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Control of immune responses towards self and non-self by Foxp3$^+$ regulatory T cells

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1. Abstract

The immune system is composed of a complex network of cells and molecules providing the body with a highly sophisticated defence system against invading pathogens. Generally, the immune system can be divided into two major parts, the innate and the adaptive immune system. The innate branch of the immune system responds mainly to so-called danger signals in an antigen non-specific manner, whereas the adaptive branch is composed of highly antigen specific cells with an enormous repertoire of different antigen receptors. Our immune system is highly evolved, but problems can still occur when immune responses to particular antigens are not balanced correctly or, going further, adaptive immune cells recognize self or harmless foreign antigens. Several regulatory mechanisms have emerged to minimize potential risks mainly provided by self-antigen specific T cells. These include several immunosuppressive mechanisms comprising many different molecules and enzymes, but also highly specialized cell types. Probably the most potent system to avoid self-destruction is negative selection of self-reactive T cells in the thymus. Nevertheless, it has been shown that negative selection is not complete, giving evidence that there must be an additional system in the periphery dealing with effector T cells that have escaped this selection. The mechanisms by which immune homoeostasis and self-tolerance are achieved can be separated into two major types called recessive and dominant. The recessive mechanisms are cell-intrinsic, leading for example to apoptosis of responding T cells. Dominant mechanisms are cell-extrinsic. Here, certain T cells actively dampen the activation and expansion of aberrant or over-reactive lymphocytes. The goal of this work is to better understand how and when the most potent suppressive cells of the body, regulatory T cells, function. As part of the adaptive immune system, regulatory T cells are potentially capable to act in an antigen-specific manner and they are believed to be an important instrument of the immune system to dampen unwanted immune responses towards certain antigens, mostly towards self. As a tool to investigate regulatory T cells,

bacterial artificial chromosome technology was chosen, allowing for monitoring and manipulation of these cells directly *in vivo* by cell type specific introduction of the appropriate reporter. Using novel transgenic mice, the role of regulatory T cells in general for the maintenance of self-tolerance was explored. In a following study, the dependence of regulatory T cells on their major transcription factor Foxp3 and their behaviour in the absence of Foxp3 was assessed. It could be shown that regulatory T cells are very efficient and also necessary to secure the body from self-destruction by the immune system. However, too many regulatory T cells would potentially be deleterious to desired immunity, circumventing, for example, anti-tumour responses or efficient vaccination. The particular role of regulatory T cells in these settings has been addressed in a work where development of protective immunity was measured following a strong vaccination protocol in presence or absence of regulatory T cells. It was further shown that tolerance induction upon certain stimuli was dependent on Foxp3$^+$ Tregs rather than mediated by other regulatory mechanisms.

2. Introduction

First evidence for existence of regulatory T cells was given in 1969 by Nishizuka and Sakakura who showed induction of autoimmunity by day-3 thymectomy of newborn mice. In 1970, Gershon and Kondo could show that regulation was performed by a specific T cell subset, termed suppressor T cells at that time [1]. 15 years later, Hall *et al.* found first evidence for the accumulation of suppressor T cells within the $CD4^{+}CD25^{+}$ T cell subset. In 1995, Sakaguchi and colleagues finally proved existence of T cells with regulatory function by adoptively transferring $CD4^{+}CD25^{+}$ into day-3 thymectomized mice. Reconstitution of the regulatory T cell subset, as they were called since then, lead to abrogation of the disease [2].

2.1. Regulatory T cell biology

Among the T cell subsets, different cells with suppressive capacity have been described. Generally, those types can be divided into two major groups, the natural occurring regulatory T cells and the adaptive regulatory T cells that are induced in the periphery in response to distinct stimuli (e.g. T_H3 and T_R1 cells) [3]. Natural regulatory T cells (Tregs) generated in the thymus are considered to be the most potent and stable T cell subset exhibiting suppression, and are capable of controlling autoimmunity through dominant tolerance [4]. Thymectomy in newborn mice and rats leads to death due to multiorgan inflammation, showing that self-reactive potentially pathogenic T cells are dominantly controlled by thymus-derived regulatory T cells in the periphery, which leads to the circumvention of chronic T cell-mediated autoimmunity and immunopathology.

Not every single T cell subtype exhibiting regulatory function expresses Foxp3 (forkhead box p3), but it is broadly accepted that this is the major transcription factor driving suppressive

capacity [5, 6], and that Foxp3 expression highly correlates with regulatory T cell function [7-9]. Not only natural regulatory T cells express Foxp3: it is also up regulated in non regulatory $CD4^+$ T cells under specific conditions, converting these cells in the periphery into induced regulatory T cells which can be as competent as natural Tregs in certain settings [10]. Consequently, Foxp3 is accepted to be the master regulator of Treg function. The high specificity of this protein for regulatory T cells in general also allows for using it as an excellent marker to investigate regulatory T cells *in vivo* (see appendix 1). Other markers (e.g. CD25, glucocorticoid-induced TNF receptor family-related gene/protein (GITR), or cytotoxic T cell associated antigen-4 (CTLA-4)) have been useful in the past for studying Treg role and function, but it is accepted that specificity is not complete, mostly due to up regulation of the different proteins in activated, non-regulatory T cells. Additionally, Foxp3-expressing regulatory T cells negative for CD25 can be found in the periphery, up-regulating the receptor only after TCR engagement [8, 11]. Since the distinction of regulatory T cell subsets is very controversial, this work will focus on the role of regulatory T cells expressing Foxp3, including both natural and induced regulatory T cell subsets.

2.1.1. Mechanisms of immune regulation by Tregs

Several mechanisms by which Tregs exert suppression are currently discussed. Depending on the site of action and the target effector cell, types of suppression may vary. Most Tregs have been shown to suppress autoreactive T cells directly in the regional lymph nodes [12] whereas they are also capable of dampening an immune response in inflamed tissues, tumours, and infectious sites [13]. *In vitro*, Tregs suppress proliferation and cytokine production by responder T cells [14, 15]. Beside the suppression of T cells, Tregs have also been shown to be capable of regulating other cell types such as dendritic cells, macrophages, B cells and natural killer cells (reviewed in [16-19]). Most likely, not only one suppressive mechanism is

used and the outcome of immune-suppression may result from the joint action of different suppressive cytokines and surface molecules. Suppression can therefore be executed by partially redundant mechanisms such as secretion of immunosuppressive cytokines, cell-contact-dependent suppression (e.g. direct lysis of target cells), suppression by metabolic disruption, and functional modification or killing of antigen presenting cells. In the following, some of the most important mechanisms are summarized.

2.1.1.1. Suppressive cytokines

By creating conditional IL-10-deficient mice and crossing these to mice expressing cre recombinase under the control of Foxp3, the role of IL-10 production by Tregs in particular could be dissected [20]. This study revealed high impact of IL-10-mediated regulation by Tregs at environmental interfaces such as skin, lung, and gut, circumventing immunologic hyper-reactivity at naturally microbe-exposed sites.

Recently, another regulatory cytokine was discovered which has been designated IL-35 [21]. IL-35 seems to be required for optimal suppressive capacity of Tregs. This novel cytokine consists of Epstein-Barr-virus-induced gene 3 (*Ebi3*) and interleukin-12 alpha/p35 (*IL12α*), both being specifically expressed in Tregs but not in effector T cells. Messenger RNA levels of both proteins are markedly up-regulated in Tregs upon co-culture with effector T cells *in vitro*, arguing for enhanced expression as a consequence of effector T cell proliferation. *Ebi3* has been shown to be a direct downstream target of Foxp3 [22] and deficiency of either *Ebi3* or *IL12a/p35* leads to significantly reduced regulatory activity *in vitro* and to failure of control of homeostatic proliferation or cure of inflammatory bowel disease *in vivo*. The fact that ectopic expression of IL-35 confers regulatory activity to naïve T cells and that recombinant

IL-35 suppresses T-cell proliferation, further underlines the regulatory capacity of this cytokine.

A more diverse function has been described for the pleiotropic cytokine TGFβ (transforming growth factor beta). Besides its expression by multiple cell types and the resulting direct function on the homoeostasis of regulatory T cells, TGFβ is also secreted by Tregs directly to suppress effector T cell proliferation. It has been shown that the absence of TGFβ signalling leads to hyperproliferation, activation and differentiation of effector T cells. This was explained on one hand by abrogated Treg cell suppression *per se*, on the other hand TGFβ has also been implicated in pre-conditioning effector T cells to be susceptible to Treg-mediated killing [16]. Interestingly, Treg numbers are not reduced in the thymus but in the periphery of TGFβ-deficient mice [23, 24], possibly caused by a failure in peripheral Treg cell induction. The transfer of wild-type Tregs restores peripheral Treg numbers but not the inflammatory phenotype of the mice [23]. Evidence that TGFβ directly produced by T cells plays a role in immune suppression was concluded from a study in which the *Tgfβ1* gene was specifically inactivated in T cells. As a consequence, mice developed wasting colitis associated with T cell hyperproliferation, activation, and effector T cell differentiation [25]. The role of Tregs in particular was underlined by the finding that TGFβ–neutralizing antibody abrogated Treg cell-mediated protection from transfer colitis [26] and that naïve CD4 T cells deficient in the relevant TGFβ receptor chain are refractory to Treg cell-induced suppression [27]. Treg-produced TGFβ has been further shown to play a role both in the control of the host immune response against Mycobacterium tuberculosis [28] and in the suppression of allergic responses [29]. On the other hand it could be demonstrated that TGFβ can cause limited anti-tumour immunity by rendering T cells unresponsive to the tumour and dampening the anti-tumour activity of cytokine-induced killer cells [30]. In contrast to the *in vivo* evidence, TGFβ-

deficient Tregs show normal suppressive capacity *in vitro* [25], leading to the conclusion that its production may be only one out of many mechanisms that Tregs utilize to regulate T cell tolerance.

Taken together, cytokines can mediate tolerance in various disease settings to various antigens, self as well as non-self. IL-10, TGFβ and IL-35 function in distinct settings which could imply a specific, non-redundant role for each individual cytokine.

2.1.1.2. Modulation of dendritic cell activation

The surface molecule CTLA-4 has been shown to exert systemical functions in immune-modulation [31, 32]. Deficiency of this molecule leads to systemic lymphoproliferative syndrome and severe pancreatic lesions. Affected mice show a large percentage of activated T cells and inflamed non-lymphoid tissues already 6 days after birth, leading to death within the first four weeks of age [33]. Both germline and Treg cell specific CTLA-4 deficiency leads to early onset of autoimmunity [34-36]. It is known that CTLA-4 and the costimulatory molecule CD28 share the ligands B7-1 and B7-2. By blocking the stimulatory ligands and thus out-competing the positive signal which would be provided by CD28, regulatory T cell-derived CTLA-4 inhibits effector T cell activation and proliferation. The surface molecule can further trigger induction of indolamine 2,3-dioxygenase (IDO) by interacting with CD80 and CD86 on dendritic cells (DCs), eliciting potent immunosuppressive effects in the local environment of the DC. In general, there is evidence that the function of Tregs on APCs plays a dominant role either by their modification (e.g. up regulation of IDO) or simply by hindering naïve T cells from forming a stable complex with antigen presenting cells (APCs) for several hours, a process that has been shown to be required for full effector T cell differentiation [37]. Neuropilin 1 is one example for a surface molecule on Tregs being

relevant for prolonged interactions between Tregs and dendritic cells [38]. Interactions between regulatory T cells and dendritic cells were also shown to result in specific down-regulation of CD86, a co-stimulatory molecule expressed by DCs, potentially contributing to dampened T cell activation [37].

2.1.1.3. Direct suppression of target cells

Galectin-1 is highly expressed on human and mouse regulatory T cells [39]. Binding to its ligands such as CD45, CD43 and CD7 on T cell surfaces [40] leads to cell cycle arrest and/or apoptosis and the inhibition of proinflammatory cytokine release (e.g. IL-2 and IFNγ) by the target T cells [41-44]. Since engagement of galectin-1 occurs *via* glycoproteins, this molecule represents an antigen non-specific suppressive mechanism. It is not clear whether galectin-1-mediated suppression requires cell contact *in vivo* or if its function can be exerted as a soluble cytokine. Although highly expressed in Tregs in comparison to naïve T cells, gene transcription of galectin-1 is not dependent on Foxp3 since ectopical expression of Foxp3 in naïve T cells does not lead to its up-regulation [45].

Another direct suppressor function by Tregs is exhibited by the transfer of cyclic adenosine monophosphate (cAMP) into responder $CD4^+$ T cells *via* gap junctions [46]. cAMP is accepted as a second messenger with high potency of inhibiting T cell growth, differentiation, and proliferation [47]. Following a related hypothesis, another study showed the importance of Treg expression of CD39 (ectonucleoside triphosphate diphosphohydrolase-1, ENTPD1), an ectoenzyme that hydrolyzes ATP/UTP and ADP/UDP to the respective nucleosides such as AMP and CD73 (ecto-5'-nucleotidase), which degrades extracellular nucleoside monophosphates to nucleosides, such as adenosine [48]. Adenosine receptors on targeted effector T cells can mediate suppressive functions [49-52].

Direct killing of target cells by Tregs could potentially be another mechanism by which these cells negatively influence the strength of an immune response. *In vitro* studies with human Tregs suggest that natural Tregs stimulated with αCD3 and αCD46 are capable of expressing high amounts of granzyme B on their surface upon stimulation, endowing them with a considerable cytotoxic T-lymphocyte (CTL) activity for almost all cell types tested [53]. Treg-mediated killing of antigen presenting cells (APCs) could induce long lasting suppression of autoimmune diseases by circumventing effector T cell activation by APCs. Tregs have also been shown to directly kill effector T cells in a perforin-independent granzyme B-dependent process [54] and to suppress effector T cell proliferation also in the absence of APCs [55], supporting the notion that this mechanism probably reflects only one out of many different ways of suppression.

2.1.1.4. Metabolic disruption

Regulatory T cells require T cell receptor (TCR) stimulation for induction of their suppressive activity. Nonetheless, Tregs are anergic, manifested by unresponsiveness to TCR stimulation, as detected by the lack of DNA replication and IL-2 secretion *in vitro*. Exposing Tregs to high amounts of exogenous IL-2 concomitantly removes the block on proliferation and abrogates suppressive capacity [14, 15]. CD25, the high affinity receptor chain of IL-2, is constitutively expressed by Tregs, suggesting the dependency on effector T cell-derived IL-2. Excess of cytokines like IL-2 and IL-7 can overcome suppression by Tregs [15, 56]. It has been shown recently that this mechanism reflects Bad- and Bim-mediated apoptosis of target cells by cytokine deprivation caused by high Treg uptake of pro-survival cytokines and not survival of the target cells mediated by pro-survival cytokine repletion [57].

2.1.2. Foxp3 as a specific transcription factor

Foxp3 is an X-chromosome encoded member of the forkhead transcription factor family. In several studies, this protein has been shown to be indispensable for regulatory T cell development as mutations or the complete lack of Foxp3 lead to a fatal, early onset development of autoimmune disease with multi-organ inflammation and wasting disease in mice (*scurfy* disease) [58, 59] and humans (IPEX = immune dysregulation, polyendocrinopathy, enteropathy, X-linked) [60, 61]. Mutations affect only hemizygous males due to the X-chromosomal location of the Foxp3 gene whereas heterozygous females as carriers of the mutant allele remain healthy. Due to different mutations of the Foxp3 gene in IPEX patients, the severity of disease varies. All affected individuals share hyperactivation of T helper cells and overproduction of proinflammatory cytokines. Classical autoimmune lesions include massive lymphoproliferation, diabetes, exfoliative dermatitis, thyroiditis, and enteropathy. Highly affected humans generally die within the first two years of life, whereas mice suffering from the so-called natural *scurfy* mutation succumb to autoimmunity within the first four weeks of life.

Initial support for Foxp3 as major transcription factor of Tregs has been made only few years ago. Before that time, Tregs have been characterized mainly by their high expression of CD25, the high affinity receptor chain for IL-2 [2]. This marker helped in the past to understand Treg biology even though it was not specific for Tregs due to is up-regulation in activated effector T cells. When Foxp3 was discovered, high correlation between the transcription factor and CD25 could be detected, facilitating interpretation of initial reports on Foxp3 *(aliases: scurfin, JM-2).* In humans, Foxp3 could also be detected transiently in effector T cells, but this expression was shown to be only short-lived and much lower compared to what could be found in Tregs [62]. Nevertheless, it could be shown recently that also in humans, Foxp3 can

be used as a specific marker for Tregs by analyzing the methylation status of the promoter region. Natural regulatory T cells show a fully de-methylated promoter region, whereas transient up-regulation of Foxp3 in effector T cells is represented by only partial de-methylation of the locus [63]. In a mouse model, it could further be shown that reduced Foxp3 expression leads to a lack of regulatory function of Tregs, underlining the requirement of high and stable Foxp3 expression for true regulatory function [64]. The importance of Foxp3 for Treg development was further supported by retroviral transduction assays, leading to the expression of the Foxp3 encoding gene in murine non-regulatory T cells. The consequence of ectopic expression of Foxp3 was the gain of suppressive function [7, 9, 65]. The transcription factor has to be maintained in the periphery in Tregs to keep suppressive functions and phenotypes. This could be shown most prominantly by usage of a mouse model allowing for conditional deletion of the Foxp3 allele in mature peripheral Tregs [66]. Under physiological conditions, Foxp3-mediated Treg differentiation is stable and hardly reversible. Foxp3 stabilizes and positively feeds back on its own expression in a fashion partially dependent on IL-2 signalling [22, 67] and by actively repressing alternative pathways, for example by overriding the T_H17 transcription factor RORγT [68]. More evidence for direct suppression of alternative pathways comes from the observation that in Foxp3-deficient mice, cells that would usually express Foxp3 have been shown to produce high amounts of IL-4 [22].

Although apparently sufficient for regulatory T cell development, some regulatory T cell characteristics have been shown to develop independently of Foxp3, e.g. the expression of granzyme B, galectin-1 or helios [22, 45, 67, 69, 70], an ikaros transcription factor family member which is discussed to be involved in Treg lineage development in the late CD4CD8 double positive stage in the thymus [71, 72]. It is under current investigation which characteristics of Tregs are strictly Foxp3 dependent and how they impact function. In this line, several mechanisms for Foxp3-mediated suppression have been described. Upon

ectopical Foxp3 expression, approximately 700-1000 genes are differentially regulated, most of these genes showing down-regulation and only few showing up-regulation. Among those are proteins known to support Treg function like G-coupled protein receptor 83 (gpr83) or extracellular matrix 1 (Ecm1) [73]. However, only around 10% of the differentially expressed genes could be shown to be directly controlled, either up-regulated or down-regulated, by Foxp3, whereas all the others respond to secondary effects [74]. Direct targets include signal transduction molecules (e.g. Zap70 [75], Ptpn22 [75]), transcription factors (e.g. Crem [22]), cytokines (e.g. IL-2 [75]), cell surface molecules (e.g. IL2Rα [75]), enzymes for cell metabolism (e.g. Pde3b [22]) and microRNAs (e.g. miR-155 [74]). In a recent study, it could be shown that Foxp3 is likely to be capable of recruiting the chromatin remodelling machinery to its binding site as a means of regulating gene expression by interaction with the histone acetyl transferase (HAT) and the histone deacetyl transferase (HDAC) complex [74, 76]. Foxp3 further has been shown to interact with the transcription factors NFκB and the nuclear factor for activated T cells (NF-AT) by cooperative binding to DNA resulting in interference with their ability to transactivate [76]. It can also directly interact with AML1/Runx1 [77]. Disruption of the binding of Foxp3 to AML1/Runx1 impairs the Foxp3-dependent suppression of IL-2 in Tregs and dampens their suppressive activity. Several studies could also demonstrate direct binding of Foxp3 to promoter regions in the IL-2, CTLA-4, GITR, CD25, CD103 and IFNγ gene loci [74-76, 78].

2.1.3. Development of natural regulatory T cells

The differentiation of the majority of regulatory T cells in naïve mice begins in the thymus and cells leave the thymus as fully functional antigen-primed natural regulatory T cells [79, 80]. Most (~5%) Foxp3 expressing Tregs can be found within the CD4 single positive stage, but also around 1% of $CD8^+$ T cells can express Foxp3 [59]. Foxp3 expression can generally

be monitored from a late $CD4^{+}CD8^{+}$ stage on [59]. However, experiments with ovarian antigens showed that ovarian ablation rendered females as responsive to the antigen as males. This suggests that self-antigen exposure in the thymus restricted to the neonatal period is not sufficient to induce tolerance, but continuous expression of self-antigen is required for physiological tolerance [81]. Nevertheless, Tregs have been shown to recognize normal self-antigens targeted in autoimmune diseases [82], tumour-associated antigens [83], and allogenic transplantation antigens [11]. Even though recent publications suggest that Tregs develop due to an enhanced recognition of self [84-87], the factors leading to lineage decision and Foxp3 up-regulation are not understood and data promoting self-recognition by Tregs remain controversial. It is still a debate whether Tregs survive selection in the thymus as a consequence of strong positive selection *via* self-recognition [88, 89] or because of resistance of differentiated $Foxp3^{+}$ cells to negative selection [90]. Findings that deficiency in LAT (linker of activated T cell) leads to the incapacity to produce natural Tregs, provided evidence that Treg development in the thymus is dependent on a strong TCR stimulus [91]. Further, RAG (recombination activating gene, required for receptor shuffling in immature thymocytes) deficiency in TCR transgenic mice also leads to the lack of natural Tregs. Regulatory T cells in TCR transgenic mice usually carry a TCR composed of the transgenic β chain and an endogenous α chain in contrast to effector T cells, which usually express both transgenic receptor chains [80]. The expression of the endogenous α chain is likely to provide the positive survival signal. In addition to signals provided by TCR-ligand interactions, the intensity of interaction between accessory molecules on the T cells with their ligands on thymic stromal cells seems to be important. Deficiency in some co-stimulatory receptors or their ligands like CD28 and B7.1 or B7.2 leads to substantially reduced Treg cell numbers in both thymus and periphery [34, 36]. This could be either a Treg cell intrinsic effect or a secondary effect due to IL-2 deficiency caused be the inability of full effector T cell stimulation [92]. CD80 and CD86 expression on DCs triggers Treg as well as effector T cell

proliferation. In the absence of an immunstimulatory signal, CD80 and CD86 expression on DCs is low. At that stage, DCs have been shown to be incapable to induce effector T cell differentiation and subsequent proliferation, whereas they still stimulate Tregs, probably due to a higher affinity of CTLA-4 to CD80 and CD86 compared to CD28, leading to the low activation threshold in Tregs (Tregs require 10-100 times less antigen to get activated than naïve T cells, [14]) [93]. In line, it has been discussed that CTLA-4 deficiency leads to the functional weakening of Tregs due to the lack of a second stimulatory signal besides TCR stimulation [4]. However, the role of CTLA-4 on Treg subsets seems to be more diverse since blocking of CTLA-4 increases Treg frequency and homoeostasis, whereas engagement of CD28 by CTLA4Ig leads to the decrease of Treg numbers [94]. Lack of CD40 and to a lesser degree lack of its ligand in mice results in substantially reduced Treg numbers [95-97]. Deficiency of CD40/CD40L however does not lead to development of autoimmunity since a defect in effector T cell differentiation overwrites the potential harm caused by Treg diminution. Treg generation is further dependent on interaction of the precursors with thymic dendritic cells and medullary thymic epithelial cells (mTECs) [98, 99]. mTECs can express tissue specific antigens under the control of *Aire*, a gene whose deficiency is reported to result in autoimmune disease development comparable to the direct depletion of regulatory T cells [100, 101].

A crucial question with regard to Treg development is whether Foxp3 is required as a survival signal circumventing negative selection in the thymus. If these cells indeed displayed a higher recognition of self, they would need mechanisms to escape negative selection in the thymus, and these mechanisms could be driven by Foxp3. However, this does not seem to be the case since very recently, two independent studies have been published using mice deleted for Foxp3 but expressing green fluorescent protein (GFP) under the control of the Foxp3

promoter. These mice did possess GFP expressing cells in the periphery, illustrating that Foxp3 is not needed for survival of Tregs [22, 69].

There is evidence that signals sent through the common γ chain-containing cytokine receptors (mostly IL-2) influence Treg development through STAT-5 dependent mechanisms [102, 103]. IL-2 or IL-2 receptor-chain-deficient mice spontaneously develop T cell-mediated fatal lymphoproliferative/inflammatory disease with autoimmune components, an effect that can also be induced by administration of blocking antibody to IL-2 [92]. Mice lacking IL-2 signalling have less Tregs when compared to wt mice, however they are not completely devoid of these cells. IL-7 has now been shown to substitute for IL-2 loss to some extent, leading to a minor Treg population in IL-2 single-deficient mice. IL-7 and IL-7R thus contribute to T cell development and peripheral homoeostasis, whereas IL-2 signalling is dominant and sufficient to restore production of Tregs in double-deficient mice [104]. It could further be shown in *in vitro* experiments that IL-2 is required to maintain Foxp3 expression in Tregs, and enhances suppressive function [17, 67]. The cytokine is also needed to sustain CD25 expression, showing that IL-2 is vital and irreplaceable for the development, survival, and function of Tregs. Since IL-2 is mainly produced by activated effector T cells, it forms a negative feedback loop by promoting regulatory T cell development and function. In a recent study, however, IL-2 was discussed as a principle driving force for proliferation of existing Tregs rather than as a Treg inducer. Here, it was shown that TGFβ signalling during the development of Tregs in the thymus was indispensable [105].

In vitro, GITR engagement on Tregs leads to vigorous proliferation of Tregs in the presence of IL-2 [106, 107]. Another potential peripheral proliferation signal is toll-like receptor (TLR) mediated activation of Tregs which has been reported to occur via TLR2, 4, 5, and 8 [108].

Interestingly, Dicer deficiency in mice leads to abrogation of regulatory T cell development resulting in inflammatory bowel disease [109]. Dicer is an RNase enzyme required for processing double-stranded RNA and thus needed for microRNA production, leading to the conclusion that microRNAs are also important for Treg development and maybe also for their function.

2.1.3. Induced regulatory T cells and antigen specificity

Aside from natural regulatory T cells directly coming from the thymus, functional regulatory T cells have also been shown to develop in the periphery from naïve T cells. The activity of induced regulatory T cells in the suppression of experimental autoimmune prostatitis has been investigated and male Tregs were almost four times more potent in suppressing disease progression compared to female cells. This education of Tregs was shown to be thymus independent [110]. Furthermore, stimulation of Tregs by their cognate antigen *in vitro* before administration led to an even higher capacity to suppress disease [111, 112]. This was explained by the hypothesis that not only effector memory T cells, but also memory Tregs can be generated by the immune system. Both findings show high antigen-specificity by induced regulatory T cells. In contrast, there is clear evidence that natural Tregs, once activated with their cognate antigen, are capable of suppressing responder T cells with distinct antigen-specificity [14]. Taken together, the pool of Foxp3$^+$ Tregs probably consists of different sub-groups. One group might preferentially recognize self and heavily proliferate in the presence of their cognate antigen, whereas the other group reflects more a resting stage in untreated mice, not being capable of recognizing self. This hypothesis was strengthened by findings that haemagglutinin-specific Tregs heavily proliferate in the pancreatic lymph-node of mice that express haemagglutinin under the control of the β-insulin promoter, but not in peripheral lymph nodes, were the antigen is not expressed [113].

Induction of regulatory T cells has been shown to be highly TGFβ-dependent [114, 115]. A number of reports could show recently that induction of the regulatory phenotype is necessary for tissue integrity at peripheral sites exposed to environmental surfaces like gut and skin, and that Treg induction preferentially takes place at these sites. Special dendritic cell subsets that can produce retinoic acid are believed to be the inducers of the induction process [116-118]. However, Tregs can also be induced *in vitro* in the presence of TGFβ, IL-2 and αCD3 as T-cell receptor stimulus. Even if Tregs are believed to carry a distinct TCR repertoire when compared to the naïve T cell pool, these *in vitro* generated induced Tregs can also cure scurfy mice from autoimmune disease. However, five to ten times more induced Tregs have to be transferred when compared to the usage of natural, thymus-derived Tregs [119].

