results in IL-2 production a few hours after stimulation (36). Thus, we hypothesized that immune responses may also affect T_{reg} cell homeostasis. As reported in other experimental systems, in T/B monoclonal mice, IL-2 production, and CD69 and CD25 expression are induced in

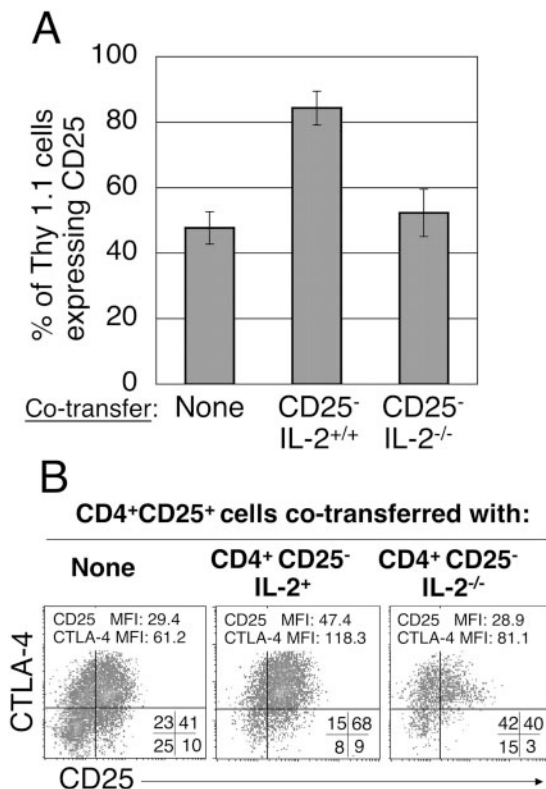


FIGURE 6. Wild-type CD4⁺CD25⁻ cells, but not IL-2-deficient CD4⁺CD25⁻ cells, help maintain a functional population of peripheral CD4⁺CD25⁺ T_{reg} cells. **A**, CD4⁺CD25⁺ cells from BALB/c Thy1.1 mice were transferred alone (0.5×10^6), or cotransferred with 2×10^6 CD4⁺CD25⁻ cells from IL-2^{+/+} or IL-2^{-/-} BALB/c Thy 1.2 mice into T/B monoclonal recipients. Ten days after transfer, the expression of CD25 in the Thy1.1⁺ donor population was determined by FACS analysis of peripheral blood samples. The figure shows the average and SD of the percentage of CD25⁺ cells within gated CD4⁺Thy1.1⁺ donor cells ($n = 3$). **B**, Mice from the groups described in **A** were killed 2 wk after transfer. The expression of CD25 and CTLA-4 in the CD4⁺Thy1.1⁺ donor population (originally CD25⁺) was analyzed in spleen samples of the recipient mice. Representative dot plots of gated CD4⁺Thy1.1⁺ cells are shown. A high percentage of Thy1.1⁺ cells expressing CD25 and CTLA-4 (upper right quadrant) were found in mice cotransferred with Thy1.2⁺ CD25⁻ IL-2^{+/+} cells, but not in mice cotransferred with Thy1.2⁺ CD25⁻ IL-2^{-/-} cells or in mice that received Thy1.1⁺ CD25⁺ cells alone.

OVA-specific T cells a few hours after immunization (24) (Fig. 7). Kinetic analysis of IL-2 mRNA expression after immunization of T/B monoclonal mice that had or had not been transferred with BALB/c splenocytes showed that IL-2 production by OVA-specific host cells peaked during the first day of immunization and decreased thereafter in the transferred and untransferred groups (Fig. 7A). In contrast, IL-4 production, a later differentiation event, was suppressed in the transferred group (24). The production of IL-2 protein by permeabilized OVA-specific T cells 14 h after immunization was verified by intracellular staining (Fig. 7B). The analysis of CD25 expression in the donor-derived polyclonal CD4⁺ cells from the transferred group demonstrates a higher CD25 expression per cell (as determined by the mean fluorescence intensity (MFI)) and a higher percentage of CD25⁺ cells (Fig. 7B, MFI of nonimmunized, 228; MFI of immunized samples, 502 and 656, respectively, for the samples represented with thinner and thicker lines).

Although most IL-2 production occurs early after immunization, the effects of immunization on the donor-derived CD4⁺ population

could be detected >1 wk later. Purified BALB/c CD4⁺CD25⁺, CD4⁺CD25⁻, or total CD4⁺ cells were transferred into T/B monoclonal mice. Half of each group was subsequently immunized with OVA-HA. The expression of CD25 in the donor-derived population was determined 9 days later by FACS analysis of peripheral blood samples. Immunization of T/B monoclonal mice with OVA-HA resulted in a higher frequency of donor-derived cells expressing CD25 (Fig. 7, B and C). The greatest effect was observed in mice transferred with purified CD25⁺ cells. Thus, immune responses of conventional CD4 cells could help sustain the pool of T_{reg} cells.

The suppressor function of CD25⁺ cells is regulated by IL-2-producing effector-type T cells

Having described the properties of CD4⁺CD25⁺ T cells that derive from CD4⁺CD25⁻ T cells upon homeostatic proliferation, we determined the properties of CD4⁺CD25⁻ T cells that originate upon transfer of CD4⁺CD25⁺ T cells. Specifically, we studied whether CD25⁻ cells derived from donor CD25⁺ cells that expanded in T/B monoclonal mice maintained the regulatory activity of their CD25⁺ predecessors. In addition, we determined whether immunization of the recipient T/B monoclonal mice with OVA-HA affected the suppressor activity of donor CD25⁺ cells. To address these issues we purified splenic CD4⁺CD25⁺ cells from BALB/c mice and transferred them into T/B monoclonal recipient mice. Half the recipient mice were immunized twice with OVA-HA (indicated I in Fig. 8), whereas the other half was not immunized (indicated NI in Fig. 8). Three days after the second immunization, donor-derived CD4⁺CD25⁻ and CD4⁺CD25⁺ cells were isolated from the recipient mice and tested in vitro for the expression of T_{reg}-associated genes, as well as for in vitro suppressor activity. Both donor-derived CD25⁺ and CD25⁻ cells expressed CTLA-4, CD103, and GITR, but expression levels were higher in the CD25⁺ population (Fig. 8A). The donor-derived CD25⁺ and CD25⁻ populations were tested in vitro for their ability to respond to anti-CD3 stimulation and to suppress the proliferation of freshly isolated CD25⁻ cells (from DO11.10 RAG^{-/-} mice). Donor-derived CD25⁺ cells from immunized mice were as anergic and suppressive as freshly isolated BALB/c CD25⁺ cells (Fig. 8B). Donor-derived CD25⁺ cells from nonimmunized mice showed somewhat lower suppressor activity than donor-derived CD25⁺ cells from immunized mice, but remained completely unresponsive to stimulation. The CD25⁻ cells derived from CD25⁺ cells displayed even lower suppressor activity, but remained largely unresponsive to stimulation. Thus, down-regulation of CD25 expression in adoptively transferred CD25⁺ cells is associated with a reduction of regulatory activity. Immunization of the recipient mice maintains the suppressor activity of the donor-derived CD25⁺ population.

Foxp3 expression is independent of IL-2 signaling

IL-2^{-/-} mice develop lymphoproliferative diseases caused by defective T_{reg} development and/or function. We showed previously that T_{reg} cells do not need to produce IL-2, although their function is highly dependent on IL-2 signaling through CD25 expression. Moreover, the fact that splenic CD4⁺ T cells from IL-2^{-/-} mice displayed normal regulatory activity when transferred to IL-2-sufficient mice indicated that IL-2 was not necessary for the development of T_{reg} cells in the thymus (26).

To investigate the IL-2 dependence of CD4⁺CD25⁺ T cells, we isolated CD25⁺ and CD25⁻ from the thymus of IL-2^{-/-} and IL-2^{+/+} littermates. The proportion of CD4⁺CD8⁻CD25⁺ was only partially reduced in the thymus of IL-2^{-/-} mice and was greatly diminished in the spleen (Fig. 9A), in agreement with published

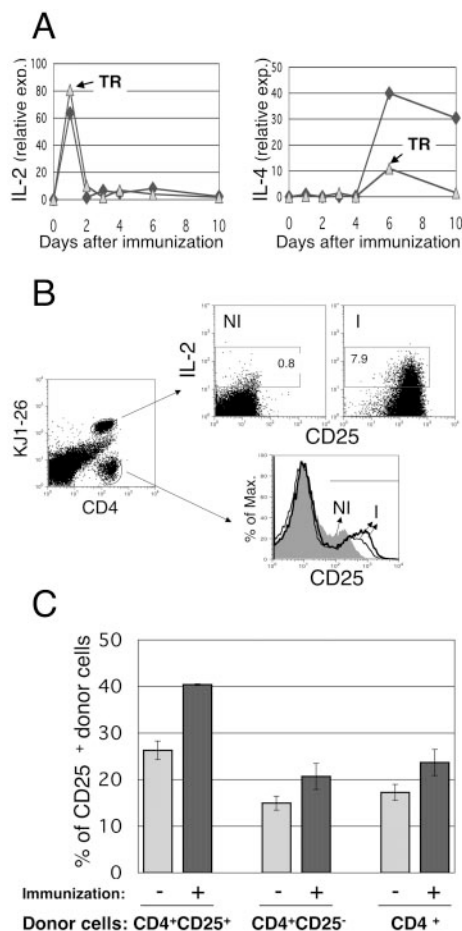


FIGURE 7. Immunization of T/B monoclonal mice with OVA-HA leads to IL-2 production by OVA-specific T cells and increased CD25 expression in T_{reg} cells. **A**, Kinetics of IL-2 and IL-4 production by endogenous OVA-specific T cells after immunization of T/B monoclonal mice. T/B monoclonal mice were transferred with 3×10^7 spleen cells from BALB/c mice. The transferred group and a nontransferred control group were immunized with OVA-HA in alum 1 day after transfer. On the indicated days after immunization, groups of three or four mice per group were killed, and OVA-specific T cells (KJ1-26⁺) were purified by magnetic sorting from the spleens of pooled group samples. After RNA extraction and cDNA synthesis, IL-2 and IL-4 expression were determined by real-time PCR. The RNA experiment is representative of two performed and is supported by extensive analysis of KJ1-26⁺ cells activation after immunization. **B**, T/B monoclonal mice were transferred with spleen cells of BALB/c mice as described in **A** and immunized with OVA-HA 20 days later. Two mice per group were killed 14 h after immunization. Spleen cells were harvested; surface-stained with anti-CD4, anti-CD25, and KJ1-26 Abs; subsequently fixed; permeabilized; and stained with anti-IL-2 Abs, without ex vivo PMA/ionomycin treatment. The dot plot on the left shows the gates for donor-derived (CD4⁺KJ1-26⁺) and endogenous cells (CD4⁺KJ1-26⁻). I, cells from immunized mice; NI, cells from nonimmunized mice. IL-2 production and CD25 expression by endogenous cells are shown in the upper middle and right plots. The histogram at the bottom shows the overlapping curves of CD25 expression by donor-derived cells from nonimmunized (solid gray) and two immunized mice samples (line). Very few IL-2-producing cells (1–2%) were detected in the donor-derived CD4⁺KJ1-26⁻ cells analyzed ex vivo at this time point (3 wk after transfer). **C**, Immunization of the recipient T/B monoclonal mice with OVA-HA partially rescues CD25 expression in transferred CD25⁺ T_{reg} cells. CD4⁺CD25⁺, CD4⁺CD25⁻, and total CD4⁺ cells were purified from spleens of BALB/c mice and transferred to T/B monoclonal mice (1×10^6 cells/mouse). Half of each group was immunized with OVA-HA. CD25 expression was analyzed in peripheral blood of T/B monoclonal mice 9 days after transfer. Shown are the percentages of CD25⁺ cells in gated donor-derived CD4⁺KJ1-26⁻ cells (average $\pm$ SD; $n = 3$).

results (37, 38). Importantly, quantitative RNA expression analysis of purified populations of CD25⁺ cells from thymus or spleen of young IL-2^{-/-} mice demonstrated high levels of expression of Foxp3, albeit somewhat lower than those in the IL-2⁺ littermates (Fig. 9B). These results indicate that the thymic generation of a Foxp3⁺CD4⁺CD25⁺ population is only marginally impaired in IL-2^{-/-} mice, supporting our assertion that IL-2 is not required for the thymic development of T_{reg} , but is crucial at later stages. Moreover, our results show that the induction of Foxp3 expression is independent of IL-2 signaling.

Discussion

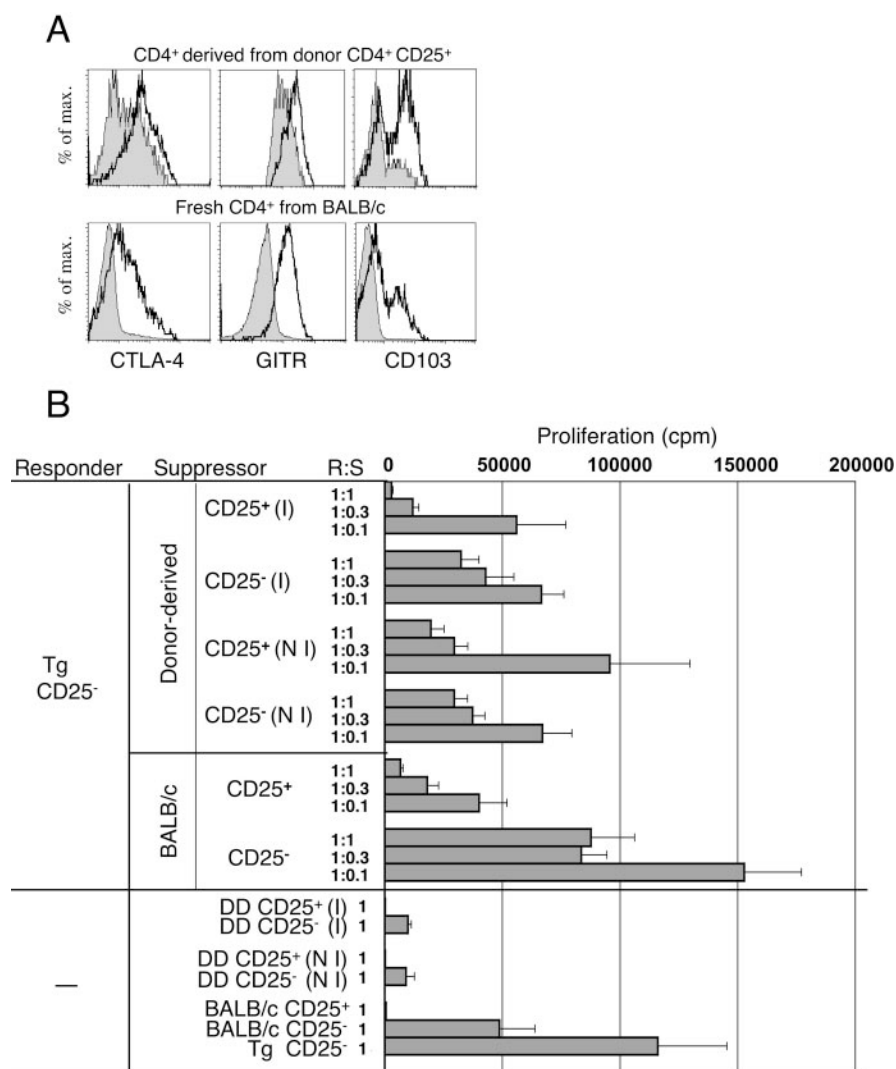
In this manuscript we showed that upon adoptive transfer of CD4⁺CD25⁻ T cells, a considerable proportion of the donor cells converted to CD25⁺ in vivo. We showed that this conversion is not the result of the outgrowth of a contaminant CD25⁺ population, and that the CD25⁻-derived CD25⁺ T cells are, phenotypically and functionally, bona fide T_{reg} cells. This conversion took place efficiently in animals that allow homeostatic proliferation of T cells, such as RAG-1^{-/-}, T/B monoclonal, or wild-type neonatal mice. In adult wild-type mice the conversion was much less efficient, although it could also be observed.

A number of recent studies reported the peripheral induction of T_{reg} cells using a variety of protocols (3, 39–51). Some of these induced T_{reg} cells are alloantigen-specific or foreign Ag-specific. The degree of phenotypic and functional resemblance between the induced regulatory cells and naturally occurring CD4⁺CD25⁺ T_{reg} cells was investigated to different degrees in the different studies. Some of the protocols led to T_{reg} cells that displayed phenotypes different from naturally occurring CD4⁺CD25⁺ T_{reg} cells (42, 52, 53), whereas other protocols led to cells indistinguishable from naturally occurring T_{reg} cells (49). The CD4⁺CD25⁺ T_{reg} cells generated through homeostatic proliferation of CD4⁺CD25⁻ T cells also behaved like naturally occurring T_{reg} cells in all parameters that we studied.

The CD25⁺ T cell-spiking experiments (Fig. 2B) demonstrated that T_{reg} cells generated through homeostatic proliferation do not represent the expansion of pre-existing CD4⁺CD25⁺ cells. On the basis that <1% of sorted human CD25⁺ cells remained alive by day 10 after activation, Walker et al. (40) concluded that the CD25⁻-derived CD25⁺ cells they obtained in vitro are not the product of the expansion of pre-existing CD25⁺ cells. In some cases, CD25⁻-cell-derived CD25⁺ T_{reg} cells were obtained from TCR-transgenic SCID or RAG^{-/-} mice (41, 49, 54). Because these mice contain virtually no CD25⁺ T cells before T_{reg} induction, outgrowth of pre-existing CD25⁺ cells is highly unlikely. Similarly, Ab depletion of CD25⁺ cells was used to show that T_{reg} cells were derived from CD25⁻ cells (43, 47, 48). In these three manuscripts it was shown or mentioned that few CD25⁺ cells were left behind after depletion, making it unlikely that contaminant CD25⁺ cells would play a role, although this possibility was not formally excluded.

CD4⁺CD25⁺Foxp3⁺ T_{reg} cells could be derived from CD25⁻ T cells in vitro by TCR stimulation in the presence of TGF- β (39, 47). Interestingly, the treatment that induced the conversion of CD25⁻ cells to CD25⁺ T_{reg} cells did not trigger expansion of pre-existing CD4⁺CD25⁺ cells (39). Thus, in this case the CD25⁺ T_{reg} cells also do not appear to be the product of outgrowth of pre-existing CD25⁺ cells. In contrast, Horwitz et al. (55) reported that TGF- β induction of human CD4⁺CD25⁺ T cells derived in vitro from naive CD45RA⁺RO⁻ T cells was markedly decreased if a pre-existing population (1% of naive CD4⁺ T cells) expressing CD25 was depleted. A subsequent study by the same group concluded that the residual CD25⁺ T cells greatly increase the number

FIGURE 8. Loss of CD25 expression in T_{reg} cells during homeostatic proliferation is accompanied by a partial loss of regulatory function. **A**, Decreased expression of T_{reg}-associated genes in CD25⁺ cells derived from CD25⁺ donor cells. CD4⁺CD25⁺ cells were purified from normal BALB/c mice and transferred to T/B monoclonal mice. One day later, half the recipient mice were immunized with OVA-HA. Two weeks after transfer, the mice were killed, spleen cells were harvested, and the expression of Treg-associated proteins, CTLA-4, CD103, and GITR, in the donor-derived population was determined by flow cytometry. The figure shows overlapping histograms of gated CD25⁺KJ1-26⁺ (line) and CD25⁺KJ1-26⁺ (solid) donor-derived CD4⁺ cells. The expressions of CTLA-4, CD103, and GITR in fresh spleen cells from a BALB/c mouse are shown for comparison. Overlapping histograms of CD4⁺CD25⁺ (line) and CD4⁺CD25⁺ (solid) BALB/c cells are shown. **B**, Partial loss of suppressor activity in CD25⁺ cells derived from CD25⁺ donor cells. The ability of donor-derived CD4⁺CD25⁺ and CD4⁺CD25⁺ cells to respond to anti-CD3 stimulation and to exert suppression was tested in vitro as described in Fig. 3C. Donor-derived CD4⁺CD25⁺ and CD4⁺CD25⁺ cells were purified from T/B monoclonal mice that were transferred with BALB/c CD4⁺CD25⁺ cells and were immunized (I) or not immunized (NI). Responder cells were DO11.10 RAG-1^{-/-} cells (Tg CD25⁺). Donor-derived cells as well as freshly isolated BALB/c CD4⁺CD25⁺ and CD4⁺CD25⁺ cells were cultured alone or were cocultured with responder cells in the presence of anti-CD3 Abs and APCs. Ratios of responders to suppressors were 1:1, 1:0.3, and 1:0.1. Proliferation was measured on the third day of culture by [³H]thymidine incorporation. Results are the average and SD of triplicate cultures. DD, donor-derived.



of CD25⁺ T cells that become CD25⁺ (44). Using a TGF- β -inducible system, Peng et al. (46) showed that TGF- β promotes expansion of the CD4⁺CD25⁺Foxp3⁺ T_{reg} pool, although conversion of CD25⁺ cells was not studied in this system.