2.2. Tregs in vaccination using TLR9 agonists

Regulatory T cell activation potently dampens immune responses in various settings. On the one hand, Treg function is extremely important in maintaining tolerance towards self, on the other hand the high potency of these cells might be detrimental by suppressing desired immune responses towards pathogens or tumours. CD8 positive cytotoxic T lymphocytes (CTLs) are the key players in host response to intracellular infections and cancer. A very encouraging approach for improving vaccination strategies triggering CD8 T cell responses evolved when toll-like receptors (TLRs) were discovered [120-122]. These pathogen recognition receptors are widely expressed in the body, mainly on antigen presenting cells, but also on most other cell types. For the human system, ten distinct functional TLRs have been reported so far, recognizing a wide range of viral and bacterial products including lipopolysaccharide (LPS), DNA containing CpG dinucleotides (CpG), double-stranded RNA, single-stranded RNA, lipoproteins, lipoteichoic acid, and flagellin (reviewed in [123]). Recognition of pathogens *via* TLRs results in priming of the innate immune system, initiating

and maintaining protective immune responses. Today, the only approved TLR agonist is Aldara, a TLR7 agonist, used to cure genital warts, human papilloma virus, actinic keratoses, and superficial basal cell carcinoma [124-126]. Other TLR ligands are under intensive investigation, including many agonists for TLR3, 4, 5, 8, and 9 and many clinical and pre-clinical trials have been published [127-130]. Often results are much less convincing than theory would predict and clinical trials were stopped before approval of a drug. Reasons for this are mostly unclear, but it is very likely that at least in some of the cases, regulatory T cell expansion dampens the establishment of a strong immune response and memory induction from the vaccine. Following depletion of CD25 positive cells in mice, primary responses have been shown to be enhanced in various settings [131-138]. However, CD25 expression is not specific for Tregs, being up-regulated also in effector T cells upon stimulation.

CpG oligonucleotides are molecules sensed by the innate immune system by so-called pathogen recognition receptors. A major role in recognizing CpGs has been shown for the intracellularly expressed toll like receptor 9 (TLR9) [139]. Most bacteria and DNA viruses contain CpG nucleotides whereas in vertebrates, they are masked by methylation ensuring that recognition by the innate immune system does not occur against self. The intracellular expression of the receptor might be an additional safety barrier [140, 141]. Engagement of TLR9 leads to increased expression of co-stimulatory molecules on the target cells (almost exclusively antigen-presenting cells), their resistance to apoptosis, upregulation of the chemokine receptor CCR7, and secretion of T_H1-promoting chemokines and cytokines [142-146]. Overall, its effect can be summarized by the induction of a strong T_H1-biased cellular and humoral immune response of both innate and adaptive immunity [147-150], resulting in the hypothesis that CpG oligonucleotides could deliver a promising approach for vaccination against both pathogens and tumours, particularly since CpG oligonucleotides have been demonstrated to effectively boost CD8 T cell responses [142, 151, 152]. Very important for *in*

vivo usage of CpG oligonucleotides is further its high toleration by the body, leading only to very mild side effects [153]. Studies using CpG oligonucleotides to activate the innate immune system revealed its potency to protect mice from various pathogens including viruses, bacteria and parasites. It was also shown to block tumour progression (reviewed in [154]). Its usage as a split vaccine was shown to be very promising, leading to the requirement of much less antigen to induce potent adaptive immune responses. Vaccinations with CpG oligonucleotides as adjuvants in comparison to others in mice demonstrated its outstanding capability of promoting T_H1 immune responses, being capable of even redirecting established T_H2 responses like allergic asthma towards a T_H1-biased response [155, 156].

Drugs containing TLR9 agonists are currently intensively investigated and included in clinical trials, where some of them have already reached preclinical status. For example, the company Coley Pharmaceuticals developed TLR9 agonist based drugs against non-small cell carcinoma or allergic asthma. Dynavax Technology uses TLR9 adjuvant activity for vaccination against Hepatitis B, influenza, or cancer. Idera investigates the potency of these agonists in renal cell carcinoma, HIV-1, and allergic asthma. Juvaris BioTherapeutics reached preclinic phase with a cationic lipid-DNA complex most probably exhibiting its adjuvant activity through the contained TLR9 agonists.

CpG oligonucleotide function is explained by synergy between TLR9 and the B cell receptor, inhibition of B cell apoptosis, and enhanced immunoglobulin G (IgG) class switch DNA recombination. Dendritic cell maturation and differentiation resulting in strong cytotoxic T-lymphocyte (CTL) generation also has a strong impact in function. The differentiation could even be shown in the absence of CD4 help [157, 158], a requirement that has been discussed to be absolutely necessary for potent CTL induction by facilitating cross-priming [159-161] and thus being necessary for long-living CD8 T cell memory [162-164]. A recent study

showed the absolute loss of protective memory upon CD4 T cell depletion in mice achieved by administering depleting antibodies, whereas the initial efficacy of priming and clonal expansion was not affected in the reported setting [162]. One discussed reason for better CTL activity and/or memory induction in the presence of CD4 helper T cells are CD40/CD40L-dependent signalling pathways that functionally modify dendritic cells to support CD8 T cell responses [159-161, 165, 166]. Combined stimulation of CD40 and a TLR agonist leads to up-regulation of CD70 [167] and the capacity of eliciting a $CD4^{+}$ T cell independent primary $CD8^{+}$ T cell response [166]. Blocking CD70 by an antibody impedes help provided by CD4 T cells [168], a mechanism that was suggested to be intrinsically CD8 dependent, but could be shown to affect help only recently [169]. Besides strictly dendritic cell dependent mechanisms, TNF/TNFR family signalling [162, 170, 171], IL-2/IL-2R interactions [172, 173], promotion of bystander survival for CD8 T cells [174], resistance to apoptosis (e.g. due to CD27 engagement by CD70 on DCs which has been shown to be upregulated by CD40 signalling [169, 175]) [171], changes in effector and central memory T cell generation [165, 176-178], and differential expression of transcriptional factors [179] could also play a role. Overall, the literature points towards an enhanced role for CD4 T cell help for sustained memory induction rather than primary response towards an antigen since under inflammatory conditions, DCs are activated *per se* and do not require T cell help for full activation.

Humoral and cellular responses towards peptides, proteins, live or inactivated viruses, dendritic cell vaccines, autologous cellular vaccines, and polysaccharide conjugates in both prophylactic and therapeutic vaccines in numerous animal models have been shown to be augmented since CpG oligonucleotides have been shown to stimulate the innate immune system [143, 180-182] and their receptor was found in 2000 [139]. Most of the studies that address helper T cell dependency of CTLs have been performed using *Listeria monocytogenes* as model infection [183], as usage of these intracellular bacteria is a well-

established system to provoke profound T cell responses towards both $CD4^+$ and $CD8^+$ T cells [184-186].

3. Aims of the study and results

In this thesis, two major topics in which regulatory T cells are considered to play an important role have been addressed. The first part of this work deals with the induction of autoimmunity by the loss of Tregs and in the second part, the role of Tregs in vaccination strategies is investigated.

3.1. Tregs in autoimmunity

A key role of Foxp3 in the development of natural Tregs has been suggested from the molecular characterization of the *scurfy* mouse mutant. These mice, which suffer from a fatal lymphoproliferative disorder, harbour a mutated *foxp3* gene coding for a product that lacks the forkhead domain [58]. *Scurfy* mice receiving CD25$^+$CD4$^+$ Tregs from WT mice early after birth remained virtually disease free [7]. Furthermore, transduction of mutant Foxp3, lacking the forkhead domain, failed to confer suppressive activity to naive CD4$^+$ T cells in contrast to full length Foxp3 [8]. Thus, it is widely accepted that an intrinsic T cell failure to generate functional CD4$^+$ Tregs is the main cause of the fatal autoimmune disease in *scurfy* mice, although an additional role of the *scurfy* mutation in non-hematopoietic cells has been suggested [187-189]. Moreover, it remains unknown whether the mere absence of functional Foxp3$^+$ Tregs is sufficient to provoke the development of the *scurfy* phenotype or whether those CD4$^+$ T cells expressing the truncated Foxp3 protein play a more active role, since depletion of Tregs with established protocols has failed to reproduce the fulminate natural disease [190, 191]. S*curfy* disease can be transplanted into nude mice by adoptive transfer of CD4$^+$ T cells [192], strengthening the hypothesis that the disease is indeed solely T cell dependent. Foxp3$^+$ Tregs preferentially recognize self-antigens with high affinity [86, 193] and activated, not naïve T cells in Foxp3-deficient mice harbor a TCR repertoire closely

resembling the repertoire of Tregs in Foxp3-sufficient mice [194]. Together, these data led to the hypothesis that "would-be" Tregs expressing self-reactive TCRs may contribute to the pathology of Foxp3-deficient mice [194].

The major aim of this thesis was to investigate whether the mere absence of regulatory T cells leads to the *scurfy* phenotype. Since αCD25 treatment does not only deplete Tregs but also activated T cells, a model that allows specific depletion of regulatory T cells has been established (appendix 1). Here, bacterial artificial chromosome (BAC) technology [195-197] was used to create a novel mouse carrying the primate high-affinity diphtheria toxin receptor (DTR) fused to enhanced green fluorescent protein (eGFP) [198] under the control of the Foxp3 promoter. BAC technology allows for specific and faithful expression of the transgene, and reporter expression highly correlates with Foxp3 expression in the novel DEREG mice (DEpletion of REGulatory T cells) (appendix 1). In adult mice, injection of 1 μg diphtheria toxin on six consecutive days led to an efficient depletion of Foxp$^+$ Tregs (appendix 1, fig. 3). Further experiments revealed that already two subsequent injections lead to comparable depletion efficiency (data not shown). To investigate the *in vivo* function of Tregs in inflammatory conditions, a delayed-type-hypersensitivity reaction (DTH) was induced in DEREG mice and Tregs were depleted after sensitization to the hapten. Highly elevated immune responses could be detected upon Treg depletion when compared to wt mice, but also in comparison to a control group that received αCD25, the current protocol for Treg depletion (appendix 1, fig. 4). Neonatal depletion of regulatory T cells by injection of diphtheria toxin (DT) led to the development of the *scurfy* phenotype (appendix 1, fig. 5) with comparable kinetics and disease severity to the natural *scurfy* mutants.

In the first study, it could not yet be ruled out that mutated, Foxp3-deficient sf Tregs somehow contributed to some extent to the fatal phenotype in *scurfy* mice. It has been shown

that under certain experimental conditions using a fixed transgenic T cell receptor (TCR) ß-chain, the Treg TCR repertoire is shifted towards self-antigen recognition [86, 194, 199], and the presence of non-functional self-reactive Tregs has been discussed as a potential co-factor for the induction of autoimmunity in *scurfy* mice [86]. By crossing DEREG mice to *scurfy* mice, it could be assessed if $GFP^{+}Foxp3^{-}$ "would-be" Tregs were present in the thymus and the periphery of natural Foxp3 mutants (appendix 2, fig. 1, 2). Further, based on expression of the fluorescent reporter, these cells could be sorted and studied functionally. Sf Tregs show most *bona fide* Treg markers as assessed by real-time PCR and FACS, but did not show any suppressive capacity when tested in an *in vitro* suppression assay (appendix 2, fig. 3). However, we could detect a difference in intracellular cAMP levels in sf Tregs and DEREG Tregs (appendix 2, fig. 3). When CD4 T cell compartments, either GFP^{+} or GFP^{-}, were cultured and cytokine production was measured, both scurfy cell subsets produced high amounts of T_H2-driving cytokines, as shown for IL-4, IL-10 and IL-13 (appendix 2, fig. 4). Nevertheless, cytokine expression did not largely interfere with the ability of GFP^{-} scurfy cells to induce Foxp3 promoter activity *in vitro* in the presence of TGFβ, IL-2, and αCD3 (appendix 2, fig. 5), as could be suggested from a recent publication claiming that T_H2 cytokines actively abrogate Treg induction in the periphery in humans [200]. By transferring Foxp3-mutated, GFP-expressing *scurfy* regulatory T cells into T and B cell-deficient RAG-1 knock out mice, their potential to transmit disease could be investigated. No pathologic consequences were observed when Sf Tregs were transferred into $RAG1^{-/-}$ mice whereas mice receiving the effector T cell population from *scurfy* donors developed the *scurfy* phenotype (appendix 2, fig. 6).

3.2. Tregs in vaccination strategies

Many clinical trials have been performed during the last years, indicating a clear potential for CpG oligonucleotides to induce a substantial clinical benefit towards both tumours and pathogens. However, the results from most trials have never been as clear as expected. Besides differential expression of TLR9 on accessory cells in humans and mice, regulatory T cells are discussed to be one cause, and the lack of regulatory T cells during vaccination could potentially increase the vaccination efficiency. Temporary down-modulation of Tregs might be an attractive target for improving the efficacy of vaccines. The positive adjuvant effect of TLR ligands could partially be linked to the Treg compartment, either by direct reversion of Treg function [201], or by TLR-induced cytokines (such as IL-6), which drive responding T cells refractory to suppression by Tregs [202]. In contrast, other studies suggested that an excessive induction of T_H1 responses during sensitization could lead to the loss of effective memory by an initially overshooting IFNγ response. Due to the lack of specific mouse models, the role of regulatory T cells during vaccination has not been investigated in detail thus far.

By using the DEREG mice (appendix 1) the role of regulatory T cells during vaccination and the effect of their depletion could be addressed directly. It has been shown by collaborators that depletion of the CD4 positive cell compartment during sensitization leads to higher CD8 frequencies, and this effect has been explained by the depletion of regulatory T cells (appendix 3, fig. 2). Those data were somewhat unexpected since CD4 T cells were implicated in providing help for a strong and sustained cytotoxic T lymphocyte (CTL) response. Nevertheless, it seemed as if the mere depletion of Tregs dominates the loss of CD4 T helper cells. Using αCD25, it could be shown that Treg depletion was indeed beneficial for vaccination efficiencies. By vaccinating the mice with MHC class II-deficient dendritic cells (which cannot prime CD4 T cells), regulatory T cell induction could also be circumvented,

again arguing for the dominant effect of regulatory T cells over CD4 help provided by CD4 effector cells (foreign work presented in appendix 3, fig. 7). Even though all these experiments point in the same direction, the final proof could not be achieved by using depleting antibodies or dendritic cells being incapable of priming CD4 T cells in general. Reasons for this are the lack of specificity and the possibility of secondary effects by DC transfer experiments. Our work using the DEREG mice could show clearly that by depleting regulatory T cells during the priming phase, the primary response towards protein-CpG can be dramatically increased (appendix 3, fig. 6A, B). Higher vaccination efficiencies are thus directly linked to the lack of regulatory T cells and the depletion of these cells after sensitization with the model antigen ovalbumin (OVA) covalently linked to CpG leads not only to higher OVA specific CD8 frequencies on day 7, but also to better protection against OVA-expressing *Listeria monocytogenes* five weeks after sensitization (appendix 3, fig. 6C). Of note, using our DEREG mice combined with another accepted *in vivo* mouse model, the DIETER mouse, it could be shown that tolerance induction by dendritic cells is indeed $Foxp3^+$ Treg dependent (appendix 4, fig. 1). Treg-depletion in this model leads to up-regulation of co-stimulatory molecules on dendritic cells, circumventing effective tolerance induction (appendix 4, fig. 2). Steady-state dendritic cells were capable of inducing effective CTL responses in the absence of Tregs.

4. Discussion

4.1. Treg control of autoimmunity

To investigate Tregs *in vivo*, a novel transgenic mouse line was established named DEREG: DEpletion of REGulatory T cells. Analysis of DEREG mice revealed selective DTReGFP expression within the $CD4^+$ T cell compartment, with highest expression within the $CD25^+$ subset (appendix 1, fig. 1B). Importantly, GFP expression highly correlated with Foxp3 expression, showing the high specificity of transgene expression in the novel mouse strain. DEREG mice showed comparable percentages of Tregs when compared to wt mice with equally high Foxp3 expression, ruling out the possibility that the BAC transgene influences regulation of the endogenous *foxp3* locus. The cells were further assessed for regulatory capacity and were proven to be as suppressive as their wild type counterparts in an *in vitro* suppression assay. Other cell populations like dendritic cells or B cells were also not altered in frequency in DEREG mice. Consequently, DEREG mice showed specific expression of the DTR-eGFP fusion protein in fully functional $Foxp3^+CD4^+$ Tregs, allowing for detailed *in vivo* investigation of Tregs. By injection of diphtheria toxin (DT) into DEREG mice, regulatory T cells could be depleted with an efficiency of 90-98%. DT treatment did not affect other lymphocyte subsets, suggesting that DT treatment of DEREG mice allows for the selective depletion of $Foxp3^+$ cells.

To assess whether the absence of Tregs during the priming phase of the adaptive immune system can augment inflammation, a delayed type hypersensitivity (DTH) reaction towards a hapten was performed. Indeed, massive footpad swelling could be observed in Treg depleted DEREG mice, whereas the control group receiving αCD25 to mimic the current Treg

depletion protocol did not show elevated swelling when compared to hapten treated Treg-sufficient wild type mice. This can be explained on the one hand by depletion of not only the Treg compartment, but also the $CD25^+$ effector T cell pool, which has been shown to constitute the main effectors in the a DTH reaction [203]. On the other hand, some Tregs have been shown to be negative for CD25 and these cells can be found mainly at peripheral sites [11, 65, 204]. By using αCD25 antibody, these cells will remain undepleted. Taken together, the DEREG mouse model is to date the most suitable tool to investigate the *in vivo* function of regulatory T cells.

Aside from suppression of immune responses to infectious pathogens [205], tumours [206, 207], and transplants [208-210] and protecting from autoimmune diseases like inflammatory bowel disease [35, 211, 212] or autoimmune diabetes [213-215], Tregs have an active role in maintaining immune homoeostasis as could be shown by the development of a wasting, lymphoproliferative *scurfy*-like disease as a result of Treg depletion in neonates (appendix 1, fig. 5). It has been suggested earlier that the mere absence of functional Tregs is sufficient to cause the fatal lymphoproliferative disease in *scurfy* mice. However, this could never be proven due to the lack of a suitable mouse model. Since lesions in DT-treated DEREG mice closely resembled those observed in *scurfy* mice and no significant differences in pathology of those particular organs were observed, we concluded that diminution of $CD4^+Foxp3^+$ Tregs is sufficient to induce the lethal lymphoproliferative autoimmune syndrome observed in the treated mice. In contrast to depletion of $Foxp3^+$ Tregs by DT injection, which consistently resulted in the development of *scurfy*-like symptoms, Treg depletion in neonatal mice with αCD25 antibodies resulted in marginal development of autoimmune disease if at all [190, 191]. These discrepancies are again best elucidated by both the existence of $CD25^-Foxp3^+$ Tregs especially within peripheral tissues [4, 216, 217], as well as the induced expression of

CD25 on activated conventional CD4$^+$ T cells, which are critically involved in the development of the *scurfy* phenotype [192].

Another publication dealing with the role of regulatory T cells for immune homoeostasis has been published at the same time as our work [218]. In contrast to our observations, the authors detect development of multiorgan disease also in adult mice depleted from Tregs by a comparable approach. In the publication by Kim *et al.*, knock-in mice were used, also carrying the DTReGFP protein under the control of the Foxp3 promoter. DEREG mice, however, do not develop autoimmunity when depleted from Tregs in an adult stage. The immune system from neonates differs from the adult immune system in many ways. One of the most likely explanations for a difference observed upon Treg depletion is that the neonatal system reflects a lymphopenic environment, and Treg-mediated homoeostasis is much more important at this stage. Further, other regulatory mechanisms could substitute for Tregs in an educated immune system (e.g. Foxp3-negative subsets of T_R1 or T_H3 cells). Discrepancies between the two studies could reflect differences in depletion efficiency, in the genetic background of the used mice (C57Bl/6 versus C57Bl/6 x 129Sv), or in the used toxin (Merck versus Sigma). In addition, differences in the SPF status of the animal facilities have been discussed to influence induction of autoimmunity [219]. When depleting Tregs in adult mice, Kim *et al.* show enhanced activation and proliferation of the DC pool. DEREG mice do not show significantly enhanced activation of DCs when CD40 and MHC class II were assessed. Subtle differences could be observed for CD86 (data not shown). However, depletion of Tregs under certain conditions can lead to up-regulation of co-stimulatory molecules also in DEREG mice and this was discussed as a reason for higher CD8 frequencies in a TCR transgenic system (appendix 4, fig. 2). Nevertheless, neither TCR transgenic nor DEREG control mice did develop autoimmunity. A very recent publication shows maturation of DCs

in presence or absence of Tregs *in vitro*, also claiming major differences for CD86 expression [37].

Kinetics on the rebound of regulatory T cells after their ablation (appendix 1, suppl. data) reveal a very fast replenishment of the Treg compartment, already starting around day three after the last injection day. This strong rebound is consistent with another report showing much higher homoeostatic proliferation of Tregs when compared to naïve CD4 T cells [94, 220]. Currently, the *in vivo* function of Tregs quickly filling up the space is assessed and it seems as if they were non-functional in the initial stage. This observation is based mainly on experiments using the DTH model and the *in vitro* suppression assay (data not shown). These findings would support earlier publications showing a lack of Treg function during the proliferation phase *in vitro* [14, 15].

Foxp3 expression by epithelial cells as claimed by another study published recently [221] could not be detected by histology (appendix 5, fig. 1) leading to the conclusion that disease development occurred due to the loss of the Treg compartment. Further, collaboration partners could show that depleted mice can be rescued by the adoptive transfer of functional Tregs, again clearly arguing against a direct effect on epithelial cells as a cause of autoimmunity (appendix 5, fig. 2). By using the commercially available monoclonal antibody against Foxp3 (clone FJK-16s, eBioscience), we did not detect any Foxp3 protein expression in epithelial cells of the lung, prostate, or thymic cortex (appendix 5, fig. 1A). The latter was in full agreement with a previous report [222]. Additionally, in contrast to another study from Liu's group that reported broad Foxp3 expression by breast epithelial cells and its role as a cancer suppressor gene in *Cell* [223], we did not detect Foxp3 expression in breast tissue nor tissue damage in depleted mice (appendix 5, fig. 1B). It is possible that the polyclonal rabbit α-Foxp3 antibody utilized by Liu *et al*. non-specifically cross-reacts with an epithelial antigen

instead of Foxp3. Theoretically this protein could be regulated by Foxp3, which would explain the lack of detection in scurfy mice [221].

According to Liu and colleagues, eliminating Foxp3 expressing cells in DEREG mice is expected to dramatically destroy the epithelial tissue architecture [221]. However, in all tissues where epithelial Foxp3 expression was reported by Liu *et al.*, high levels of Foxp3 protein were detected in cells with lymphoid but not epithelial morphology (appendix 5, fig. 1A, B). Furthermore, eliminating Foxp3-expressing cells in DEREG mice resulted in the loss of Foxp3 signal among lymphoid cells whereas very weak background levels of cytoplasmic Foxp3 staining in epithelial cells remained unchanged. Importantly, the tissue architecture that is supported by epithelial cells was unperturbed in the DT-treated DEREG mice.

Since all studies by Liu *et al.* were performed in BALB/c mice, we crossed our C57Bl/6 DEREG mice onto the BALB/c background for ten generations to exclude strain-specific differences [188, 189, 221, 223]. Identical to our results on a C57Bl/6 background, non-hematopoietic tissues were not affected by DT administration in BALB/c DEREG mice (appendix 5, fig. 1A, B).

As arguably the strongest argument for their contention, Liu and collegues used their earlier observation that the transfer of Foxp3-deficient bone-marrow into Foxp3-sufficient Rag-deficient recipient mice did not induce autoimmunity [188]. This result suggested that Foxp3 deficiency in bone marrow-derived cells was not primary to autoimmune disease instigation, challenging current dogma. In a stark contrast to these findings, it has been reported that bone marrow transfer from *Foxp3*-deficient mice into *Rag*$^{-/-}$ recipients leads to autoimmune lymphoproliferative disease [8, 224, 225]. Additionally, wild type bone marrow transferred into *Foxp3*$^{-}$ *Rag*$^{-/-}$ mice did not cause a disease [226]. The disease was not caused by mature pathogenic T cells possibly contaminated within the *Foxp3*$^{-}$ donor bone marrow cells because

transfer of fetal liver cells from *Foxp3*$^{-}$ mice or bone marrow cells from Foxp3^{-} *nude* mice, both of which do not contain any mature pathogenic T cells, also caused identical disease in irradiated *Rag*$^{-/-}$ recipients [222, 226]. The usage of recipient mice with a genetic deficiency in T cell generation is essential for assessing the contribution of Foxp3 expression in hematopoietic cells [226]. However, the utilization of lymphocyte-deficient recipients is necessary because radiation-resistant Treg cells in wild-type recipients rapidly reconstitute the Treg cell compartment after irradiation [226]. Thus, expanded host-derived Treg cells spare irradiated recipient mice from lethal autoimmunity, irrespective of the donor bone marrow genotype. Notably, an early bone-marrow transfer study used as an additional independently obtained evidence in support of a model for Foxp3 action in non-hematopoietic tissues utilized irradiated wild-type mice as recipients of Foxp3-deficient bone marrow [187]. Therefore, the result obtained by Liu and colleagues has not been independently reproduced thus far. It should be also pointed out that the bone marrow transfer study by Liu and colleagues lacked a positive control group, i.e. bone marrow transfer from *Foxp3*$^{-}$ mice into *Foxp3*$^{-}$ *Rag*$^{-/-}$ mice, obfuscating the interpretation of their findings [188].Another study, including all the combinations of donor and host Foxp3 genotypes, clearly showed no contribution of Foxp3-deficiency in non-hematopoietic tissues to the disease development [226]. Finally, bone marrow transplantation serves as an effective treatment for IPEX patients in agreement with the bone marrow chimera studies in mice [227].

Besides bone marrow transfer studies, a large body of genetic evidence established that Foxp3 expression in T cells and more specifically in Treg cells is required to prevent catastrophic autoimmunity. Mice with a Foxp3 deficiency restricted to the T cell lineage through CD4-Cre-mediated deletion of a conditional Foxp3 allele are indistinguishable from mice with the germ-line ablation of the *Foxp3* gene [7, 222]. In contrast, near complete deletion of *Foxp3* in thymic epithelial cells was inconsequential, i.e. no changes in thymocyte development and no

signs of autoimmunity were observed [222]. To accommodate these findings, Liu and colleagues suggested that CD4-Cre is expressed and mediates *Foxp3* deletion in thymic epithelial cells, and that the latter, not Foxp3 deficiency in T cells causes the autoimmune syndrome in these mice [188]. However, extensive genetic analyses of Cre-mediated recombination in CD4-Cre transgenic mice using a highly sensitive reporter allele failed to detect Cre expression in thymic epithelial cells and genetically controlled immunhistochemical analysis failed to detect Foxp3 protein expression in thymic epithelial cells [222]. It has further been demonstrated that Treg cell-specific Foxp3 deletion *via* retroviral Cre delivery to purified Treg cells isolated from conditional Foxp3 knockout mice abrogates Treg cell suppression activity, causing fulminating autoimmunity when transferred into lymphopenic recipients either alone or together with T cells from *Foxp3*$^-$ mice [66]. Together, catastrophic autoimmunity in mice and humans with Foxp3 mutations or engineered mice to inducibly eliminate Foxp3-expressing cells is ascribed to the essential role of Foxp3 in Treg cells.

If autoimmunity in Foxp3-deficient mice and humans is indeed caused by the lack of Treg cells, restoration of the Treg cell compartment is predicted to cure disease. In agreement with this notion, different groups have demonstrated that injecting purified Treg cells into Foxp3-deficient mice is sufficient to prevent life-threatening autoimmunity [7, 119, 225, 226, 228, 229]. Additionally, transfer of Treg cells into *Foxp3*^{DTR} (here: mouse-line from the Rudensky-group) mice treated with diphtheria toxin to eliminate Foxp3-expressing cells, inhibits tissue pathology in the liver, lung, and skin (foreign work in appendix 5, fig. 2). In contrast to these results, Liu and colleagues recently demonstrated that injecting sorted Treg cells into *scurfy* mice did not alleviate morbidity [189]. Thus, these results and the published data from several groups are at odds with those generated in the Liu laboratory.

Taken together, we have been unable to independently confirm Liu's assertion that Foxp3 is highly expressed by epithelial cells. From our experiments we conclude that DT-mediated depletion of non-hematopoietic cells in our mice does neither lead to an onset of autoimmune disease nor occurs at all.

4.2. The role and phenotype of Foxp3 mutated Tregs

Based on the previous findings, a contribution of Foxp3-mutated Tregs in the natural *scurfy* (sf) mouse mutant could not yet be ruled out. For this reason, DEREG mice were crossed to sf mice (appendix 2) to allow for monitoring of sf mutated Tregs.