Our results have several implications. First, homeostatic proliferation of lymphocytes is believed to play an important role in the immune systems of young (newborn) and aging normal individuals. In addition, several cancer treatments cause partial lymphocyte ablations that trigger homeostatic proliferation, and chronic infections can alter thymic output and cause homeostatic proliferation. Low level homeostatic proliferation is likely to be continuously taking place. Thus, in vivo conversion of CD4⁺CD25⁺ T cells into CD4⁺CD25⁺ T_{reg} cells under homeostatic proliferation conditions could play an important balancing role in the immune system.

Although naive CD25⁺ T cells can eventually give rise to CD25⁺ T_{reg} via homeostatic proliferation (Fig. 5), the capacity of CD25⁺ cells to generate CD25⁺ T_{reg} cells is not limitless. Re-transfer experiments in which CD25⁺ T cells were injected, and the donor-derived CD25⁺ cells were purified and reinjected into secondary recipients showed a 5-fold decrease in the fraction of secondary donor-derived CD25⁺ cells compared with the fraction of CD25⁺ cells that arises upon transfer of fresh CD25⁺ T cells (data not shown). Although fewer CD25⁺ cells were generated upon these retransfers, the CD25⁺ cells displayed normal T_{reg} properties, such as elevated expression of Foxp3 (data not shown).

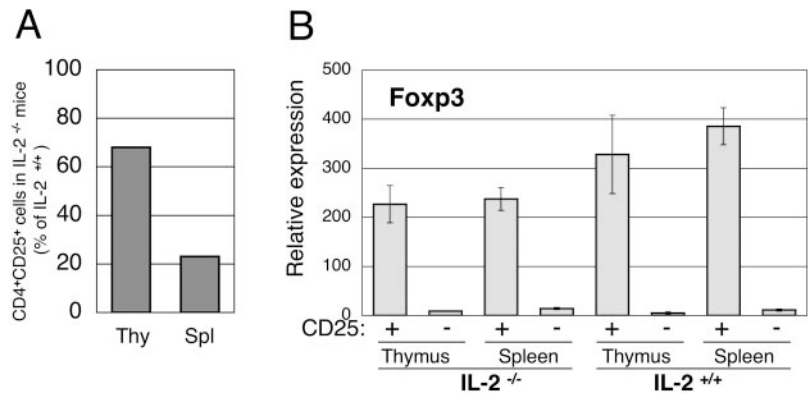
Therefore, it appears that the generation of CD25⁺ cells is not a stochastic event by which any CD25⁺ T cell has a given probability of becoming CD25⁺. Rather, many CD25⁺ cells are, seemingly, not able to generate CD25⁺ cells in these experimental conditions. These data are consistent with a crucial role of TCR specificity in the generation of T_{reg} cells, whereby only a fraction of CD25⁺ T cells would express TCR chains that are compatible with T_{reg} generation under particular stimuli (19, 56).

Another implication of the in vivo generation of CD4⁺CD25⁺ T_{reg} cells from CD4⁺CD25⁺ cells is that, considered together with the inability of genetically deficient CD25^{-/-} T cells to exert suppression (26), it provides a model to explain the protective activity of CD4⁺CD25⁺ T cells in models of autoimmunity and allergy; the ability of CD4⁺CD25⁺ cells to protect in these disease models would depend on the generation of CD25⁺ T_{reg} cells in the periphery.

In this manuscript we also determined that acquisition/loss of CD25 expression correlated with the acquisition/loss of suppressor phenotype, and found that IL-2, produced by cells other than T_{reg}, is one of the key factors in determining the maintenance of CD25 expression. Cells that do not produce IL-2, such as nonexpanding CD25⁻ T cells or IL-2^{-/-} T cells, do not support CD25 expression.

In vitro, CD4⁺CD25⁺ T_{reg} cells exert their suppressive activity by preventing IL-2 production by effector cells (57, 58); however,

FIGURE 9. $CD4^+CD8^-CD25^+Foxp3^+$ cells develop in the thymus of $IL-2^{-/-}$ mice. **A**, The expression of CD25 in $CD4^+CD8^-$ single-positive cells from thymus and $CD4^+$ cells from spleen of 3-wk-old $IL-2^{-/-}$ and $IL-2^+$ BALB/c littermates was determined by flow cytometry. The figure shows the CD25⁺ cells in $IL-2^{-/-}$ mice as a percentage of the $IL-2^{+/+}$ wild-type littermates (100%). **B**, CD25⁺ and CD25⁻ $CD4^+CD8^-$ cells were purified from thymus and spleen of 3-wk-old $IL-2^{-/-}$ and $IL-2^{+/+}$ littermates. The expression of Foxp3 in the samples was determined by real-time PCR.



the situation in vivo is quite different. Indeed, the early burst of IL-2 production by OVA-specific T cells upon immunization of T/B monoclonal mice is unaffected by the presence of T_{reg} cells (Fig. 7A) despite the striking suppressive effect of T_{reg} cells on IL-4 production and IgE switching (Fig. 7A) (24). Thus, as proposed by Furtado et al. (26), IL-2 produced by activated effector T cells fuels the peripheral conversion of $CD25^-$ to $CD25^+$ cells and sustains CD25 expression, thus establishing an autoregulatory loop during immune responses.

Our observations emphasize the interdependence of T_{reg} and conventional T cells to maintain a peripheral balanced immune system. Although $CD25^+$ cells down-regulate immune responses, self or Ag-specific conventional T cells help maintain a functional T_{reg} compartment.

Acknowledgments

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Chapter IV

Regulatory T cells inhibit stable contacts between CD4⁺ T cells and dendritic cells in vivo

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Regulatory T cells inhibit stable contacts between CD4⁺ T cells and dendritic cells in vivo

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Regulatory T (T reg) cells exert powerful down-modulatory effects on immune responses, but it is not known how they act in vivo. Using intravital two-photon laser scanning microscopy we determined that, in the absence of T reg cells, the locomotion of autoantigen-specific T cells inside lymph nodes is decreased, and the contacts between T cells and antigen-loaded dendritic cells (DCs) are of longer duration. Thus, T reg cells can exert an early effect on immune responses by attenuating the establishment of stable contacts during priming of naive T cells by DCs.

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Several recent studies have revealed the dynamics of live T cell interactions in explanted intact LNs, or intravitaly in the LNs of live mice, using laser-scanning microscopy (1–4). T cell interactions with antigen-presenting DCs in LNs are different from those observed in vitro, and it is becoming increasingly apparent that an intact lymphoid organ structure is fundamental for the development of normal immune responses (2, 5). These studies indicated that in the absence of immunization T cells move extensively within the T cell zones of LNs, and this movement facilitates the encounter of rare antigen-specific T cells with antigen-loaded DCs. Upon antigen encounter, T cells partake in a variable period of dynamic interactions and then arrest their locomotion for several hours before regaining motility by 36 h (2–4, 6).

Another component that may play a role, but has not been studied in vivo thus far, is the regulatory T (T reg) cell compartment. T reg cells are major components of immune responses, down-modulating Th1 or Th2 responses against auto, allo, or foreign antigens (7–10). Despite their prominent role, little is known about how T reg cells control immune responses in vivo. In this study, we used intravital two-photon laser-scanning microscopy to visualize the behavior of autoantigen-specific T cells in the presence or absence of naturally

occurring T reg cells in intact LNs. We found that 20 h after immunization with autoantigen-loaded DCs, contacts between T cells and antigen-loaded DCs are of shorter duration in the presence of T reg cells than in their absence, indicating an early effect of T reg cells during immune responses.

RESULTS AND DISCUSSION

Demonstration of T reg cell function in the popliteal LN (PLN)

To assess how T reg cells affected encephalitogenic CD4⁺ T cells in vivo, we used an experimental system in which the host either contains or lacks T reg cells. The experimental system is based on myelin basic protein (MBP) Ac1-11-specific TCR transgenic mice (11). Despite the high frequency of functional MBP-specific T cells, the hemizygous transgenic T/R⁺ mice do not develop experimental autoimmune encephalomyelitis (EAE) because of the presence of T reg cells. Genetic ablation of T reg cells, which can be accomplished by crossing T/R⁺ mice to RAG^{-/-} mice, to TCR $\alpha^{-/-}\beta^{-/-}$ mice, or by breeding the TCR transgenes to homozygosity (Tg/Tg), leads to the spontaneous development of EAE (11–13). In this study, we used hemizygous T/R⁺ as mice harboring T reg cells and Tg/Tg as mice lacking T reg cells. On a C57BL/10.PL genetic background, EAE spontaneously develops in T reg cell-deficient mice (including Tg/Tg mice) beginning

The online version of this article contains supplemental material.

at ~45 d of age, with 50% EAE incidence reached at 60 d and 100% by 120 d of age (12, 13).

We first characterized the cellularity and organization of the PLN in T/R⁺ and Tg/Tg mice. At 5 wk old or younger, both T/R⁺ and Tg/Tg mice harbor an equally high number of MBP-specific T cells, the vast majority of which display naive phenotype (Fig. S1 a, available at <http://www.jem.org/cgi/content/full/jem.20050783/DC1>; reference 11). T/R⁺ and Tg/Tg mice also have similar proportions of DC subpopulations (Fig. S1 c). The most striking difference is the presence of Foxp3-expressing T reg cells in T/R⁺ mice but not Tg/Tg mice (Fig. S1 b). The naive status of MBP-specific T cells in the 5-wk-old T/R⁺ and Tg/Tg mice (11), despite the presence of MBP in the central nervous system, suggests that DCs in PLNs are not presenting endogenous MBP autoantigen (14). Immunohistology was used to confirm that in both types of recipient mice, PLNs were structurally normal and the transferred CFSE-labeled T cells homed to the T cell areas (Fig. S1 d).

Both T reg cell-deficient and -sufficient MBP-specific TCR transgenic mice undergo fulminant EAE upon immunization with MBP emulsified in complete Freund's adjuvant and pertussis toxin, indicating that T reg cells can be overwhelmed or disengaged by strong inflammatory signals (12). Therefore, we immunized with MBP Ac1-11 in IFA to provide an in situ depot for autoantigen delivery to DCs without overwhelming T reg cells. This was an effective strategy because subcutaneous injection in the foot pad with MBP

Ac1-11 in IFA triggered IL-2 production by MBP-specific T cells in the PLNs in the Tg/Tg mice, but not the T/R⁺ mice, at 20 h (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20050783/DC1>). Thus, MBP Ac1-11 peptide in IFA triggered an immune response in the PLN that was suppressed by endogenous T reg cells.

Influence of T reg cells on the movement of CD4 effector cells in vivo

We next used two-photon intravital microscopy to study the movement of MBP-specific T cells in the presence or absence of T reg cells. CFSE-labeled MBP-specific CD4 T cells purified from Tg/Tg mice (naive effector precursor T cells) were transferred i.v. into T/R⁺ or Tg/Tg recipient mice (scheme depicted in Fig. 1 a). PLNs from anesthetized mice were exposed and imaged by two-photon laser-scanning intravital microscopy 20 h after T cell transfer. We analyzed the images to determine the speed and arrest coefficient of MBP-specific T cells under both conditions. The arrest coefficient is the proportion of time in which a T cell does not move (threshold <2 μ m). The arrest coefficient is generally low when T cells are not engaged in stable contacts with DCs (6). In the absence of immunization, MBP-specific T cells migrated at a mean speed of 6.72 μ m/min in T/R⁺ mice and 6.34 μ m/min in Tg/Tg mice, but this difference was not significant (Fig. 1, b and c, and Videos S1 and S2, available at <http://www.jem.org/cgi/content/full/jem.20050783/DC1>). The mean arrest coefficients were also similar in the presence

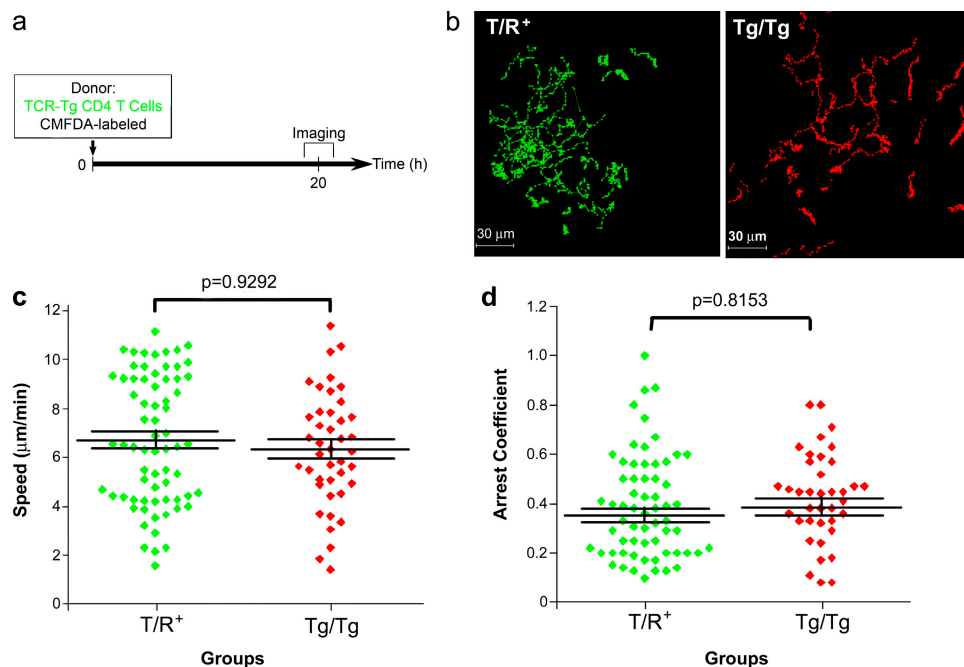


Figure 1. In the absence of immunization, T reg cells do not influence the movement of MBP-specific CD4⁺ T cells. (a) Experimental protocol. T/R⁺ or Tg/Tg mice, which have or lack endogenous T reg cells, respectively, received 10^7 CFSE-labeled MBP Ac1-11-specific CD4⁺ T cells by tail vein injection. 20 h later, PLNs were imaged.

(b) Representative tracks of MBP-specific T cells in T/R⁺ and Tg/Tg recipients. (c) Mean speeds of MBP-specific CD4⁺ T cells in both types of recipient mice. (d) Arrest coefficient for CD4⁺ T cells in both types of recipient mice. Results are representative of three independent experiments.

and absence of T reg cells (0.35 and 0.39, Fig. 1 d). Thus, baseline T cell migration is similar in the presence and absence of T reg cells.

We next asked if the effect of autoantigen on MBP-specific CD4⁺ T cell movement is altered in the presence or absence of T reg cells. Mice were immunized in the hind footpad with MBP Ac1-11 emulsified in IFA, and, at the same time, naive T cells from Tg/Tg mice were transferred i.v. (Fig. 2 a). We then imaged the draining PLNs at 20 h, as this has been shown to be appropriate to observe foreign antigen effects on CD4⁺ T cells (2, 6, 15). Immunization slowed the MBP-specific T cells substantially only in the Tg/Tg mice (Fig. 2 b and Videos S3 and S4, available at <http://www.jem.org/cgi/content/full/jem.20050783/DC1>). In the presence of T reg cells, MBP-specific T cells did not significantly change their speed in response to autoantigen, which remained at 6.72 $\mu\text{m}/\text{min}$ (Fig. 2 c), but significantly increased their arrest coefficient to 0.49 (Fig. 2 d, $P = 0.0011$). Earlier studies with foreign antigen-specific T cells showed larger effects of antigen on migration in this time frame, but autoantigens are often weaker agonists than foreign antigens, which may account for the smaller effect. In contrast, in the absence of T reg cells, MBP-specific T cells significantly decreased their speed to 3.13 $\mu\text{m}/\text{min}$ (Fig. 2 c, $P < 0.0001$) and increased their arrest coefficient to 0.70 (Fig. 2 d, $P < 0.0001$).

To have an internal control for the integrity of the PLN preparations in Tg/Tg mice where antigen-specific T cells sometimes were fully arrested, we cotransferred and imaged WT polyclonal (WT) CD4⁺CD25⁻ T cells from nontransgenic syngeneic chicken β -actin promoter cyan fluorescence protein (CFP) transgenic mice (16). 20 h after transfer, we imaged CFSE-labeled MBP-specific T cells and CFP-expressing WT T cells in the draining PLNs of Tg/Tg mice immunized with MBP Ac1-11 peptide in IFA. The same fields that contained arrested MBP-specific cells had actively moving WT T cells. The latter cells displayed a speed of 5.51 $\mu\text{m}/\text{min}$ (Fig. 2 c and Video S5, available at <http://www.jem.org/cgi/content/full/jem.20050783/DC1>) and an arrest coefficient of 0.50, values that are similar to MBP-specific T cells in unimmunized mice. Thus, we conclude that the presence of T reg cells significantly increased the speed and decreased arrest of antigen-specific CD4⁺ T cells in the presence of autoantigen.

Interactions between CD4 effector cells and DCs in the presence or absence of T reg cells

One possible explanation for our results could be that in the presence of T reg cells the stable contact between antigen-specific CD4⁺ naive T cells and DCs was diminished. To directly test this possibility, we transferred CFSE-labeled

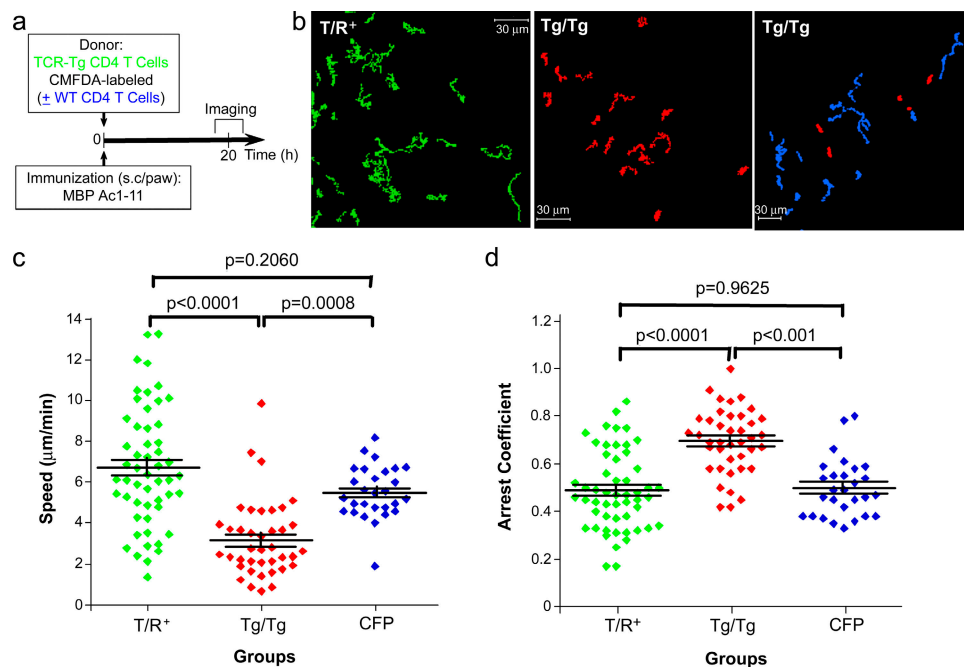


Figure 2. T reg cells release antigen-specific CD4⁺ T cells in the presence of antigen. (a) Experimental protocol for b–d. T/R⁺ or Tg/Tg mice, which have or do not have endogenous T reg cells, respectively, received 10^7 CFSE-labeled MBP Ac1-11-specific CD4⁺ T cells via the tail vein. As endogenous control, Tg/Tg animals received, by tail vein injection, $1\text{--}2 \times 10^7$ CFSE-labeled MBP-specific T cells from Tg/Tg mice together with 10^7 CD4⁺CD25⁻ T cells from syngeneic CFP-expressing mice. On the same day, mice received 50 μg MBP Ac1-11 peptide emulsified

in IFA in the footpad. 20 h after immunization, draining PLNs were imaged. (b) Representative tracks of MBP-specific T cells (green) in T/R⁺ and Tg/Tg immunized recipients. (c) Mean speeds of MBP-specific CD4⁺ T cells in both types of recipient mice. (d) Arrest coefficient for CD4⁺ T cells in both types of recipient mice. Results are representative of three independent experiments. Representative tracks of WT CD4⁺CD25⁻ T cells (blue) are shown in b. Arrest coefficients for WT CD4⁺ T cells (blue) are shown in d.