$Foxp3^{-}$ sf Tregs do exist in male sf mice, as could be assessed by GFP expression, showing that DEREG x sf mice that did not express the functional Foxp3 gene (sf allele on the X chromosome) still express factors activating the additional Foxp3 promoter in the transgenic mice (GFP expressed as BAC transgene instead of Foxp3 exon1). Interestingly, sf Tregs could be found as $GFP^{+}Foxp3^{-}$ cells in similar percentages as $GFP^{+}Foxp3^{+}$ cells from DEREG control mice. Assessment of several *bona-fide* Treg markers revealed comparable expression levels when compared to DEREG Tregs.

Sf Tregs also showed a distribution of TCR Vβ chain expression comparable to wild type Tregs (not shown). Moreover, selection of a defined TCR specificity was not altered by the absence of functional Foxp3 as shown by a TCR transgenic approach. Thus, it appeared that Foxp3 is not required for thymic selection of Tregs and that sf mice possess "would be" Tregs (appendix 2, fig. 1 and 2). This is consistent with recent data from the laboratories of Rudensky and Chatila. Here, genetic models have been used to detect non-functional Tregs in BALB/c mice harbouring a truncated version of Foxp3 [69] or in 129Sv/C57Bl/6 mice where

the *foxp3* gene was completely knocked out and replaced by GFP [22]. Both studies also indicated that thymic selection of Tregs is independent of functional Foxp3 expression [69].

IPEX disease, the human homologue of *scurfy* disease, has an identical aetiology and IPEX pathogenesis can also be explained by mutations affecting the transcription factor Foxp3 [230, 231]. However, autoimmune phenotypes of affected patients vary depending on the site of mutation and possibly additional environmental factors [231]. Given the different outcomes of IPEX disease in humans depending on different sites and forms of mutations, it is intriguing to study non-functional sf Tregs in the natural *scurfy* mouse mutants. In the recently published study of Lin *et al.* [69], exon 11 was knocked out, resulting in expression of a stable Foxp3 protein, lacking the C-terminal forkhead domain. The truncated Foxp3 protein was non-functional, since the forkhead domain drives the translocation of the protein to the nucleus and enables DNA binding. Nevertheless, dominant-negative effects of the truncated protein could not completely be ruled out [69], a fact that gained importance after Ono *et al.* published that an N-terminal region of the Foxp3 protein is needed to bind AML1/Runx1 [77]. Loss of this interaction results in an abrogation of the anergic state of the Tregs and attenuation of their suppressive capacity [76, 77, 232]. Additionally, the N-terminal half of the protein has been shown, in human cells, to be necessary and sufficient for Foxp3-mediated suppression of a NF-AT inducible luciferase reporter [233]. Therefore, it cannot be excluded that the truncated protein could still bind AML1/Runx1 and directly compete with NF-AT binding, of which the latter is thought to be at least one of the mechanisms resulting in inhibition of activation-induced cytokine expression [76]. Furthermore, a forkhead box independent interaction between Foxp3 and the T_H17-driving transcription factor RORγT has been published recently. In the presence of RORγT, a truncated version of Foxp3 lacking the forkhead box was detectable in the nucleus [234].

As expected, sf Tregs did not suppress in an *in vitro* suppression assay (appendix 2, fig. 3A). However, *bona-fide* Treg markers used to characterize natural regulatory T cells were detectable (appendix 2, fig. 3B). Often, these molecules are also brought into context with regulatory T cell function. Data from RT-PCR was confirmed by FACS analysis in order to assess the expression levels of known suppression-related molecules such as CTLA-4 or GITR on protein level. The knock-out of CTLA-4 results in a phenotype comparable to the *scurfy* mouse mutant, suggesting overlapping functions of Foxp3 and CTLA-4 [31, 235]. GITR interaction with its ligand GITR-L on accessory cells has been linked to induction of regulatory properties and proliferation of plasmacytoid dendritic cells [236]. While Foxp3, as expected, was not detectable on protein level (appendix 2, fig. 1, 3C), CTLA-4 and GITR expression patterns were not altered in sf Tregs compared to wild type Tregs, indicating that the expression of both molecules is not dependent on Foxp3 expression and is also not able to provide suppressive activity when Foxp3 is absent. The surface molecule CD103 is a marker for effector/memory-like Tregs [237, 238] and analysis of this adhesion molecule showed a direct dependency of CD103 on Foxp3 expression. Female sf x DEREG mice stained negative for CD103 in all $GFP^{+}Foxp3^{-}$ sf Tregs, whereas the Foxp3-competent Tregs of the same mice had normal CD103 expression (appendix 3, suppl. fig. 1). This is in accordance with the recent finding of Marson *et al.*, showing a direct binding of Foxp3 to the CD103 promoter [75, 225]. However, loss of suppressive capacity of sf Tregs cannot simply be explained by loss of CD103 expression, since Tregs from $CD103^{-/-}$ mice display a comparable suppressive capacity as their wild type counterparts [239]. Furthermore, Foxp3-dependent CD103 expression can be overcome by overt inflammation since CD103 expression could be recovered in male *scurfy* mice (appendix 2, fig. 3A, B).

Recently, intracellular cAMP has been shown to be highly abundant in Tregs and its delivery into responder cells contributes to the suppressive capacity of Tregs [46]. Intracellular cAMP

levels in sf Tregs are much lower when compared to wild type Tregs (appendix 2, fig. 3D). It will have to be investigated if intracellular cAMP levels are directly dependent on Foxp3 expression. The substantial reduction of cAMP in sf Tregs might be one reason for the loss of suppressive capacity.

"Would-be" Tregs in female heterozygous *scurfy* mice were less abundant than their Foxp3-competent counterparts (appendix 3, suppl. fig. 1), indicating that Foxp3 might influence survival or homoeostasis of Tregs even though Foxp3 does not seem to be directly required for survival. Using quantitative RT-PCR and FACS analysis (appendix 2, fig. 2, 3B) it was demonstrated here that sf Tregs fail to up-regulate the high affinity IL-2 receptor chain (CD25), consistent with the finding that Foxp3 induces CD25 expression [75]. Consequently, the survival disadvantage of "would-be" Tregs in healthy female *scurfy* mice could be due to decreased signalling of IL-2, a cytokine known to be crucial for the homoeostasis of Tregs [75, 92, 240, 241]. This effect might play a negligible role in *scurfy* male mice suffering from severe autoimmunity, since it has been shown that the highly activated status in those mice leads to elevated IL-2 expression levels [242].

Since sf Tregs were shown to be non-suppressive, they might have acquired effector functions. Regarding the cytokine expression profiles, the most striking differences between GFP^+ sf Tregs and GFP^+ wt Tregs were observed for the production of T_H2-type cytokines, such as IL-4, IL-13, and IL-10, which were markedly up-regulated in sf Tregs. In earlier studies, the T_H2-type cytokine expression in sf mice has already been linked to $CD4^+CD25^+$ T cells, which under inflammatory conditions mainly comprised activated effector T cells [9]. Here, using DEREG mice crossed with *scurfy* mice, the enhanced cytokine expression could for the first time be detected in both GFP^+ sf Tregs and GFP^- effector T cells. Dysregulation of T_H2-type cytokines in the latter cell type can be best explained by the absence of functional

$Foxp3^+$ Tregs. In sf Tregs, however, expression of T_H2 cytokines might be affected more directly by the lack of Foxp3, since Foxp3 has been reported to negatively influence IL-10 and IL-4 production by direct binding to their promoter or by binding to the transactivator NF-AT [69, 74, 76, 225, 243]. Interestingly, as shown by RT-PCR, Galectin-1 appeared to be expressed independently of Foxp3 since it was highly up-regulated on sf T cells (appendix 2, fig. 3B). This elevated expression might have a compensatory effect because of the high T cell activation in these mice and could even play a deleterious role. It has been shown that galectin-1 can induce apoptosis in T_H1 and T_H17 cells, which could result in a bias towards T_H2-type immunity [244]. Furthermore, we observed both on message level (appendix 2, suppl. fig. 2), as well as by FACS analysis (appendix, fig. 4C), increased levels of the master regulator of T_H2 cells, GATA-3. Interestingly, IL-17 as a cytokine often brought into context with various autoimmune diseases, was not elevated in supernatants of sf T cell cultures. RT-PCR data revealed a drop of mRNA of the major Th17 transcription factor in both GFP^+ and GFP^- sf T cells. These findings are in accordance with a previously published report, even though underlying mechanisms are yet unclear [245]. It appears that when Foxp3 is mutated, cells which are prone to produce high amounts of cytokines are unleashed and a dominant T_H2 phenotype emerges. Importantly, the T_H2 environment of male sf mice did affect, but not completely prevent generation of induced sf Tregs (appendix 2, fig. 5), as predicted from studies where GATA-3 was overexpressed [200, 246]. Differentiation of naïve $CD4^+$ T cells into T_H2 effector cells has been shown to be dependent on CD28/B7 co-stimulation [247, 248]. Since $CD28^{-/-}$ *scurfy* mice show a substantially prolonged life span and significantly less cytokine production when compared to *scurfy* mice with same level of lymphoproliferation [249], it is conceivable that the observed T_H2 shift in *scurfy* $CD4^+$ T cells is an essential factor for disease development. Also for the human form of the disease, IPEX, high correlations between disease progression and increased IgE and eosinophilia could be observed [231]. In *in vitro* proliferation studies using Ki-67 staining, we could observe that IL-2 was dispensable

for a basal proliferation of sf Tregs which were not anergic, and that exogenous IL-2 promoted proliferation to a much higher extent (appendix 2, fig. 4D). Accumulation of sf Tregs in affected organs may reflect increased proliferation and migration towards the site of inflammation than the cause of autoimmune pathology. This hypothesis is further strengthened by the fact that the adoptive transfer of sf Tregs did not cause *scurfy*-like multiorgan inflammatory disease, while sf CD4 effector cells led to massive lymphocyte infiltration and tissue damage of all observed organs within 8 weeks (appendix 2, fig. 6).

Since Foxp3 expression was shown to be strictly Treg specific, the reason why sf effector T cells transmit the disease whereas DEREG effector T cells do not, are not yet clear. Possible explanations are the inflammatory status of *scurfy* mice at the time of donor cell purification. Self-recognizing effector T cells might have already proliferated extensively, resulting in a T cell repertoire highly shifted towards self. The other important difference might be the incapability of sf effector T cells to adapt regulatory properties upon conversion into Tregs, a phenomenon that has been shown for effector T cells *in vivo* [250] and *in vitro* [114, 251]. In line, GFP^{+} cells can be detected in the recipients at the time of analysis, clearly demonstrating Treg induction in the donor cell compartment from DEREG mice (appendix 2, suppl. fig. 3B). Colitis might develop due to the highly sophisticated immune system in the gut which is constitutively exposed to commensal flora, being dependent on sustained Treg function to maintain integrity, whereas skin or liver might compensate for a temporal loss of Tregs by other mechanisms. In a transfer colitis model, it has been shown that induced Tregs cannot prevent disease-induction in the gut [252]. This has been explained by a deficiency of IL-10 production by the peripherally induced Tregs consistent with the hypothesis that IL-10-mediated immune regulation is particularly important in the gut.

In summary, the novel BAC-transgenic DEREG mouse is a new tool to analyze Foxp3$^+$ Tregs *in vivo*. The previous limitations of antibody based depletion protocols will be avoided since Foxp3$^+$ Tregs can be selectively depleted in DEREG mice at any time of the immune response. Thus, the model will allow more precise insights into the role of Tregs in tumours, autoimmune disease, transplantation, and infection models. Furthermore, depletion of Foxp3$^+$ Tregs in neonatal DEREG mice also shed light on the cellular players involved in the development of the fatal lymphoproliferative disease in *scurfy* mice since our data unequivocally show that depletion of Foxp3$^+$ cells is sufficient to induce a *scurfy*-like phenotype. From the data using sf x DEREG mice, it can be concluded that Foxp3 mutated "would-be" sf Tregs can be found in *scurfy* mice but appear to be neither the initiators, nor the major contributors mediating autoimmunity.

4.3. Tregs in vaccination

Using DEREG mice, it could be shown for the first time that the enhancement of the CTL response generated in the absence of CD4 T cell help is due to the temporal loss of Tregs, which otherwise suppress the differentiation towards protective CD8$^+$ memory T cells (appendix 3, fig. 6). CD4 depleting antibodies have been used to show that depletion of the CD4 T cell subset leads to increased frequencies of antigen specific CTLs upon vaccination with live vaccines or split vaccines (appendix 3, fig. 2). In an additional approach, vaccination with MHC class II-deficient dendritic cells has been carried out to suppress *de novo* production of induced Tregs (appendix 3, fig. 7). Based on the findings observed using the DEREG mouse model that allows for specific depletion of Tregs only, the conclusion can be drawn that, although conventional CD4$^+$ T cell help participates in memory T cell generation during protein vaccination [162-164, 253, 254], the absence of this pathway is more than compensated for by the potency of Treg depletion. Tregs may limit CD8 T cell priming by

dampening dendritic cell activation. This leaves the possibility that CD4 T cell help is usually needed by the DC compartment to overcome Treg-mediated suppression [255, 256]. The likelihood of interference of Tregs with priming of CTLs by DCs is supported by the fact that depletion one day after sensitization is effective. By day seven, the day when the CTL response towards OVA-CpG peaks, at least half of the Treg compartment is replenished (see appendix 1, supplementary information). This argues for a most prominent effect of Treg depletion during the priming phase of the immune response. Furthermore, the recall response is induced *via L. monocytogenes* infection five weeks after sensitization, when the Treg compartment is fully replenished for around three weeks (see appendix 1, supplementary information).

A role for Tregs in interfering with the priming of the immune system could be shown in a different study, using DEREG mice crossed to DIETER [257] mice. In DIETER mice, steady-state dendritic cells induce tolerance by presenting a CTL epitope derived from LCMV in the absence of inflammatory stimuli. In initial studies performed to characterize these mice, it could be shown that tolerance induction is indeed mediated by dendritic cells and dependent on CTLA-4 and PD-1 [258]. Regulatory T cell depletion in DEREG crossings led to up-regulation of co-stimulatory molecules on dendritic cells, increased numbers, and efficient priming of a CTL response (appendix 4).

Immediately after homoeostatic proliferation, Tregs have been shown to exhibit their highest regulatory potential [220]. It still has to be investigated if the fast rebound of Tregs upon depletion is due to true *de novo* generation of Tregs in the thymus with subsequent homoeostatic proliferation, or due to conversion of naïve T cells into Tregs in the periphery. As pointed out in the introduction, natural or induced Tregs show distinct behaviours. It

remains to be shown that Tregs are induced in mice receiving OVA-CpG in an undepleted setting and the absence of these particular Tregs during recall causes better protection.

The finding that depletion of regulatory T cells after sensitization with OVA-conjugate lead to better protection against OVA-expressing *Listeria monocytogenes* is extremely important, given that CD8 T cell frequencies (although being specific for the antigen) do not necessarily correlate with the control of intracellular pathogen replication or tumour growth [259-262] and that efficacy depends on qualitative rather than quantitative parameters. One important qualitative parameter known so far is the avidity of a CD8 T cell receptor for its cognate antigen. The higher the sensitivity, the lower is the antigen dose which can be recognized by the cell. High antigen sensitivity leads to more rapid effector function, often leading to better virus clearance or better target cell elimination [263-269]. Antigen concentration has been shown to be directly related to the functional program of the cells, further strengthening the importance of T cell receptor avidity [270-273]. TCR avidity has not been addressed yet in this work, but it is likely that regulatory T cell depletion also results in a pool of CD8 T cells with higher avidity.

The high primary responses towards the model antigen OVA in the presence of CpG as an adjuvant achieved here do not necessarily correlate with vaccination efficacy, where long-term memory is needed. This implicates that effective memory depends not solely on higher frequency of CD8 memory clones of a given specificity, but also to intrinsic characteristics of CD8 memory T cells, involving increased capacity for cell division, cytokine secretion, and cell survival [274, 275]. In contrast, highly activated and heavily proliferating cells have been shown to have a limited replicating life span [276]. Theoretically, excessive stimulation could result in irreversible clonal depletion [277-279]. Further, it has been shown that high IFNγ levels can have a negative influence on the development of effective immunological memory

[280]. Showing that Treg depleted mice are better protected towards *Listeria monocytogenes* infection 5 weeks after sensitization may suggest that upon Treg depletion, memory responses are elevated. Evaluating immune responses five weeks after sensitization does not, however, necessarily reflect “true” memory, implicating that additional studies are required with a longer timeframe between sensitization and challenge phase. One other study also showing advantages towards *Listeria* infection upon CD4 T cell depletion shows similar kinetics, but also in this study, the read-out was performed at a relatively early time point to interpret results as “true” immunological memory [281]. It will further be necessary to evaluate memory induction upon depletion of the whole CD4 T cell compartment in more detail, considering that these cells are broadly discussed to play a major role for “true” memory induction by programming memory T cells for secondary expansion, rather than during primary sensitization. Regulatory T cells could be of higher hierarchy, leading to stronger responses if absent in general, but Treg modulation could also be a direct opponent of CD4 T cell help. In any case, the Treg compartment is an attractive target for improving the efficacy of *in vivo* cross-priming during vaccination, especially by increasing the number of long-living (at least for 5 weeks) $CD8^+$ memory T cells (appendix 3). It has been shown that Tregs are largely dependent on interactions with MHC class II and require interactions with self-MHC-peptide complexes for *in vivo* homoeostasis [220]. Given that memory T cells, either CD4 or CD8, are not dependent on MHC-peptide complexes for activation in a lymphopenic environment [282-285], it would be interesting to analyze the potential of MHC class II-deficient dendritic cell vaccination in recall responses and to compare the resulting frequencies to those generated by the mere depletion of Tregs in the challenge phase. Theoretically, the generation of efficient memory CTLs should then be augmented since memory CD4 T cells might still provide help to some extent even in a non-lymphopenic environment. In contrast, Tregs would not be stimulated in this setting. If this hypothesis holds true and if memory CD8 T cells, as reported on the basis of CD25 depletion [286], *per*

se respond better in the absence of Tregs will have to be addressed in future work. It will be interesting to further investigate the role of Treg depletion in the challenge phase in light of publications showing that IL-2 is important to program memory CD8 T cells for secondary expansion [173, 287] and one of the discussed Treg-mechanisms is cytokine deprivation of IL-2. In line, CD4 T cell help has even been shown to contribute to epigenetic remodelling of the IL-2 and IFNγ loci in $CD8^+$ T cells [288]. Tregs might also play a role for affinity maturation of CD8 memory cells, which has been shown to only occur after secondary infection [289]. It is very likely that T cells with different peptide specificity and thus different avidity for a certain pathogen are differentially regulated by Tregs. Understanding the mechanisms of regulation in this context would help to design efficient vaccines against pathogens and tumours without risking tissue destruction by autoreactive CTLs. It could be shown recently that the immune system might harbour self-specific CD8 T cells with low affinity for self-antigens, while still being able to recognize those antigens in the context of an infection [290]. Tuning the balance of CD8 T cell frequency and avidity by interfering with the regulatory T cell compartment would thus be a promising approach for vaccinology.

5. References

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6. Appendices

1 - **Lahl, K.**, C. Loddenkemper, Drouin, C., J. Freyer, J. Arnason, G. Eberl, A. Hamann, H. Wagner, J. Huehn, T. Sparwasser. Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. *J Exp Med* 2007 204(1):57

2 - **Lahl, K.**, Mayer, C., Bopp T., Huehn, J., Loddenkemper, C., Dornmair, K., Eberl, G., Geffers, R., Buer, J., Schmidt, E., T. Sparwasser. Development of "would-be" regulatory T cells and defective control of TH2-cytokine production in natural Scurfy mutant mice. *JI (submitted)*

3 - Heit, A.*, Gebhardt, F.*, **Lahl, K.***, Neuenhahn, M., Schmitz, F., Wagner, H., Sparwasser, T., Busch, D., K. Kastenmueller. Circumvention of regulatory CD4+ T cell activity during cross-priming strongly enhances T cell-mediated immunity. *shared first authorship. *EJI* 2008 38:1585-1597

4 - Schildknecht, A., Brenner, C., **Lahl, K.**, Sparwasser, T., Probst, H. C., M. van den Broek. FoxP3+ regulatory T cells essentially contribute to peripheral CD8+ T cell tolerance induced by steady state dendritic cells. *PNAS (under revision*)

5 - Kim, J.*, **Lahl, K.***, Loddenkemper, C., Hori, S., Rudensky A., T. Sparwasser. Letter to JI: Foxp3 expression is restricted to hematopoietic cells and deficiency leads to autoimmune disease caused by the lack of Treg cell development. *shared first authorship. *JI (submitted)*

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Selective depletion of Foxp3$^+$ regulatory T cells induces a scurfy-like disease

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The scurfy mutant mouse strain suffers from a fatal lymphoproliferative disease leading to early death within 3–4 wk of age. A frame-shift mutation of the forkhead box transcription factor Foxp3 has been identified as the molecular cause of this multiorgan autoimmune disease. Foxp3 is a central control element in the development and function of regulatory T cells (T reg cells), which are necessary for the maintenance of self-tolerance. However, it is unclear whether dysfunction or a lack of T reg cells is etiologically involved in scurfy pathogenesis and its human correlate, the IPEX syndrome. We describe the generation of bacterial artificial chromosome–transgenic mice termed "depletion of regulatory T cell" (DEREG) mice expressing a diphtheria toxin (DT) receptor–enhanced green fluorescent protein fusion protein under the control of the *foxp3* gene locus, allowing selective and efficient depletion of Foxp3$^+$ T reg cells by DT injection. Ablation of Foxp3$^+$ T reg cells in newborn DEREG mice led to the development of scurfy-like symptoms with splenomegaly, lymphadenopathy, insulitis, and severe skin inflammation. Thus, these data provide experimental evidence that the absence of Foxp3$^+$ T reg cells is indeed sufficient to induce a scurfy-like phenotype. Furthermore, DEREG mice will allow a more precise definition of the function of Foxp3$^+$ T reg cells in immune reactions in vivo.

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Naturally occurring CD25$^+$CD4$^+$ regulatory T cells (T reg cells) are currently intensively characterized because of their major importance in modulating host responses to tumors and infections, in preventing transplant rejection, and in inhibiting the development of autoimmunity and allergy (1–3). Originally, CD4$^+$ T reg cells were identified exclusively by the constitutive expression of CD25, and many in vivo experiments have been performed using depleting antibodies directed against CD25 (4, 5). However, both the existence of CD25$^-$ T reg cells, especially within peripheral tissues (1, 6, 7), as well as the expression of CD25 on activated conventional T cells, which precludes discrimination between T reg cells and activated conventional T cells, limits the interpretation of data obtained by the use of anti-CD25 depleting antibodies (7–9). The most specific T reg cell marker currently known is the forkhead box transcription factor Foxp3, which has been shown to be expressed specifically in mouse CD4$^+$ T reg cells and acts as a master switch in the regulation of their development and function (10). Moreover, activated conventional mouse CD4$^+$ T cells or differentiated Th1/Th2 cells fail to induce Foxp3 expression (11, 12).

A key role of Foxp3 in the development of natural T reg cells has been suggested from the molecular characterization of the scurfy mouse mutant. These mice, which suffer from a fatal lymphoproliferative disorder, harbor a mutated *foxp3* gene coding for a product that lacks the forkhead domain (13). Scurfy mice receiving CD25$^+$CD4$^+$ T reg cells from WT mice remained virtually disease free (11). Furthermore, transduction of mutant Foxp3, lacking the forkhead domain, failed to confer suppressive activity

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to naive CD4+ T cells, in contrast to full-length Foxp3 (12). Thus, it is widely accepted that an intrinsic T cell failure to generate functional CD4+ T reg cells is the main cause of the fatal autoimmune disease in scurfy mice, although an additional role of the scurfy mutation in nonhematopoietic cells has been suggested (14, 15). Moreover, it remains unknown whether the mere absence of functional Foxp3+ T reg cells is sufficient to provoke the development of the scurfy phenotype or whether those CD4+ T cells expressing the truncated Foxp3 protein play a more active role, because depletion of T reg cells with established protocols has failed to reproduce the fulminant natural disease (4, 5). The latter hypothesis is supported by the fact that scurfy disease could be transplanted into nude mice through adoptive transfer of CD4+ T cells (16). Furthermore, Foxp3+ T reg cells preferentially recognize self-antigens with high affinity (17, 18), and activated, not naive, T cells in Foxp3-deficient mice preferentially use TCRs found in the TCR repertoire of T reg cells in Foxp3-sufficient mice (19). Collectively, these data led to the hypothesis that these "would-be" T reg cells expressing self-reactive TCRs may contribute to the pathology of Foxp3-deficient mice (19).

To address the question of the in vivo role of T reg cells in immunopathology, we have generated bacterial artificial chromosome (BAC)–transgenic mice termed "depletion of regulatory T cell" (DEREG) mice, which express a diphtheria toxin receptor (DTR) enhanced GFP (eGFP) fusion protein under the control of the *foxp3* locus, allowing both detection and inducible depletion of Foxp3+ T reg cells. Phenotypic characterization of DEREG mice not only revealed an eGFP expression pattern similar to that of previously published Foxp3 reporter mice (20, 21) but also showed that DT treatment, in contrast to conventional T reg cell depletion strategies, allows for efficient and selective depletion of Foxp3+ cells without affecting CD25+ effector T cells. Interestingly, ablation of Foxp3+ T reg cells in newborn DEREG mice led to the development of scurfy-like symptoms, thus providing direct evidence that Foxp3+ T reg cells are critically involved in controlling homeostasis of the immune system and in preventing the development of autoimmune diseases.

RESULTS AND DISCUSSION

Generation of BAC-transgenic DEREG mice

BACs are large fragments of genomic DNA cloned into bacterial vectors that allow for stable propagation in bacteria. Given the large size of BACs, most if not all regulatory sequences of a gene are present and can be used to direct faithful and tissue-specific expression of heterologous genes in vivo in BAC-transgenic mice. We modified a BAC containing the *foxp3* locus by introducing the gene coding for a DTR-eGFP fusion protein into the first exon of the *foxp3* gene (Fig. 1 A). The modified 150-kb BAC was injected into the pronuclei of fertilized C57BL/6 oocytes. Transgenic mouse lines with high transgene expression were established. The transgenic mice were termed DEREG mice. Analysis

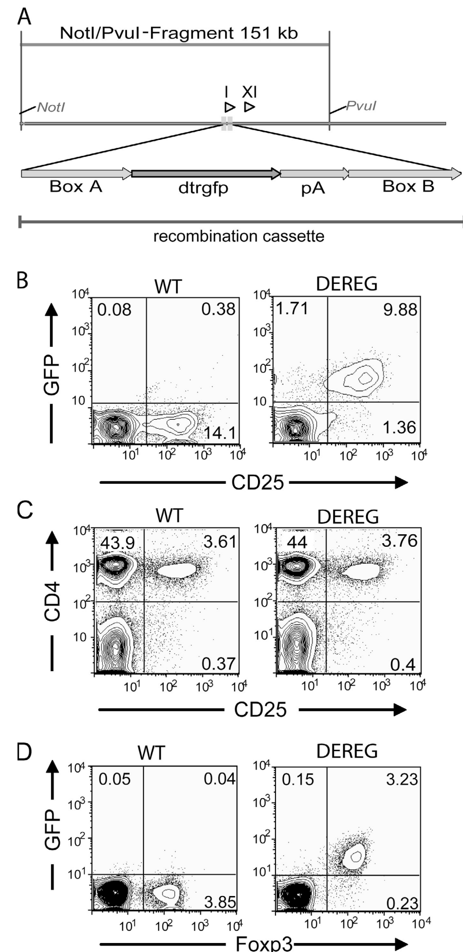

Figure 1. DEREG mice specifically express DTR-eGFP in T reg cells. (A) Map of BAC construct used for generation of transgenic mice. I and XI indicate the positions of exons I and XI of the *foxp3* gene. 24 bp of exon I were replaced by the gene coding for DTR-eGFPpA by homologous recombination with a 1-kb 5′ and 3′ homologous sequence (Boxes A and B). (B) Transgene expression is specific for CD25+CD4+ T cells in naive DEREG mice. Flow cytometric analysis of live-gated CD4+ LN cells of the indicated genotype reveals GFP expression mainly in CD25+CD4+ T cells. (C) Analysis of CD25+CD4+ T cell subsets in LNs from DEREG mice and WT littermates. Plots show similar frequencies of the cell subset in live-gated cells. (D) Foxp3 and GFP expression of LN cells. Flow cytometric analysis reveals a specific transgene expression in T reg cells. Cells are live gated. The percentage of cells in each quadrant (B–D) is indicated.

of DEREG mice revealed selective DTR-eGFP expression within the CD4+ T cell compartment, with the highest expression within the CD25+ subset (Fig. 1 B). Importantly, similar frequencies and total numbers of CD25+CD4+ T reg cells were observed between DEREG and WT mice (Fig. 1 C and not depicted), and Foxp3 levels in CD25+CD4+ T reg cells from DEREG mice were comparable to T reg cells from WT mice (Fig. 1 D), ruling out the possibility that the BAC transgene influences regulation of the endogenous *foxp3* locus. Histological and flow cytometry analysis of spleen, thymus, and LN revealed that the BAC transgene,

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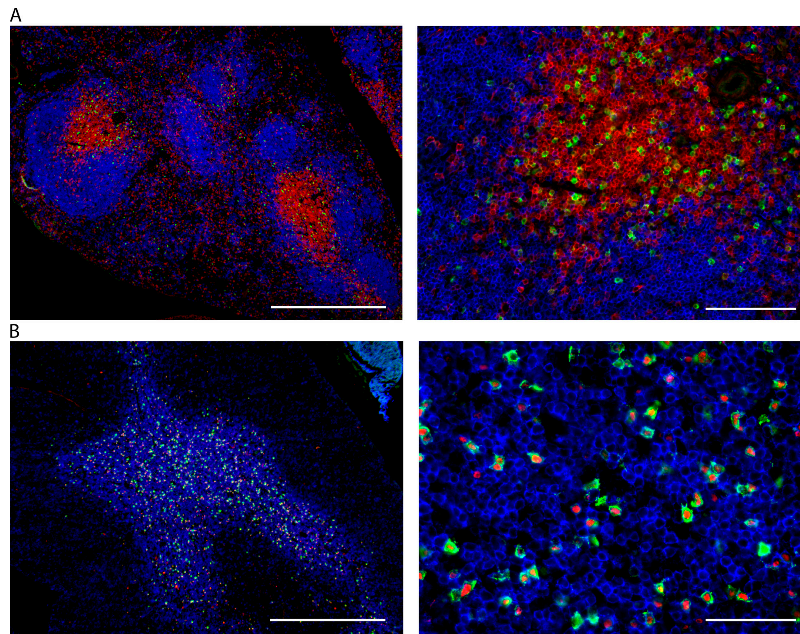

Figure 2. Localization of Foxp3⁺ cells in the spleen and thymus. (A) Spleen sections were stained with αB220-allophycocyanin (blue), αCD3–Alexa Fluor 555 (red), and αGFP–Alexa Fluor 488 (green). (B) Thymus sections were stained with αCD3–Alexa Fluor 647 (blue), αFoxp3–Alexa Fluor 555 (red), and αGFP–Alexa Fluor 488 (green). Bars: (A and B, left) 500 μm; (A, right) 100 μm; (B, right) 50 μm.

encoding the DTR-eGFP fusion protein, is faithfully expressed in Foxp3⁺CD4⁺ T cells and is absent from thymic epithelial as well as B220⁺ and CD8⁺ cells (Fig. 2 and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20061852/DC1). Furthermore, no gross alteration in the frequency and total number of CD4⁺ and CD8⁺ T cells, B220⁺ B cells, and CD11c⁺ DCs was observed in LNs and spleen of DEREG mice when compared with WT controls (Fig. S1 and not depicted). In addition, CD25⁺CD4⁺ T reg cells from DEREG and WT mice displayed a similar in vitro suppressive capacity (not depicted). Collectively, DEREG mice showed specific expression of the DTR-eGFP fusion protein in fully functional Foxp3⁺CD4⁺ T reg cells.

DT injection allows selective depletion of Foxp3⁺ T reg cells

Next we assessed whether injection of DT leads to selective depletion of Foxp3⁺ T reg cells in vivo. To achieve this, 6-wk-old DEREG mice were injected with 1 μg DT per mouse for six consecutive days. On day 7, we analyzed Foxp3 expression in various lymphoid organs. Among splenocytes and LN-derived cells, we observed an almost complete obliteration in the frequency and absolute number of Foxp3⁺CD4⁺

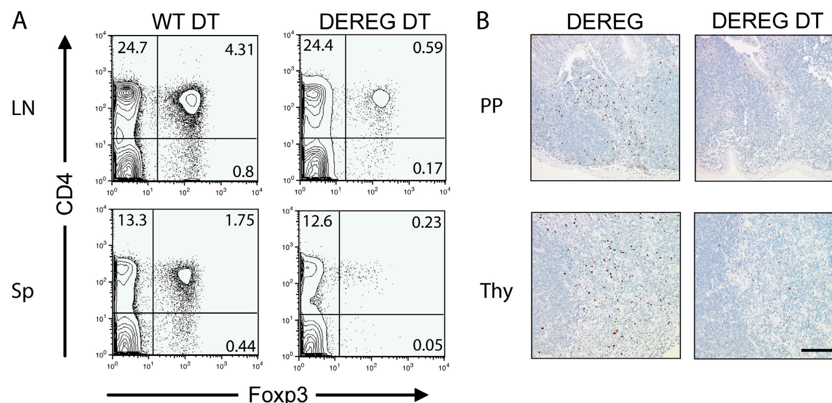

Figure 3. Depletion of Foxp3⁺ cells with DT in DEREG mice. After six consecutive days of DT injection (1 μg/day), mice were killed on day 7, and lymphoid organs were removed. (A) Flow cytometric analysis of LN-derived cells and splenocytes shows efficient depletion of Foxp3⁺ cells in DT-treated DEREG mice compared with DT-treated WT control mice. The percentage of cells in each quadrant is indicated. (B) Foxp3 staining (brown) of thymic and Peyer's patch (PP) sections shows effective depletion of Foxp3⁺ cells in DT-treated compared with untreated DEREG mice. Bar, 100 μm.

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T cells in DT-treated DEREG mice compared with untreated DEREG mice or WT control mice (Fig. 3 A). A similar degree of depletion was observed in the thymus and in Peyer's patches (Fig. 3 B and not depicted). The daily dose of 1 μg DT depleted up to 90% of Foxp3$^+$ T reg cells after 7 d. Importantly, DT treatment did not affect other lymphocyte subsets (Fig. 3 and not depicted), suggesting that DT treatment of DEREG mice allows for the selective depletion of Foxp3$^+$ cells.

Selective depletion of T reg cells leads to an enhanced and prolonged delayed-type hypersensitivity (DTH) response

Previous attempts to analyze the role of CD4$^+$ T reg cells in vivo have used anti-CD25 depleting antibodies (1). In those experiments, the antibodies remained in the circulation for a considerable time, and recent evidence has indicated that CD25$^+$ effector T cells are also affected (unpublished data). In this report, we investigated whether selective depletion of Foxp3$^+$ T reg cells in DEREG mice would provide a method to analyze the role of CD4$^+$ T reg cells during an established immune response. We chose a DTH model in which T reg cells were depleted after hapten-specific sensitization during the phase when CD25$^+$ effector T cells driving the DTH response were generated (22).

Both untreated DEREG and WT control mice developed a weak DTH response after challenge with the hapten, as indicated by footpad swelling. DT treatment resulted in a substantially increased DTH response in DEREG mice 24, 48, and 72 h after challenge, suggesting that Foxp3$^+$ T reg cells are involved in the regulation of the local inflammatory response (Fig. 4 A and not depicted). The increased footpad swelling in DT-treated DEREG mice was accompanied by an increased cellular immune response within the draining LN (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20061852/DC1). In contrast to the increased footpad swelling after DT treatment in DEREG mice, WT control mice receiving anti-CD25 antibodies showed a weak DTH response comparable to challenged mice without T reg cell depletion (Fig. 4 A). Analysis of CD4$^+$ T cells from peripheral blood before challenge revealed a very efficient depletion of both CD25$^+$ and CD25$^-$GFP$^+$ T reg cells in DT-treated DEREG mice, whereas a sufficient fraction of CD25$^+$GFP$^-$ cells remained (Fig. 4 B). Anti-CD25 antibody treatment completely removed CD25$^+$ cells from circulation, suggesting that this treatment not only led to the depletion of CD25$^+$CD4$^+$ T reg cells but also removed CD25$^+$ effector T cells (Fig. 4 C). Additionally, the same treatment has recently been shown to efficiently deplete CD25$^+$CD4$^+$ T reg cells but not CD25$^-$Foxp3$^+$ T reg cells (23). Therefore, our data suggest that selective removal of total Foxp3$^+$ T reg cells by the use of DEREG mice represents a major progress in the analysis of T reg cell function during the effector phase of an ongoing immune response.

Neonatal depletion of Foxp3$^+$ T reg cells leads to the development of scurfy-like symptoms

Lack of functional T reg cells has been suggested to cause the fatal lymphoproliferative disease in scurfy mice. The DEREG mice described in this report have now allowed us to assess whether diminution of Foxp3$^+$ T reg cells is sufficient to induce scurfy-like symptoms or whether dysfunctional T reg cells expressing the truncated Foxp3 protein contribute to pathology.

The scurfy mutation is manifested by several clinical disorders and is evident primarily in the lymphoid organs and skin 14 d after birth (24). The disease is characterized by runting; scaliness and crusting of the eyelids, ears, and tail; gross internal lesions including marked splenomegaly; enlarged LNs;

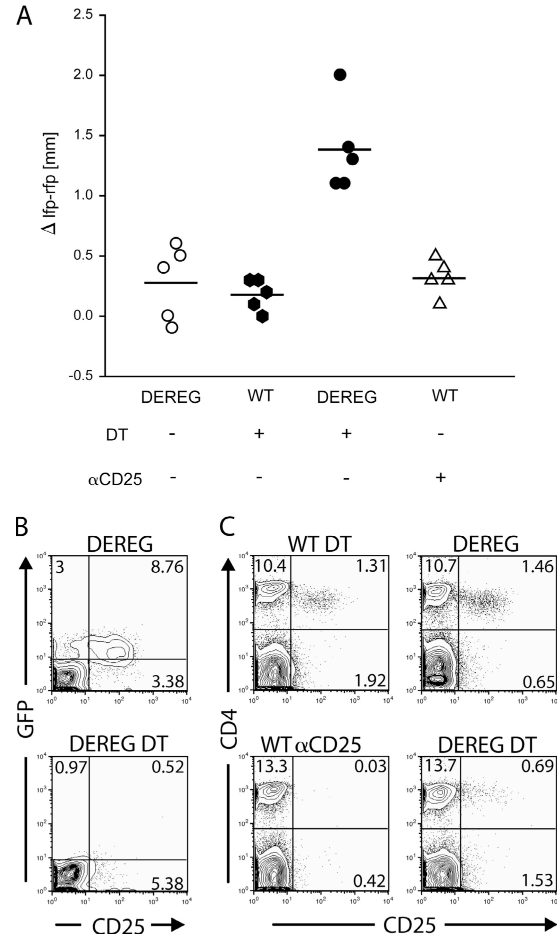

Figure 4. Depletion of Foxp3$^+$ cells in DEREG mice results in enhanced DTH reaction. (A) Differences in thickness between challenged and PBS-injected footpads in the indicated groups of mice 24 h after challenge. Horizontal lines represent the means. (B) Flow cytometric analysis of CD25 and eGFP expression in live-gated peripheral blood CD4$^+$ T cells after DT treatment of DEREG mice (before challenge) and in untreated DEREG control mice reveals that, after depletion, most of the remaining CD25$^+$CD4$^+$ T cells are GFP negative. (C) Frequency of peripheral blood CD25$^+$CD4$^+$ T cells before challenge. Plots show the absence of double-positive cells in mice that received 500 μg αCD25 and a reduction of CD25$^+$CD4$^+$ T cells in DEREG mice injected with 5 × 1 μg DT (bottom). Control groups show the normal frequency of CD25$^+$CD4$^+$ T cells (top). Cells were live gated. Data are representative of one out of three independent experiments with five mice per group. The percentage of cells in each quadrant (B and C) is indicated.

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and severe anemia. These symptoms result in a wasting syndrome that generally leads to death by 15–24 d of age (24). When newborn DEREG mice were treated with 500 ng DT i.p. (or 100 ng DT s.c.) followed by a second DT injection on day 7, which led to an almost complete depletion of Foxp3$^+$CD4$^+$ T cells (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20061852/DC1), these mice developed an aggressive lymphoproliferative autoimmune syndrome strongly resembling the observed pathology in scurfy mice. The spleens of DT-treated DEREG mice were significantly enlarged and resembled spleens from age-matched scurfy mice (Fig. 5 A). Histologically, they displayed a disrupted architecture with hyperplasia of the white and red pulp (not depicted). DT-treated DEREG mice showed markedly enlarged LNs as well as increased total cell numbers when compared with both DT-treated WT control and scurfy mice (Fig. 5 B). Histological analysis of DT-treated DEREG mice revealed massive inflammatory infiltrates in various organs (Fig. 5 C). The skin overlying the hyaline cartilage of the ear was extensively thickened with epidermal hyperplasia and a dense infiltrate of lymphocytes—including large numbers of CD3$^+$ T cells (Fig. S4)—with admixed neutrophils and macrophages in the dermis compared with DT-treated WT control mice. The lungs of DT-treated DEREG mice showed similar infiltrates as scurfy mice with peribronchial and perivascular distribution when compared with the normal lung with thin and delicate alveolar walls in control mice. The acini of the pancreas displayed infiltrates with destruction of the islets (insulitis), and the liver exhibited portal aggregates (Fig. 5 C). Because lesions in DT-treated DEREG mice closely resembled those observed in scurfy mice and no significant differences in the pathology of those particular organs were observed, we concluded that diminution of CD4$^+$ T reg cells is sufficient to induce the lethal lymphoproliferative autoimmune syndrome and that an active contribution of pathogenic autoreactive CD4$^+$ T cells expressing the truncated Foxp3 protein is not necessarily required (19). Indeed, immunohistochemical staining for Foxp3 showed that in the thymi and spleens of DT-treated DEREG mice the vast majority of Foxp3$^+$ T reg cells were absent (Fig. 5 D and not depicted).

In contrast to depletion of Foxp3$^+$ T reg cells by DT injection, which consistently resulted in the development

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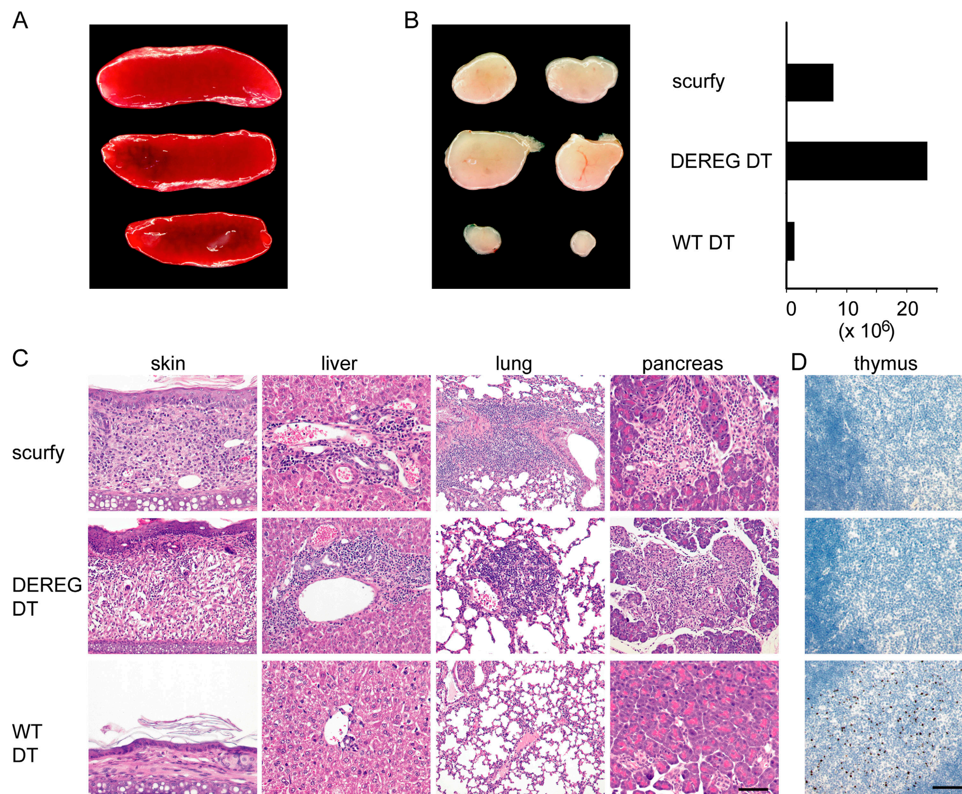

Figure 5. Depletion of Foxp3$^+$ cells in neonate DEREG mice results in a scurfy-like phenotype. Neonates were injected i.p. on days 1 and 7 after birth with 500 ng DT and analyzed after 21 d. (A) Splenomegaly in scurfy mice (top) and depleted DEREG mice (middle) compared with a DT-injected WT littermate (bottom). (B) Enlargement and increase of LN cellularity (left, scapular LN; right, inguinal LN; the mean of total cell numbers from four pooled peripheral LN is shown) in scurfy and depleted DEREG mice compared with DT-treated WT littermates. (C) Histological analysis of different organs. Figures show hematoxylin and eosin staining of the indicated organs in the different mice. Bar, 100 μm. (D) Foxp3 staining (brown) of thymus sections shows remaining Foxp3$^+$ expression only in WT littermates. Data are representative of 1 out of 10 independently analyzed mice in which a total of 12 organs each (spleen, LN, Peyer's patches, thymus, liver, pancreas, salivary gland, heart, lung, kidney, gastrointestinal tract, and skin) was evaluated. Bar, 50 μm.

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of scurfy-like symptoms, T reg cell depletion in neonatal mice with anti-CD25 antibodies resulted in marginal development of autoimmune disease, if at all (4, 5). These discrepancies are again best elucidated by both the existence of $CD25^-Foxp3^+$ T reg cells, especially within peripheral tissues (1, 6, 7), as well as the induced expression of CD25 on activated conventional $CD4^+$ T cells, which are critically involved in the development of the scurfy phenotype (16). Interestingly, in adult mice neither T reg cell depletion with anti-CD25 antibodies (8) nor depletion of total $Foxp3^+$ T reg cells by DT treatment (not depicted) resulted in the development of autoimmune diseases. This finding can be explained by the lack of lymphopenia-induced proliferation preventing the activation of autoreactive T cells (5). Furthermore, within 2 wk after the last DT injection, we observed a significant rebound of $Foxp3^+$ T reg cells in adult but not neonatal DEREG mice (Fig. 5 D; Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20061852/DC1; and not depicted), which might participate in the control of pathogenic effector cells.

In summary, the novel BAC-transgenic DEREG mouse is a new tool to analyze $Foxp3^+$ T reg cells in vivo. The previous limitations of antibody-based depletion protocols will be avoided because $Foxp3^+$ T reg cells can be selectively depleted in DEREG mice at any time of the immune response. Thus, the model will allow more precise insights into the role of T reg cells in tumors, autoimmune disease, transplantation, and infection models. Furthermore, depletion of $Foxp3^+$ T reg cells in neonatal DEREG mice also shed some light on the cellular players involved in the development of the fatal lymphoproliferative disease in scurfy mice, because our data unequivocally show that depletion of $Foxp3^+$ cells is sufficient to induce a scurfy-like phenotype.

MATERIALS AND METHODS

Mice. DEREG and WT C57BL/6 mice were bred at the animal facility of the Institut für Medizinische Mikrobiologie, Immunologie und Hygiene at the Technische Universität München. Scurfy mice (B6.Cg-Foxp3 sf/J) were purchased from Charles River Laboratories. All animal experiments were performed under specific pathogen-free conditions and in accordance with institutional, state, and federal guidelines.

Generation of DEREG mice. Transgenic mice were generated using BAC technology, as previously described (25, 26), with the following modifications. As a transgene, the coding sequence of the DTR-eGFP fusion protein was used (27). The BAC encoding the complete mouse *foxp3* gene locus (RP23-267C15) was obtained from the BACPAC Resources Center at Children's Hospital Oakland Research Institute. In contrast to the published overlap PCR strategy (26), homologous regions named Box A and Box B were ligated to DTR-eGFP via AscI (Box A) or PmeI sites (Box B). The polyA fragment was amplified from the TOPO Tools SV40 pA 3′ element kit (Promega) using primers, adding a SpeI site 5′ and a PmeI site 3′. The WT BAC was recombined using the pLD53.SC1 shuttle vector provided by N. Heintz (The Rockefeller University, New York, NY), gel purified, and injected into the pronuclei of fertilized C57BL/6 oocytes. Two transgenic mouse lines with high transgene expression were established (nos. 16.1 and 23.2). DEREG mice were genotyped by PCR using the primers P442 (5′-CCCAGGTTACCATGGAGAGA-3′) and P443 (5′-GAACTTCAGGGTCAGCTTGC-3′).

Antibodies, staining, and sorting reagents. The following antibodies and secondary reagents were purchased from eBioscience: αCD4 (H129.19), αCD25 (PC61), αCD8 (53–6.7), αB220 (RA3-6B2), αTCR-β (H57-597), αCD11c (HL3), αCD3ε (500A2), αB220 (RA3-6B2), αFoxp3 (FJK-16s), a T reg cell staining kit, streptavidin, and appropriate isotype controls. Rabbit αGFP and FITC-conjugated goat α rabbit polyclonal antibodies were obtained from Invitrogen. The antibody αCD25 (PC61) was produced in our laboratory. For immunohistochemistry, the polyclonal αCD3 obtained from DakoCytomation was used.

Flow cytometry. Cytometric analysis was performed using a CyAn (DakoCytomation) or a FACSCalibur (BD Biosciences) and FlowJo software (Tree Star, Inc.). Dead cells were excluded by propidium iodide staining or ethidium monoazide (Sigma-Aldrich). Intracellular Foxp3 staining was performed with the PE α mouse Foxp3 staining set (Bioscience), according to the manufacturer's instructions.

Depletion of T reg cells. To deplete T reg cells, DEREG mice were injected with DT (Merck) diluted in endotoxin-free PBS. In our standard protocol, 1 μg DT was injected i.p. on a daily basis for four to six consecutive days unless otherwise indicated. Newborn mice were injected twice either i.p. with 500 ng or s.c. with 100 ng at days 1 and 7 after birth. Anti-CD25 (PC61) was administered i.p. at 500 μg/mouse.

Microscopy, immunofluorescence staining, histology, and immunohistochemistry. LNs and spleens from scurfy and DT-treated mice were photographed individually with the same magnification using a stereo microscope (MZ-APO; Leica). Immunofluorescent stainings were performed as previously described (28). Slides were examined under a fluorescence microscope (AxioImager M1; Carl Zeiss MicroImaging, Inc.) equipped with a CCD camera (AxioCam MRm; Carl Zeiss MicroImaging, Inc.) and processed with Axiovision software (Carl Zeiss MicroImaging, Inc.). Hematoxylin and eosin stainings were performed on formalin-fixed and paraffin-embedded organs. Foxp3 and CD3 immunohistochemistry was performed as previously described (29).

DTH model. DTH reactions were performed as previously described using 2% and 0.1% NP-O-Su (Biosearch Technologies) for sensitization and challenge, respectively (30). For depletion of T reg cells, mice were injected i.p. with 1 μg DT per mouse for five consecutive days, starting on day 2 after sensitization. Alternatively, CD25-expressing cells were depleted via i.p. injection of 500 μg anti-CD25 (PC61) on day 5 after sensitization. Mice were challenged on day 7 after sensitization, and the inflammatory response was determined 24 h later by the measurement of footpad thickness. For in vitro restimulation, mice were killed 48 h after challenge. 4×10^5 cells from draining popliteal and inguinal versus control LNs were stimulated with 3×10^5 modified bone marrow GM-CSF–cultured DCs from naive mice. For modification, DCs were incubated for 3 min at 37°C with 0.02% NP-O-Su. After 4 d, proliferation and IFN-γ production were measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) and the DuoSet ELISA (R&D Systems), according to the manufacturer's instructions, respectively.

Online supplemental material. In Fig. S1, flow cytometry analysis reveals that DTR-eGFP expression in DEREG mice is confined to $TCR\beta^+$ T cells and precluded from $B220^+$, $CD11c^+$, and $CD8^+$ cells. Fig. S2 depicts the increased cellular immune response in the local draining LN after DT treatment in hapten-challenged mice. Fig. S3 depicts the degree of depletion of $Foxp3^+$ T reg cells on day 8 in neonatal DEREG mice upon the second DT treatment on day 7. Fig. S4 shows $CD3^+$ T cell infiltrates in different organs of DT-treated DEREG mice compared with scurfy and DT-treated WT control mice. Fig. S5 shows the rebound of $Foxp3^+$ T reg cells in DT-treated DEREG mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20061852/DC1.

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Non-functional regulatory T cells and defective control of TH2 cytokine production in natural *scurfy* mutant mice

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Abbreviations used in this paper: Treg, regulatory T cell; BAC, bacterial artificial chromosome; sf, *scurfy*; IPEX: immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome; DEREG: depletion of regulatory T cells

Abstract

Foxp3$^+$ Tregs are crucial for preventing autoimmunity. We have demonstrated that depletion of Foxp3$^+$ Tregs results in the development of a *scurfy*-like disease, indicating that Foxp3$^-$ effector T cells are sufficient to induce autoimmunity. It has been postulated that non-functional Tregs carrying potentially self-reactive T cell receptors may also contribute to *scurfy* pathogenesis due to enhanced recognition of self by Treg lineage committed cells. Those cells, however, could not be identified in *scurfy* (sf) mutants due to the lack of Foxp3 protein expression. To address this question, we crossed the natural *scurfy* mouse mutant with BAC transgenic DEREG mice. Since DEREG mice express GFP under the control of an additional Foxp3 promoter, those crossings allowed proving the existence of "would-be" Tregs, which are characterized by GFP expression in the absence of functional Foxp3. Sf Tregs lost their *in vitro* suppressive capacity. This correlated with a substantial reduction of intracellular cAMP levels, whereas surface expression of Treg markers was unaffected. Both GFP$^+$ and GFP$^-$ sf cells produced high amounts of Th2-type cytokines, reflected also by enhanced Gata-3 expression, when tested *in vitro.* Nevertheless, sf Tregs can be induced *in vitro*, although with lower efficiency than DEREG Tregs. Transfer of GFP$^+$ sf Tregs, in contrast to GFP$^-$ sf T cells, into RAG-deficient animals did not cause the scurfy phenotype. Taken together, natural and induced Tregs develop in the absence of Foxp3 in sf mice, which lack both suppressive activity and autoreactive potential, but rather display a Th2-biased phenotype.

(246 words)

Introduction

Central tolerance is mediated by deletion of self-reactive T cells in the thymus, while small numbers of potentially pathogenic T cells, which escape central tolerance, can be controlled in the periphery by regulatory T cells (Tregs) (1, 2). Foxp3 has been shown to be the major transcription factor associated with Treg development and function (3-5) and mutations within the *foxp3* gene locus can lead to IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) disease, a rare autoimmune disease in humans associated with eczema, severe enteropathy, type I diabetes, thyroiditis, haemolytic anemia, and thrombocytopenia (6, 7). Severity and outcome of the disease are highly dependent on the site of mutation and different grades of autoimmunity have been observed for complete loss, truncated forms of Foxp3 protein, or single point mutations (8). It is evident that disease severity can be closely linked to mutations in different functional regions of the protein, however, environmental factors may also contribute to IPEX pathogenesis (8).