MBP-specific T cells into T/R⁺ or Tg/Tg animals and, 24 h later, transferred 5-(and-6)-((((4-chloromethyl) benzoyl) amino) tetramethylrhodamine (CMTMR)-labeled antigen-loaded DCs into the foot pad of the same recipient mice (Fig. 3 a). Because the complex between I-A^b and the naturally occurring MBP Ac1-11 is highly unstable, an MBP Ac1-11 mutant peptide (referred to as Ac1-11[4Y]) was used (14, 17). The PLNs were then imaged 20 h later, which allows time for DC migration and therefore should provide similar timing of T–DC interaction as the IFA immunization experiments described above. Antigen-specific T cell movement in PLNs that received antigen-pulsed DCs was qualitatively (Fig. 3 b vs. Fig. 2 b) and quantitatively (Fig. 3 d vs. Fig. 2 d) similar to the movement observed in peptide-immunized animals. Labeling antigen-loaded DCs with a vital dye allowed us to determine the duration of their interaction with MBP-specific T cells in the presence and absence of T reg cells. Contact time was measured by analyzing frame-by-frame individual T cell–DC interactions while viewing the rendered three-dimensional datasets from the most optimal angles. The mean contact time in the absence of T reg cells was 995 s, which was close to the entire time of the datasets, whereas this was reduced to 340 s in the presence of T reg cells (65% reduction, $P = 0.0001$; Fig. 3 c; see also Videos S6 and S7, available at <http://www.jem.org/cgi/content/full/jem.20050783/DC1>). Thus, the difference in duration of interactions in the presence or

absence of T reg cells was about threefold. The arrest coefficient for antigen-specific T cells was significantly lower in the T/R⁺ recipients compared with the Tg/Tg⁺ recipients with transferred DCs (36%, $P < 0.0001$; Fig. 3 d). CFP-expressing polyclonal CD4⁺ T cells used as internal controls had a contact time and arrest coefficient similar to MBP-specific cells transferred to animals that harbor T reg cells (Fig. 3, c and d).

Reduction of CD4⁺ T cell–DC interaction after adoptive transfer of T reg cells

To exclude other possible differences between T/R⁺ mice and Tg/Tg mice, we transferred T reg cells into Tg/Tg mice. We previously showed that both CD4⁺CD25⁺ and CD4⁺CD25[−] T cells from T/R⁺ mice suppressed EAE development, but later it was found that in T reg cell-deficient experimental systems some of the transferred CD4⁺CD25[−] T cells acquire the CD4⁺CD25⁺Foxp3⁺ T reg cell phenotype and function in vivo, in an IL-2-dependent manner (18, 19). Because this process of peripheral T reg cell induction from CD25[−] T cells takes several days, it is possible to examine acute (<36 h) effects of T reg cells by transfer of CD4⁺CD25⁺ cells with CD4⁺CD25[−] cells as a negative control. To prove that T reg cells were responsible for the reduction in the duration of contacts between naive antigen-specific CD4⁺ T cells and antigen-loaded DCs, we transferred purified WT splenic CD4⁺CD25⁺ T cells (T reg cells)

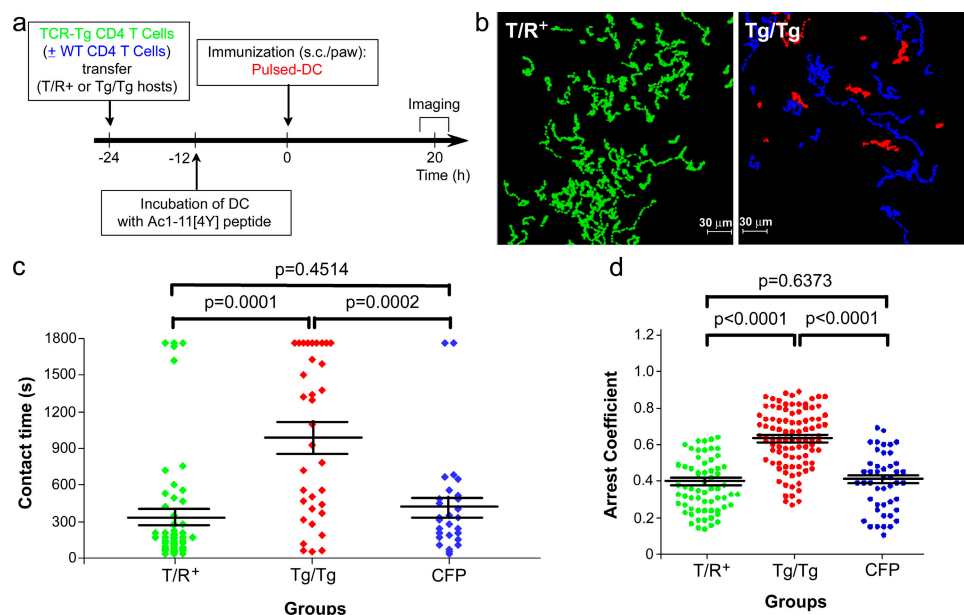


Figure 3. The presence of endogenous T reg cells results in shorter contact time between CD4⁺ T cells and peptide-pulsed DCs. (a) Experimental protocol. T/R⁺ or Tg/Tg mice, which have or lack endogenous T reg cells, respectively, received 10^7 CFSE-labeled MBP Ac1-11-specific CD4⁺ T cells together with 10^7 CD4⁺CD25[−] T cells from syngeneic CFP mice by tail vein injection and, 24 h later, received $5\text{--}10 \times 10^5$ CMTMR-labeled DCs pulsed with MBP Ac1-11[4Y] peptide in the footpad.

20 h after DC transfer, PLNs were imaged. (b) Representative tracks of MBP Ac1-11-specific CD4⁺ T cells in T/R⁺ and Tg/Tg DC-transferred recipients. Trackings of CFP-expressing WT CD4⁺ T cells are represented in blue. (c) Contact time between MBP-specific or WT CD4⁺ T cells and antigen-loaded DCs. (d) Arrest coefficient for transgenic (green or red) or WT (blue) CD4⁺ T cells in T/R⁺ and Tg/Tg DC-transferred recipients. Results in b–d are representative of three independent experiments.

or control $CD4^+CD25^-$ T cells into Tg/Tg animals and observed the interactions between CFSE-labeled $CD4^+$ naive MBP-specific T cells and CMTMR-labeled Ac1-11[Y4]-loaded DCs 12 h after DC injection (Fig. 4 a). Transfer of T reg cells to Tg/Tg mice recapitulated the qualitative (Fig. 4 b vs. Fig. 2 b and Videos S8 and S9, available at <http://www.jem.org/cgi/content/full/jem.20050783/DC1>) as well as quantitative (Fig. 4, c and d, vs. Fig. 3, c and d) features of T/R⁺ mice. Tg/Tg animals that received T reg cells displayed significantly shorter $CD4$ -DC interaction time compared with Tg/Tg mice that received $CD4^+CD25^-$ T cells (29%, $P = 0.004$). Note that the data shown in Fig. 4 correspond to a 20-min observation experiment (maximum interaction time, 1,200 s). It was not unexpected that the effect of T reg cells transferred into Tg/Tg mice was not as great as the effect of T reg cells endogenously generated in the T/R⁺ mice (Fig. 4 c vs. Fig. 3 c) because of issues related to the purification of T reg cells with antibodies, followed by transfer and engraftment. These issues notwithstanding, WT T reg cell transfer into Tg/Tg mice decreased the arrest coefficient of antigen-specific T cells compared with $CD4^+CD25^-$ cell transfer (34%, $P < 0.0001$; Fig. 4 d). Observation at later time points in $CD25^+$ cell-transferred Tg/Tg mice, but not $CD25^-$ cell-transferred mice, showed a decreased number of DCs (not depicted), suggesting that antigen⁺ DCs may have a shorter half-life in the PLN in the presence of T reg cells, an observation that requires further experimentation.

One of the most sought-after answers in the T reg cell field is to the question of how these powerful cells exert their function in vivo. Our real-time intravital microscopy imaging showed that one of the ways in which T reg cells affect immune responses is by decreasing the contact time between DCs and the precursors of $CD4$ effector cells. This was observed in two ways, one in which T reg cells were present in the host (T/R⁺ mice), and one in which WT T reg cells were transferred into T reg cell-deficient Tg/Tg recipients. Absence of T reg cells always resulted in prolonged, more stable contacts between $CD4$ cells and DCs. This phenomenon required autoantigen, as T reg cells did not change the movements of MBP-specific T cells in the absence of the autoantigen (Fig. 1). Furthermore, T reg cells did not affect the speed or arrest coefficient of polyclonal $CD4^+$ cells (Fig. 2, c and d). While our paper was under review, a complementary study by Tang et al. (20) obtained similar findings in vitro. Using two-photon imaging of explanted pancreatic LNs in the nonobese diabetic mouse model, they found that the presence of endogenous T reg cells decreased the duration of autoantigen-specific naive and T reg cell interactions with pancreatic islet emigrant DCs. Direct imaging of T reg cells and pancreatic islet emigrant DCs demonstrated the capacity for long-lived interactions of T reg cells with DCs.

An important distinction between T/R⁺ mice and T reg cell-transferred Tg/Tg mice is that T/R⁺ mice harbor several MBP-specific $CD4^+CD25^+$ T reg cells. In this and other

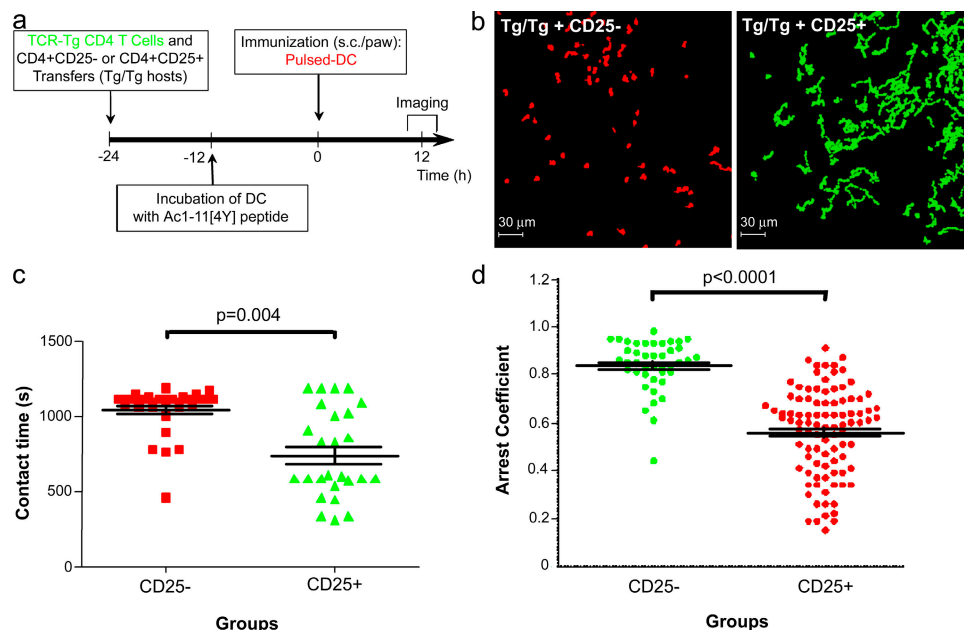


Figure 4. Reconstitution of Tg/Tg animals with T reg cells diminishes the contact time between $CD4^+$ T cells and peptide-pulsed DCs. (a) Experimental protocol. Tg/Tg mice received 10^7 CFSE-labeled MBP-specific $CD4^+$ T cells via tail vein and, on the same day, $5-10 \times 10^5$ $CD4^+CD25^+$ T cells (T reg cells) or $CD4^+CD25^-$ T cells (non-T reg cells) from a syngeneic WT animal in the footpad. 24 h later, CMTMR-labeled DCs pulsed with MBP Ac1-11[Y4] peptide were

injected in the footpad, and PLNs were imaged 12 h after DC transfer. (b) Representative tracks of $CD4$ effector T cells in Tg/Tg animals reconstituted with T reg or non-T reg cells. (c) Contact time between $CD4$ effector T cells and DCs in Tg/Tg mice that received T reg or non-T reg cells. (d) Arrest coefficient of $CD4^+$ T cells in Tg/Tg mice that received T reg or non-T reg cells. Results in b–d are representative of three independent experiments.

TCR transgenic RAG⁺ systems, it has been shown that these CD4⁺CD25⁺ T reg cells are dual TCR-expressing cells that can be used as antigen-specific T reg cells (21, 22). However, CD4 T cell/DC contact time was also decreased when WT polyclonal CD4⁺CD25⁺ T cells were transferred, indicating that uniform MBP Ac1-11 specificity was not required by T reg cells for their function in the model that we studied.

Our intravital data are consistent with previous reports indicating that T reg cells exert their suppressive function by inhibiting DC maturation and/or function (23, 24), although the existence of such a mechanism does not exclude several other means by which T reg cells could function in vivo. The fact that CD4⁺CD25⁺ T cells are able to prevent spontaneous EAE—although it required several days before these cells converted to CD4⁺CD25⁺Foxp3⁺ T reg cells in vivo (18)—supports the existence of additional mechanisms that act at later time points.

Thus, our data show an early effect of T reg cells on CD4 T cell responses in vivo. The manner in which T reg cells achieve steady-state regulation of DC interaction with T cells is not clear. Inhibition of arrest is consistent with decreasing the quality of the TCR stop signal or increasing the level of competing chemokinetic signals (25). The intravital approach that we have developed is ideally suited to study this question.

MATERIALS AND METHODS

Mice. MBP Ac1-11-specific TCR transgenic mice (T/R⁺) have been described previously (11). In brief, genomic TCR- α and - β constructs were coinjected into C57BL/6 zygotes, and the mice were subsequently backcrossed with C57BL/10.PL (The Jackson Laboratory) to incorporate the I-A^b restriction element. Tg/Tg mice are T/R⁺ mice in which the TCR transgenes have been bred to homozygosity. Tg/Tg mice develop EAE spontaneously due to a defect in T reg cells (12). T/R⁺ mice always refer to mice hemizygous for the TCR α/β integration. C57BL/6 mice expressing CFP under the transcriptional control of the chicken β -actin promoter and CMV enhancer (16) were purchased from The Jackson Laboratory and crossed to the C57BL/10.PL background for two generations to incorporate the MHC H-2^{u/a}. Mice were kept under specific pathogen-free conditions in individually ventilated cages (Thoren) at the Skirball Institute Central Animal Facility, New York University Medical Center. All protocols involving live mice were approved by New York University's Institutional and Animal Care and Use Committee.

Peptides, antibodies, and other reagents. The N-acetylated MBP 1-11 peptide (AcASQKRPSQRSK) and the I-A^b-stable Y4 variant (AcASQYR-PSQRSK) were purchased from Invitrogen. The anti-TCR clonotypic antibody 3H12 was described previously (13). Other monoclonal antibodies were purchased from BD Biosciences. CFSE and CMTMR were purchased from Invitrogen.

Cell purification, labeling, transfer, immunization protocols, and intracellular IL-2 staining. Naive MBP-specific CD4⁺ T cells were obtained after negative selection in MACS columns (Miltenyi Biotec), staining splenocytes from Tg/Tg animals with anti-CD8, anti-CD11b, anti-B220, anti-CD11c, and anti-Ter119 FITC-labeled antibodies and incubation with anti-FITC magnetic beads (Miltenyi Biotec). DCs were obtained after positive selection of splenocytes from B10.PL animals using anti-CD11c magnetic beads and columns (Miltenyi Biotec). CD4⁺CD25⁺ or CD4⁺CD25⁺ T cells were obtained from splenocytes of B10.PL animals after a first round of negative selection (as described to obtain naive MBP-specific CD4⁺ T cells)

and a second round of positive selection of CD4⁺CD25⁺ cells, staining these CD4 cells with anti-CD25 PE antibody and anti-PE magnetic beads (Miltenyi Biotec). The purities were always $\geq 90\%$. Positive selection of CD4⁺CD25⁺ cells does not alter their T reg cell activity.

Naive MBP-specific CD4⁺ T cells from Tg/Tg spleens were labeled for 10 min at 37°C with 5 μ M CFSE. 10⁷ cells were injected i.v. into recipient T/R⁺ or Tg/Tg mice via the tail vein. In experiments with immunized animals, shortly after injection of CFSE-labeled CD4⁺ cells, each animal received subcutaneously in the footpad 20 μ l of an emulsion containing 2 mM MBP Ac1-11 peptide diluted in IFA 1:1 (vol/vol). Splenic DCs were purified from syngeneic animals and were pulsed overnight with MBP 10 μ M Ac1-11[4Y] peptide, washed, and labeled for 10 min at 37°C with 5 μ M CMTMR. These DCs were not subjected to further maturation or activation signals. 5–10 $\times 10^5$ DCs in 20 μ l PBS were injected into each footpad of recipient mice 24 h after the labeled CD4 cells. In experiments in which Tg/Tg animals were reconstituted with CD4⁺CD25⁺ or CD4⁺CD25⁺ T cells, 5–10 $\times 10^5$ cells were injected in both footpads of each animal on the same day that they received the MBP-specific CD4⁺ cells. CFP-expressing WT CD4⁺ T cells were injected as controls in the relevant experiments. Intracellular staining for IL-2 detection was performed as described previously (19).

Intravital two-photon microscopy data collection and analysis. 20 or 12 h after immunization or DC injection, mice were anesthetized by an initial intraperitoneal injection of 50 mg/kg ketamine, 10 mg/kg xylazine, and 4 mg/kg acepromazine, and the PLN was prepared microsurgically for intravital microscopy as described previously (2). The prepared LN was immobilized between the edges of two glass slides coated with clay. Each LN was then submerged in normal saline and covered with a glass coverslip. An objective heater (with a temperature probe) was used to maintain and monitor local temperature, which was maintained at 33°C. Care was taken to spare blood vessels. Two-photon imaging was performed in a Bio-Rad Laboratories radiance multiphoton microscope equipped with a 40 $\times$, 0.8 numerical aperture objective (Nikon). This microscope was fitted with a Tsunami Ti:Sapphire-pulsed laser (Spectra-Physics) tuned to 800 nm and controlled by LaserSharp2000 software (Bio-Rad Laboratories). All the images were acquired in a depth between 50 and 100 μ m. To create time-lapse sequences, we scanned volumes of tissue measuring 50 $\times$ 170 $\times$ 170 μ m (Videos S1–S4) or 50 $\times$ 256 $\times$ 256 μ m (Videos S5–S10) at 4- μ m z spacing for 30 min (only the experiment shown in Fig. 4 had a duration of 20 min). To detect CFSE-, CMTMR-, and CFP-labeled cells, 525/50-nm, 620/100-nm, and 510/30-band-pass filters were used, respectively. Image and cell movement analysis was performed with Velocity software (Improvision), which was also used for semi-automated tracking of cell motility. Determination of speeds and arrest coefficients were made using a displacement threshold of 2 μ m as described previously (26). Arrest coefficients were calculated as the ratio between the time that a cell was not moving (instantaneous speed < 2 μ m/min) and the total time the cell was observed. Interactions between T cells and DCs were defined as physical contacts lasting > 30 s. Overall, we analyzed 3,820 cell tracks from 46 imaging fields in 24 mice for these studies. Statistical significance was determined using the Mann-Whitney test and ANOVA. p-values < 0.01 were considered significant. Each dot plot graphic contains horizontal lines representing mean and standard errors.

Immunofluorescence. PLNs from animals submitted to intravital microscopy or similarly treated were harvested and frozen in Tissue-Tek O.C.T. media (Sakura Finetek), and 8- μ m-thick slices were cut. Slides were fixed in 1% paraformaldehyde and blocked with 10% normal mouse serum for 1 h at 24°C. Slides were then stained with the corresponding fluorescent antibodies for another 2 h at 24°C. After washing with PBS, slides were mounted with Fluoromount G mounting media (SouthernBiotech).

Online supplemental material. Fig. S1 illustrates the differences between T/R⁺ and Tg/Tg mice regarding the presence of T reg cells in the former

mouse type but not the latter, and the overall similarities between the two mouse types in all other lymphocyte compartments, as well as antigen-presenting cell subsets and LN architecture. Fig. S2 shows the difference in IL-2 production in the T/R⁺ and Tg/Tg mice immunized with MBP Ac1-11 in IFA. The time-lapsed Videos S1 and S2 illustrate MBP Ac1-11-specific CD4⁺ T cell movement in Tg/Tg or T/R⁺ PLNs, respectively. Videos S3 and S4 illustrate the same cells transferred to Tg/Tg or T/R⁺ immunized animals, and Video S5 shows MBP-specific CD4⁺ T cells (green) and WT CD4⁺ T cells (blue) in the PLN of an MBP-immunized Tg/Tg animal. Videos S6 and S7 illustrate the interactions between antigen-specific CD4 T cells and antigen-loaded DCs in Tg/Tg or T/R⁺ animals, and Videos S8 and S9 illustrate the same T-DC interactions in Tg/Tg animals adoptively transferred with CD4⁺CD25⁻ or CD4⁺CD25⁺ T cells.