In the genetically equivalent mouse-model named s*curfy* (sf), a natural frame-shift mutation in exon 8 (insertion of two additional adenosines) causes a premature stop and truncation of the forkhead box region of the protein, resulting in a highly unstable and non-detectable Foxp3 protein (9). The sf mutation leads to the development of a severe lymphadenopathy (10) with a similar spectrum of immune-mediated tissue inflammation as observed in IPEX patients (9, 11). The *scurfy* phenotype has in part been associated with typical Th2 pathology, for example eosinophilia, hyper IgE syndrome and blepharitis also occurring in Th1-prone C57Bl/6 mice (12-14) and IPEX patients (10). Massive autoimmunity in sf mice results in death during the first month after birth and can only be cured by neonatal adoptive transfer of functional Foxp3$^+$ Tregs (5, 15, 16). The disease has been shown to be mediated by CD4$^+$ T cells (14) although it was not possible to distinguish between activated effector T cells and lineage-committed Tregs due to a lack of specific Treg markers. In previous studies we and others were able to demonstrate in transgenic mouse models that specific ablation of Tregs in

neonates was sufficient to induce fatal autoimmunity to a comparable severity with the naturally occurring s*curfy* phenotype (17, 18). We thereby concluded that the mere absence of Tregs was sufficient for the development of the disease. Nevertheless, it could not be ruled out that the mutated, Foxp3 deficient sf Tregs also contributed to the fatal phenotype in *scurfy* mice. It has been shown that under certain experimental conditions using a fixed transgenic T cell receptor (TCR) ß-chain, the Treg TCR repertoire is shifted towards self-antigen recognition (19-21), and the presence of non-functional self-reactive Tregs has been discussed as a potential co-factor for the induction of autoimmunity in *scurfy* mice (19).

By crossing DEREG mice (mice expressing DTR-eGFP under the control of the Foxp3 promoter in a BAC-transgenic approach (17)) to sf mice, we were able to assess $GFP^{+}Foxp3^{-}$ "would-be" Tregs in the thymus and periphery of natural Foxp3 mutants. We observed that, although these cells lost their suppressive capacity, they still expressed *bona-fide* Treg markers such as CD25, GITR and CTLA-4. The only observed difference between functional Tregs and non functional sf Tregs was intracellular cAMP, which was highly diminished in sf Tregs when compared to DEREG Tregs. Regardless that sf Tregs could no longer suppress naive T cell proliferation and were non-anergic in *in vitro* cultures, they did not elicit any pathogenic consequences when transferred into $RAG1^{-/-}$ mice whereas mice receiving the effector T cell population from scurfy donors developed the scurfy phenotype. Furthermore, we show that sf Tregs and sf $CD4^{+}GFP^{-}$ T cells, here on a C57Bl/6 background, display a substantial shift towards a Th2 phenotype as measured by their capacity to produce high amounts of IL-4, IL-10 and IL-13, correlating with high Gata-3 production.

Material and Methods

Mice

DEREG and C57Bl/6 mice were bred at the animal facility of the Institute for Medical Microbiology, Immunology and Hygiene (TU Munich, Germany). *Scurfy* mice (B6.Cg-Foxp3 sf/J) were purchased from Charles River. TCR-HA and pgk-HA mice were bred at the Research Institute for Molecular Pathology (Vienna, Austria). All animal experiments were performed under specific pathogen-free conditions and in accordance with institutional, state and federal guidelines.

Antibodies, staining and sorting reagents

The following antibodies and secondary reagents were purchased from eBioscience: αCD4 (GK1.5), αCD25 (PC61), αCD3ε (500A2), αCD69 (H1.2F3), αCD62L (MEL-14), αCD127 (A7R34), αCD44 (IM7), αCD103 (M290), αCD25 (PC61.5), αCD45.1 (A20), αFoxp3 (FJK-16s), fix/perm, streptavidin, and appropriate isotype controls. αGITR and αCTLA-4 were generated in our own laboratory. Rabbit αGFP and Cy3-conjugated goat α rabbit polyclonal antibodies were obtained from Jackson ImmunoResearch. TCR-HA transgenic T cells were detected by a FITC conjugated monoclonal Ab (6.5) generated in the laboratory (22). For *in vitro* restimulation, αCD3ε (145-2C11) and αCD28 (37.51) from eBioscience or PMA/Ionomycin (Sigma) were used. For intracellular staining, αIL-4 and αIFNγ antibodies from eBioscience and αGATA-3 antibody from BD bioscience were used.

Cytometric analysis and sorting were performed using a CyAn (Dako Cytomation) or an Aria (BD) and the FlowJo software (Treestar). Dead cells were excluded by propidium iodide staining or ethidium monoazide (Sigma).

Microscopy, immunofluorescence staining, histology and immunohistochemistry

Immunofluorescent stainings were performed as described previously (23). Histologic stainings on various organs were performed on formalin-fixed and paraffin-embedded organs. Foxp3 immunohistochemistry was performed as described (24).

***In vitro* suppression assay**

GFP^+ Tregs and "would-be" GFP^+Foxp3^- Tregs were purified by fluorescent-based sorting from lymph nodes of DEREG and sfxDEREG mice, respectively. $CD25^-CD4^+$ responder T cells were purified from lymph nodes of C57Bl/6 mice by CD4 negative depletion followed by negative selection via αCD25-PE staining and αPE-microbeads (Miltenyi Biotech). Purification of responder T cells was followed by CFSE-labelling. Antigen presenting cells (APCs) were purified from C57Bl/6 mice using αCD90-microbeads (Miltenyi Biotech). Cultures were established in 96-well round bottom plates using $1x10^5$ responder cells and $2x10^5$ APCs per well. Tregs were added at a 1:1 ratio. Cells were activated using 1µg/ml αCD3 monoclonal antibody and CFSE dilution was assessed by flow cytometry after 4 days.

Real time RT-PCR

RNA was isolated with the RNeasy kit (Qiagen) following cDNA synthesis using the Superscript II Reverse Transcriptase (Invitrogen). Quantitative real-time RT-PCR was performed on LightCycler480 (Roche) using Roche´s SYBR Green Probe Master PCR kit and specific primers optimized to amplify 90–250 bp fragments from the different genes analyzed. A threshold line was set in the linear part of the amplification curve to determine the corresponding crossing point (Cp) for every gene. Relative mRNA levels were determined by using included standard curves for each individual gene and further normalization to RPS9. Melting curves established the purity of the amplified band. Primer sequences are: Foxp3 (5'-CTG-GCGAAG-GGC-TCG-GTA-GTC-CT-3', 5'-CTC-CCA-GAG-CCCATG-GCA-GAA-

GT-3'); Il-10 (5'-ATT-TCT-GGG-CCA-TGC-TTC-TCT-GC-3', 5'-CTG-GAC-AAC-ATA-CTG-CTA-ACC-GAC-TC-3'); Itgae (5'-GCT-GGG-CCC-TCC-TTG-TGC-TCT-3', 5'-GAA-CTG-CCG-ATC-CTT-GGT-GAA-ATA-3'); Gpr83 (5'-GGC-CAC-AAC-GGG-TTC-CAC-AGA-T-3', 5'-ACC-CTC-CCC-AGT-TCC-TTC-CTT-CAG-3'); Tnfrsf18 (5'-CAT-AGG-GCC-CAA-TCG-TAA-CTC-ACC-3', 5'-GAA-CGC-GGG-GAG-CAG-ACA-GA-3'); Ctla-4 (5'-CTT-CCT-GTG-GCA-TTA-ACT-TTG-TGT-3', 5'-GGG-CTG-GGT-CTT-TAC-ACT-CAT-TTT-3'); Lgals1 (5'-TCC-GCC-GCC-ATG-TAG-TTG-AT-3', 5'-AAC-CTG-CCT-TCC-CCT-TCC-3').

For GATA-3 and RORγT following method was used: cDNA was prepared using the μMACS One-Step cDNA kit and a ThermoMACS magnetic separator (both from Miltenyi Biotec) according to the manufacturer's instructions. Validated intron-spanning primer sets for several target genes were designed employing the Universal Probe Library Assay Design Center (www.roche-applied-science.com). The following primer pairs were used: GATA-3 (5'-TTATCAAGCCCAAGCGAAG-3', 5'-TGGTGGTGGTCTGACAGTTC-3'); RORgT (5'-ACCTCTTTTCACGGGAGGA-3', 5'-TCCCACATCTCCCACATTG-3'). Quantitative real-time PCR was performed using the Mouse Universal Probe Library, the Light Cylcer 480 Probes Master Kit and a LightCycler480 (all from Roche) according to the manufacturer's instructions. Integrated system software was used to obtain second derivative crossing point (C_P) values. Data were normalized to the hypoxanthine phosphoribosyl-transferase (Hprt) housekeeping gene.

***In vitro* restimulation and cytokine production**

$5x10^4$ T cells plus equal number of irradiated APCs per 96 round-bottom well were cultured in RPMI medium (10% FCS supplemented). αCD3 and αCD28 were supplemented in a concentration of 1 μg/ml and recombinant human IL-2 was used in a final concentration of 100 U/ml. Cultures were incubated for four days. All ELISAs were purchased from R&D. For

intracellular cytokine staining, cells were washed after four days of αCD3/αCD28 stimulation and further incubated for three days with complete medium containing 100U/ml IL-2. On day 7, cells were restimulated with PMA/Ionomycin in presence of Brefeldin A, harvested and stained intracellularly for IL-4 and IFNγ.

In vitro Treg induction assay

$CD4^{+}GFP^{-}$ cells were isolated from peripheral lymph nodes (LNs) of male sf x DEREG and DEREG transgenic mice by FACS sorting. $1x10^{5}$ cells per well were seeded in RPMI medium (10% FCS supplemented) in presence of 100 U/ml huIL-2 (Roche), 2 ng/ml TGF-β1 (Peprotech) and 0.01 μg/ml α-CD3ε. Treg induction was assessed by FACS analysis for expression of GFP, CD25 and CD4 after 2 days.

Adoptive cell transfer

To assess the pathogenicity of Foxp3-deficient T cell populations, GFP^{+} and $GFP^{-}CD4^{+}$ T cells were sorted from scurfy x DEREG and DEREG mice. 3 x 10^{5} cells were transferred intraveneously into RAG-$1^{-/-}$ recipients. Mice were monitored closely and sacrificed for analysis after signs of skin disease development (8 to 12 weeks).

Results

Foxp3$^-$GFP$^+$ "would-be" Tregs can be detected in *scurfy* mice

We set out to establish an experimental system to study T cells from *scurfy* (sf) natural mutant mice, which were programmed to be Tregs but did not express functional Foxp3 protein due to a frameshift mutation within exon 8 of the *foxp3* gene (9). Since none of the commercially available antibodies detect Foxp3 protein in sf T cells, it was unclear if sf mice suffer from autoimmune disease solely because they lack Tregs or whether, in addition, non-functional, self-reactive Tregs may contribute to disease severity. By crossing BAC-transgenic DEREG mice (DEpletion of REGulatory T cells) to sf mice, we wanted to investigate if sf Tregs can be identified in sf x DEREG male mice (carrying the mutated copy of the *foxp3* gene) based on Foxp3 promoter-driven GFP expression mediated by the additional BAC-encoded promoter copy.

By using DEREG mice as reporter, we could for the first time detect Foxp3$^-$GFP$^+$ "would-be" Tregs (designated as sf Tregs) in sf x DEREG male mice within different lymphatic organs (Fig. 1). Foxp3 antibody staining failed in sf x DEREG double mutant male mice, however, we could detect GFP$^+$ cells in spleens from those mice by immunofluorescence (Fig. 1A). To test if relative numbers of sf Tregs were reduced in comparison to DEREG Tregs, FACS analysis of GFP$^+$ cells from different lymphatic organs was performed (Fig. 1B). Analysis of GFP$^+$ cells from the periphery (lymph nodes and spleens) of sf x DEREG male mice and DEREG control mice revealed similar percentages of GFP$^+$ cells (Fig. 1B). Given the severe lymphadenopathy of *scurfy* mice, the absolute number of lymph node GFP$^+$Foxp3$^-$ sf Tregs was significantly increased compared to the numbers of GFP$^+$Foxp3$^+$ Tregs in DEREG controls (not shown).

Tregs can be generated both in the periphery from Foxp3$^-$ progenitors (induced Tregs, iTregs), and within the thymus (natural Tregs) (reviewed in (25)). The thymic selection process has

been discussed to be potentially dependent on functional Foxp3 expression and peripheral conversion may require a self-amplification loop for Foxp3 (26, 27). When we analyzed the development of thymic natural "would-be" Tregs (Fig. 1B), we observed comparable proportions of GFP^+ cells in sf x DEREG male mice versus DEREG control mice, suggesting that potentially self-reactive natural sf Tregs could indeed be selected in the absence of functional Foxp3 protein expression. To test whether differences in the specificity of T cells from sf and DEREG mice can be observed, we analyzed in detail the T cell receptor repertoires of LN GFP^+ and GFP^- cells and thymic GFP^+ cells from both genotypes by CDR3 spectratyping. This technique allows the detection of expanded clones by measuring the length distributions of the CDR3 regions for each possible Vβ-Jβ combination (reviewed by (28). Expanded clones yield single peaks, whereas polyclonal populations yield Gaussian length distributions. For each sample, we tested all possible Vβ-Jβ rearrangements using 24 V-element specific forward, 13 J-specific (29), and one C-specific reverse primer (30) in all combinations. In none of the samples did we observe any major difference between GFP^+Foxp3^- sf and GFP^+Foxp3^+ DEREG Tregs selected in the absence or presence of functional Foxp3 protein (data not shown). Furthermore, we could not detect any alterations in the TCR repertoire of the corresponding GFP^- populations.

We then wanted to address whether the selection of T cells with a defined TCR specificity may be impaired due to the lack of functional Foxp3 expression. Thereto, we made use of a TCR-transgenic system, where co-expression of a TCR specific for influenza hemagglutinin (TCR-HA) and its cognate antigen (pgk-HA) as corresponding neo-self antigen in the thymus leads to the efficient selection of $TCR\text{-}HA^+$ cells into the Treg lineage (22). Lack of functional Foxp3 did not impair this process, as shown by the equal percentages and absolute numbers of $TCR\text{-}HA^+$ $CD25^+$ cells in the thymus of WT and sf TCR-HA x pgk-HA mice (Figs. 2A and B). To test whether general up-regulation of CD25 expression in sf mice was a consequence of Treg development and not merely a result of T cell activation, Foxp3 mRNA

expression was assessed by real-time PCR detecting both WT and mutated Foxp3 mRNA. Foxp3 mRNA was expressed both in WT and sf TCR-HA$^+$ cells of TCR-HA x pgk-HA mice (Fig. 2C), indicating that the selection of a defined TCR-specificity into the Treg lineage is not impaired by the absence of functional Foxp3. Although the percentage of TCR-HA$^+$CD25$^+$ cells was comparable, Foxp3 mRNA levels were somewhat decreased in sf mice when compared to their WT counterparts, possibly reflecting a Foxp3-mediated positive feedback mechanism and self-stabilizing function of the Treg phenotype (27)}.

Sf Tregs lack suppressive activity but express most *bona-fide* Treg markers

Since Foxp3 expression was reported to be necessary for the suppressive phenotype of natural Tregs, we wanted to assess the *in vitro* suppressive capacity of Foxp3-mutated sf Tregs. Functional properties of sf Tregs were analyzed in an *in vitro* suppression assay. Whereas GFP$^+$Foxp3$^+$ Tregs from DEREG control mice clearly suppressed the proliferation of CD4$^+$CD25$^-$ responder T cells (Fig. 3A), sf Tregs displayed no suppressive capacity. These findings indicate that expression of functional Foxp3 is not only sufficient (5), but also necessary for the suppressive capacity of Tregs.

To seek for differentially expressed suppression-involved molecules in sf and WT Tregs, we sorted GFP$^+$ cells from sf x DEREG and DEREG control mice and performed RT-PCR and flow cytometry analysis (Figs. 3B and C). RT-PCR data were normalized to *hprt* as housekeeping gene and further to conventional GFP$^-$CD4$^+$ T cells from DEREG mice (Fig. 3B). No gross differences in the mRNA expression levels of typical Treg markers also associated with regulatory function such as Foxp3, GITR and CTLA-4 could be observed when peripheral GFP$^+$ cells from sf x DEREG and DEREG control mice were compared. Activation markers of Tregs such as CD103 and Galectin-1 were upregulated on sf Tregs. Galectin-1, a lectin upregulated in Tregs upon TCR activation, has been recently implied to be

a candidate effector molecule since its blockade can abrogate *in vitro* suppression by Tregs (31). However, mRNA levels of this molecule were markedly up-regulated in both Tregs (Fig. 3B) and effector T cells (not depicted) from sf x DEREG mice when compared to the expression level in DEREG Tregs, most likely attesting to the constant immune activation in male sf mice. Another molecule, which has been recently linked to Treg function, is the G protein-coupled receptor 83 (GPR83) (32). RT-PCR revealed that this molecule was slightly down-regulated in sf Tregs compared to their DEREG counterparts (Fig. 3B). IL-10 as putative effector cytokine from Tregs was up-regulated in sf Tregs when compared to DEREG Tregs.

We then performed FACS analysis of sf x DEREG Tregs and DEREG Tregs in order to measure expression of several markers on the protein level (Fig. 3C). Intracellular Foxp3 protein expression was - as expected - clearly absent from the sf Treg population. The activation and memory markers CD69 and CD103 were found to be marginally up-regulated in male sf Tregs compared to DEREG Tregs (Fig. 3C), again reflecting the autoimmune phenotype in male mice. Notably, CD103 expression was absent on $Foxp3^-$ Tregs in female individuals (Suppl. Fig. 1). No significant differences for CD44 and CD127 could be observed for sf Tregs compared to DEREG Tregs (Fig. 3C). For the Treg markers GITR and CTLA-4, only a slight reduction of CTLA-4 expression in *scurfy* Tregs could be detected, while equal levels could be detected in Foxp3 deficient and sufficient Tregs from female sf x DEREG mice (Suppl. Fig. 1). CD25, as a marker of both, Treg-lineage and also T cell activation marker, was nevertheless down-regulated in sf Tregs in both genders (Fig. 3C and Suppl. Fig. 1).

Recently, cAMP expression by Tregs was reported to be a potent mechanism for suppressing responder cells (33). In order to test whether a lack of intracellular cAMP in sf Tregs could be the cause for disrupted regulatory capacity, we assessed cAMP levels in sf Tregs and DEREG

Tregs in comparison to GFP$^-$CD4$^+$ cells from both mouse strains. Indeed, cAMP levels were significantly lower in sf Tregs when compared to DEREG Tregs whereas both effector T cell populations did not contain measurable amounts of intracellular cAMP (Fig. 3D).

Sf Tregs express high amounts of Th2 cytokines, are not anergic, and can be induced *in vitro*

We and others have previously shown that the mere absence of Foxp3$^+$ Tregs was sufficient to induce a *scurfy*–like phenotype (17, 18). To assess whether sf Tregs, in addition to GFP$^-$ cells, might further contribute to disease severity in sf mice, we first analyzed the cytokine expression profile of sf Tregs and sf effector T cells. A high percentage of both cell types stained positive for interleukin 4 after in *vitro* culture (Fig. 4A). We could also detect IFNγ producing cells, although at a lower percentage. As controls, CD4 T cells from DEREG and WT mice were cultured in the presence of diphtheria toxin (DT) to exclude Treg specific effects. Both cultures contained less IFNγ producing cells and almost no IL-4 producing cells when compared to sf cultures. Upon restimulation, *ex vivo* isolated sf Tregs as well as sf effector T cells, did express higher levels of the Th2 transcription factor GATA-3 compared to DEREG cells as assessed by FACS staining (Fig. 4B). This was confirmed by RT-PCR on cDNA from directly *ex vivo* sorted sf Tregs and DEREG Tregs (Suppl. Fig. 2).

In order to dissect Th2 cytokine production from sf Tregs and sf effector T cells, both populations were sorted and subjected to *in vitro* stimulation. GFP$^+$ WT Tregs and GFP$^-$ effector cells from DEREG mice served as controls. We could measure high amounts of IL-4, IL-10 and IL-13 in cell-culture supernatants from *in vitro* restimulated purified sf Tregs and sf effector T cells (Fig. 4C). IL-17 and TGF-β production were not elevated. Th17 cells are known to play a major role in the development of autoimmune diseases and a reciprocal relationship between Tregs and Th17 cells has been suggested (reviewed in (34)). To confirm

the absence of high levels of IL-17 in sf T cells, we performed RT-PCR for the main transcription factor for Th17 cells (Suppl. Fig. 2). RORγT was markedly down regulated in both sf Tregs and sf effector T cells when compared to DEREG control cells.

Since sf Tregs showed a Th2-biased phenotype closely resembling Th2 effector cells, we analyzed whether the absence of Foxp3 rendered these cells non-anergic. Upon stimulation of sorted GFP^{+} cells with αCD3 and αCD28, we were able to recover a substantial amount of sf Tregs, whereas Tregs from DEREG control mice died in the absence of exogenous IL-2 (not shown). To confirm active proliferation of sf Tregs, we stained cultured cells for the nuclear proliferation marker Ki-67 (Fig. 4D). Upon stimulation of the cultures with IL-2 alone, DEREG Tregs survived but did not proliferate in contrast to sf Tregs. When a TCR stimulus was provided in the absence of exogenous IL-2, DEREG Tregs did not survive the 4-day culture period, whereas sf Tregs not only survived but concomitantly underwent active proliferation. As control, proliferation of conventional $GFP^{-}CD4^{+}$ T cells upon stimulation with αCD3 and αCD28 was also assessed, and no striking differences were observed between cells derived from sf x DEREG and DEREG control mice. Thus, our data suggest that GFP^{+} sf Tregs do not harbour an anergic phenotype *in vitro*.

GATA-3-driven Th2 responses have been demonstrated to inhibit TGF-β-induced Foxp3 expression and therefore induction of Tregs (iTregs) both in mice and man (35, 36). To test if the sf mutation may affect the generation of induced Tregs, we cultivated GFP^{-} effector T cells from male sf x DEREG mice *in vitro* in the presence of IL-2, TGF-β and αCD3 according to published protocols (37, 38) (Fig. 5). We could generate GFP^{+} induced sf Tregs in the absence of Foxp3 expression albeit with lower efficacy when compared to conventional $GFP^{-}CD4^{+}$ T cells from DEREG control mice.

Sf Tregs infiltrate sites of inflammation but do not cause autoimmune pathology

Previous studies suggested that Tregs show a T cell receptor repertoire shifted towards the recognition of self antigens (21). Since we demonstrated that Foxp3-mutated "would-be" Tregs from sf mice are able to escape from negative selection within the thymus, display an activated Th2-biased phenotype, and are not anergic *in vitro*, we next asked whether sf Tregs could be potentially harmful and could induce autoimmune pathology.

First, we tested for the presence of GFP^+ sf Tregs in inflamed peripheral organs of sf x DEREG mice. We could detect high numbers of GFP^+ sf Tregs in conjunction with GFP^- effector T cells in the inflamed ears of male sf x DEREG mice, while T cell infiltrates in healthy tissues from DEREG control mice were negligible (Suppl. Fig. 3A).

Since GFP^+ non-suppressive, potentially self-reactive sf Tregs could be found in tissues displaying autoimmune pathology, we next performed adoptive transfer experiments into RAG-$1^{-/-}$ mice to investigate whether sf Tregs would lead to the onset of the typical autoimmune pathology observed in scurfy mice. Interestingly, sf Tregs survived in RAG-$1^{-/-}$ hosts (Suppl. Fig. 3B) but did not induce any signs of inflammation, as observed in skin, pancreata, and colon (Fig. 6). In contrast, massive lymphocyte infiltration and tissue destruction occurred in recipients of effector sf T cells. Naïve T cells from DEREG mice served as a control and did not induce the classical scurfy autoimmune pathology but, as expected, led to transfer colitis in the recipients. Thus, sf Tregs, despite their activated Th2-effector like phenotype and their presence in affected organs, were not sufficient to cause inflammatory reactions upon transfer in RAG-$1^{-/-}$ mice.

Discussion

We and others could demonstrate recently that the lack of Foxp3$^+$ Tregs is sufficient to cause severe autoimmunity in mice (17, 18). Given that Tregs can express self-reactive TCRs (19-21), it could not be ruled out from those studies that non-functional self-reactive Tregs may additionally contribute to induction of autoimmunity in sf mice (19). On the other hand, self-reactivity of Tregs has been questioned (39) and various infection models (summarized in(40)) suggest that cross-reactive or specific induction and expansion of Tregs can also occur in response to foreign antigen. To test the hypothesis that *scurfy* (sf) disease is exclusively caused by the lack of natural Foxp3$^+$ Tregs, we asked whether cells expressing mutated Foxp3, designated here as "would-be" *scurfy* (sf) Tregs, exist in sf mice and, if so, could eventually contribute to the autoimmune disease.

In this study we could demonstrate for the first time that GFP$^+$Foxp3$^-$ sf Tregs do exist in sf mice. By crossing BAC-transgenic DEREG mice to sf mutant mice, the additional copy of the Foxp3 promoter together with the GFP reporter gene could be used to detect cells which did not express the functional Foxp3 gene (sf allele on the X chromosome) but may express factors activating the additional Foxp3 promoter (GFP expressed as BAC transgene instead of Foxp3 exon1). Interestingly, sf Tregs could be found as GFP$^+$Foxp3$^-$ cells in similar percentages as GFP$^+$Foxp3$^+$ cells from DEREG control mice, and expressed *bona-fide* Treg markers, clearly indicating that sf Tregs do exist in *scurfy* mice.

Sf Tregs showed a distribution of TCR Vβ chain expression comparable to WT Tregs, indicating that no particular T cell clone proliferated extensively in sf mice (data not shown). Moreover, selection of a defined TCR specificity was not altered by the absence of functional Foxp3, as shown by a TCR transgenic approach. Thus, it appeared that Foxp3 is not required for thymic selection of Tregs and that sf mice possess "would be" Tregs (Figs. 1 and 2). This is consistent with recent data from the laboratories of Rudensky and Chatila. Here, genetic

models have been used to detect non-functional Tregs in BALB/c mice harbouring a truncated version of Foxp3 (26) or in 129Sv/C57Bl/6 mice where the *foxp3* gene was completely knocked out and replaced by GFP (27). Both studies also indicated that, in those genetic models, thymic selection of Tregs is independent of functional Foxp3 expression (26).

IPEX disease, the human equivalent of *scurfy* disease, has an identical aetiology and IPEX pathogenesis can also be explained by mutations affecting the transcription factor Foxp3 (6, 8). However, autoimmune phenotypes of affected patients vary depending on the site of mutation and possibly additional environmental factors (8). Given the different outcomes of IPEX disease in humans depending on different sites and forms of mutations, we found it intriguing to study non-functional sf Tregs in *scurfy* mice. In the recently published study of Lin *et al.* (26), exon 11 was knocked out, resulting in expression of a stable Foxp3 protein, lacking the C-terminal forkhead domain. The truncated Foxp3 protein was non-functional, since the forkhead domain drives the translocation of the protein to the nucleus and enables DNA-binding. Nevertheless, dominant-negative effects of the truncated protein could not completely be ruled out (26), a fact that gained importance after Ono *et al.* published that an N-terminal region of the Foxp3 protein is needed to bind AML1/Runx1 (41). Without this interaction there is an abrogation of the anergic state of the Tregs and attenuation of their suppressive capacity (34, 41, 42). Additionally, the N-terminal half of the protein has been shown in human cells to be necessary and sufficient for Foxp3-mediated suppression of a NF-AT inducible luciferase reporter (43). Therefore, it cannot be excluded that the truncated protein could still bind AML1/Runx1 and directly compete with NF-AT binding, of which the latter is thought to be at least one of the mechanisms resulting in inhibition of activation-induced cytokine expression (42). Furthermore, a forkhead box independent interaction between Foxp3 and the Th17 driving transcription factor RORγT has been published recently. In the presence of RORγT, a truncated version of Foxp3 lacking the forkhead box was detectable in the nucleus (44).

As expected, Foxp3-mutated sf Tregs did not suppress in an *in vitro* suppression assay (Fig. 3A). Interestingly, we nevertheless detected all *bona-fide* Treg markers tested by RT-PCR (Fig. 3B). We asked whether the lack of suppressive capacity of $GFP^{+}Foxp3^{-}$ sf Tregs could be explained by changes in the protein expression levels of known suppression-related molecules such as CTLA-4 or GITR. CTLA-4 has been suggested to be an essential molecule for Treg mediated suppression and the knock-out of CTLA-4 results in a phenotype comparable to the *scurfy* mouse mutant, suggesting overlapping functions of Foxp3 and CTLA-4 (45-47). GITR interaction with its ligand GITR-L on accessory cells has been linked to induction of regulatory properties and proliferation of plasmacytoid dendritic cells (48). While Foxp3, as expected, was not detectable on protein level (Figs. 1 and 3B), GITR expression patterns were not altered in sf Tregs compared to WT Tregs and CTLA-4 was down-regulated only in a minor subset of Sf Tregs, probably a consequence of high proliferation and T cell activation (49). CTLA-4 expression was not altered in female Foxp3-deficient Tregs (Fig. 3B and Suppl. Fig. 1B), indicating that the expression of both molecules is not dependent on Foxp3 expression and, furthermore, is not able to provide suppressive activity when Foxp3 is absent. The surface molecule CD103 is a marker for effector/memory-like Tregs (50, 51) and analysis of this adhesion molecule showed a direct dependency of CD103 on Foxp3 expression. Female sf x DEREG mice stained negative for CD103 in all $GFP^{+}Foxp3^{-}$ sf Tregs, whereas the Foxp3-competent Tregs of the same mice had normal CD103 expression (Suppl. Fig. 1). This is in accordance with the recent finding of Marson *et al.*, showing a direct binding of Foxp3 to the CD103 promoter (52, 53). However, loss of suppressive capacity of sf Tregs cannot simply be explained by loss of CD103 expression, since Tregs from $CD103^{-/-}$ mice display a comparable suppressive capacity as their WT counterparts (54). Furthermore, Foxp3-dependent CD103 expression can be overcome by overt inflammation since CD103 expression could be recovered in male *scurfy* mice (Figs. 3A and B).

Recently, intracellular cAMP has been shown to be highly abundant in Tregs and its delivery into responder cells essentially contributes to the suppressive capacity of Tregs (33). As shown in Fig. 3D, intracellular cAMP levels in sf Tregs are significantly reduced when compared to WT Tregs. It will have to be adressed in future studies if intracellular cAMP levels are directly dependent on Foxp3 protein expression, as could be shown recently for Pde3b, one potential cAMP-degrading enzyme (27). Hence, the substantial reduction of cAMP levels in sf Tregs might be one reason for the loss of suppressive capacity.

"Would-be" Tregs in female heterozygous *scurfy* mice were less abundant than their Foxp3-competent counterparts (Suppl. Fig. 1), indicating that Foxp3 might influence survival or homeostasis of Tregs even though we could show that Foxp3 is not directly required for survival. Using quantitative RT-PCR and FACS analysis (Fig. 2, Fig. 3C), we demonstrated here that sf Tregs fail to up-regulate the high affinity IL-2 receptor chain (CD25), consistent with the finding that Foxp3 induces CD25 expression (52). Consequently, the survival disadvantage of "would-be" Tregs in healthy female *scurfy* mice could be due to decreased signalling of IL-2, a cytokine known to be crucial for the homeostasis of Tregs (52, 55-57). This effect might play a negligible role in *scurfy* male mice suffering from severe autoimmunity, since it has been shown that the highly activated status in those mice leads to elevated IL-2 expression levels (12).

We next asked the question whether non-suppressive sf Tregs might have acquired effector functions. The classical sf phenotype has been partially attributed to the increased production of Th2 cytokines and typical Th2-type pathology (12-14). Regarding the cytokine expression profiles, the most striking differences between GFP^+ sf Tregs and GFP^+ WT Tregs were observed for the production of Th2-type cytokines, such as IL-4, IL-13, and IL-10, which were markedly up-regulated in sf Tregs. In earlier studies, the Th2-type cytokine expression in sf mice has already been linked to $CD4^+CD25^+$ T cells, which under inflammatory

conditions mainly comprised activated effector T cells (4). Here, using Foxp3-GFP-reporter mice crossed with *scurfy* mice, the enhanced cytokine expression could for the first time be detected in both GFP^{+} sf Tregs and GFP^{-} effector T cells. Dysregulation of Th2-type cytokines in the latter cell-type can be best explained by the absence of functional $Foxp3^{+}$ Tregs. The absence of IL-4 positive cells in Treg depleted cultures from DEREG mice clearly shows that elevated IL-4 production is not directly dependent on the absence of $Foxp3^{+}$ cells within the cultures. In sf Tregs, however, expression of Th2 cytokines might be affected more directly by the lack of Foxp3, since Foxp3 has been reported to negatively influence IL-10 and IL-4 production by direct binding to their promoter or by binding to the transactivator NF-AT (26, 42, 53, 58, 59). Interestingly, as shown by RT-PCR, Galectin-1 appeared to be expressed independently of Foxp3 since it was highly up-regulated on sf T cells (Fig. 3B). This elevated expression might have a compensatory effect because of the high T cell activation in these mice and could even play a deleterious role. It has been shown that galectin-1 can induce apoptosis in Th1 and Th17 cells, which could result in a bias towards Th2-type immunity (60). Furthermore, we observed both on mRNA (Suppl. Fig. 2) and protein level (Fig. 4C) increased levels of the master regulator of Th2 cells, GATA-3. Interestingly, IL-17, a cytokine augmented in various autoimmune diseases, was not elevated in supernatants of sf T cell cultures. This is in contrast to the model by Rudensky and collegues who showed elevated IL-17 levels in “would-be” Tregs. RT-PCR data revealed a reduction of mRNA of the major Th17 transcription factor in both GFP^{+} and GFP^{-} sf T cells. These findings are in accordance with a previously published report, even though underlying mechanisms are yet unclear (61). It appears that when Foxp3 is mutated, cells which are prone to produce high amounts of cytokines are unleashed and a dominant Th2 phenotype emerges. Importantly, the Th2 environment of male sf mice did affect, but not completely prevent, generation of induced Tregs (Fig. 5) as predicted from studies where GATA-3 was overexpressed (35, 36). Differentiation of naïve $CD4^{+}$ T cells into Th2 effector cells has been

shown to be dependent on CD28/B7 co-stimulation (62, 63). Since $CD28^{-/-}$ *scurfy* mice show a substantially prolonged life-span and significantly less cytokine production when compared to *scurfy* mice with same level of lymphoproliferation (64), it is conceivable that the observed Th2 shift in *scurfy* $CD4^{+}$ T cells is an essential factor for disease development. Also, for the human form of the disease, IPEX, high correlations between disease progression and increased IgE and eosinophilia could be observed (8). In *in vitro* proliferation studies using Ki-67 staining, we could observe that exogenous IL-2 was dispensable for a basal proliferation of sf Tregs which were not anergic, and that additional IL-2 promoted proliferation to a much higher extent (Fig. 4D). Accumulation of sf Tregs in affected organs may reflect rather increased proliferation and migration towards the site of inflammation than the cause of autoimmune pathology. This hypothesis is further strengthened by the fact that the adoptive transfer of sf Tregs did not cause scurfy-like multiorgan inflammatory disease, while sf CD4 effector cells led to massive lymphocyte infiltration and tissue damage of all observed organs within 8 weeks (Fig. 6). In summary, we conclude that Foxp3 mutated "would-be" sf Tregs can be found in *scurfy* mice but appear to be neither the initiators, nor the major contributors mediating autoimmunity.

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Figures

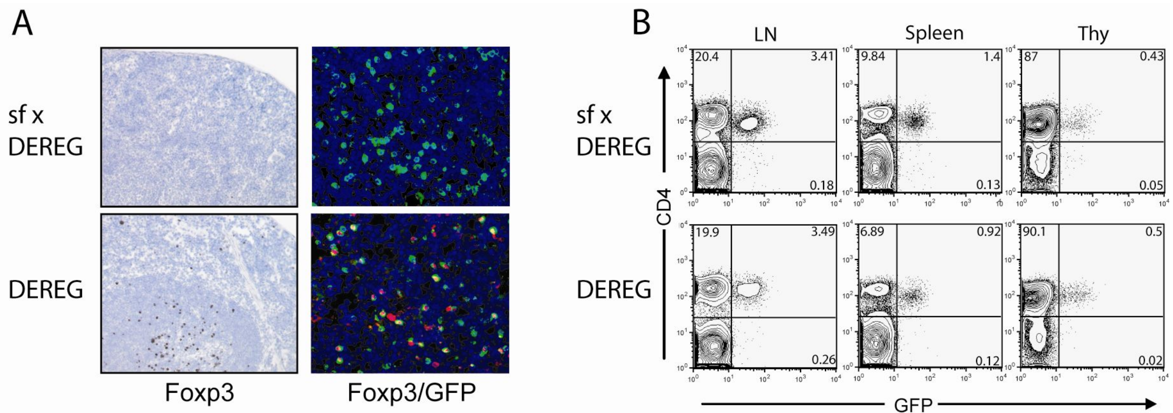


Fig. 1:

„Would-be" Tregs survive negative deletion in the thymus and can be found in the periphery. Splenic sections were stained for Foxp3 by histochemistry (left panel) and for Foxp3 and GFP by immunofluorencence (GFP-Alexa488 in green and Foxp3-Alexa555 in red) to demonstrate absence of Foxp3 in sf x DEREG mice (A). Lymph nodes, spleens and thymus from sf x DEREG and DEREG mice were stained for CD4 and analyzed by flow-cytometry to compare GFP^+ cell percentages in the different genotypes. Plots show live gated cells (B).

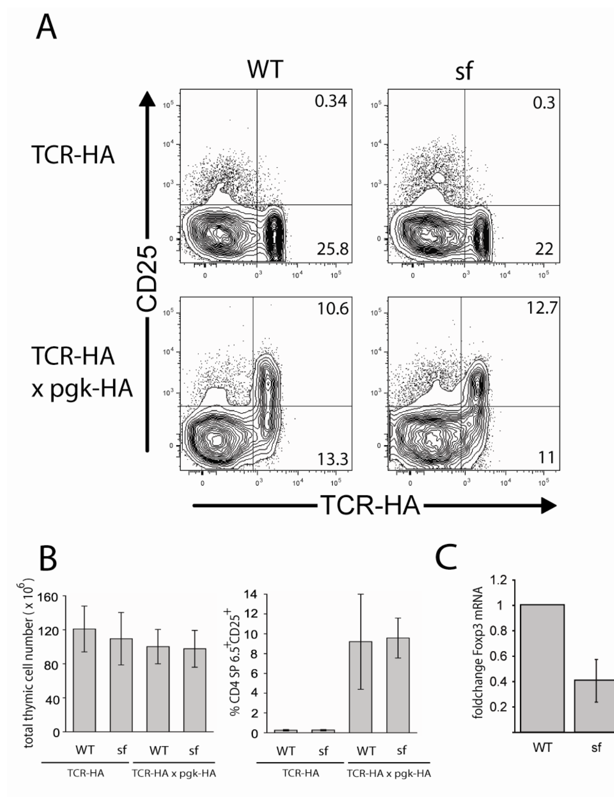


Fig. 2:

TCR-HA^{+}CD25^{+} cells can be found in TCR-HA x pgk-HA mice lacking functional Foxp3. Expression of CD25 and TCR-HA by CD4 single positive thymocytes from 15 day old TCR-HA or TCR-HA x pgk-HA WT or sf mice is depicted (A). Numbers indicate percentages within the respective gates. Total thymic cellularity and percentage of thymic TCR-HA^{+}CD25^{+} cells from 15 day old TCR-HA or TCR-HA x pgk-HA WT or sf mice is shown (B). Foxp3 mRNA expression of TCR-HA^{+}CD25^{+} from TCR-HA x pgk-HA sf mice is reduced compared to WT levels (normalized to the expression level of WT TCR-HA^{+}CD25^{+} cells) (C).

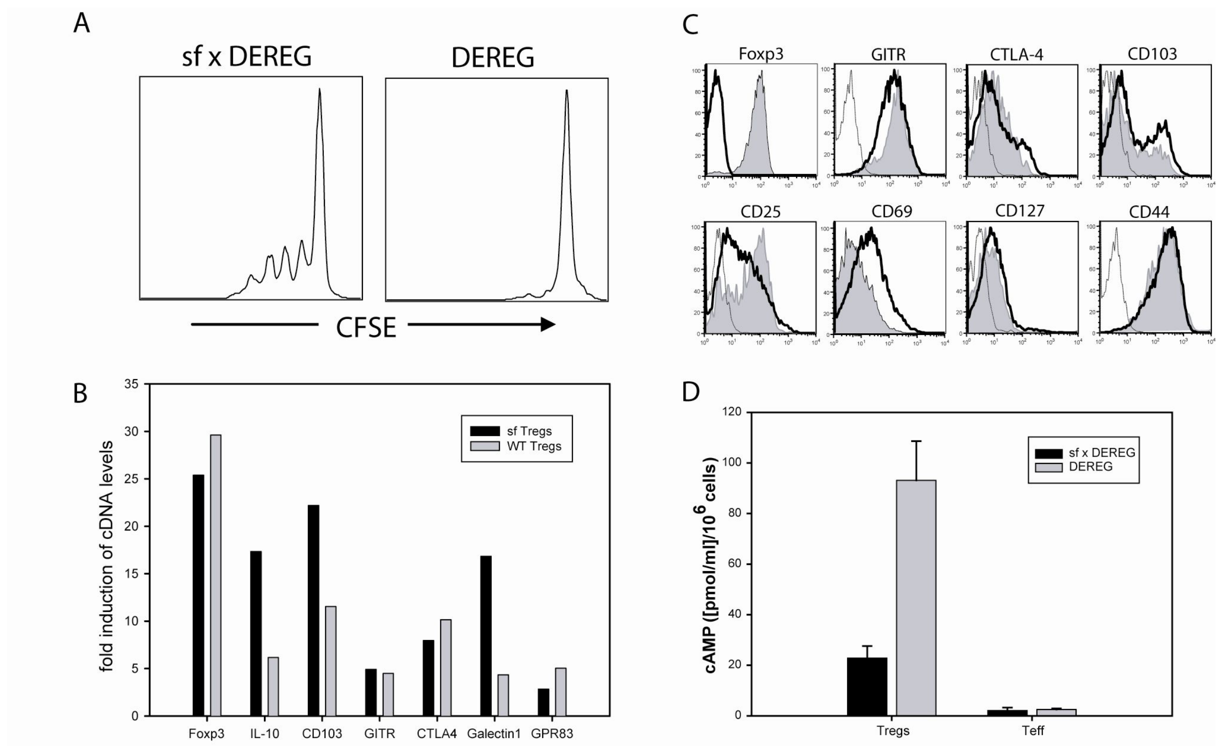


Fig. 3:

Foxp3 control of suppression related genes.

Foxp3 deficient Tregs from sf x DEREG mice were co-cultured with $CD4^{+}CD25^{-}$ T cells from congenic CD45.1 mice in a ratio of 1:1 and antigen-presenting cells *in vitro* in the presence of αCD3 as stimulus. Histograms show CFSE dilution in $CD4^{+}CD45.1^{+}$ T cells after four days of culture (A).

Quantitative PCR was performed for indicated genes on cDNA from GFP^{+} and $GFP^{-}CD4^{+}$ cells from sf x DEREG (black bars) versus DEREG (gray bars) mice. Data were normalized to expression levels of the house-keeping gene *hprt* and then to naïve $CD4^{+}GFP^{-}$ T cells from DEREG mice (B).

Lymph node $CD4^{+}GFP^{+}$ cells from male sf x DEREG mice (thick black, open histograms) compared to DEREG mice (gray, filled histograms) were FACS-stained for Treg markers and gated on living GFP^{+} cells. Isotype controls are shown in thin black histograms (C).

Sorted GFP^{+} and $GFP^{-}CD4^{+}$ cells from DEREG x sf (black bars) and DEREG mice (gray bars) were lyzed, and intracellular cAMP levels were assessed by ELISA (D).

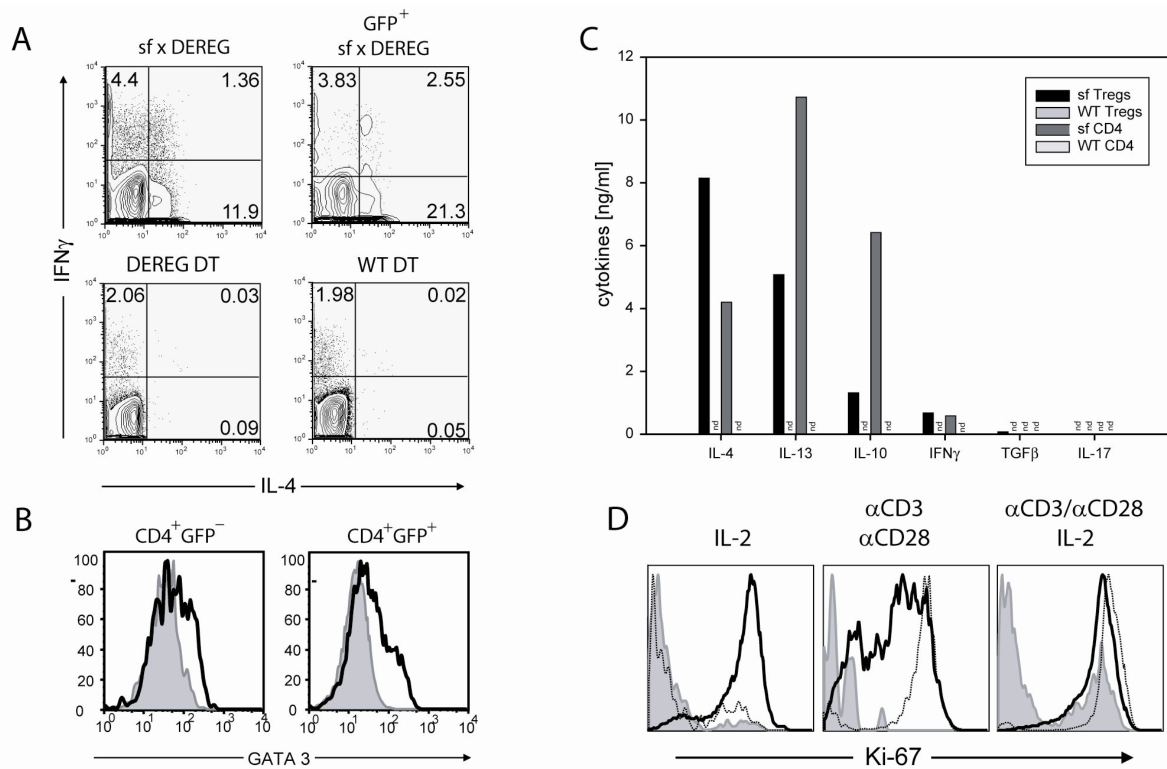


Fig. 4:

Sf Tregs cells shift towards a Th2 phenotype after *in vitro* culture and are not anergic.

CD4 T cells from sf x DEREG mice and DEREG mice or DEREG and WT mice as controls in the presence of diphtheria toxin (DT) to exclude Treg effects were cultured under non-polarizing conditions with αCD3 and αCD28 (1µg/ml each) for three days and kept another 4 days in medium containing 100U/ml huIL-2. On day 7, cells were harvested, restimulated with PMA/Iono for 6 hours and stained for IL-4 and IFNγ intracellularly. Plots show live gated $CD4^+$ cells (A). DEREG and sf x DEREG lymph node suspensions were directly restimulated for 5 hours *ex vivo* with PMA/Ionomycin to stain for Gata-3. Histograms show live gated GFP^+ versus GFP^-CD4^+ cells from DEREG (filled gray histograms) versus sf x DEREG (black open histograms) cells (B). GFP^+ and GFP^-CD4^+ cells were sorted from sf x DEREG (GFP^+ = black, GFP^- = dark gray) and DEREG mice (GFP^+ = light gray, GFP^- = white). Cells were cultured for four days with αCD3, αCD28 and IL-2. Supernatants were taken and ELISAs for IL-4, IL-13, IFNγ, TGFβ, IL-17 and IL-10 were performed (C).

Cultures from GFP^+CD4^+ sf x DEREG cells (black lines) and GFP^+CD4^+ DEREG cells (gray filled histograms) were stimulated with IL-2 alone, αCD3 and αCD28 or αCD3, αCD28 and IL-2. GFP^-CD4^+ cells from DEREG mice serve as controls (gray open histograms). Histograms show intracellular Ki-67 expression of live cells after four days of culture (D).

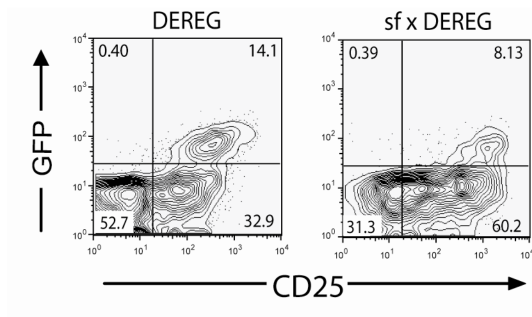


Fig. 5:

Induced Tregs can arise from Foxp3-deficient T cells.

Sorted GFP$^-$ CD4$^+$ lymph node (LN) cells from sf x DEREG or control DEREG males were cultured for 2 days under Treg-inducing conditions. Cells were then analysed for expression of GFP, CD25 and CD4 to quantify Treg induction. Living and CD4$^+$ cells are shown. Plots indicate expression of CD25 and GFP.

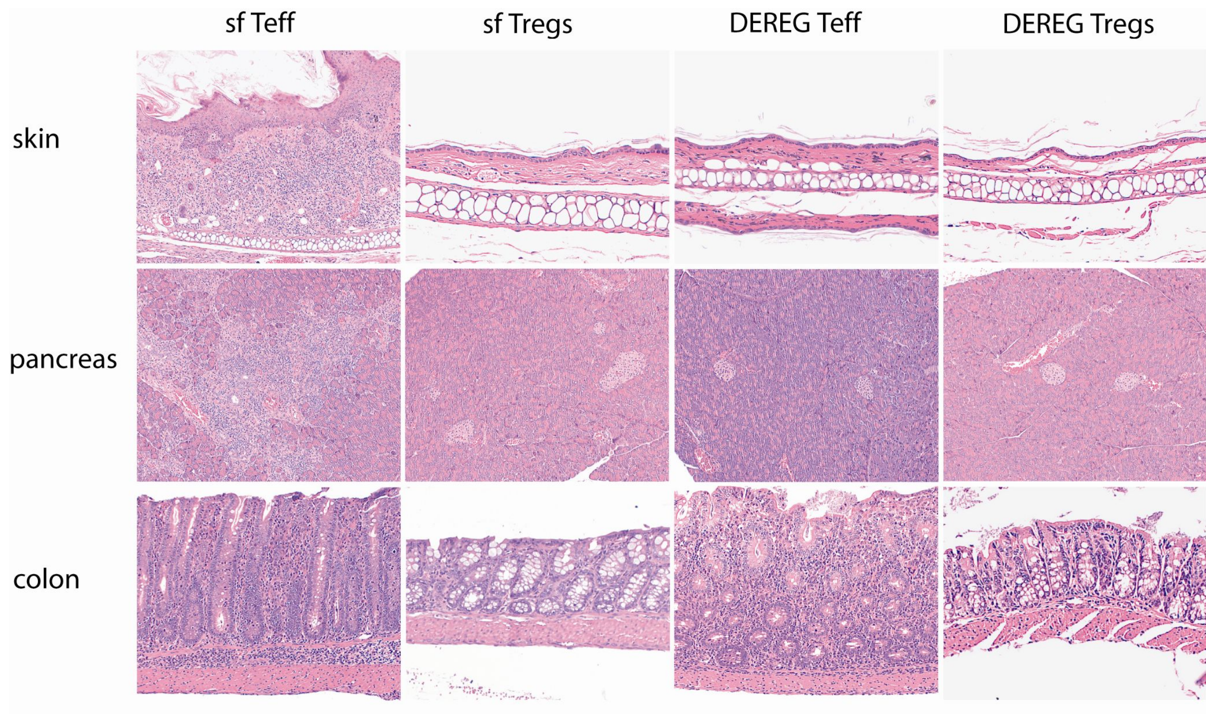
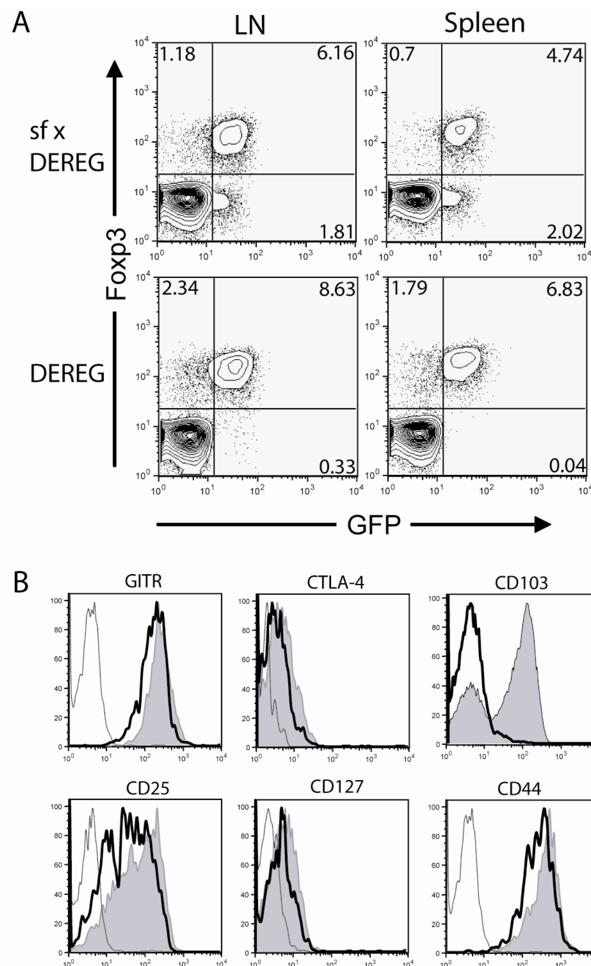


Fig. 6:

Scurfy Tregs are not pathogenic.

As indicated, 3 x 10^5 GFP^+ (Treg) or GFP^- (Teff) from either sf x DEREG or DEREG mice were transferred into RAG-1 deficient recipients. Mice were sacrificed when pathologic consequences were visible, occurring between 8 and 12 weeks after transfer. Organs were collected and embedded for histologic analysis. Representative H&E stainings of skin, pancreas and colon are displayed.

Supplementary data



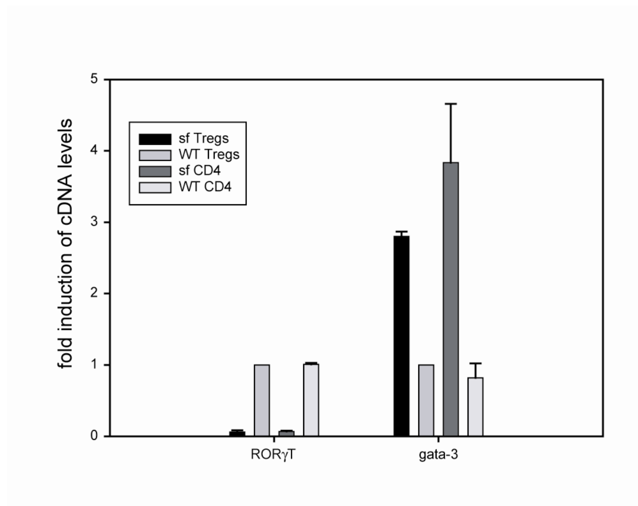
Supplementary figure 1:

(A) Heterozygous sf x DEREG females harbour small percentages of Foxp3-mutated Tregs.

LN and spleen cells from sf x DEREG and DEREG mice were harvested and stained for Foxp3. Plots show live gated cells.

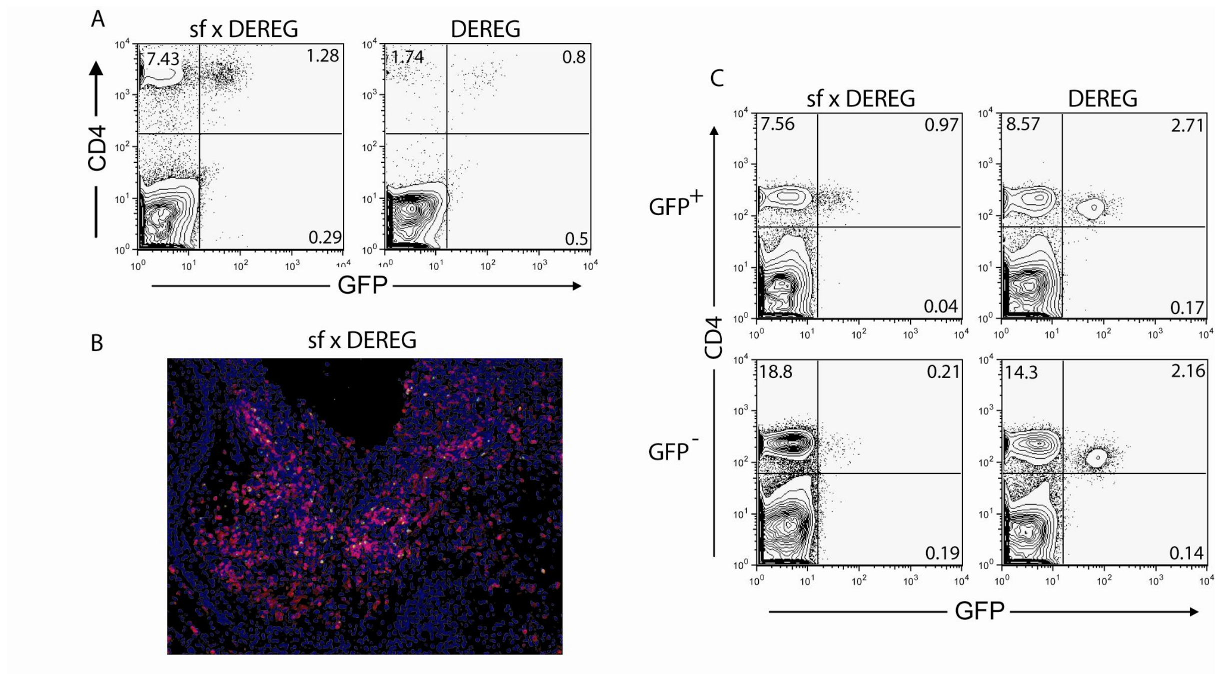
(B) Differential control of *bona fide Treg* markers.