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Supplement material

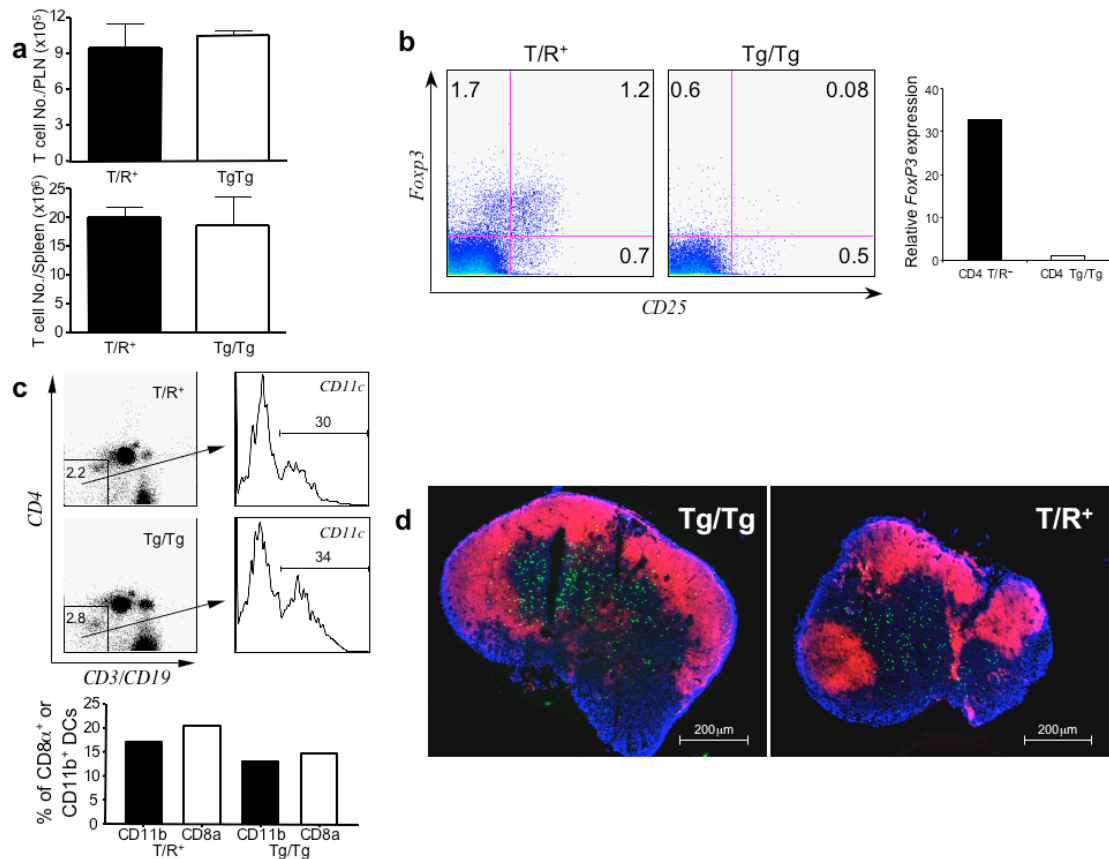


Figure S1. Lymphoid organs of T/R⁺ and Tg/Tg mice are very similar but differ in the presence of T reg cells in T/R⁺ mice.

(a) T/R⁺ and Tg/Tg mice harbor a very similar number of MBP-specific T cells. Absolute number of MBP-specific T cells in the PLN (top) and spleen (bottom) of unimmunized T/R⁺ and Tg/Tg mice. Single cell suspensions were counted in hemocytometer and stained with the anti-clonotypic antibody 3H12 (13) for FACS analysis. The fraction of live cells (determined by forward and side scatter gating) that stained with 3H12 was multiplied by the absolute number of live cells determined by hemocytometer count with exclusion of trypan blue⁺ cells. (b) T/R⁺ mice have Foxp3-expressing T reg cells, which are greatly reduced in Tg/Tg mice. Left panels show FACS analysis of CD4, CD25, and Foxp3 staining (CD4⁺ gate) in the PLNs of T/R⁺ and Tg/Tg mice. The rightmost panel shows real-time quantitative RT-PCR analysis of Foxp3 mRNA expression in bead-purified CD4⁺ T cells from T/R⁺ and Tg/Tg splenocytes. Primers used were Foxp3 forward, ACTGGGGTCTTCTCCCTCAA, and Foxp3 reverse, CGTGGAAGGTGCAGAGTAG. (c) Similar proportion of DC subpopulations in Tg/Tg and T/R⁺ mice. Single cell suspensions from the spleen were stained with anti-CD3, anti-CD19, anti-CD11c, anti-CD11b, and anti-CD8. Among non-T, non-B cells, CD11c⁺ cells (DC) were gated to determine the proportion of CD11c⁺CD11b⁺ or CD11c⁺CD8⁺ cells (d). Similar distribution of transferred MBP-specific T cells in the PLNs of Tg/Tg or T/R⁺ mice. PLNs were harvested 20 h after transfer of CFSE-labeled MBP-specific T cells (green). Frozen sections were labeled with anti-B220 PE mAb (red) and DAPI (blue).

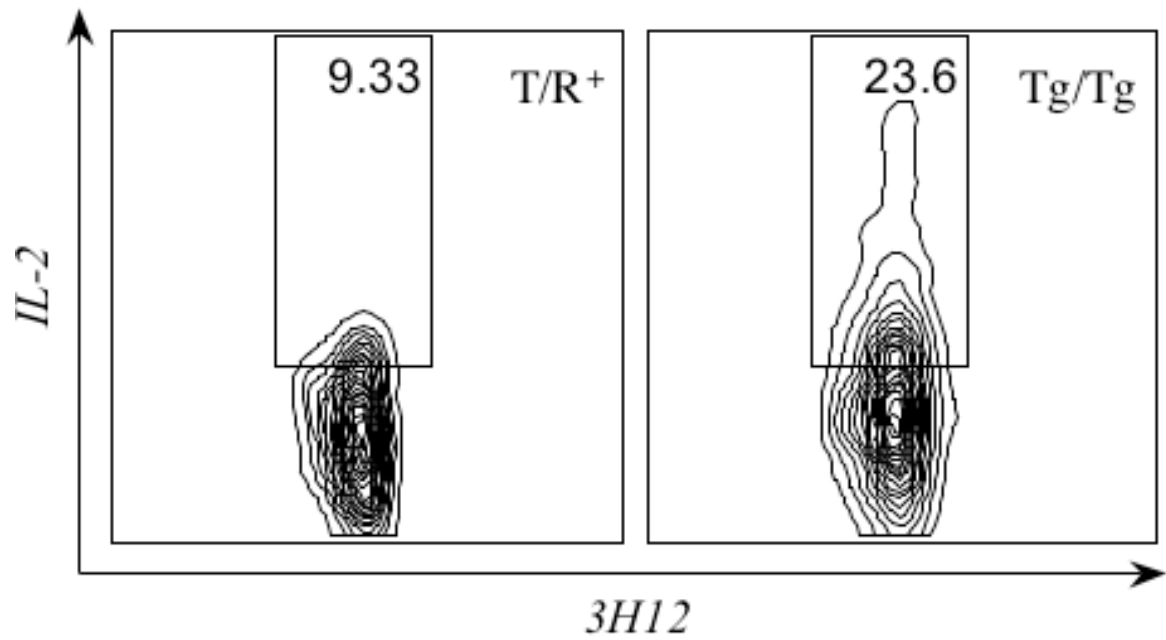


Figure S2. Enhanced IL-2 production by MBP-specific T cells in the absence of T reg cells. T/R+ or Tg/Tg animals received 50 µg MBP Ac1-11 peptide emulsified in IFA in the footpad (protocol as in Fig. 2). 20 h after immunization, draining PLNs were harvested and cell suspensions were evaluated for IL-2 production by intracellular staining. Gated on MBP-specific (3H12+) T cells.

Chapter V

Regulatory T cells induce apoptosis of antigen-loaded dendritic cells *in vivo*

By: Andreia C. Lino, Carlos E. Tadokoro, Nino Kutchukhidze, Maria A. Curotto de Lafaille, & Juan J. Lafaille

Abstract

Foxp3⁺ regulatory T cells (Treg) are essential to maintain immune tolerance, but the mechanism(s) of their action are still not fully understood. One of the possible targets of Treg cell action is the antigen-presenting dendritic cell (DC). We noticed that the number of DC recovered after adoptive transfer was always lower in recipient animals harboring Foxp3⁺ Treg cells than in animals that lack Foxp3⁺ Treg cells. While in steady state the number of DC in mice with or without Treg cells was approximately the same, BrdU-labeling experiments indicated that DC turnover was faster in mice that harbored Treg cells. Furthermore, there were more apoptotic CD11c⁺ cells in animals harboring Treg cells. We also found that Treg cells induced apoptosis of DC loaded with OVA, but not of DC loaded with an irrelevant antigen when Teff cells are OVA-specific. We propose that one of the mechanisms for Treg-dependent peripheral tolerance is the induction of apoptosis of antigen-loaded DC.

Introduction

Naturally-occurring Treg cells are a subset of CD4⁺ T cells generated in the thymus that express the transcription factor Foxp3 (1-7); the majority of Foxp3⁺ T cells also express CD25 (8, 9). In addition to the naturally-occurring Treg cells, adaptive Foxp3⁺ Treg cells can be peripherally generated *in vivo* by conversion of conventional T cells (10-16). Oral exposure to antigen induces the development of adaptive antigen-specific Foxp3⁺ regulatory T cells in mice that lack naturally-occurring Foxp3⁺ Treg cells; these adaptive Foxp3⁺ Treg cells are able to prevent experimental asthma *in vivo* and suppress proliferation of T cells *in vitro* (13). Foxp3⁺ Treg cells are essential to maintain self tolerance and immune homeostasis, as exemplified by the fact that mice (17) and humans (18) lacking Foxp3 develop a rapidly fatal autoimmune pathology. Although the importance of Foxp3⁺ Treg cells in immune tolerance has been clearly established and a great deal of information have been amassed over the past years, several important questions about Treg cell biology remain unanswered, chief among them is the mechanism of action.

One considered target of Treg cell action has been the antigen-presenting cell (APC). It has been shown that CD4⁺CD25⁺ T cells down-modulate the levels of the co-stimulatory molecules CD80 and CD86 on the APC surface, and that this down-modulation is dependent on the contact between CD4⁺CD25⁺ T cells and APC. Furthermore, both APC number and quality influenced Treg-mediated *in vitro* suppression (19). Recently, it has been shown that *in vitro*-generated Foxp3⁺ Treg cells induce down-modulation of CD80 and CD86 expression on antigen-loaded dendritic cells (DC), and that the Treg cell-induced down-modulation is much more pronounced in the presence of effector T cells (Teff) (20).

Recent work using intravital microscopy (21) or explanted lymph nodes (22) brought some light into the mechanism of Treg action *in vivo*. Using two-photon microscopy to visualize the interactions between DC and Teff cells in presence and absence of Treg cells, these studies showed that the contact times between Teff cells and antigen-presenting DC were of shorter duration when Treg cells were present than when they were absent. By reducing the contact time between Teff cells and DC, acquisition of the full repertoire of effector T cell functions would be hampered by Treg cells. These results suggested that Treg cells could suppress autoimmune responses by interacting with DC *in vivo*.

Here we demonstrate that when regulatory T cells are absent, dendritic cells have a slower turnover than when Treg cells are present. Furthermore, Treg cells can mediate the induction of apoptosis of antigen-loaded dendritic cells. Both phenomena are antigen dependent. Our results indicate that one of the mechanisms by which Treg maintain immune tolerance is through the elimination of DC loaded with self-antigen.

Results

The presence of regulatory T cells results in lower recovery of dendritic cells in vivo

In order to determine whether Treg cells can affect DC *in vivo*, we used a TCR transgenic mouse model in which animals either have naturally-occurring regulatory T cells (T/R⁺) or lack them due to their genetic makeup (Tg/Tg). Both T/R⁺ and Tg/Tg mice harbor myelin basic protein (MBP) Ac1-11-specific T cells; due to the absence of regulatory T cells, all Tg/Tg animals spontaneously develop experimental autoimmune encephalomyelitis (EAE) starting at 6 weeks of age, whereas T/R⁺ mice remain healthy throughout life (21, 23-25). CD11c⁺ cells purified from spleens of wild-type mice were preloaded with MBP Ac1-11[4Y], an MBP peptide that contains a mutation in position 4, an MHC contact residue, that enables the formation of stable complexes with I-A^u (26). MBP Ac1-11[4Y]-loaded DC were labeled with carboxyfluorescein succinimidyl ester (CFSE) and transferred by subcutaneous injection into the footpad of 4-5 week old Tg/Tg or T/R⁺ mice. Twenty hours later, popliteal lymph nodes (PLN) were stained for CD11c expression and examined for the recovery of CFSE-labeled DC. Even though we transferred the same number of DC into T/R⁺ or Tg/Tg mice, we repeatedly recovered fewer transferred CD11c⁺ cells in the PLN of T/R⁺ than Tg/Tg mice (Figure 1a). These results suggested that, in the presence of Treg cells, recovery of live antigen-loaded DC was reduced. Although the most conspicuous difference between T/R⁺ and Tg/Tg mice is the presence of Treg cells in the former but not the latter strain, it remained possible that a factor other than the presence of Treg cells could be responsible for the decreased recovery of DC in T/R⁺ mice. To confirm that regulatory T cells were responsible for the lower recovery of transferred DC from the popliteal LN, we reconstituted Tg/Tg animals with Treg cells by intravenous injection of CD4⁺CD25⁺ Treg or CD4⁺CD25⁻ cells purified from spleens of syngeneic wild-type mice. Although CD25 expression is not exclusive of regulatory T cells, it can be used as an enrichment method since about 90-95% of CD4⁺CD25⁺ cells in WT mice from SPF animal facilities are Foxp3⁺. Twenty four hours after Treg cell reconstitution, CD11c⁺ cells pre-loaded with MBP Ac1-11[4Y] were transferred into the footpad of animals with Treg (Tg/Tg + CD25⁺) or lacking Treg (Tg/Tg + CD25⁻) (Figure 1b). Consistent with the results obtained with unmanipulated mice that harbor or lack Treg cells, a reduced DC recovery was observed in mice that were reconstituted with Treg cells (Tg/Tg + CD25⁺) compared with Tg/Tg (+ CD25⁻) that received cells that have reduced Treg activity (Figure 1b). These observations demonstrate that Treg cells are responsible for the lower recovery of transferred DC.

Faster turnover of dendritic cells in the presence of Treg cells

Since the presence of regulatory T cells brought about a reduced recovery of transferred CD11c⁺ cells, we sought to determine if there were any effects on the endogenous DC in mice with and without Treg cells. We reported previously that Tg/Tg and T/R⁺ mice were similar in regards to the number and subsets distribution of DC in a steady-state situation (21). Although steady-state numbers of DC are similar in the presence (T/R⁺ mice) or absence (Tg/Tg mice) of naturally-occurring regulatory T cells, DC turnover rate could be affected by the presence of Treg cells. We therefore measured BrdU incorporation by endogenous CD11c⁺ DC in Tg/Tg and T/R⁺ mice. Groups of 4-5 week old Tg/Tg and T/R⁺ animals were injected i.p. with 1mg of BrdU twice a day, and were sacrificed 3, 22, and 46 hours after the first BrdU injection to quantify BrdU incorporation in CD3⁻CD19⁻CD11c⁺ cells. Animals that lack Treg cells (Tg/Tg) displayed significantly lower percentages of BrdU⁺ CD11c⁺ cells than animals that harbor Treg cells (T/R⁺), and this was the case at all time points analyzed (Figures 1c and 1d), indicating that endogenous DC have a faster turnover in the presence of regulatory T cells. Figure 1c displays representative histograms of each time point for Tg/Tg and T/R⁺ mice, while Figure 1d shows the average percentage of BrdU⁺ cells. The turnover of CD11b⁺ DC (often referred as myeloid DC) was accelerated by the presence of Treg cells, whereas the turnover of CD8⁺ DC (often referred as lymphoid DC) was much less affected (Supplementary Figure S1). Taken together with the fact that the steady-state number of DC in T/R⁺ and Tg/Tg is similar, the lower BrdU incorporation on DC of Tg/Tg mice suggests that endogenous dendritic cells die faster in the presence of regulatory T cells. Since we studied spleen and multiple lymph nodes in the same mice, it is unlikely that DC migrate more (rather than die) in the presence of Treg cells, although it remains a possibility that DC may migrate to tissues that we have not surveyed.

In order to check the maturation and activation status of dendritic cells in Tg/Tg and T/R⁺ animals, we stained spleen and LN of these mice for CD11c, MHC-II, CD80 and CD86. We found that, in mice that lack Treg cells, the fraction of DC that displays high expression of CD80 was modestly but reproducibly higher than in mice that harbor Treg cells (Figure 1e). Thus, in the absence of Treg cells the fraction of DC with an activated phenotype is slightly increased. A point to be considered is that at this moment it is not possible to evaluate the proportion of endogenous DC that are presenting MBP in these mice due to the aforementioned instability of the MBP Ac1-11 complex with I-A^u.

Although we used young Tg/Tg mice, which at the age that were used had no signs of T cell activation, proinflammatory cytokine production, or spontaneous EAE, the possibility

that an undetectable disease process was affecting the DC survival needed to be addressed by a different experimental system.

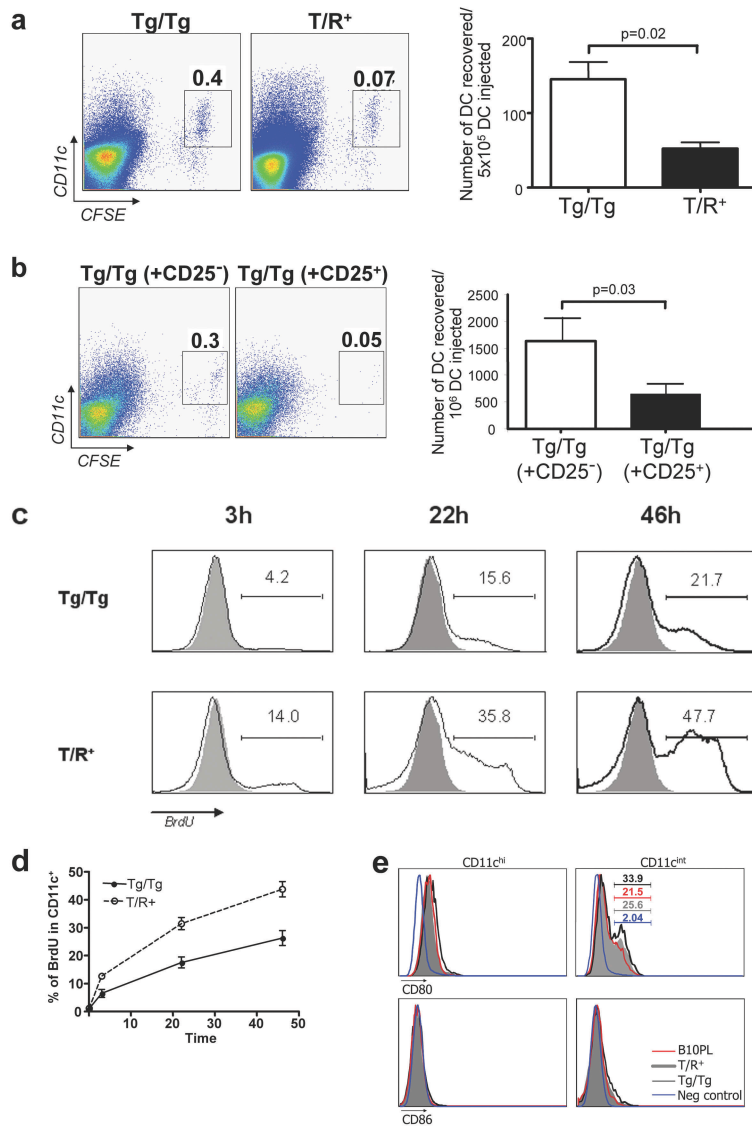


Figure 1. Lower dendritic cell recovery in mice that harbor regulatory T cells.

(a) Flow cytometry of CFSE-labeled CD11c⁺ wild-type spleen DC that were injected into the footpad of animals that have Treg (T/R⁺, right) or don't have Treg (Tg/Tg, left). Twenty hours later, the draining popliteal LN were harvested and the transferred DC were quantified. On the rightmost panels the number of DC recovered per 5x10⁵ injected DC is shown (t student test). (b) Flow cytometry of CFSE-labeled CD11c⁺ wild-type spleen DC that were injected into the footpad of Tg/Tg mice reconstituted with polyclonal Treg (+CD25⁺, right) or with cells that have reduced Treg activity (+CD25⁻, left) using a protocol similar to the one described above. On the rightmost panels the number of DC recovered per 10⁶ injected DC is shown (t student test). (c) 4-6 week old T/R⁺ and Tg/Tg mice were injected with BrdU (1mg/mouse) twice a day and spleens analyzed by flow cytometry at the indicated times after the first BrdU injection. Representative BrdU histograms of gated CD11c⁺ cells at each time point are shown for Tg/Tg mice (top) and T/R⁺ mice (bottom). The filled histograms represent an animal that did not receive any BrdU. (d) Same as panel (c) but showing the integrated data from a group of T/R⁺ mice (dashed line) or Tg/Tg mice (full line). Each data point ($\pm$ s.e.m) represents three or four mice. (e) Histogram analysis of CD80 (top) and CD86-stained (bottom) spleen cells from 4-6 week old wild-type, T/R⁺, and Tg/Tg mice gated on CD11c⁺ cells and CD4⁺ T cells as negative control. The numbers on the top right quadrant represent the frequency of CD80^{hi} DC in the three mouse strains and in CD4⁺ T cells. The nomenclature in the bottom right panel applies to the four panels of this figure. Presented data are representative of three to five experiments.