Sf x DEREG LN cells were gated on Foxp3^{+}GFP^{+} cells (gray filled histograms) or Foxp3^{-} GFP^{+} cells (thick black open histograms) to compare protein expression by sf Tregs and WT Tregs from one donor. Isotype controls are shown by thin black histograms.



Supplementary Figure 2:

Gata-3 and RORγT are differentially expressed in sf x DEREG T cells and DEREG T cells. GFP^{+} and GFP^{-} $CD4^{+}$ cells were sorted from sf x DEREG and DEREG mice and cDNA was prepared from the purified populations. Bars show expression of the indicated transcription factors normalized to DEREG Tregs.



Supplementary figure 3:

(A) Foxp3 deficient Tregs infiltrate affected organs in *scurfy* mice.

Ears from sf x DEREG and DEREG mice were digested with collagenase for 1 hour. Cell suspensions were stained for CD4 to monitor cell infiltration by effector T cells and Tregs. Plots show live gated cells.

(B) Ears were stained histologically with DAPI (blue), αCD3 (red) and αGFP (green) (magnification x 100).

(C) Foxp3 deficient Tregs can survive transfer into RAG-1 knock-out mice.

Mediastinal lymphnodes were stained for CD4 to assess T cell repopulation 66 days after adoptive transfer of indicated cell types (Sf x DEREG-donor: left plots, DEREG-donor: right plots; GFP^+ cells: upper plots, GFP^- cells: lower plots). Plots show live gated cells and are representative for three individual mice per group.

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Circumvention of regulatory CD4+ T cell activity during cross-priming strongly enhances T cell-mediated immunity

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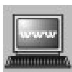
Immunization with purified antigens is a safe and practical vaccination strategy but is generally unable to induce sustained CD8+ T cell-mediated protection against intracellular pathogens. Most efforts to improve the CD8+ T cell immunogenicity of these vaccines have focused on co-administration of adjuvant to support cross-presentation and dendritic cell maturation. In addition, it has been shown that CD4+ T cell help during the priming phase contributes to the generation of protective CD8+ memory T cells. In this report we demonstrate that the depletion of CD4+ T cells paradoxically enhances long-lasting CD8-mediated protective immunity upon protein vaccination. Functional and genetic *in vivo* inactivation experiments attribute this enhancement primarily to MHC class II-restricted CD4+ regulatory T cells (Treg), which appear to physiologically suppress the differentiation process towards long-living effector memory T cells. Since, in functional terms, this suppression by Treg largely exceeds the positive effects of conventional CD4+ T cell help, even the absence of all CD4+ T cells or lack of MHC class II-mediated interactions on priming dendritic cells result in enhanced CD8+ T cell immunogenicity. These findings have important implications for the improvement of vaccines against intracellular pathogens or tumors, especially in patients with highly active Treg.

Key words: CD8+ T cells · Cross-priming · Protective immunity · Regulatory T cells



Supporting Information available online

Introduction

CD8+ T cells are crucial for protection against intracellular pathogens, including the “big three” epidemiologically most relevant human infections: HIV, tuberculosis and malaria. Most vaccines that aim to induce long-lasting CD8+ T cell responses are based on living intracellular organisms, which are believed to best mimic the complex host-microbe interactions required for the development of protective CD8+ T cells [1]. However, antigen-expressing vectors for live vaccines are relatively labor-intensive to generate and are potentially harmful, especially for immune-compromised individuals. Furthermore, the immune response

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towards the vector itself is often very strong, limiting the use of the same vector for different antigenic targets. Efforts to make vaccines easier to generate as well as safer, *e.g.* by using non-replicating antigen in the form of recombinant proteins, usually result in substantial loss of immunogenicity.

Progress has been made in the identification of adjuvants that can support $CD8^+$ T cell responses; most of the adjuvants target germline-encoded pattern recognition receptors, especially Toll-like receptors (TLR). TLR recognize conserved microbe-derived products and initiate signaling cascades, causing activation of the innate host defense system [2–4]. In addition to being the primary sensor for invading pathogens, TLR are an important component within the complex interplay between innate and adaptive immune responses that supports the development of protective immunity [5]. Especially the TLR-mediated induction of costimulatory molecules and various cytokines and chemokines by antigen-presenting cells, primarily dendritic cells (DC), is involved in the strong adjuvant effects of TLR ligands on T cell responses. TLR9 recognizes unmethylated CpG oligodenucleotides (CpG-ODN) [6], and the role of CpG-ODN as a potent adjuvant for $CD8^+$ T cell responses has been demonstrated in various vaccination studies [7–9].

Priming of $CD8^+$ T cells by vaccination with purified pathogen-derived proteins (so-called "split vaccines") requires cross-presentation and cross-priming [10]. Only specialized antigen-presenting cells, primarily DC and perhaps only some DC subsets [11], are capable of efficiently accomplishing this specific immune function *in vivo*. TLR ligation most likely supports cross-priming through cumulative effects on the initiation of maturation programs, which include changes within the antigen processing and presentation machinery [12].

$CD4^+$ T cell help is also an important factor affecting the efficiency of cross-priming *in vivo* [13–15]. There are different models describing how the interaction of $CD4^+$ T cells and antigen-presenting cells might enhance $CD8^+$ T cell responses; most of these models suggest that interactions between CD40 (expressed on antigen-presenting cells or $CD8^+$ T cells) and CD40L (expressed on $CD4^+$ T cells) mediate a crucial helper cell stimulus. Interestingly, it has recently been shown that $CD4^+$ T cell help early during infection with intracellular pathogens is essential for the generation of long-living $CD8^+$ memory T cells [16–18]. A lack of $CD4^+$ T cells, achieved experimentally by antibody-mediated depletion, did not affect the efficacy of priming and clonal expansion of $CD8^+$ T cell populations during the effector phase (first week post-infection) but caused a complete loss of protective memory responses towards a secondary challenge with the pathogen [16].

The exact mechanism of how $CD4^+$ T cells are capable of modulating the differentiation program of naive $CD8^+$ T cells towards memory T cells is not well understood, but crucial involvement of CD40/CD40L-dependent signaling pathways [19] and access to IL-2 [20] have been suggested. Most of the studies that have demonstrated strict T helper (Th) cell dependency of $CD8^+$ memory T cell responses have been performed in the infection model using *Listeria monocytogenes (L.m.)* [21]. In this experimental model, the quality of protective immunity can be precisely quantified, and long-lasting protective immunity relies almost exclusively on $CD8^+$ memory T cell responses [22]. Major target antigens for protection have been identified, *e.g.* the virulence factor listeriolysin O (LLO) and the murein hydrolase p60 [23, 24].

Primary immune responses are also characterized by the activation of counter-regulatory mechanisms, which are necessary to prevent overzealous T cell expansion or maintenance of potentially harmful cell populations, such as autoreactive T cells [25]. Regulatory T cells (Treg) seem particularly to be an adept at controlling immune responses to self antigens as well as pathogens. At least two major types of Treg exist, "naturally occurring Treg" and "antigen-induced Treg". Naturally occurring Treg develop in the thymus, require IL-2 for their survival, and depend on constitutive expression of a specialized transcription factor, Foxp3, for their function [26]. Most of these cells reside in secondary lymphoid organs and are defined by the expression of CD4 and CD25 [27]. Naturally occurring Treg are believed to recognize self peptide/MHC molecules, which are constitutively expressed on all nucleated cells (MHC class I) or only on specialized antigen-presenting cells (MHC class II) [25]. In contrast, antigen-induced Treg can recognize foreign antigens and develop during prolonged antigen exposure such as chronic infections. There are at least two different types of antigen-induced Treg, referred to as Th3 [28] and Tr1 cells [29].

The mechanisms used by Treg to suppress immune responses are not yet well understood. In some cases predominant involvement of IL-10 (Tr1) or TGF-β (Th3) has been demonstrated, but suppression often appears to be a multifactorial event including direct cell contact-dependent mechanisms. Because of their potent suppressive activity on immune responses *in vivo*, temporary down-modulation of Treg has been suggested as an attractive target to improve the efficacy of vaccines. In fact, the positive adjuvant effect of TLR ligands has been partially linked to the Treg compartment, either by direct reversion of Treg function [30] or by TLR-induced cytokines (such as IL-6), which make responding T cells refractory to suppression by Treg [31]. However, in infection models, control of antigen-specific immune responses by Treg activity has been shown to be most relevant for recall responses upon secondary infection [32]. On the other hand, it has been established for various vaccination strategies that anti-CD25 treatment can enhance primary immune responses [33–40], which has been interpreted to be caused mainly by inactivation of Treg. A specific role of Treg in controlling the generation of long-living memory T cells, the major aim of T cell-based vaccination, has not been described.

Results

$CD4^+$ T cell depletion enhances long-lasting immunity

In order to study requirements for protective $CD8^+$ T cell responses upon protein vaccination in more detail, we generated highly

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purified recombinant proteins for two *L.m.*-derived antigens: recombinant LLO (r-LLO) and p60 (r-p60). In addition, a mutated variant of r-LLO lacking hemolytic activity (r-LLO492$_{T\text{-}A}$) [41] was generated. 'Single-shot' vaccinations with r-LLO alone were tested within a wide range of protein concentrations (1–100 μg) and different routes of application [intravenously (i.v.) or subcutaneously (s.c.)], but did not result in any measurable long-term protection against infection with living *L.m.* (Fig. 1 and data not shown). Co-administration of CpG-ODN during protein vaccination only modestly improved the induction of long-lasting protective immunity (>1 $\log_{10}$ bacterial load reduction is regarded significant in this model), and this effect was only detectable upon s.c. vaccination (Fig. 1).

Next we evaluated to what extent these memory responses are dependent on CD4$^+$ T cell help, as recent reports have indicated that CD4$^+$ T cell help during the early priming phase is a prerequisite for the development of long-living CD8$^+$ memory T cells [16–18]. CD4$^+$ T cells were depleted shortly before and during vaccination by administration of the anti-CD4 antibody GK1.5. This procedure resulted in effective depletion of CD4$^+$ T cells for at least 1 wk (Supporting Information Fig. 1), followed by a rapid restoration of this compartment within a few weeks (data not shown and [42, 43]). Unexpectedly, we found that 5 wk after protein/CpG-adjuvant vaccination, the group of CD4$^+$ T cell-depleted mice showed much superior protection against *L.m.* challenge as compared to the non-depleted control group. Similar results were obtained for vaccination with r-LLO492$_{T\text{-}A}$ or r-p60 (data not shown). These results not only demonstrate that protein/CpG-adjuvant mixtures generate long-lasting protective immunity in the absence of CD4$^+$ T cells, but also point at the presence of a strong suppressive component on CD8$^+$ memory T cell generation within the CD4$^+$ T cell compartment.

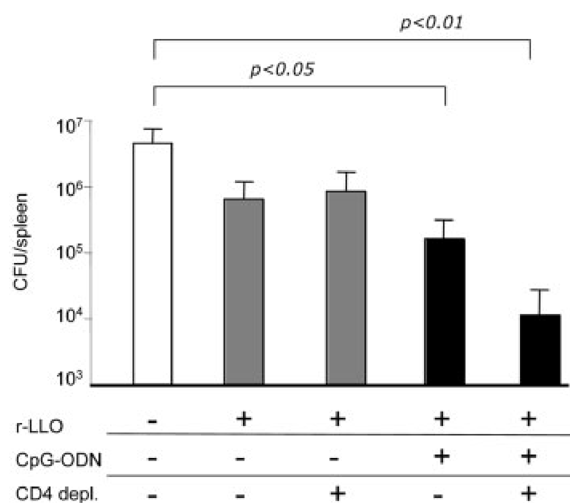

Figure 1. Enhanced protection against *L.m.* by CD4 depletion during r-LLO immunization. Mice were s.c. immunized with r-LLO in the presence (black bars) or absence of CpG-ODN (grey bars). Where indicated, mice were additionally treated with anti-CD4 mAb during immunization. After 5 wk, mice were rechallenged with 2×LD50 *L.m.*, and protective immunity was measured after three more days by determining colony-forming units (CFU) in the spleen. Naive mice that received *L.m.* only served as a control (white bar; *n*=5 per group).

Increased burst size of the effector phase upon CD4$^+$ T cell depletion

Since antigen-specific CD8$^+$ T cells have been identified as the major component required for protection against *L.m.* infection, we analyzed the CD8$^+$ T cell responses elicited under the different vaccination conditions described above. Seven days after s.c. vaccination with r-LLO, the positive effect of CpG-ODN adjuvant can be visualized by MHC multimer staining as an increase in the expanded LLO$_{91–99}$-specific T cell population; thereby, most of these cells acquired typical effector functions, such as the production of IFN-γ (Fig. 2A). Upon additional CD4$^+$ T cell depletion, the burst size of antigen-specific effector CD8$^+$ T cells increased to larger frequencies (Fig. 2A), an observation that was further confirmed by calculating absolute cell numbers per organ and by determining epitope-specific cytokine production as well as *in vivo* cytotoxicity (Supporting Information Fig. 2).

These effects were not restricted to LLO, as similar results were found for vaccination with a variety of different antigens, including r-p60 (Fig. 2B) and OVA (Supporting Information Fig. 3). In addition, it appeared unlikely that the observed effects of CD4 depletion were just caused by the generation of space, as no evidence of homeostatic effects on CD8$^+$ T cells could be observed (Supporting Information Fig. 4). Interestingly and reminiscent with published data [17], CD4 T cell depletion during the priming phase of *Listeria* infection leads to opposite effects (Fig. 2). Obviously, priming conditions and dependencies on accessory factors like T cell help or suppression are substantially different in this infection model. These distinct dependencies on CD4 depletion during primary infection or protein vaccination are also translated in T cell recall expansion upon rechallenge with *Listeria* (Supporting Information Fig. 5).

Overall, our data indicate that during s.c. protein vaccination, CD8$^+$ T cell responses are actively suppressed by a CD4$^+$ population. Even in the absence of CpG-ODN as an adjuvant, CD4 depletion was capable of slightly enhancing the size of the responding CD8$^+$ T cell population (Fig. 2). In contrast, depletion of CD4$^+$ T cells during primary infection with *L.m.* did not result in a measurable enhancement of CD8$^+$ T cell responses, confirming previous results [16–18] and demonstrating that a detectable suppressive effect of CD4$^+$ T cells is dependent on the type of immunization.

Enhancement of CD8$^+$ T cell responses by anti-CD25 treatment

Since it is known that CD4$^+$ Treg are specialized in the down-modulation of immune responses, we suggested that the enhancement observed after anti-CD4 treatment is mediated by the loss of Treg. Naturally occurring Treg are characterized by their

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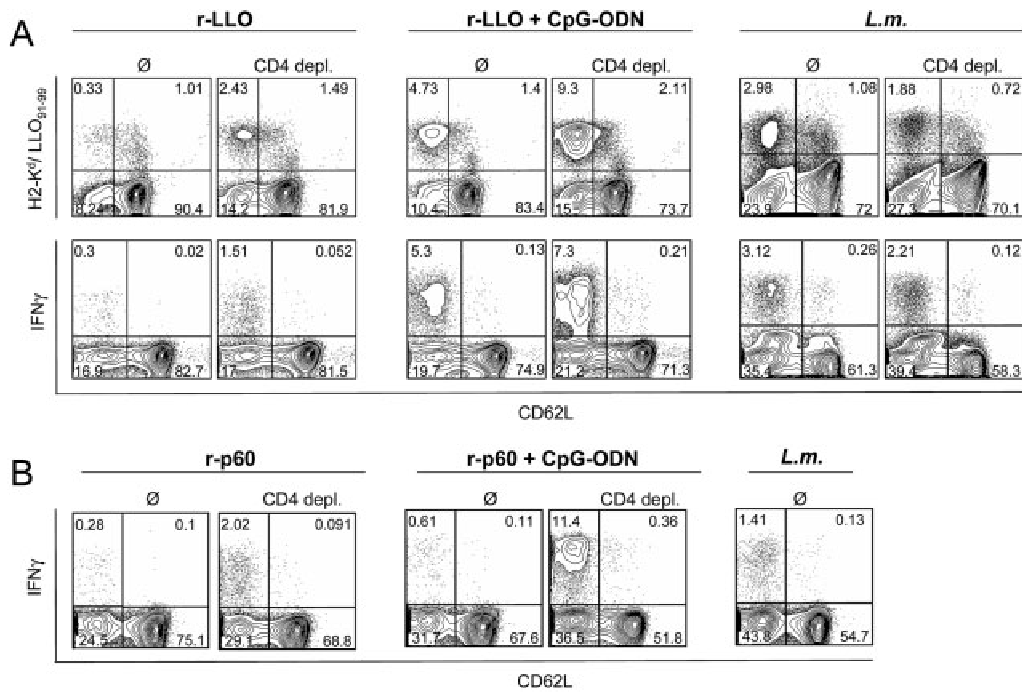

Figure 2. CD4 depletion leads to increased epitope-specific CD8$^+$ T cell frequencies. Untreated (∅) or CD4-depleted mice were s.c. immunized with r-LLO (A) or r-p60 (B) with or without CpG-ODN. Control mice were infected with a sublethal dose of *L.m.* After 7 days, splenocytes were analyzed for epitope-specific CD8$^+$ T cells. Representative density-dot plots of splenocytes (gated on live CD8$^+$ cells) stained with (A) H2-K^d/LLO$_{91–99}$ multimers or IFN-γ after restimulation with LLO$_{91–99}$ peptide or (B) IFN-γ after restimulation with p60$_{217–225}$ are shown.

constitutive expression of the IL-2 receptor α-chain (CD25), and treatment of mice with anti-CD25 antibodies results in depletion or functional impairment of the Treg compartment *in vivo* [44]. We used this approach to address the question of whether anti-CD25 treatment can mimic the effects of CD4 depletion upon protein vaccination.

As shown in Fig. 3A and reported previously [44], anti-CD25 treatment resulted in a substantial loss of Foxp3$^+$ CD4$^+$ cells, and the remaining Foxp3$^+$ cells lost their CD25 surface expression. Importantly, anti-CD25 treatment induced an enhancement comparable to what we observed following CD4 depletion (Fig. 3B). In most experiments, the frequencies and absolute numbers (Supporting Information Fig. 6) of expanded cells were even higher upon anti-CD25 treatment than in CD4-depleted mice. Similar results were obtained for vaccination with other antigens such as OVA (Supporting Information Fig. 3). In summary, these data suggest that the depletion of Treg is the major cause for the positive effects of CD4 depletion on CD8$^+$ T cell responses upon protein vaccination.

CD4$^+$ T cell depletion enhances the generation of protective effector memory T cells

The increased burst size of CD8$^+$ T cell responses following anti-CD4 or anti-CD25 treatment on day 7 after vaccination does not necessarily correlate with enhanced numbers of cells entering the memory T cell pool. Therefore, we analyzed the generation of long-living memory T cells in more detail. We [19] and others [45] recently reported that the IL-7 receptor α-chain (CD127) is constitutively expressed on memory T cells. The expression pattern of L-selectin (CD62L) further enables discrimination between memory T cells preferentially migrating to lymphoid organs (CD62L^{hi}, so-called central memory T cells) and cells migrating to non-lymphatic tissues (CD62L^{low}, so-called effector memory T cells) [46]. Protection against rapidly replicating intracellular pathogens, such as *L.m.* or vaccinia virus, is crucially dependent on the presence of sufficient numbers of effector memory T cells [23, 47]. Since the lineage commitment towards short- or long-living T cells occurs early during an immune response, the staining patterns of subtype-associated markers provide important information about the subsequent memory T cell pool.

Phenotyping of LLO$_{91–99}$-specific T cells 12 days following LLO/CpG-ODN vaccination revealed a bias towards CD127/CD62L double-positive cells, the subset containing precursors for central memory T cells (Fig. 4A and B). In sharp contrast, anti-CD25 treatment induced a strong switch towards CD127^{high}CD62L^{low} cells, the subset from which cells can be maintained as protective effector memory T cells. CD4 depletion also showed a differentiation shift towards CD127^{high}CD62L^{low} cells, although not as strong as was found for anti-CD25 treatment. Obviously, CD4 depletion is not identical to the effects of anti-CD25 treatment. This finding is in accordance with recent reports that CD4$^+$ T cell help can positively support the differentiation into effector memory T cells

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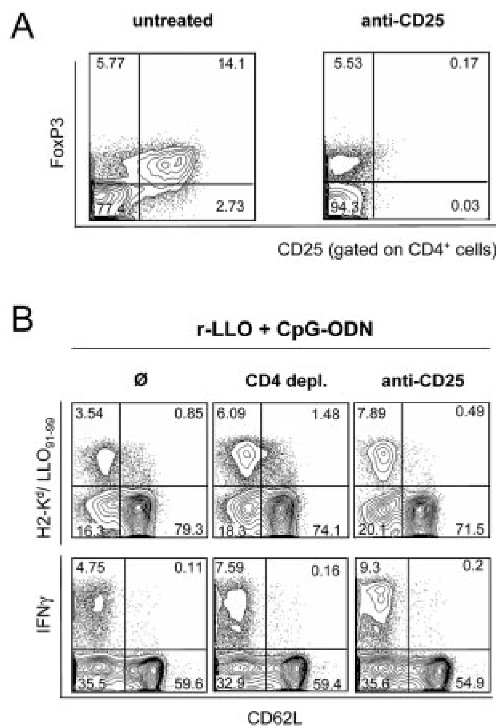

Figure 3. Treatment with anti-CD25 mAb further enhances epitope-specific $CD8^+$ T cell frequencies. Mice either untreated (∅), CD4-depleted or treated with anti-CD25 were s.c. immunized with r-LLO and CpG-ODN. (A) Efficiency of anti-CD25 treatment. Seven days after immunization, splenocytes of untreated (left) or anti-CD25-treated (right) mice were stained for Foxp3 (y axis) and CD25 (x axis). Representative density-dot plots gated on live $CD4^+$ cells are shown. Depletion efficacy controls were performed for every single animal. (B) On day 7 after immunization, splenocytes were stained with CD62L (x axis) and $H2\text{-}K^d/LLO_{91–99}$ multimers or IFN-γ after stimulation with $LLO_{91–99}$ peptide (y axis). Density-dot plots are gated on live $CD8^+$ cells.

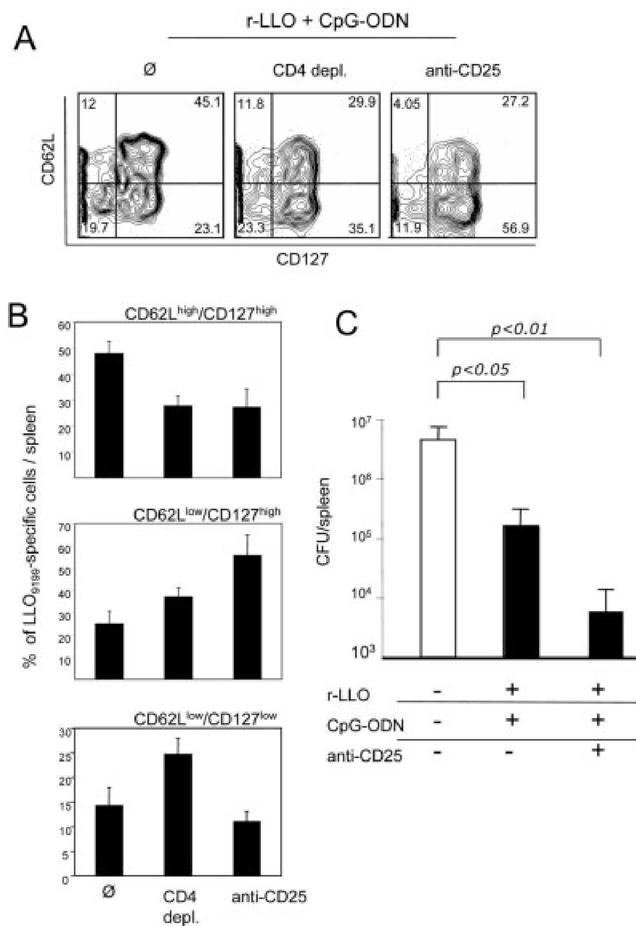

Figure 4. Influence of anti-CD25 treatment on T cell differentiation and long-term protection. (A) On day 12 after s.c. immunization with r-LLO and CpG-ODN in the presence or absence of anti-CD4 or anti-CD25 antibodies, splenic antigen-specific $CD8^+$ T cells were analyzed for their expression of CD127 (x axis) and CD62L (y axis). Density-dot plots are gated on live $CD8^+$ $H2\text{-}K^d/LLO_{91–99}$ multimer$^+$ cells. (B) Data from five mice per group are summarized. Bars represent mean values of percentages of $CD62L^{high}CD127^{high}$, $CD62L^{low}CD127^{high}$ and $CD62L^{low}$ $CD127^{low}$ subsets among $LLO_{91–99}{}^+$ cells. (C) Mice either left untreated or treated with anti-CD25 were s.c. immunized with r-LLO and CpG-ODN (filled bars). After 5 wk, mice were rechallenged with *L.m.* (2×LD50), and long-term protection was assessed by determination of viable bacteria in the spleen 3 days later. Naive mice served as a control (open bar). Bars represent five mice per group. Results are representative of at least three independent experiments.

[19]. CD4 depletion eliminates this pathway, which might explain the difference compared to the more Treg-specific anti-CD25 treatment.

Phenotypical analyses of differentiation patterns early after vaccination can only serve as predictors or indicators for the efficacy of subsequent memory responses. In order to directly evaluate the quality of memory T cells generated, challenge experiments were performed. As summarized in Fig. 1 and 4C, mice were significantly more protected against challenge with living *L.m.* following anti-CD4 or anti-CD25 treatment as compared to mice that received only protein and adjuvant. These data directly correlate with *in vivo* cytotoxicity experiments >5 wk after vaccination (Supporting Information Fig. 7), in which anti-CD25-treated mice had the highest values for epitope-specific lysis. Taken together, these data suggest that the depletion of Treg not only enhances the overall size of clonally expanding T cell populations, but also specifically supports the differentiation into protective effector memory T cells.

Depletion of MHC class II-restricted Treg increases the number of protective effector memory T cells

The interpretation of antibody-mediated depletion experiments has limitations, especially since expression of most antigens is not restricted to one lymphocyte subpopulation. This is also the case for CD25, which is not exclusively expressed on Treg; *e.g.* recently activated T cells and some antigen-presenting cells also temporarily express CD25. We therefore explored other experimental systems in order to more conclusively demonstrate the origin of the enhancement effect during protein vaccination.

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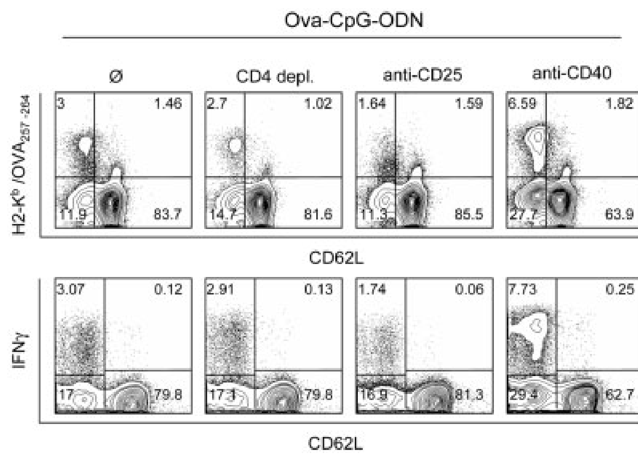

Figure 5. Immunization of MHC class II-deficient mice treated with anti-CD4 or anti-CD25 mAb. MCH class $II^{-/-}$ mice were left untreated or treated with anti-CD4, anti-CD25 or anti-CD40 mAb, and were s.c. immunized with OVA-CpG-ODN conjugate. Seven days later splenocytes were stained with H2-K^b/$OVA_{257–264}$ multimers (upper row) or for intracellular IFN-γ after restimulation with $OVA_{257–264}$ (lower row) in combination with cell surface CD62L expression (x axis). Density-dot plots are gated on live $CD8^+$ cells.