Treg cell-dependent high DC turnover is reverted in Foxp3-deficient mice

We sought to confirm in an independent manner that the presence of Foxp3⁺ regulatory T cells was responsible for the lower recovery and faster turnover of DC. Toward that end, we used Foxp3-deficient (scurfy) mice crossed with another strain of TCR transgenic mice, hereafter referred to as T/Bmc. In T/Bmc, the T cells (DO11.10) recognize the foreign antigen chicken Ovalbumin (OVA) and the B cells (17/9) recognize the foreign antigen influenza hemagglutinin peptide (YPYDVDPYASLRS) (27). Due to the cross with RAG^{-/-} mice, T/Bmc mice also lack naturally occurring regulatory T cells and develop an exacerbated Th2/IgE response after a single i.p. immunization with OVA-HA (27). In fact, all TCR transgenic RAG^{-/-} mouse lines that have been analyzed thus far lack naturally-

occurring Treg cells, as first determined functionally (23, 24) and later by the absence of Foxp3⁺ T cells (28). In contrast to Tg/Tg mice, which develop EAE spontaneously, non-immunized T/Bmc only harbor naive T and B cells due to the absence of antigen. Unless specified otherwise, the nomenclature T/Bmc refers to RAG1^{-/-} T/Bmc mice.

We have previously shown that when OVA is administrated orally to T/Bmc mice, hyper IgE and asthma-like responses are suppressed due to the generation of OVA-specific adaptive Foxp3⁺CD25⁺ regulatory T cells converted from Foxp3⁻CD25⁻ T cells (13). Furthermore, the oral route of OVA entry does not lead to the generation of T effector cells, even if adaptive Foxp3⁺CD25⁺ Treg cells cannot be generated due the cross of T/Bmc with scurfy mice (Curotto de Lafaille et al., submitted).

Unlike soluble OVA, which is rapidly eliminated from the mice, adaptive CD25⁺Foxp3⁺ Treg cells can be found in T/Bmc mice at least 5 weeks after oral OVA treatment and i.v. administration of OVA (Supplemental Figure S2). We generated adaptive OVA-specific Treg cells following the protocol schematized in Figure 2a. In brief, T/Bmc mice were given OVA in the drinking water for 8 days, and 3 days later were given OVA intravenously. To monitor DC turnover, at the time of the OVA i.v. injection the mice received the first BrdU i.p. injection; BrdU injections were carried out twice a day for the following 3 days, and at the end of the third day spleen cells were analyzed.

Confirming our previous report (13), both oral OVA treatment and i.v. administration of OVA without prior OVA feeding resulted in the appearance of CD25⁺Foxp3⁺ Treg cells, which are virtually absent in untreated animals. Further increase in the proportion of CD25⁺Foxp3⁺ Treg cells was achieved when OVA i.v. injection followed OVA feeding (Figure 2b). This is likely due to the fact that the injection of antigen generates IL-2 production by the effector T cells. IL-2 has been shown to have a role in adaptive Treg cell generation and Treg homeostasis *in vivo*, as well as the stability of CD25 expression on Treg cells (11, 29, 30).

When we analyzed BrdU incorporation by endogenous DC (CD3⁻CD19⁻CD11c⁺) in the four groups of mice, we observed that the percentage of CD11c⁺ BrdU⁺ cells was significantly higher when the frequency of Treg was the highest (mice treated with Oral OVA + i.v. OVA, Figure 2c). Thus, in this experimental system a higher DC turnover also correlated with the presence of Foxp3⁺ regulatory T cells. However, there was not a perfect correlation between the frequency of Treg cells and DC turnover, as DC turnover appeared to be also enhanced by the i.v. immunization alone, as discussed below.

In the experiment described above it was possible that OVA i.v. administration, through the activation of naive T cells, could have contributed, to an unknown degree, to the

observed effect on DC turnover. In order to demonstrate that the described effect was indeed dependent on the Foxp3⁺ Treg cells that were generated in T/Bmc mice, we used T/Bmc mice crossed with Foxp3-deficient scurfy mice. Although carrying the X-linked Foxp3 mutation, male T/Bmc Foxp3^{sf} mice are completely healthy because their monoclonal T and B cells do not recognize self-antigens. Therefore in these mice there is no detectable T, B, or DC activation (Curotto de Lafaille et al, submitted). We fed OVA to T/Bmc Foxp3^{WT} mice and T/Bmc Foxp3^{sf} mice. This treatment leads to Treg cell but not Teff cell generation in T/Bmc Foxp3^{WT} mice, and results in neither Treg cell nor Teff cell generation in T/Bmc Foxp3^{sf} mice (Curotto de Lafaille et al, submitted). We subsequently administered OVA i.v. and BrdU i.p. to the mice and sacrificed them to determine the turnover rate of unmanipulated, endogenous DC. Only mice capable of generating Foxp3⁺ regulatory T cells (T/Bmc Foxp3^{WT}) and to which antigen was given (+ i.v. OVA) displayed a high turnover of DC, as determined by the higher percentage of BrdU incorporated by CD11c⁺ cells at 72 hours (Figure 2d). These experiments confirm in an unrelated experimental setup that Foxp3⁺ regulatory T cells induce higher turnover of DC. The experiments further suggest that the loading of DC with antigen may be required, a point addressed in the next sections.

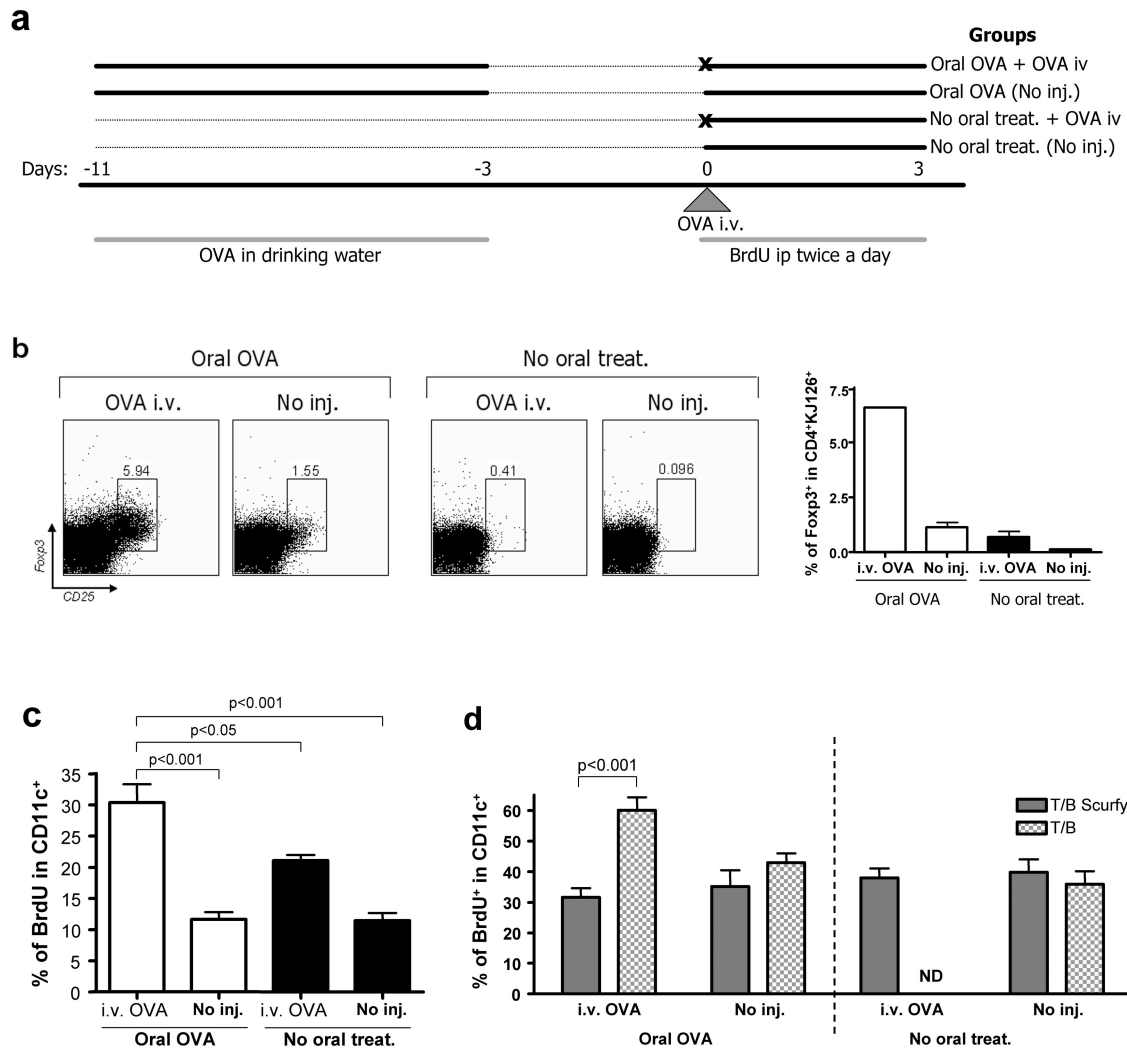


Figure 2. Higher turnover of DC in the presence of Treg cells and antigen.

(a) Experimental protocol and experimental groups. Groups of 3-5 T/Bmc mice were administered a 1% OVA solution in drinking water for 8 consecutive days (days -11 to -3). Three days later, mice were given 100mg/mouse OVA i.v. (day 0). At day 0, administration of BrdU i.p. (twice a day) was started. 72 hours after the first BrdU injection, spleens were harvested for analysis. (b) Adaptive Treg induction. Representative dot plots of CD25 and Foxp3 staining on gated CD4⁺KJ1-26⁺ T cells from mice of all four experimental groups, 72h after the OVA i.v. administration (wherever indicated). Right side, quantification of Treg cells found in all four experimental groups, 72h after the OVA i.v. administration (wherever indicated). (c) Percentage of splenic CD11c⁺BrdU⁺ cells in mice from all four experimental groups at 72h after the administration OVA i.v. (wherever indicated). (d) The experimental protocol described in (a) was used for groups of 3-5 T/Bmc and T/Bmc Foxp3sf (scurfy) mice. Bars indicate the percentage of splenic CD11c⁺BrdU⁺ cells 72h after the administration OVA i.v. (wherever indicated). P values only shown for data that reached statistical significance.

Faster turnover of dendritic cells in the presence of Treg is antigen-dependent

T cells in Tg/Tg and T/R⁺ mice recognize the autoantigen MBP, a protein that is abundant in the central nervous system, peripheral nervous system, and is present in peripheral lymphoid tissues (31, 32). It is therefore not possible to study the role of antigen recognition in the Treg-mediated decrease of DC in this system. The data shown in

Figure 2 suggests that antigen-loaded DCs are more affected by the presence of Treg cells than DCs that are not loaded with antigen. However, a caveat of this assay is that the antigen both loads dendritic cells and induces and boosts adaptive Treg cell development. It was therefore important to assess the turnover rate of DC in the absence of antigen but in the presence of regulatory T cells. Toward that end, we determined the kinetics of BrdU incorporation in T/Bmc $RAG^{-/-}$ and $RAG^{+/-}$ mice. Analogously to T/R⁺ mice, T/Bmc mice on a $RAG^{+/-}$ background do have Foxp3⁺ regulatory T cells (28). Thus, by employing the T/Bmc system in the $RAG^{-/-}$ and $RAG^{+/-}$ backgrounds, we can use mice with or without naturally-occurring Treg cells, but in a condition in which OVA is absent. In contrast to the results obtained in the presence of the self antigen MBP, in the absence of OVA we observed the same proportion of CD11c⁺BrdU⁺ cells in both groups of T/Bmc mice at 24 and 72h after the first BrdU injection regardless of the presence of Treg cells (Figure 3a). The comparison of DC turnover in experimental systems in which antigen is absent or present is shown in Figure 3b. Our results indicate that regulatory T cells have little or no effect on DC turnover when the antigen is absent.

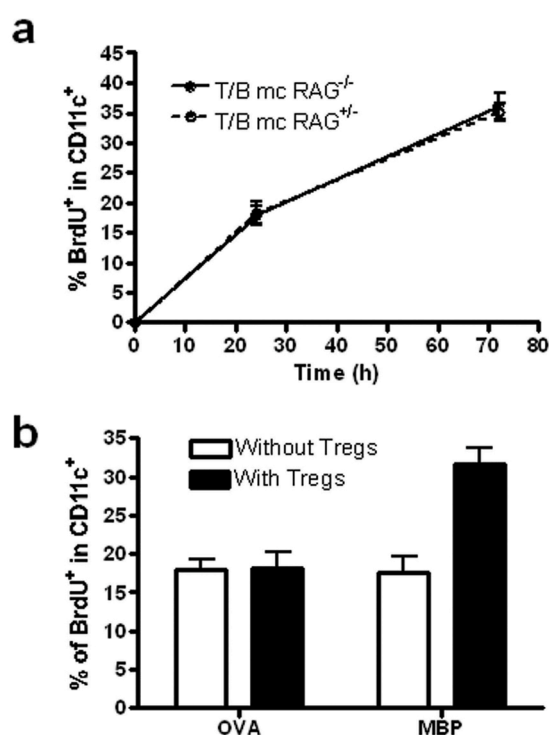


Figure 3. The faster DC turnover only occurs when antigen is present.

(a) OVA-specific TCR transgenic mice that have (T/Bmc $R^{+/-}$) or lack (T/Bmc $R^{-/-}$) regulatory T cells were injected BrdU i.p., twice a day. The graph indicates the percentage of CD11c⁺BrdU⁺ cells in spleens of T/Bmc $R^{-/-}$ and T/Bmc $R^{+/-}$ mice at the indicated times. Note that no mouse received OVA by any route. Three to five mice per group per time-point were analyzed. (b) Comparison of BrdU incorporation by CD11c⁺ cells in mice that have or lack Treg cells, in the absence of antigen (OVA) or the presence of the self antigen MBP.

Regulatory T cells preferentially eliminate dendritic cells loaded with antigen

To test the hypothesis that Treg-mediated DC elimination requires antigen recognition, we used a transfer system schematized in Figure 4a. First, we transferred OVA-specific Treg cells, generated as shown in Figure 2c, group labeled “Oral OVA + OVA i.v.” into

naïve T/Bmc mice. In order to check if Treg cells, generated as described above, could efficiently reconstitute naïve T/Bmc mice, one group of mice was injected only with CFSE-labeled OVA-specific Treg cells. This group of mice was analyzed 30 hours after Treg cell transfer for the presence of CFSE⁺Foxp3⁺ cells in spleen and mLN. Indeed, we could detect the transferred CFSE⁺ Treg (Suppl. Figure S3a upper panels). The vast majority of CFSE⁺ cells were Foxp3⁺, and all Foxp3⁺ cells were transferred (CFSE⁺) cells (Suppl. Figure S3a, lower panels). Having validated the experimental system, a group of T/Bmc mice (T/Bmc + Treg) was injected with unlabeled Treg cells. Twenty four hours after Treg cell injection, we transferred mixes of DC in which 50% had been loaded with OVA₃₂₃₋₃₃₉ and the other 50% had been loaded with the I-A^d-binding peptide HA₁₂₆₋₁₃₈. The latter peptide is irrelevant in this system because it is not recognized by the DO11.10 T cells (33) or the B cells from T/Bmc mice. In the first DC mix, DC loaded with OVA were labeled with CFSE at 0.25mM (CFSE^{lo}) and HA-loaded DC were labeled with CFSE at 2.5mM (CFSE^{hi}). To control for differential toxicity, we included the reciprocal mix in which the OVA-loaded DC are the CFSE^{hi} and the HA-loaded DC are the CFSE^{lo}. The DC mixes were injected into three types of recipient mice. The first group consisted of RAG^{-/-} mice, the second group consisted of naïve T/Bmc mice (T/Bmc), and the third group consisted of T/Bmc mice to which OVA-specific Treg cells had been transferred (T/Bmc + Treg). Six hours after DC transfer, the fate of injected DC in the recipients' spleens was determined by FACS analysis of gated CD11c⁺ cells and exclusion of KJ1-26⁺ T cells.

The mixtures of antigen-loaded DC could be recovered at approximately 50-50 ratio in lymphocyte-empty RAG^{-/-} mice, showing that the labeling with one or other peptide did not differentially affect the viability of the transferred DC (Figures 4b and 4c and Suppl. Figures S3b and S3c). A similar result was obtained when the DC mixtures were injected into naïve T/Bmc mice. Strikingly, when the same mixtures were transferred into T/Bmc mice that had received OVA-specific Treg cells, there was a significant (p=0.003) reduction in the recovery of OVA-loaded DC compared to HA-loaded DC. The reduced recovery of OVA-loaded DC in relation to HA-loaded DC was observed both when OVA-loaded DC were labeled as CFSE^{high} (Figures 4b and 4c) or labeled as CFSE^{low} (Suppl. Figures S3b and S3c).

Finally, to ensure that the transferred OVA-loaded DC were seen by OVA-specific T cells, we performed a similar experiment to the one described above in which we analyzed CD69 expression on OVA-specific CD4⁺ cells 6h after injection of the DC mixture. Six hours after the transfer of either DC mix into naïve T/Bmc mice, there was a robust CD69

upregulation on T cells, in contrast to very little CD69 expression observed in uninjected T/Bmc mice (Suppl. Figure S3d).

Our results show that Treg cells preferentially affect the recovery of DCs that are presenting the relevant antigen.

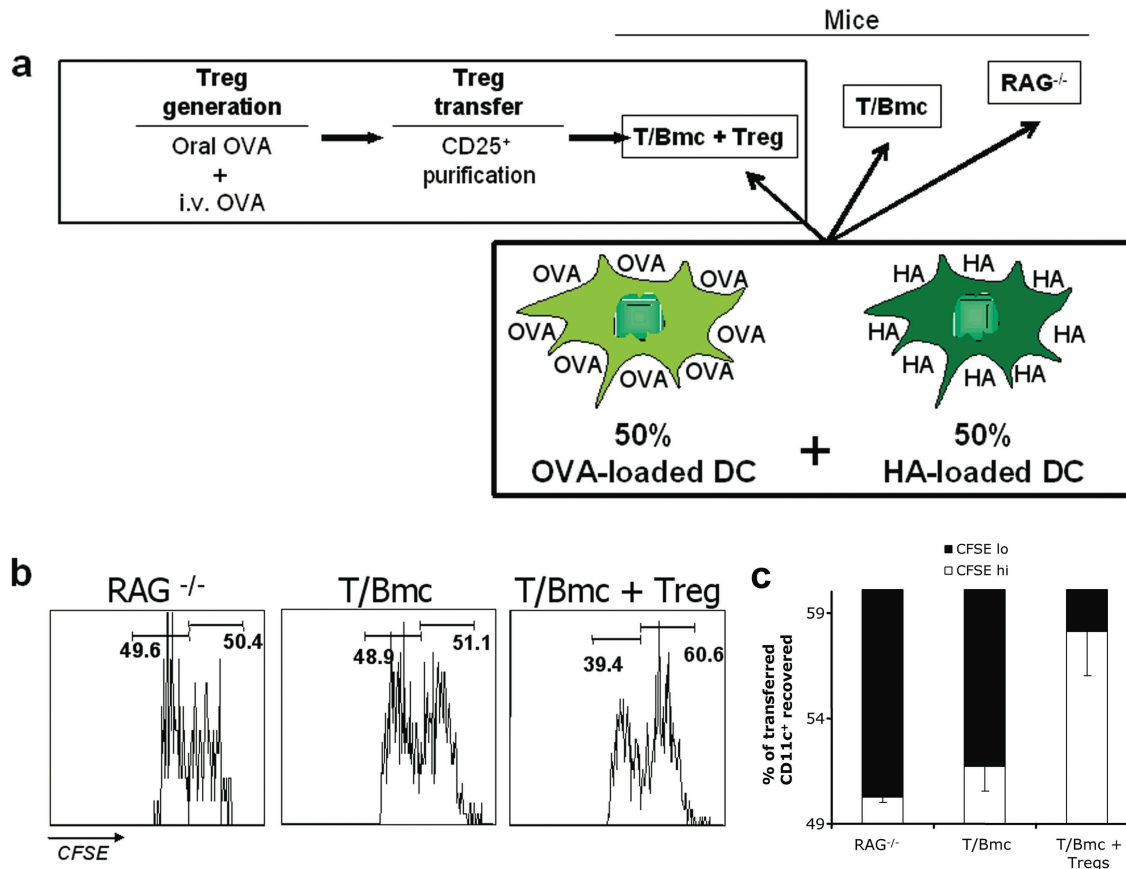


Figure 4. Lower Treg cell-mediated recovery of DC loaded with the relevant antigens.

(a) Experimental protocol and experimental groups. DC were isolated from spleens of WT syngeneic mice. Half of the DC were loaded with OVA peptide and labeled with 0.25mM CFSE (CFSE^{lo}), and the other half was loaded with HA peptide and labeled with 2.5mM of CFSE (CFSE^{hi}). OVA-loaded and HA-loaded DC were then mixed at a 1:1 ratio and injected into three groups of recipient mice. One recipient group (T/Bmc + Treg) consisted on naïve T/Bmc mice that 24 h earlier had received adaptive OVA-specific Foxp3⁺ Treg cells that were purified from other T/Bmc mice treated as schematized in Figure 2a. The second recipient group consisted of naïve T/Bmc mice that did not receive Treg cells (T/Bmc), and the third group consisted of syngeneic RAG-1^{-/-} mice (RAG^{-/-}). All mice were analyzed for the presence of transferred DC 6h later. (b) representative histograms of each group gated on transferred (CD11c⁺CFSE⁺) cells (c) Percentage (± SEM) of OVA-loaded CD11c⁺CFSE^{low} (black) and HA-loaded CD11c⁺CFSE^{high} (white) CD11c⁺ cells recovered from the spleens of mice each experimental group, six hours after DC mix transfer. Note the partial scale. Representative of four experiments with three mice per group per experiment.

Antigen specific T effector cell engagement is required for elimination of dendritic cells in presence of regulatory T cells

OVA-loaded DC were depleted after transfer to animals with Tregs (Figure 4) and we now decided to check if the interaction with effector T cells could protect DCs from death by

increasing the expression of the anti-apoptotic gene Bcl-X_L. To investigate gene expression changes experimented by dendritic cells that are or not engaged by effector T cells in presence or absence of regulatory T cells, we used the experimental system described in the previous section and schematized in figure 4. Briefly, DCs were isolated from spleens of WT syngeneic mice (as schematized in Figure 4). Half of the DC were loaded with OVA peptide and labeled with 0.25mM CFSE (CFSE^{hi}), and the other half was loaded with HA peptide and labeled with 2.5mM of CFSE (CFSE^{lo}). OVA-loaded and HA-loaded DC were then mixed at a 1:1 ratio and injected into groups of recipient mice. The first recipient group consisted of naïve T/Bmc mice that did not receive Treg cells (No Tregs). The second recipient group consisted of T/Bmc mice treated as schematized in Figure 2a (Tregs). 6 hours after DC transfer, remaining transferred DC were sorted using CD11c⁺CFSE^{lo}, CD11c⁺CFSE^{hi} and CD11c⁺CFSE⁻. RNA was extracted from transferred DC (OVA) and (HA), endogenous DC (Endog) as well as from DC loaded with OVA and HA before transfer (pre-injected). The levels of mRNA for Bcl-X_L were determined by real-time PCR (figure 5a). We observed that, in absence of Tregs, the expression of Bcl-X_L in the remaining transferred DC loaded with OVA, is approximately 600 times higher than the one of transferred DC loaded with HA or of the empty endogenous DC (Endog) (figure 5a). The Bcl-X_L relative expression by pre-injected loaded DC (both HA and OVA loaded) is equivalent to the one observed by the transferred DC loaded with HA or the unloaded endogenous DC (Endog) (figure 5a). This result suggest that the increase in Bcl-x_L expression by transferred DCs loaded with OVA results of the engagement by effector T cells. When Tregs are present, the increase of Bcl-X_L expression by the remaining OVA loaded DC is 3 fold lower than when Tregs are absent (figure 5a). Our results suggest that Tregs can act by decreasing the anti-apoptotic signals given by effector T cells to DC loaded with relevant antigen.

To this point our experiments do not discriminate whether antigen engagement by effector T cells and/or regulatory T cells is required, but do show that engagement of effector T cells in the absence of Treg cells does not result in lower DC recovery. To clarify the importance of Teff and Treg cells specificity to this phenomena we transferred a mixture containing DC loaded with OVA and labeled with 2.5μM of CFSE (CFSE^{hi}) together with DC loaded with HA and labeled with 0.25μM of CFSE (CFSE^{lo}) to 5 groups of recipient mice (figure 5b). The first recipient group (T/Bmc with OVA specific Treg) consisted on T/Bmc mice that were treated as schematized in Figure 2a. The second recipient group (T/Bmc with polyclonal Treg) consisted on T/Bmc mice that 24 h earlier had received polyclonal Foxp3⁺ Treg cells that were purified from BALB/c mice. The third group consisted of syngeneic RAG-1^{-/-} mice (RAG^{-/-}) and serves the propose of defining

the relative constitution of the DC mixtures injected in a particular experiment. The fourth recipient group consisted of BALB/c mice (BALB/c with OVA-specific Tregs) that 24 h earlier had received OVA-specific Foxp3⁺ Treg cells that were purified from T/Bmc mice treated as schematized in Figure 2a and the fifth recipient group consisted of BALB/c mice (BALB/c with polyclonal Tregs). All mice were analyzed for the presence of transferred DCs 6h later. Representative histograms of each group gated on transferred (CD11c⁺CFSE⁺) cells are shown on top of figure 5b and averages of percentage ($\pm$ SEM) of OVA-loaded CD11c⁺CFSE^{hi} (black) and HA-loaded CD11c⁺CFSE^{lo} (white) cells recovered from the spleens of mice of each experimental group, six hours after DC mix transfer are on the bottom of figure 5b. The results shown on figure 5b show that the reduction in OVA loaded DC only happened in recipient mice that carry OVA specific Teff cells (T/Bmc) but not in mice that have a polyclonal population of Teff cells (BALB/c). The ratio of recovered DC were of 40% of CFSE^{hi} (OVA loaded) to 60% of CFSE^{lo} (HA loaded) for RAG^{-/-} and BALB/c recipient mice (BALB/c with polyclonal Tregs and BALB/c with OVA-specific Tregs) and of 30% of CFSE^{hi} (OVA loaded) to 70% of CFSE^{lo} (HA loaded) for T/Bmc recipient mice (T/Bmc with OVA specific Treg and T/Bmc with polyclonal Treg) (figure 5b). Our results suggest that is the specificity of Teff that determines the elimination of DC loaded with relevant antigen in presence of regulatory T cells and that the specificity of these last cells do not play a role.

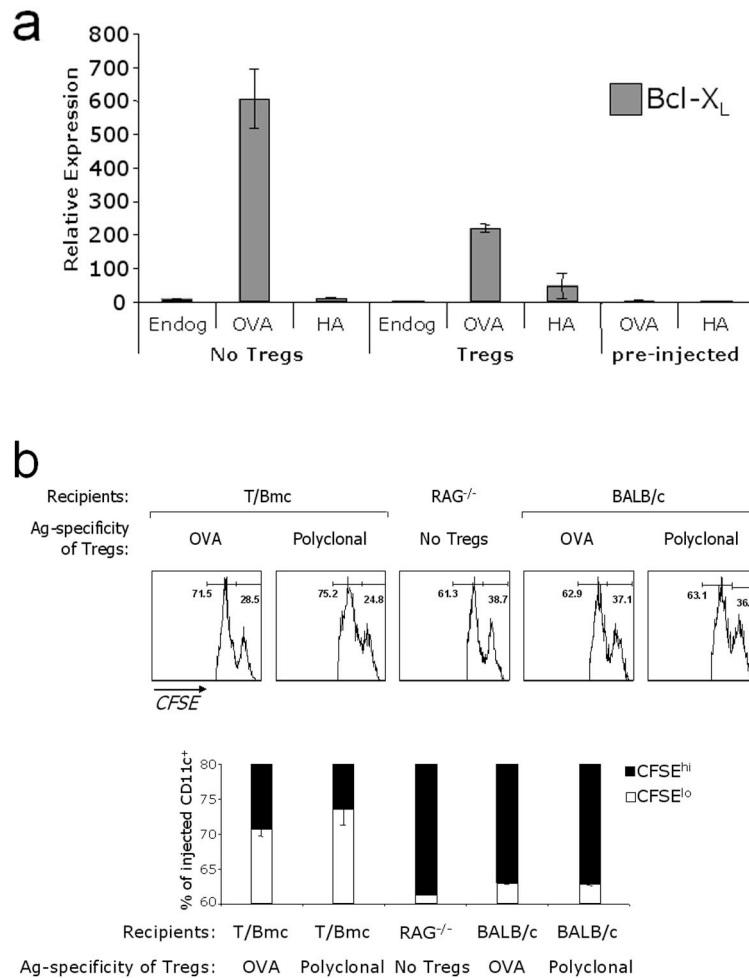


Figure 5. T effector cells specificity determines the elimination of dendritic cells in presence of regulatory T cells.

Using the experimental design described in figure 4. Briefly, DC were isolated from spleens of WT syngeneic mice. Half of the DC were loaded with OVA peptide and labeled with 0.25mM CFSE (CFSE^{hi}), and the other half was loaded with HA peptide and labeled with 2.5mM of CFSE (CFSE^{lo}). OVA-loaded and HA-loaded DC were then mixed at a 1:1 ratio and injected into groups of recipient mice. (a) The first recipient group consisted of naïve T/Bmc mice that did not receive Treg cells (No Tregs). The second recipient group consisted of T/Bmc mice treated as schematized in Figure 2a (Tregs). 6 hours after DC transfer, remaining transferred DC were sorted using CD11c⁺CFSE^{lo} and CD11c⁺CFSE^{hi}. RNA was extracted from transferred DC (OVA) and (HA), endogenous DC (Endog) as well as from DC loaded with OVA and HA before transfer (pre-injected). The levels of mRNA for Bcl- X_L were determined by real-time PCR. Expression levels (average $\pm$ SD) relative to β -actin are shown. (b) One recipient group (T/Bmc with OVA specific Treg) consisted on T/Bmc mice that were treated as schematized in Figure 2a. The second recipient group (T/Bmc with polyclonal Treg) consisted on T/Bmc mice that 24 h earlier had received polyclonal Foxp3⁺ Treg cells that were purified from BALB/c mice. The third group consisted of syngeneic RAG-1^{-/-} mice (RAG^{-/-}). The fourth recipient group consisted of BALB/c mice that 24 h earlier had received OVA-specific Foxp3⁺ Treg cells that were purified from T/Bmc mice treated as schematized in Figure 2a and the fifth recipient group consisted of BALB/c mice. All mice were analyzed for the presence of transferred DC 6h later. Representative histograms of each group gated on transferred (CD11c⁺CFSE⁺) cells are shown on top and percentage ($\pm$ SEM) of OVA-loaded CD11c⁺CFSE^{hi} (black) and HA-loaded CD11c⁺CFSE^{lo} (white) CD11c⁺ cells recovered from the spleens of mice each experimental group, six hours after DC mix transfer are on the bottom. Note the partial scale. Representative of two experiments with three or two mice per group per experiment.

Regulatory T cells induce apoptosis of dendritic cells loaded with relevant antigen

Dendritic cells loaded with a peptide recognized by the effector cells and/or regulatory T cells are recovered in lower proportions in the secondary lymphoid organs. This could be

due to the induction of differential migration of dendritic cells by regulatory T cells or to the direct or indirect induction of apoptosis. The first explanation was unlikely as we failed to find DC in other locations in experiments similar to the one described in Figure 1a and 1b. To determine whether dendritic cells were eliminated by induction of apoptosis in mice harboring Treg cells, we determined the annexin V expression level in transferred CD11c⁺ cells that had either been loaded with antigen MBP[4Y] or not. We found twice as many annexin V⁺ DCs when antigen-loaded DCs were transferred into mice that had regulatory T cells (T/R⁺) than when the same cells were transferred into mice that lacked Treg cells (Tg/Tg) (p=0.01, Figure 5a). In contrast, when unloaded DCs were transferred, we observed no statistically significant difference in the proportion of annexin V⁺ DC (Figure 5b).

To further confirm that Treg cells induce apoptosis of DCs we perform a TUNEL assay using T/R⁺ and Tg/Tg mice that had been crossed with CD11c-YFP reporter mice (34). Using immunofluorescence as a TUNEL readout we found that there were more TUNEL positive CD11c⁺ cells in T/R⁺ than in Tg/Tg animals (Figure 5c, left). In order to quantify these differences we performed FACS analysis of cells from T/R⁺ CD11c-YFP and Tg/Tg CD11c-YFP animals subjected to TUNEL assay. We observed a significant higher percentage (p=0.03) of TUNEL positive dendritic cells in T/R⁺ than in Tg/Tg recipient mice. In conclusion, our data demonstrate that in the presence of Foxp3⁺ Treg cells there is higher apoptosis and a faster turnover of antigen-loaded DC.

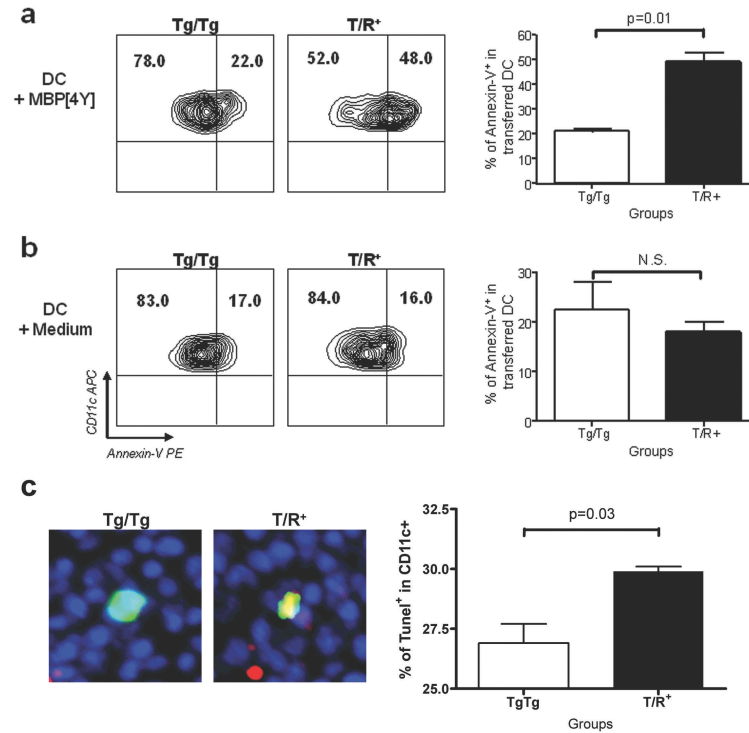


Figure 6. Dendritic cells are eliminated by apoptosis.

(a,b) CD11c⁺ purified from spleen of WT mice were loaded with MBP[4Y] (a) or left unloaded (b), labeled with CFSE and transferred into footpad of TgTg (left dot plots) and T/R⁺ (right dot plots). 12h later, popliteal LN were harvested and the cells were stained for annexin V. Represented dot plots are of gated transferred CFSE⁺ cells. On the right panels, the averages of % of annexin V positive cells within the transferred cells are shown ($\pm$ SEM). (c) Left panels, TUNEL of spleen tissue sections TgTg CD11c-YFP (left) and T/R⁺ CD11c-YFP (right). Right panel, TUNEL⁺ CD11c⁺ cells in TgTg CD11c-YFP and T/R⁺ CD11c-YFP mice were quantified by flow cytometry ($\pm$ SEM). A representative experiment out of two (six mice) is shown.

Discussion

Several mechanisms have been postulated to explain how regulatory T cells suppress immune responses (35, 36). In this manuscript we focus on the effect of regulatory T cells on antigen-presenting dendritic cells *in vivo*. We have used experimental systems in which we studied endogenous, unmanipulated DC in mice that have or lack Treg cells, or mice in which Tregs were transferred. We have done the reverse type of experiments in which the Treg compartment was not manipulated and DC were transferred. Finally, we have used experimental systems in which both Treg cells and DC were transferred. In all experimental systems, the common feature is that, in the presence of regulatory T cells, the turnover of DC is higher than in the absence of Treg cells, or the recovery of DC is lower than in the absence of Treg cells.

Most *in vitro* suppression assays are carried out in the presence of APC. However, it has been suggested that APC may not be strictly necessary in the assays, in particular if the Treg cells have been pre-activated. It has been shown that, *in vitro*, pre-activated regulatory T cells can suppress the proliferation of responder CD8⁺ T cells in the absence of APC (37). It has also been postulated that APC are not necessary for Treg cell suppression of CD4⁺ T cells. However, in the case of CD4⁺ T cell responders it is clear that the presence of APC dramatically enhances the effectiveness of Treg-mediated suppression. Parallel assays run by one research group showed that Treg cells lowered the anti-CD3 and APC-induced proliferation of responder T cells by about six fold, whereas the same proportion of Treg cells lowered the proliferation of responder T cells by less than two fold in the absence of APC (38). It remains possible that a low level of contamination of T cells with APC on either CD4⁺ T cell preparation could explain the comparatively weak level of suppression that was observed when APC were not added to the assays.

From early on in Treg cell research, the relative ineffectiveness of suppression observed in assays conducted in the absence of APC pointed toward an effect of regulatory T cells on APC. Cederbom *et al* found that, in a cell contact-dependent manner, CD4⁺CD25⁺ regulatory T cells down-regulated the expression of CD80 and CD86 in dendritic cells *in vitro* (19). CD80-low DC would be poor stimulators of naive T cells thus offering a mechanism for Treg cell-mediated suppression. This mechanism was consistent with the loss of Treg-dependent suppression in assays in which the number of CD86-transfected APC was increased. Furthermore, addition of excess non-transfected APC reduced the suppressive effect of Treg cells (39). In a recent manuscript, Shevach and co-workers

determined that antigen-loaded DC co-cultured with adaptive Foxp3⁺ Treg cells displayed lower expression of CD80 and CD86, and that the down-modulation was most marked when Teff cells were also present in the co-cultures. In these *in vitro* assays, no preferential apoptosis of antigen-presenting DC occurred (20). Using human DC differentiated *in vitro* from blood monocytes, Misra *et al* reported that co-culture with Treg resulted in a reduction in CD86 and CD83 positive DC compared to co-culture with CD4⁺CD25⁻ T cells. The same trend was observed for CD80 but it did not reach statistical significance (40). In all three papers it was noted that the expression of costimulatory molecules on APC was augmented by co-culture with CD4⁺CD25⁻ T cells. Thus, *in vitro* experiments showed an impact of Treg on APC.

In vivo, the effect of Treg on APC-Teff cell interactions has also been described. Using intravital two-photon microscopy we have reported that, twenty hours after administration of antigen-loaded DC, the stable contact time between APC and responder T cells was reduced when regulatory T cells were present (21). Similar results were reported by Tang *et al* upon *ex vivo* analysis of explanted lymph nodes (22). Thus, *in vivo* there is an early effect of Treg cells that, depending on other conditions, may manifest as a reduced Teff expansion phase or may manifest at a later stage of the immune response.

Our data show that, in the presence of regulatory T cells, DC loaded with the antigen recognized by the effector T cells are targeted to die by apoptosis. We show here that in presence of regulatory T cells the survival signals received by DC when engaging Teff cells are reduced. A major unresolved question is whether the DC that undergo apoptosis are those in simultaneous contact with both regulatory and effector T cells. The net result of this mechanism is the reduction of activating antigen presentation to the precursors of effector T cells. The dying activated DC are constantly being replenished with new DC. This enhanced DC turnover in the presence of Treg cells, together with the observation that similar numbers of DC are found in mice with and without Treg cells, lends support the view that steady-state DC numbers are maintained by homeostatic mechanisms such as availability of DC growth factors. The proposed mechanism is also consistent with a publication showing a reduced recovery of OVA-loaded DC compared to unpulsed DC in draining lymph nodes of mice that also had received DO11.10 OVA-specific T cells (41). Although not the focus of the referred study, the reduced recovery of DC was observed in situations in which both OVA-specific (dual TCR-expressing) and polyclonal Treg cells were present. Our study shows that antigen recognition by regulatory T cells is not necessary. This important finding opens doors to the existence of an alternative mechanism of Treg action that does not require TCR activation.

Treg-mediated apoptosis of DC was not noted *in vitro* (20), but occurs *in vivo*. It is becoming increasingly apparent that, while the *in vitro* suppression assays are very useful tools to determine the existence of regulatory T cell activity in a particular population of cells, they may not reflect the mechanisms employed by Treg cells to control autoimmune or allergic diseases *in vivo*. For instance, we have shown some time ago that regulatory T cells are able to suppress the development of a Th2 response *in vivo* without affecting the initial expansion of responding T cells (27). The late manifestation of Treg action *in vivo* was also reported by Klein *et al* who observed that CFSE dilution of effector T cells was not impeded by transferred regulatory T cells (42). However, it is possible that under other conditions, in particular when the microenvironmental ratio of Treg to T effector cells is higher, the initial activation and proliferation of effector T cells could be affected by Treg also *in vivo*.

The longevity of DC has a great influence in immune responses. Mice bearing a DC-specific defect in Fas-mediated cell death develop systemic autoimmunity (43). DC from mice over-expressing a human Bcl-2 transgene under the CD11c promoter have a slower turnover, and immune responses in these mice are augmented (44). Similarly, mice expressing on their DC the caspase inhibitor baculovirus p35 protein driven by the CD11c promoter display enhanced immune responses, and old mice develop signs of systemic autoimmunity such as the accumulation of anti-DNA antibodies (45). Many factors affect DC longevity, such as the TNF-family member RANKL (TRANCE) (46), or the cytokine IL-10 which decreases the expression of anti-apoptotic genes (47). Regulatory T cells can produce IL-10. Tr1 cells are Foxp3⁻ adaptive regulatory T cells that work through secretion of IL-10, although regarding Foxp3⁺ Treg cells IL-10 production is required for their suppressive function in some conditions but not others (48-50).

Although we observed a strong correlation between the presence of Treg cells and the induction of DC apoptosis *in vivo*, we do not know if Treg cells are themselves killing the DC. We also shown that the survival signals received by DC loaded with relevant antigen are reduced in presence of Tregs. It has been shown that pre-activated Treg cells induce the apoptosis of B cells *in vitro*, and that preferentially kill those B cells capable of presenting antigen to Treg cells (51). The latter as well as other reports have shown that Treg cells do express molecules that could induce apoptosis in target cells (51-53).

At this moment we do not know the pathway(s) involved in the Treg cell-dependent DC apoptosis. Granzyme-B is a possible candidate, as it has been shown that in mouse regulatory T cells Granzyme-B expression is quickly upregulated on activation, and Granzyme-B-deficient Treg cells are severely hampered in their ability to suppress the proliferation of responder T cells *in vitro* (53). Fas-mediated apoptosis has been proposed

as a tolerizing mechanism exerted by *in vitro*-anergized human T cells, which share some properties with regulatory T cells (54). In support of the importance of the Fas pathway, it has been shown that mice that have deleted the Fas gene only in CD11c⁺ DC display signs of autoimmune disease, such as high titers of anti-nuclear antibodies, hyperimmunoglobulinemia, and splenomegaly (43). Another potential Treg cell effector mechanism could be mediated by Galectin-1, a lectin that recognizes N-acetyl-lactosamine residues and can induce cell death (55, 56). Galectin-1 is highly expressed by regulatory T cells and Galectin-1-deficient regulatory T cells have a decreased suppressive efficiency in *in vitro* assays (57). It is thus possible that DC are additional targets of Galectin-1-induced death.

Kim *et al* observed a remarkable increase in the number of DC seven days after diphtheria toxin-mediated elimination of Foxp3-expressing Treg cells (58). They also noted an augmented number of DC in lymph nodes of Foxp3-deficient mice or mice with life-long depletion of Foxp3-expressing Treg cells (58). Given the mutual activation between effector T cells and DC (19, 20, 40, 59), some of the dramatic results may be explained by the uncontrolled activation of effector T cells that occurs in the absence of Foxp3, activation which results in systemic accumulation of a number of cytokines (60). Consistent with this interpretation, in addition to dendritic cells, a number of hematopoietic lineages were greatly expanded seven days after Treg cell depletion (58). Furthermore, when the same authors analyzed TCR transgenic mice in which most T cells recognize a foreign antigen and are in a naive state, the absence of regulatory T cells did not result in an increased steady-state frequency of DC (58), a result also reported by our group (21). Lewkowich *et al* described the unexpected observation that, in a model of allergen-induced pulmonary inflammation, treatment of C3H and A/J mice with anti-CD25 antibody resulted in increased immune responses in C3H mice but not in A/J mice, although the antibody was equally effective in reducing Treg cell numbers in both mouse strains. Interestingly, in the C3H strain, anti-CD25-treated mice had increased numbers of pulmonary CD11b⁺ DC with elevated expression of MHC, CD80, and CD86 (61). Our analysis of DC turnover indicates that BrdU incorporation was higher in mice that have Treg cells than in mice that lack Treg cells, but only when the antigen recognized by the T cells was present. This occurred when T cells recognized an ubiquitous autoantigen, or, in the case of T cells recognizing a foreign antigen, when this antigen was administered to the mice.

It thus appears that Foxp3⁺ regulatory T cells carry out their suppressive function by a number of mechanisms, some acting on APC, and some more likely to directly affect responding T cells (62). We showed here that one of the mechanisms *in vivo* is the

induction of apoptosis of dendritic cells, a mechanism that explains many observations in the literature. It remains to be determined under which conditions each of the different mechanisms is utilized.

Material and Methods

Mice

MBP Ac1-11-specific TCR transgenic mice (T/R⁺ and Tg/Tg) have been described previously (21, 23, 25). Tg/Tg mice are T/R⁺ mice in which the TCR transgenes have been bred to homozygosity. Tg/Tg mice develop EAE spontaneously due to the lack of naturally-occurring Treg cells. The nomenclature T/R⁺ mice always refer to mice hemizygous for the TCR α/β integration.

T/Bmc were previously described (27). T/Bmc mice harbor monoclonal populations of T and B lymphocytes that are specific for chicken OVA₃₂₃₋₃₃₉ and HA of influenza virus, respectively. T/Bmc were crossed to Foxp3-deficient scurfy mice (Curotto de Lafaille et al., submitted) [note: attached for reviewers' perusal].

Mice expressing enhanced yellow fluorescent protein (YFP) under the control of the CD11c promoter have been described (34).

All mice were kept under specific pathogen-free conditions in individually ventilated cages (Thoren) at the Skirball Institute Central Animal Facility, New York University Medical Center. All protocols involving live mice were approved by New York University's Institutional and Animal Care and Use Committee.

Peptides and other reagents

The I-A^u-stable Y4 variant (AcASQYRPSQRSK) of the *N*-acetylated MBP 1–11 peptide (AcASQKRPSQRSK); chicken OVA₃₂₃₋₃₃₉ (ISQAVHAAHAEINEAGR) and HA₁₂₆₋₁₃₈ (HNTNGVTAASSHE) peptides were purchased from Invitrogen.

Oral antigen administration, immunization and BrdU administration

Oral tolerance to OVA was induced by offering to the animals, *ad lib*, a 1% OVA (grade II; Sigma-Aldrich) solution dissolved in drinking water for 8 consecutive days. Immunization with OVA protein was carried out performed with 100 μ g/mouse of OVA in PBS, i.v., 10-14 days after the end of the oral treatment.

Mice were injected twice a day with 1mg of BrdU (Sigma B5002 – 5G) i.p.

Cell preparation and transfer

DCs were obtained after positive selection of splenocytes from WT animals using anti-CD11c magnetic beads and columns (Miltenyi Biotec). The purities were always 90% or greater. DCs were transfer intravenously or subcutaneously, as described in each experiment.

Treg cells were purified from spleens of OVA-treated T/Bmc mice or from WT mice using anti-B220, CD8 and CD11c magnetic beads depletion followed by anti-CD25 PE anti-PE beads positive selection (Miltenyi Biotec).

FACS analysis and RNA analysis

Lymph nodes and spleens were made into single-cell suspensions in staining buffer (PBS containing 2% FCS and 0.1% NaN₃). Cells were filtered and stained for 45 minutes at 4°C with the antibody cocktails. Samples were analyzed in a FACScalibur instrument (BD) or LSRII instrument (BD). All analysis was based on a live lymphocyte gate.

For annexin V (Pharmingen) analysis, samples were stained with monoclonal antibody cocktails as described above, and, after washing, samples were incubated in a buffer solution containing Ca²⁺ (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂) and annexin V-PE (BD Biosciences/Pharmingen) for 10 minutes at 4°C, in the dark. All samples were analyzed in a FACScalibur, as described above.

For Foxp3 staining, samples were first stained with the antibody against the relevant surface markers, then permeabilized with Cytofix/Cytoperm at room temperature for 20 minutes, followed by another round of permeabilization with 1% paraformaldehyde plus 0.5% Tween-20 in PBS at room temperature for 30 minutes, then stained with anti-Foxp3-PE antibody (eBioscience) at room temperature for 1 hour.

For anti-BrdU staining, samples were treated as described for Foxp3 staining up to after second round of permeabilization, then the samples were treated with DNase I (Sigma D4263) at 50 krut units in DNase I buffer (150mM NaCl, 4.2mM MgCl₂, pH 5) for 15 minutes at 37°C and then stained with anti-BrdU antibody (CALTAG Laboratories) at room temperature for 1 hour. All samples were analyzed in a LSRII (BD) instrument, as described above.

For quantitative mRNA expression analysis transferred DC previously loaded with OVA or HA or endogenous DC were MoFlo-sorted. RNA was extracted from samples using TRIzol, and cDNA was synthesized using standard procedures. The expression of Bcl-X_L and β -actin was determined by quantitative real-time PCR using the primers below.

Expression was normalized to the level of β -actin in each sample: Bcl-X_L, GTCGCCGGAGATAGATTTGA, TGTCCAAAACACCTGCTCAC; β -actin, ACGGCCAGGTCATCACTATTG, CACAGGATTCCATACCCAAGAAG.

In situ apoptosis

TUNEL assay (Roche) was performed. Slides containing 5- μ m cryosections of spleen were fixed in 1% paraformaldehyde in PBS, for 30 minutes at room temperature. Slides were then washed 3 times with PBS, and a permeabilization buffer (1% vol/vol Triton X-100 and 1% wt/vol of sodium citrate, in deionized and distilled H₂O) was added over each slide for 2 minutes, at 4°C. Subsequently, each slide was washed twice, and a labeling solution (In Situ Cell Detection Kit, Fluorescein; Roche Diagnostics Corp.) was added following the manufacturer's instructions. At this point, slides were incubated for 1 hour, at 37°C, in a humidified dark chamber. At the end of this incubation, each slide was washed twice with PBS and blocked for 30 minutes at room temperature with PBS plus 10% normal mouse serum (Cedarlane Laboratories Ltd.) plus Fc Block (BD Biosciences/Pharmingen). After blocking, APC-labeled CD11c monoclonal antibody (CALTAG Laboratories) was added, and slides were incubated for 2 hours at room temperature. Finally, each slide was washed again with PBS and mounted with Fluoromount-G (SouthernBiotech). The blocking and labeling were performed in a humidified dark chamber. Images were acquired using SlideBook version 3.0.9.0 software (Intelligent Imaging Innovations Inc.) in an Axioplan 2 Zeiss microscope.

TUNEL assay (Roche) was performed for FACS analysis in a similar way than described above.

CFSE labeling

CFSE (Molecular Probes; Invitrogen Corp.) was added to DC suspensions (1×10^7 cells/ml) at a final concentration of 1 μ M, 37°C, for 10 minutes; then reaction was stopped with FCS at a final concentration of 10%, and cells were washed twice with PBS and injected intravenously. For DC mixture experiments, CFSE was used at final concentration of 2.5 μ M or 0.25 μ M.

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Supplement material

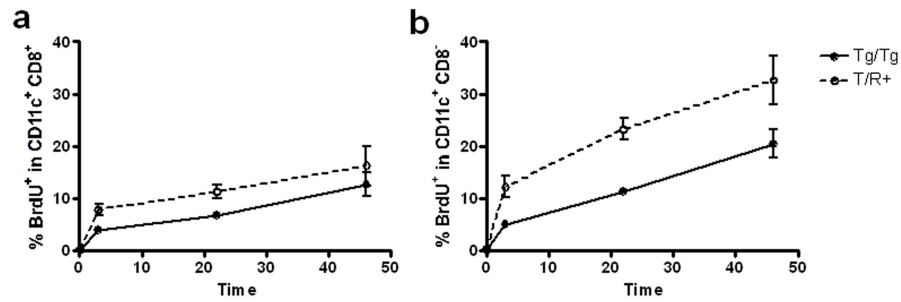


Figure S1. The turnover of CD8⁻ DC is more affected by the presence of Treg cells than the turnover of CD8⁺ DC.

Four to six week old T/R⁺ mice (dashed line) and Tg/Tg (full line) mice were injected with BrdU (1mg/mouse) twice a day and spleens analyzed at the indicated times after first BrdU injection. BrdU incorporation profiles of (a) CD11c⁺CD8⁺ DC, and (b) CD11c⁺CD8⁻ (mostly myeloid) DC.

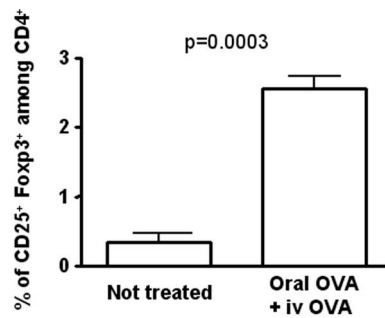


Figure S2. Adaptive Treg cells persist in T/Bmc mice.

Adaptive Treg cells were induced as shown in Figure 2a and mesenteric lymph nodes (mLN) from the mice were analyzed five weeks later. Controls mice did not receive any treatment, p=0.0003, p value with or without adaptive Treg cell induction. Average of 4-5 mice per group is presented.

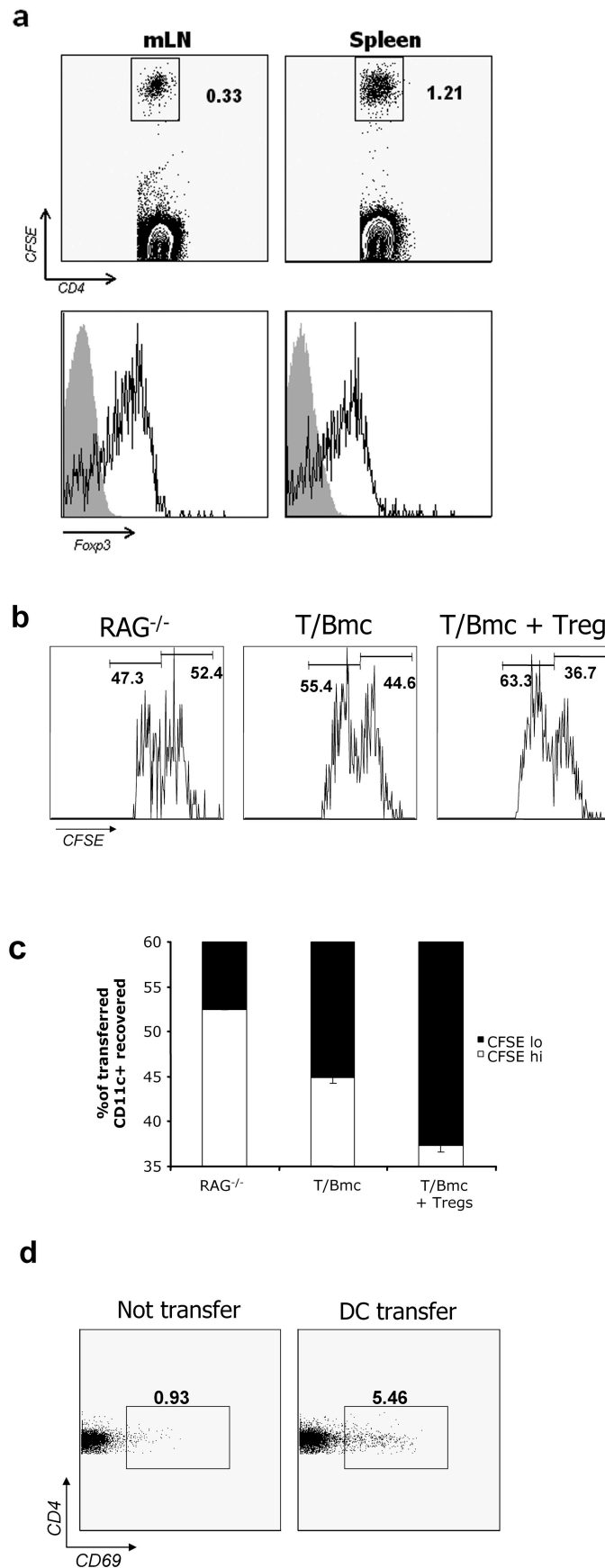


Figure S3. Treg cell transfer into T/Bmc mice impacts on antigen-loaded DC recovery.

(a) Control experiment for Treg generation and transfer. Flow cytometry of CFSE-labeled adaptive OVA-specific Treg cells purified from T/Bmc mice spleens and injected i.v. into naïve T/Bmc. Adaptive OVA-specific Treg cells were generated as described in figure 2a. Four days after the OVA i.v. injection, CD4⁺CD25⁺ cells were purified from spleens, labeled with CFSE, and transferred into naïve T/Bmc mice. Thirty hours after transfer spleens and mLN were analyzed for the presence of transferred Treg cells. Upper panels represent the percentages of CFSE⁺ within CD4⁺ cells. Bottom panels show histograms of Foxp3 expression. Filled histograms represent CD4⁺CFSE⁺ (recipient) cells and back line histograms represent CD4⁺CFSE⁺ (donor) cells. (b) Representative histograms of DC isolated from the 3 recipient groups six hours after being transferred with the inverse DC mixture as in Figure 4b: in this case, DC loaded with OVA peptide were labeled with 2.5 μ M of CFSE (CFSE^{high}) and DC loaded with HA peptide were labeled with 0.25 μ M of CFSE (CFSE^{low}). Recipient mouse groups were: RAG-1^{-/-} mice; T/Bmc mice, and T/Bmc mice that received adaptive OVA-specific T reg cells (T/Bmc + Treg). (c) Percentage ($\pm$ SEM) of OVA-loaded CD11c⁺CFSE^{high} (black) and HA-loaded CD11c⁺CFSE^{low} (white) CD11c⁺ cells recovered from the spleens of mice each experimental group, six hours after DC mix transfer. Note the partial scale. (d) Analysis of CD69 expression on gated CD4⁺ splenic T cells six hours after DC mix transfer. Left panel: control mice, untreated T/Bmc. Right panel: T/Bmc mice that received the DC mixture.

Chapter VI

General Discussion

During the past few years we performed several experiments that are now in this thesis that aim to contribute to clarify several aspects of regulatory T cell (Treg) biology. One of the most remarkable aspects, and one of the few that is beyond doubt, is the fact that Tregs are a small population among the CD4⁺ T cells. Due to their importance in controlling autoimmune diseases, resolving immune responses to non-self and beneficial role in transplant tolerance (reviewed in [1-3]) a number of approaches have been tried in order to establish a way to expand or generate them from naïve CD4⁺ T cells [4-10], but Treg use for therapeutic purposes remains challenging.

In chapter 2, we showed that a way to use regulatory T cells in therapy could be by enhancing their survival. We showed that stabilization of β -catenin enhances regulatory T cell survival. β -catenin have a central role in the Wnt signaling pathway, reported to control homeostatic self-renewal in adult tissues and to have a role in almost every aspect of embryonic development, maintaining the stem cell pluripotency (reviewed in [11]). The use of a β -catenin with four point mutations results in the increase of its half-life and so its concentration in the nucleus where it performs transcriptional activity [12]. We showed that transducing regulatory T cells with this stabilized form of β -catenin resulted in up-regulation of Bcl-X_L, that results in enhance survival of the cells both *in vitro* and *in vivo*. The β -catenin-transduced regulatory T cells are anergic and suppressive *in vitro* and *in vivo*. Using the inflammatory bowel disease (IBD) model, we showed that fewer β -catenin-transduced regulatory T cells were needed to control IBD than the non-transduced T cells. Moreover, we showed that stabilization of β -catenin in CD4⁺CD25⁻ non-regulatory T cells induced anergy in these cells, resulting in a similar anti-inflammatory effect. The induction of anergy in these later cells did not depend on Foxp3 up-regulation, since anergy of naïve T cells from *scurfy* mice was induced by stabilization of β -catenin in these cells.

The findings reported in chapter 2 open the possibility for clinical use of regulatory T cells by the pharmacological control or modification of certain pathways (for instance Wnt- β -catenin pathway) or by using gene therapy, adding suicide genes, to enhance regulatory T cell survival and consequently their activity. Our findings allow the possibility of using autologous transfusions to control inflammatory pathologies, transducing regulatory T cells with stabilized form of β -catenin in the collected blood. By adding a suicide gene to the genetic construct, transduced cells can be eliminated when not needed. When thinking in clinical uses for this pathway it's important to mention the absence of malignancy in mice that receive the stable β -catenin-expressing T cells. This fact can be

explained by the observed decrease in c-Myc mRNA levels that correlated with Bcl-X_L up-regulation.

As mentioned in chapter 1, CD4⁺CD25⁻ cells have been shown to have regulatory T cell activity in several experimental systems, such as animal models of autoimmune encephalomyelitis [13, 14], allergy [15], diabetes [16-18] and inflammatory bowel disease [19]. In chapter 3, we shed light into this apparent paradox by demonstrating that upon adoptive transfer of CD4⁺CD25⁻ T cells, some of these cells become CD4⁺CD25⁺ regulatory T cells by homeostatic proliferation. These later cells displayed markers of freshly isolated regulatory T cells and are anergic and suppressive *in vitro*. Furthermore, we also showed that the maintenance of CD25 expression by transfer CD4⁺CD25⁺ cells is dependent on IL-2 secreted by other cells.

A considerable number of recent studies reported that regulatory T cells can be induced in the periphery using different protocols [7-10, 20-30]. Some of these protocols produce cells that are virtually indistinguishable from naturally occurring regulatory T cells [8]. In chapter 3, we showed that CD4⁺CD25⁺ T cells generated by homeostatic proliferation of transferred CD4⁺CD25⁻ T cells also behave like naturally occurring regulatory T cells. We showed that donor-derived CD4⁺CD25⁺ T cells, but not donor-derived CD25⁻, display high levels of Foxp3 mRNA and express GITR, CTLA-4, and CD103 and are anergic and suppressive *in vitro*. Therefore, phenotypically and functionally, CD4⁺CD25⁺ T cells arising from CD4⁺CD25⁻ T cells that undergo homeostatic proliferation were indistinguishable from freshly isolated CD4⁺CD25⁺ T cells.

The results presented in chapter 3 allow us to explain why CD4⁺CD25⁻ T cells produced by depletion of CD25⁺ T cells can exert regulatory T cell activity but CD25^{-/-} T cells were unable to do so [31]. CD25 must be expressed for T cells to exert suppression. Antibody depletion of CD25⁺ cells was used to show that CD25⁻ T cells generated regulatory T cells [10, 26, 28, 29]. The anti-CD25 antibody treatment leave behind few CD25⁺ T cells, making it very unlikely that these few contaminants are the ones giving rise to CD25⁺ T cells, although this possibility can not be formally excluded. Similarly, it is very unlikely that CD25⁺ T cells generated by peripheral expansion of CD25⁻ T cells that were obtained from TCR-transgenic SCID or RAG^{-/-} mice [8, 23], with virtually no CD25⁺ T cells before Treg induction, come from the outgrowth of pre-existing CD25⁺ T cells. In chapter 3, we demonstrate by contaminating the donor CD25⁻Thy1.2⁺ cells with 0.5% of CD25⁺Thy1.1⁺ that Tregs are indeed generated through homeostatic proliferation of CD25⁻ T cells and do not result from an outgrowth of pre-existing CD25⁺ T cells.

In chapter 3, we also showed that the CD45RB^{low} fraction of CD25⁻ T cells is kinetically more efficient than CD45RB^{hi} fraction (naïve cells and a larger fraction of CD25⁻ T cells) to generate CD25⁺ regulatory T cells. This result indicates that CD45RB^{low} fraction of CD25⁻ is enriched in regulatory T cell precursors. The fact that CD25⁺ T cells give rise to CD25⁻ T cells upon homeostatic proliferation exclude the possibility that the CD45RB^{low} fraction of CD25⁻ T cells were in a later differentiation state that led to regulatory T cell than the CD45RB^{hi}. The donor-derived CD25⁻ T cells displayed lower suppressor activity, but remained largely unresponsive to *in vitro* stimulation.

The capacity of CD25⁻ cells to generate CD25⁺ Tregs doesn't seem to be limitless. Retransfer of donor-derived CD25⁻ T cells into secondary lymphopenic recipients generates CD25⁺ T cells but the secondary recipients showed a 5-fold decrease in the fraction of donor-derived CD25⁺ cells when compared with the first recipients (data not shown). The fewer donor-derived CD25⁺ generated by homeostatic proliferation of retransferred CD25⁻ displayed high levels of Foxp3 mRNA and were similar to naturally occurring Tregs (data not shown). The appearance of fewer CD25⁺ T cells in the secondary recipients could be explained by the possibility that only some TCR chains are compatible with regulatory T cell generation [13, 32]. Therefore, the population of donor-derived CD25⁻ that is retransferred has been purged of the majority of T cell clones that have TCRs compatible with regulatory T cell generation. Being a possibility, this explanation is not fully satisfactory, and there are some existing data argue against it. For example, FACS analysis of TCR V α and V β gene usage of the periphery converted CD25⁺ showed a broad diversity of TCR chains usage, similar to the freshly isolated CD4⁺ T cells from BALB/c mice. Other studies showed that conventional and regulatory T cells display overlapping TCR repertoires [33-35]. Moreover, transgenic expression of a TCR cloned from a regulatory T cell isolated from the thymus do not generate mice with regulatory T cells only (S. Shen and J. Lafaille unpublished observations). And when these mice were further crossed into RAG^{-/-} background they behave like all the other TCR transgenic mice in RAG^{-/-} background, they are devoid of regulatory T cells [36].

In vitro, IL-2 has proliferative properties and CD4⁺CD25⁺ T cells exert their suppressive activity by preventing IL-2 production by effector T cells [37-39]. In chapter 3 we showed that, *in vivo*, the situation is not quite the same. The production of IL-2 by cells other than Tregs, is necessary for the maintenance of CD25 expression and for the maintenance of suppressor phenotype. The co-transfer of CD4⁺CD25⁻ T cells that are IL-2 sufficient together with CD4⁺CD25⁺ T cells allow for the majority of these later cells to maintain the CD25 expression, whereas when CD4⁺CD25⁻ T cells from IL-2 deficient mice were used a substantial fewer number of CD4⁺CD25⁺ T cells were able to maintain CD25 expression.

Therefore, conventional T cells help maintaining a functional Treg compartment, through the production of IL-2.

Knowing what happen with lymphocytes, and with regulatory T cells in particular, under conditions of homeostatic proliferation is important and can have therapeutic implications. Homeostatic proliferation is likely to be continuously taking place. The peripheral conversion of CD25⁻ cells into cells with regulatory T cells activity has probably an essential role in maintaining the balance of the immune system. Interestingly enough, Tregs play a major role in the control of homeostatic proliferation. Naïve T cells transferred into animals with regulatory T cells showed no proliferation and enhanced apoptosis, whereas these naïve cells were able to proliferate in TCR Tg animals that lack Tregs [40]. Homeostatic proliferation plays a relevant role in a number of physiological and pathologic situations, like in newborn or aging immune systems as well as in individuals undergoing chemotherapy or with chronic infections that alter the thymic output. Certain cancer treatments cause partial lymphopenic state, causing homeostatic proliferation. As referred before, IL-2 is essential in the generation of CD25⁺ from CD25⁻ by homeostatic proliferation. IL-2 therapy can be used in cancer immunotherapy leading to significant improvements [41, 42].

Despite the fact that regulatory T cells are essential to maintain immune tolerance and have a prominent role in the down-modulation of immune responses, little is know about how they act *in vivo*. In chapters 4 and 5 we presented data that aim to shed light into the mechanism of action of regulatory T cells *in vivo*.

In chapter 4, we used intravital two-photon laser scanning microscopy to actually be able to visualize an *in vivo* immune response happening in an intact lymphoid organ in presence or absence of regulatory T cells. We found that in the presence of regulatory T cells, T cells and antigen-loaded DC establish encounters of shorter duration than when regulatory T cells are absent. This result indicates that regulatory T cells exert an effect during priming of naïve T cells by DCs.

In the study on chapter 4, we used the myelin basic protein (MBP) Ac1-11-specific TCR transgenic mice [32]. In particular, the hemizygous T/R⁺ animals harboring regulatory T cells and homozygous TgTg mice lacking them. The use of TgTg mice as mice lacking regulatory T cells instead of T/R⁻ mice (mice that also lack Tregs), was due to the nature of this study. It has been shown that an intact lymphoid organ structure is of enormous importance for a normal immune response [43, 44]. Due to the presence of B cells (absent in T/R⁻ mice) TgTg animals displayed a normal structural organization of the lymphoid organs, in particular, the popliteal lymph nodes (pLN). Moreover, we showed

that pLN of TgTg and T/R⁺ are very similar in terms of cellularity. They displayed similar proportions of DC sub-populations and equal numbers of MBP-specific T cells with naïve phenotype, suggesting that endogenous MBP is not presented in the pLN of TgTg or T/R⁺ mice at the age that the experiments were done. Therefore, this situation allowed an external delivery of the antigen. The delivery of MBP Ac1-11 emulsified in incomplete Freund's adjuvant (IFA) by subcutaneous injection in the foot pad was proved to be an effective strategy to delivery antigen to DC preserving regulatory T cell function in T/R⁺ mice. This protocol triggered IL-2 production by MBP-specific T cells in the pLN of TgTg mice but not in T/R⁺ mice, due to the presence of Foxp3 expressing cells in the later, but not in the previous mice.

Being well established a strategy that allowed regulatory T cells to perform suppression in vivo when present, we used two-photon intravital microscopy to compare cell mobility and cell interaction in mice that have or lack regulatory T cells. We showed that in absence of immunization MBP-specific CD4⁺ T cell migration is similar in the presence or absence of regulatory T cells. In absence of antigen, T cells do not establish stable contacts with dendritic cells. By the same token, in presence of MBP, antigen-specific CD4⁺ T cells slowed their motion but only in absence of regulatory T cells. In T/R⁺ mice, MBP-specific CD4⁺ T cells did not significantly change their speed, but they increased the time that they are not moving, measured by the arrest coefficient, that is low when T cells do not recognize peptide-MHC complexes on DCs. In TgTg mice, MBP-specific CD4⁺ T cells decrease their speed and increase their arrest coefficient. These results suggested that regulatory T cells act in presence of antigen, avoiding the establishment of contacts of longer duration between MBP-specific CD4⁺ T cells and antigen-loaded DCs. We showed that this was indeed the case by imaging directly MBP-specific CD4⁺ T cells and antigen-loaded DCs. We showed that transfer of antigen-loaded DCs did not changed qualitatively or quantitatively the MBP-specific CD4⁺ T cell movement when compared with the movement of these cells in MBP-immunized animals. Therefore, the transfer of antigen-loaded DCs allowed us to measure and compare the contact time between antigen-loaded DCs and MBP-specific CD4⁺ T cells in presence or absence of regulatory T cells. We showed that there is a threefold reduction in contact time in presence of regulatory T cells. We confirmed the reduction of MBP-specific CD4⁺ T cell and antigen-loaded DC interaction in presence of regulatory T cells by reconstituting regulatory T cell deficient mice (TgTg) with WT polyclonal CD4⁺CD25⁺ T cells or control CD4⁺CD25⁻ T cells. We showed that TgTg animals that received CD4⁺CD25⁺ T cells displayed shorter CD4-DC interaction time than TgTg animals that received CD4⁺CD25⁻ T cells. The effect of polyclonal Tregs was not as great as the effect observed in T/R⁺ mice, with

endogenous generated regulatory T cells. This difference can be explain by issues related with purification of the transferred cells, or even regulatory T cells numbers present in the lymph node or the increase effect observed with endogenously generated Tregs compared with WT polyclonal CD4⁺CD25⁺ T cells can be due to the presence of MBP-specific regulatory T cells in T/R⁺ mice. It has been shown that some CD4⁺CD25⁺ Treg cells in T/R⁺ mice are double TCR-expressing cells, therefore MBP-specific Treg cells [45]. It is although very clear that WT polyclonal CD4⁺CD25⁺ T cells were able to diminish the duration of the contact time between MBP-specific CD4⁺ T cell and MBP-loaded DC. Moreover, we showed that this phenomenon is antigen dependent, since, in absence of antigen the baseline T cell migration is similar in T/R⁺ and TgTg mice. This question will be further clarified when discussing the data presented in chapter 5.

Similar findings to the ones presented in chapter 4 were reported by others [46]. Using two-photon *in vitro* imaging of explanted pancreatic LNs and the nonobese diabetic mouse model, they showed that endogenous regulatory T cells decrease the duration of the interaction between autoantigen-specific T cells and DCs bearing islet antigen. By direct imaging the Tregs the authors could not detect any stable associations between regulatory T cells and autoantigen-specific T cells during suppression. In contrast, Tregs cells established long-lived interactions with dendritic cells bearing islet antigen.

In chapter 4, using real-time intravital microscopy imaging we showed that one of the ways that Treg cells act *in vivo*, affecting the immune response, is by decreasing contact time between antigen-loaded DCs and effector T cell precursors that can recognize and be activated by these antigen-loaded DCs. Regulatory T cells did not affect the speed or the arrest coefficient of polyclonal CD4⁺ T cells that were co-transferred with MBP-specific CD4⁺ T cells.

In chapter 5, we presented data that resulted from further investigation of a reoccurring observation: we repeatedly recovered fewer transferred DCs in the pLN of T/R⁺ than Tg/Tg mice. We showed that while in steady state the number of DC in mice with or without Treg cells was approximately the same, BrdU-labeling experiments indicated that DC turnover was faster in mice that harbored Treg cells. Furthermore, there were more apoptotic dendritic cells in animals harboring Treg cells. We also found that Treg induced apoptosis of DC loaded with OVA, but not of DC loaded with an irrelevant antigen when T_{eff} cells were OVA-specific. We proposed that one of the mechanisms of Treg-dependent peripheral tolerance is the induction of apoptosis of antigen-loaded DC.

From early on in Treg cell research, the relative ineffectiveness of *in vitro* suppression observed in assays conducted in the absence of APC as well as the difference in quality

of suppression depending on the cells used as APC pointed toward an effect of regulatory T cells on APC [47]. This idea become even more appealing after the imaging studies presented in chapter 4 as well as the direct imaging of Tregs in which the authors could not detect any stable associations between regulatory T cells and autoantigen-specific T cells during suppression, but Treg were found to establish long-lived interactions with dendritic cells bearing islet antigen [46]. These later studies indicated that modulation of APCs could be a main mechanism of regulatory T cell action *in vivo*.

In chapter 5, we showed that, in the presence of regulatory T cells, DC loaded with the antigen recognized by the effector T cells are targeted to die by apoptosis. We showed that in presence of regulatory T cells the survival signals received by DC when engaging Teff cells are reduced. A major unresolved question is whether the DC that undergo apoptosis are those in simultaneous contact with both regulatory and effector T cells. Although we showed a strong correlation between presence of Tregs and the induction of antigen-loaded DC apoptosis our results do not allowed us to know if regulatory T cells are directly killing DC. But this is certainly a possibility that is supported by reports showing that regulatory T cells express molecules that could induce apoptosis in target cells [48-50].

The net result of the mechanism we described in chapter 5 is the reduction of active antigen presentation to the precursors of effector T cells. The dying activated DC are constantly being replenished with new DC. This enhanced DC turnover in the presence of Treg cells, together with the observation that similar numbers of DC are found in mice with and without Treg cells, lends support the view that steady-state DC numbers are maintained by homeostatic mechanisms such as availability of DC growth factors. The proposed mechanism is also consistent with a publication showing a reduced recovery of OVA-loaded DC compared to unpulsed DC in draining lymph nodes of mice that also had received DO11.10 OVA-specific T cells. Although not the focus of the referred study, the reduced recovery of DC was observed in situations in which both OVA-specific (dual TCR-expressing) and polyclonal Treg cells were present [51]. Using two different experimental systems our study clearly showed that antigen recognition by regulatory T cells is not necessary, since in mice transferred with polyclonal Tregs we could observed a preferential reduction of DC that were loaded with antigen recognize by T effector cells. This important finding opens doors to the existence of an alternative mechanism of Treg action that does not require TCR activation. Further experimentation is needed to identify the molecule and/or pathway involved in this process. Its identification and manipulation can constitute a therapeutic tool in the treatment of autoimmune diseases, since it opens

the possibility of transfer of pre-activated regulatory T cells. It has been shown that *in vitro* pre-activated regulatory T cells can suppress CD8⁺ T cells responses [52].

It's important to note that regulatory T cell mediate suppression by induction of apoptosis of antigen loaded-DC is an antigen dependent and specific process. We did not observe increase in BrdU incorporation by endogenous DC in mice harboring regulatory T cells (T/B mc R^{+/-}) in absence of antigen. Moreover, we only observed preferential reduction of DC when Teff cells were able to associate with antigen-loaded DC through TCR, but not when polyclonal T effectors cells were present.

We showed that polyclonal Treg could induce apoptosis of antigen loaded-DC that are recognized by effector T cells. It remains to be determined if antigen specific regulatory T cells are somehow more effective than polyclonal Treg. Although our results suggested that this is not the case, this possibility was not formally discarded.

Leon et al [53] proposed and reviewed [54] a model that describes the peripheral dynamics of antigen presenting cells, effector T cells and regulatory T cells. This three-partner crossregulation model imposes that regulatory T cell persistence and expansion depends on specific interactions they establish with APCs and effector T cells. The data presented in chapter 5, support and stresses the importance of the tri-directional crosstalk between the tree cell types predicted by the crossregulation model. Furthermore, our data suggest that there is another molecular mechanism for APC-dependent suppression of effector T cells by regulatory T cells that does not depend on regulatory T cell specificity.

The induction of apoptosis of antigen-loaded DC by regulatory T cells is likely to be a mechanism by which regulatory T cells prevent autoimmunity. Supporting this idea is the fact that mice with a DC-specific defect in Fas-mediated cell death develop systemic autoimmunity, reveled by high titers of anti-nuclear antibodies, hyperimmunoglobulinemia and splenomegaly [55]. Similar observations were reported for mice expressing a caspase inhibitor driven by CD11c promoter [56]. Mice over-expressing a Bcl-2 transgene under the control of CD11c promoter display augmented immune responses and DC show a slower turnover [57]. The longevity of DC has an obvious influence in immune responses. We showed here that, in presence of an autoantigen such as MBP, or in the case of T cells recognizing a foreign antigen, such as OVA, the presence of antigen increased BrdU incorporation by DC in presence of regulatory T cells. This result suggests that regulatory T cells can prevent autoimmunity by limiting the longevity of antigen loaded-DC. At this moment we do not know the pathway(s) involved in Treg cell-dependent DC apoptosis.

Kim *et al* observed a remarkable increase in the number of DC seven days after diphtheria toxin-mediated elimination of Foxp3-expressing Treg cells [58]. They also noted an augmented number of DC in lymph nodes of Foxp3-deficient mice or mice with life-long depletion of Foxp3-expressing Treg cells [58]. Given the mutual activation between effector T cells and DC [47, 59-61], some of the dramatic results may be explained by the uncontrolled activation of effector T cells that occurs in the absence of Foxp3, activation which results in systemic accumulation of a number of cytokines [62]. Consistent with this interpretation, in addition to dendritic cells, a number of hematopoietic lineages were greatly expanded seven days after Treg cell depletion. Furthermore, when the same authors analyzed TCR transgenic mice in which most T cells recognize a foreign antigen and are in a naive state, the absence of regulatory T cells did not result in an increased steady-state frequency of DC [58].

In chapter 5, we showed that induction of apoptosis of antigen loaded-DC is one of the mechanisms of regulatory T cell dependent suppression (Figure1). It remains to be determined under which conditions this mechanism is effective.

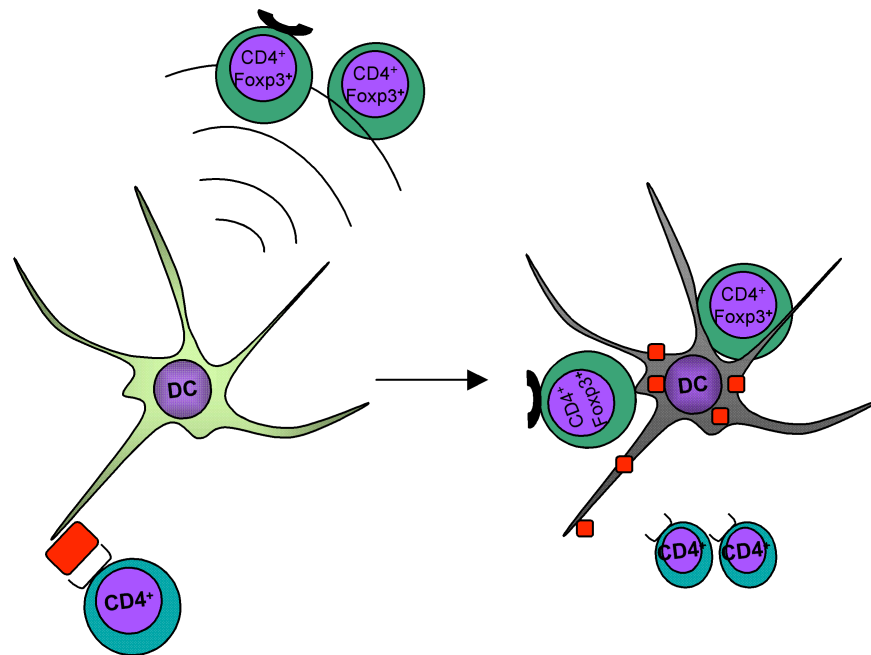


Figure 1: Induction of apoptosis of antigen loaded-DC is one of the mechanisms of regulatory T cell mediated suppression. Dendritic cells (DC) loaded with antigen recognized by CD4⁺ T cells are target to die by apoptosis by CD4⁺Foxp3⁺ regulatory T cells, in a TCR independent manner. Thus, due to the elimination of DCs, regulatory T cells eliminate the activating stimulus for effector T cell proliferation and expansion suppressing the immune response.

In conclusion, we showed that Beta-catenin stabilization extends regulatory T cell survival and induces anergy in nonregulatory T cells, opening the possibility of using this pathway for therapeutic purposes; that CD25⁻ T cells generate CD25⁺Foxp3⁺ regulatory T cells by peripheral expansion; that regulatory T cells inhibit stable contacts between CD4⁺ T cells and dendritic cells *in vivo* and that one of the mechanisms by which regulatory T cells carry out their suppressive function, *in vivo*, is the induction of apoptosis of antigen-loaded dendritic cells.

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