MHC class II-deficient mice lack MHC class II-restricted naturally occurring Treg as well as conventional $CD4^+$ T cells. Therefore, if the enhancement effect of anti-CD4 or anti-CD25 antibody treatment is mainly caused by MHC class II-restricted Treg, this effect should not be detectable in MHC class II-deficient mice. This is exactly the case (Fig. 5, Supporting Information Fig. 8); anti-CD4 treatment did not increase the burst size of the $CD8^+$ T cell response upon vaccination with OVA-CpG-ODN (OVA had to be used as antigen, since MHC class $II^{-/-}$ mice were only available on the C57BL/6 background). Anti-CD25 treatment even slightly reduced the number of responding T cells in MHC class $II^{-/-}$ mice, which might be explained by depletion of activated $CD25^+$ T cells. When a typical Th cell signal *via* a stimulatory anti-CD40 antibody was provided, $CD8^+$ T cell responses in MHC class $II^{-/-}$ mice were enhanced (Fig. 5, Supporting Information Fig. 8), showing that when the need for physical $CD4^+$ T cell presence is bypassed, this classical 'helper cell pathway' can still be operative.

In order to directly demonstrate that Treg represent the main cell population suppressing $CD8^+$ T cell responses upon protein-CpG vaccination, we took advantage of a recently developed model of *in vivo* depletion of $Foxp3^+$ lymphocytes [48]. Transgenic expression of the high-affinity diphtheria toxin (DT) receptor under control of the Foxp3 promoter (DEREG mice) enables specific depletion of Treg upon DT treatment, leaving the Th cell compartment intact. As shown in Fig. 6A and B, depletion of $Foxp3^+$ Treg during the priming phase upon protein-CpG vaccination dramatically enhanced the burst size of responding $CD8^+$ T cell populations. Similar to CD4 depletion or anti-CD25 treatment, DEREG mice demonstrated 5 wk after short-term DT treatment during protein vaccination improved protection towards challenge with *L.m.* (Fig. 6C).

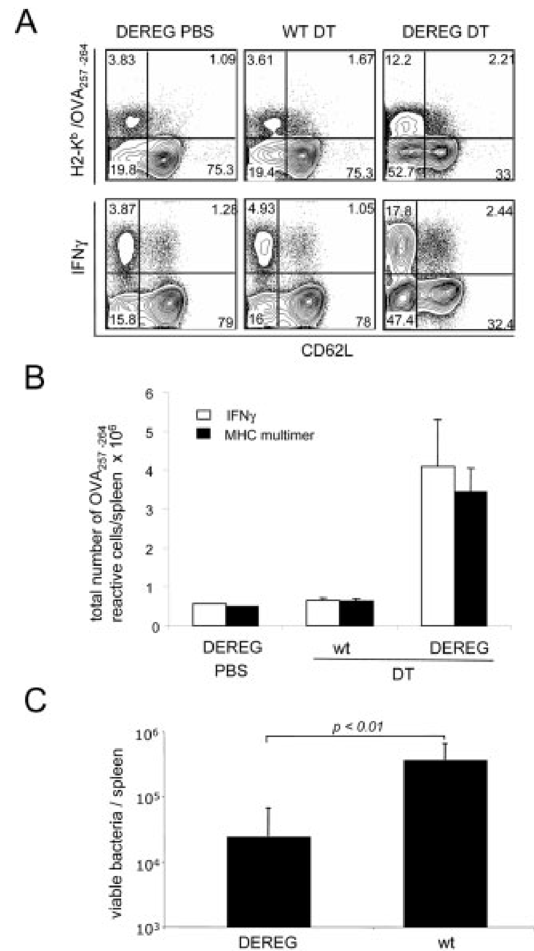

Figure 6. Depletion of Treg leads to an elevated CD8 T cell response to OVA-CpG-ODN complex. DEREG mice were immunized with OVA-CpG-ODN and subsequently treated with 1 μg DT i.p. on the three following days to deplete Treg (DEREG DT); as a control, non-DT-responsive wild-type mice were treated similarly (WT DT). (A) On day 7 post-immunization, splenic cells were analyzed for epitope-specific $CD8^+$ T cells by staining with antibodies specific for CD62L (x axis) as well as H2-K^b/$OVA_{257–264}$ multimers or anti-IFN-γ (intracellular) after *in vitro* peptide restimulation and fixation. Representative density-dot plots from each DT-treated group and a PBS-treated DEREG control group (DEREG PBS) are shown. Data are gated on $CD8^+$ T cells. (B) Absolute numbers of $OVA_{257–264}$-specific T cells per spleen were calculated; mean values and SD are shown. (C) Five weeks after immunization, cohorts of mice were infected with 2×10^5 OVA-expressing *L.m.* and sacrificed 3 days later. Protective immunity was measured by determination of viable bacteria in the spleen. Means of eight DEREG mice and five wild-type control mice (all treated similar with DT) out of two independent experiments are depicted in the graph.

Priming with MHC class II-deficient DC enhances $CD8^+$ T cell responses

Our data demonstrate that MHC class II-restricted $CD4^+$ Treg physiologically suppress $CD8^+$ T cell responses and their differentiation into long-living effector memory T cells during protein vaccination. Therefore, temporary *in vivo* depletion of Treg strongly enhances the protective efficacy of otherwise weak $CD8^+$

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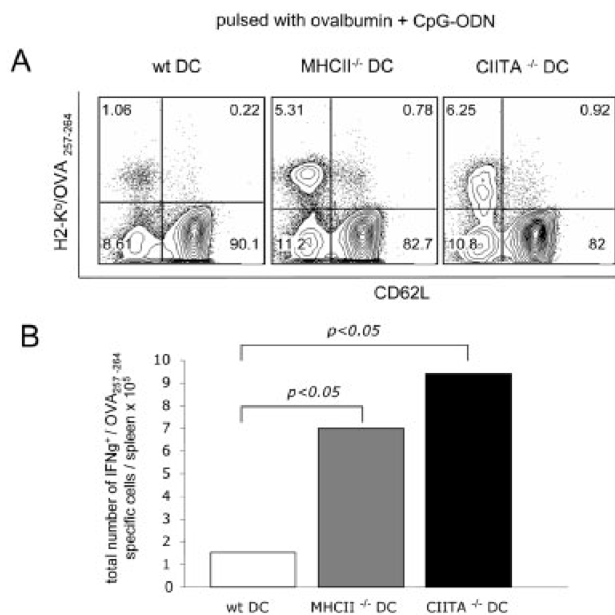

Figure 7. Immunization with DC lacking MHC class II expression. (A) BMDC were generated from C57BL/6 (wt), MHCII$^{-/-}$ or CIITA$^{-/-}$ mice, pulsed *in vitro* with OVA and CpG-ODN, and used to s.c. immunize wild-type mice. Seven days later, splenocytes were stained with H2-K^{b}/OVA$_{257-264}$ multimers (y axis) and CD62L (x axis). Representative dot plots are gated on live CD8^{+} cells. (B) Absolute numbers of IFN-γ^{+} cells upon OVA$_{257-264}$ restimulation per spleen. Data are presented as mean $\pm$ SD of five mice per group.

T cell-based split vaccines. Since in functional terms, the potency of Treg depletion largely exceeds the supportive effects provided by conventional CD4^{+} T cell help, long-term protective CD8^{+} T cell responses are generated even in the complete absence of conventional CD4^{+} T cell help during the priming phase.

In order to translate our findings into clinical applications, it would be ideal to circumvent the suppressive Treg activity selectively at the site of T cell priming. Since CD4^{+} Treg require interaction with MHC class II complexes to exert their suppressive function, our data would predict that vaccination protocols with antigen-presenting cells that lack MHC class II molecules would be more efficient inducers of CD8^{+} (memory) T cell responses. We tested this prediction by generating DC from the bone marrow of MHC class II$^{-/-}$ and wild-type mice. The DC were pulsed briefly with OVA and CpG-ODN to induce maturation and cross-presentation of CD8^{+} T cell epitopes before *in vivo* application. Extensive comparative examinations of antigen uptake, DC maturation, cytokine profiles, and epitope presentation could not identify any differences between the two DC populations (Supporting Information Fig. 9). Nevertheless, just as predicted, s.c. vaccination with MHC class II$^{-/-}$ DC induced significantly stronger antigen-specific CD8^{+} T cell responses as compared to wild-type DC (Fig. 7). Loss of MHC class II expression on DC can also be achieved by down-modulation of the MHC class II transactivator (CIITA); this was confirmed by the observation that DC from CIITA-deficient mice are as potent in cross-priming as DC from MHC class II$^{-/-}$ mice (Fig. 7).

The enhancement of CD8^{+} T cell responses translated into superior long-term protection against *Listeria* infection (Supporting Information Fig. 10), similar to the observations made for anti-

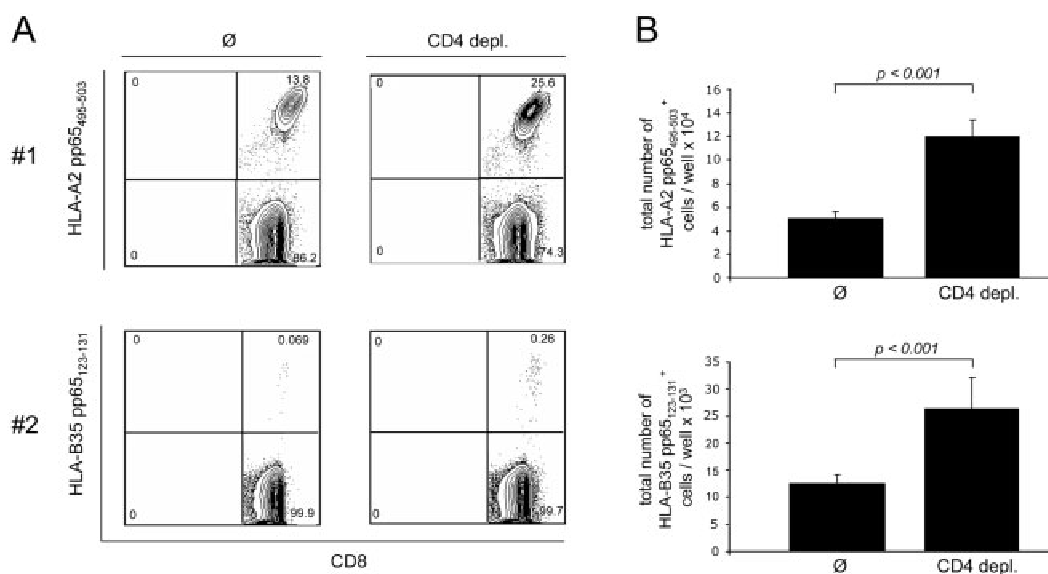

Figure 8. CD4 depletion enhances *in vitro* expansion of human CMV-reactive CD8^{+} T cells. Human PBMC from two different CMV-positive donors were CD4-depleted or left untreated ($\varnothing$). Cells were adjusted to similar CD8^{+} cell numbers and stimulated with pp65 together with imiquimod. (A) Staining on day 7 with HLA-A2 pp65$_{495-503}$ (donor #1) or HLA-B35 pp65$_{123-131}$ multimers (donor #2). Dot plots are gated on live CD8^{+} cells and are representative for three individual experiments.(B) Absolute numbers of HLA-A2 pp65$_{495-503}$- (upper row) or HLA-B35 pp65$_{123-131}$-specific cells (lower row) per well. Bar graphs represent mean $\pm$ SD of six values per group.

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CD4 or anti-CD25 treatment during protein vaccination. These data not only support the general notion that circumvention of Treg interactions at the level of priming antigen-presenting cells is a powerful strategy to improve split vaccines, even in the absence of T cell help, but also provide a first example of how this knowledge can be translated into the design of more effective DC-based vaccines.

In order to provide more evidence that basic aspects of our findings are transferable to humans, we performed depletion experiments with human PBMC from CMV-seropositive donors stimulated with recombinant CMV protein antigen (pp65) *in vitro*. According to our previous data, also under these conditions CD4 depletion should improve the expansion of pp65-specific $CD8^+$ T cells stimulated by cross-presented antigen. As shown in Fig. 8, this is indeed the case. Complete or CD4-depleted PBMC were cultured together with recombinant antigen and imiquimod for 7 days; frequencies of pp65-specific $CD8^+$ T cells were determined by MHC multimer staining or intracellular cytokine staining (data not shown). As shown for two different HLA restrictions (PBMC from two different donors), in both cases CD4 depletion resulted in higher frequencies of pp65-specific T cells (Fig. 8A). This could be further confirmed by calculating absolute numbers of expanded antigen-reactive T cells in each well, which were significantly higher in the CD4-depleted group. Although these *in vitro* data cannot prove that CD4 depletion would have similar effects *in vivo,* and although the experimental setting is limited by the fact that pp65-reactive T cells were already antigen-experienced, they suggest that our basic observations made in mice should be transferable to humans.

Discussion

With this study we addressed the question of how antigens purified from *L.m.*, which alone are not sufficient to induce protective immunity, can be converted into efficient vaccines. We found that co-application of CpG-ODN, while enhancing $CD8^+$ T cell frequencies during the effector phase, only modestly improved long-term protective immunity. Unexpectedly, we found that additional depletion of $CD4^+$ T cells, which are thought to be required for the generation of $CD8^+$ memory T cells, strongly enhanced protective immunity. Using DEREG mice, we can demonstrate for the first time that this enhancement is due to the temporal loss of $Foxp3^+$ Treg, which otherwise suppress the differentiation towards protective $CD8^+$ memory T cells.

According to the current literature, it is well accepted that the generation of long-term maintained $CD8^+$ memory T cells requires signals provided by $CD4^+$ T cells early during the priming phase [16–18]. Therefore, we were surprised to find that depletion of $CD4^+$ T cells during protein vaccination is a strong enhancer of long-lasting $CD8^+$ T cell-mediated protective immunity. This finding, although seeming contradictory at the first glance, can be attributed to the removal of suppressive MHC class II-restricted Treg. Although conventional $CD4^+$ T cell help participates in memory T cell generation during protein vaccination, the absence of this pathway is more than compensated for by the potency of Treg depletion.

It has been noted by other groups that *in vivo* anti-CD25 treatment can enhance primary immune responses [33–39]. However, it was not possible to directly show that depletion-based results are truly a consequence of the loss of Treg, since anti-CD25 antibodies can interfere with the *in vivo* function of a variety of different immune cells, and the depletion efficacy of anti-CD25 treatment has been questioned [44]. With the help of a novel genetic approach which targets *in vivo* cell depletion exclusively to $Foxp3^+$ cell populations (DEREG mice [48]), we could for the first time directly show that the enhancement is indeed caused by the elimination of suppressive effects coming from the $Foxp3^+$ Treg compartment.

Although the DEREG model pinpoints the observed effects to the Treg compartment, the exact mechanisms mediated by $CD4^+$ T cell or Treg depletion could still be multi-factorial and different from each other. For example, CD4 depletion generates a large volume of 'open space'. Although we could not find any evidence for homoeostatic effects of CD4 depletion on $CD8^+$ T cells (Supporting Information Fig. 4), it is difficult to fully exclude any influence of space issues on our observations. Furthermore, anti-CD25 treatment or Treg depletion in DEREG mice could lead to a general activation of (autoreactive) $CD4^+$ T cells, which might affect the overall quality of T cell help on $CD8^+$ T cell responses.

Previous studies have interpreted the effects of *in vivo* anti-CD25 treatment as a general liberation of immune responses from suppressive control by Treg, which increases the overall size of responding T cell populations, including subsets entering the memory pool [25]. However, our data show that Treg not only determine the overall size of responding T cell populations, but also affect the differentiation pattern and subsequent memory phenotype. Upon Treg depletion, a larger proportion of expanded T cells acquired the phenotype of $CD8^+$ effector memory T cells, indicating that under physiological conditions Treg control T cell subtype differentiation and prevent overly exuberant generation of effector memory T cells. To our knowledge this is the first report of a specific contribution of Treg activity to memory T cell differentiation.

Effector memory T cells have been identified in different infection models as the major subset providing immediate protective immunity against intracellular pathogens [23, 47]. Thus, down-modulation of Treg activity specifically supports generation of the most effective memory T cell subtype. In addition, this observation might explain why general defects in the Treg compartment cause severe autoimmune diseases [25]. Temporary autoimmune responses are frequently detectable during acute infections in otherwise healthy individuals [49], but these responses disappear shortly after the inflammation is resolved. Under physiological conditions, the majority of auto-reactive effector cells are obviously unable to enter the pool of long-term maintained memory T cells. This transition to long-living memory cells is facilitated in the absence of Treg, at least for $CD8^+$ T cells, which could be an important factor contributing to the maintenance of autoimmunity and disease development.

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The exact mechanisms used by Treg to suppress antigen-specific immune responses are currently not known. It was recently proposed that TLR ligands, such as LPS or CpG-ODN, can stimulate DC to produce factors such as IL-6, which make newly primed T cells refractory to the suppressive effects of Treg [31]. However, at least for the immunization conditions used in this study, an indirect effect of CpG-ODN on Treg activity was not a dominating factor in the efficacy of cross-priming; otherwise, depletion of Treg should not have had such a strong impact on protein vaccination in the presence of CpG-ODN as adjuvant. There is evidence that interactions of Treg with DC can inhibit DC maturation and activation of the antigen processing machinery [50]. These findings could imply a scenario in which the functional *in vivo* status of DC is constitutively under the control (*i.e.* suppression) of Treg. In this case, Treg depletion alone should enable DC to support cross-priming. This interpretation might be supported by our observation that Treg depletion enhanced cross-priming of $CD8^+$ T cells, even when immunizing with purified proteins in the absence of additional CpG adjuvant (although these responses did not result in measurable protective immunity; Fig. 1 and 2).

It was recently described that *in vivo* depletion of Treg for more than 1 wk can result in a large systemic increase of activated DC [51]. This observation was first interpreted as a possible loss of constitutive suppression of DC by Treg, but in the same study it was shown that *in vivo* DC activation is more likely to be an indirect consequence of the presence of autoreactive effector T cells. We also analyzed potential changes in the DC compartment during anti-CD4 or anti-CD25 treatment, but alterations in overall DC cell numbers, subset distribution, or expression of maturation markers could not be identified (Supporting Information Fig. 11). Together with our observation that DC from MHC class $II^{-/-}$ mice, while not detectably different from wild-type DC in cross-presentation or activation status (Supporting Information Fig. 9), still induced more potent $CD8^+$ T cell responses upon *in vivo* application, it is unlikely that the increased cross-priming upon Treg depletion is simply caused by liberation of DC from constitutive Treg suppression.

Other groups favor a model in which the suppressive effects of Treg on responder T cells are mainly a result of rapid depletion of IL-2 in their direct vicinity [52]. In this case, depletion of Treg would allow recently activated T cells to get easier access to IL-2, which could be provided by $CD4^+$ T cells or by priming antigen-presenting cells [53]. With this model it would be possible to integrate the recent observation that signaling *via* the IL-2 receptor is required early during T cell priming for the generation of memory T cells [20]. Our finding of a strong influence of Treg depletion on the differentiation towards memory T cells would also fit well to this model. However, further experiments are needed to provide more insights into the underlying mechanisms.

We show that the Treg compartment is an attractive target for improving the efficacy of *in vivo* cross-priming during vaccination, especially by increasing the number of long-living (at least for 5 wk) $CD8^+$ memory T cells. However, since the application of anti-CD4 or anti-CD25 depleting antibodies is not a practicable clinical approach, we searched for alternatives to translate these novel findings into more realistic vaccination strategies. The surprising finding that abrogation of Treg suppression fully compensates for the dependency of memory T cell generation on conventional T cell help stimulated us to test an unusual and otherwise seemingly nonsensical vaccination protocol. We hypothesized that vaccination with protein antigen- and CpG-ODN-pulsed DC that lack MHC class II molecules should be more effective in the induction of MHC class I-restricted $CD8^+$ T cell responses than normal DC. This procedure eliminates conventional MHC class II-restricted T cell help, but it also does not allow MHC class II-restricted Treg to interact directly with the priming DC. The results confirmed our prediction: vaccinations with MHC class $II^{-/-}$ or $CIITA^{-/-}$ DC were indeed significantly more effective in cross-priming than vaccinations with MHC class II-expressing DC.

Although these data are not a formal demonstration of $CD4^+CD25^+$ circumvention upon use of MHC class $II^{-/-}$ DC, they fit well into the context of our previous data. For example, these experiments not only further support the view that close interactions between the priming antigen-presenting cells, responding T cells and Treg determine the outcome of cross-priming *in vivo*, but also provide a strategy for the effective circumvention of Treg suppression without the need for depletion or functional impairment of the Treg compartment itself. Perhaps these findings also help to explain why DC-based vaccinations, which have been explored in several clinical studies [54], failed to correlate with clear clinical results.

Combination of DC vaccination with approaches to circumvent Treg activity might be a successful way to substantially improve its efficacy. Down-modulation of MHC class II on DC could be such a strategy. Since MHC class II haplotypes are usually highly variable, interference with the CIITA, which could be achieved by small interfering RNA-mediated knockdown or transgenic expression of dominant-negative CIITA variants, might be a way to achieve haplotype-independent down-modulation of MHC class II. Our vaccination results using DC from CIITA-deficient mice unquestionably demonstrate that loss of CIITA translates into a similar enhancement of cross-priming upon protein vaccination as observed for MHC class II-deficient DC. Whether this approach would have similar effects on T cell expansion and memory subset differentiation in humans has to be addressed in future studies. *In vitro* depletion experiments with human PBMC stimulated with protein antigen (Fig. 8) suggest that the basic principle is transferable to human leukocytes. However, since generation of T cell memory is very complex and because of the ongoing controversy whether human memory T cells fall into the same categories of 'central' and 'effector' memory T cell subsets as found in mouse models, any cross-species comparisons have to be interpreted with caution.

Importantly, our data demonstrate that it is possible to define vaccination conditions that permit the generation of protective $CD8^+$ effector memory T cells in the absence of $CD4^+$ T cell help. This might be of special relevance for the success of vaccination in patients with severe defects in the $CD4^+$ T cell compartment (*e.g.*

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AIDS [55]). In addition, it has been reported that chronic viral [56] or parasitic infections as well as aging [57] can cause a systemic shift towards Treg activity. It might be even more difficult to induce protective immunity by conventional vaccination strategies in these groups of individuals, and circumvention of Treg activity could be essential to overcome these problems. However, we cannot rule out that DC functionality could be altered in some chronic virus diseases, which might limit the efficacy of this novel approach in such patients. Furthermore, the quality of protection has been determined exclusively for challenges with intracellular bacteria in this present study and could differ for some viruses.

In summary, we show that *in vivo* depletion of Treg dramatically increases the efficacy of cross-priming and the induction of protective immunity against intracellular pathogens, even in the absence of conventional $CD4^+$ T cell help during the priming phase. These data identify Treg as an excellent target in strategies to improve T cell vaccines. Vaccination with DC lacking MHC class II expression provides a first example of how this knowledge can be translated into clinical applications without interference with the important systemic functions of Treg.

Materials and methods

Mice and bacteria

Age-matched BALB/c and C57BL/6 mice were obtained from Harlan Winkelmann (Borchen, Germany). $CIITA^{-/-}$ mice were purchased from The Jackson Laboratories (Bar Harbor, ME). $A\alpha^{-/-}$ and DEREG mice were derived from in-house breeding under specific pathogen-free conditions.

Infection experiments were performed by i.v. injection of wild-type *L.m.* strain 10403s (BALB/c mice) or *Listeria* expressing recombinant OVA (kindly provided by H. Shen, University of Pennsylvania, Philadelphia, PA) for experiments with C57BL/6 mice. A dose of 2×10^4 i.v. injected *L.m.* 10403s represents the LD50 for BALB/c mice. Animal experiments were approved by the local authorities (209.1/211–2531–100/04).

Generation of recombinant LLO and p60

Full-length gene sequences for LLO, $LLO492_{T\text{-}A}$ and p60 were cloned into pET27b expression vectors (Novagen) and periplasmatically over-expressed in BL21/DE3 hosts. His tags were used for primary affinity enrichment, followed by extensive gel filtration purification over a Superdex 200HR (Amersham) column. Purity largely exceeded 95% for all proteins used in this study.

Cell depletion and immunization

Depletion or inactivation of $CD4^+$ and $CD25^+$ cells *in vivo* was performed by i.p. administration of 150 μg GK1.5 antibody or 200 μg PC61 antibody in a total volume of 200 μL at each of the first 3 days before immunization and on days 2 and 5 thereafter. Treg depletion in DEREG mice was achieved by i.p. injection of 1 μg DT (Merck) for three consecutive days upon vaccination.

Mice were immunized i.v. or s.c. at the base of the tail with 1–100 μg (usually 10 μg) LLO or OVA in a volume of 50 μL without or together with CpG-ODN 1668 (TCCATGACGTTCCTGATGCT; TIB Molbiol, Berlin, Germany) at a concentration of 5 nmol in 50 μL. For some experiments, direct OVA-CpG-ODN conjugates (10 μg) were used for s.c. vaccination as described [58].

Cell surface staining

$H2\text{-}K^d/LLO_{91-99}$ and $H2\text{-}K^b/OVA_{257-264}$ multimer reagents were generated as described [24, 59]. For live/dead discrimination, cells were incubated with 1 mM ethidium monazide bromide (Molecular Probes, NL) in the presence of anti-FcR antibody (clone 2.4G2; BD PharMingen, San Diego, CA), followed by staining with multimers and the mAb anti-CD8α (clone 53-5.8), anti-CD8α-Alexa Fluor 405 (clone 5H10; Caltag Laboratories GmbH, Hamburg, Germany), anti-CD4 (clone RM4-5), anti-CD62L (clone MEL-14), anti-CD127 (clone SB/14); all from BD Pharmingen. For costaining with $H2\text{-}K^b$/SIINFEKL tetramers, anti-CD8α-specific mAb was used (clone CD8α; Caltag, South San Francisco, CA). Data were acquired on a FACSCalibur (Becton Dickinson) or a CyAn analyzer (DakoCytomation). Acquired data were further analyzed with CellQuest or FlowJo (Tree Star) software.

Intracellular cytokine staining

Splenocytes were incubated for 5 h with 10^{-6} M peptide (LLO_{91-99} or $OVA_{257-264}$) or DMSO (Sigma) in the presence of brefeldin A (Golgi Plug; BD Pharmingen). Cells were stained subsequently with Fc block (2.4G2; BD Pharmingen) and ethidium monazide (Molecular Probes). Surface staining (CD8, CD62L), fixation, permeabilization and intracellular staining (IFN-γ, XMG1.2; eBioscience) were performed using the Cytofix/Cytoperm Plus Kit (BD Pharmingen) as recommended.

Bacterial clearance

Mice were challenged with a high dose (2×LD50) *L.m.*, and 3 days later viable bacteria in the spleen were determined by plating out lysed cell suspensions in serial dilutions on brain heart infusion broth agarose.

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In vivo cytotoxicity assay

Erythrocyte-free splenocytes were either pulsed for 1 h with LLO_{91-99} peptide (10^{-7} M) and labeled with 5 μM CFSE (Molecular Probes) as targets or left untreated and labeled with 0.5 μM CFSE as controls for 10 min at 37°C in PBS. After stopping the reaction (10% FBS), the cells were washed and mixed in a 1:1 ratio. Each mouse was injected a total of 1×10^7 cells i.v. in 200 μL PBS. At various time points, peripheral blood was analyzed by flow cytometry. The percentage of specific lysis was calculated as followed: % lysis = 100 – [(% $CFSE^{high}$ / % $CFSE^{low}$ ×100) × (% $CFSE^{low}$ / % $CFSE^{high}$ of naive control)].

Generation of BMDC and DC immunization

Bone marrow was isolated from C57BL/6, $CIITA^{-/-}$ and $A\alpha^{-/-}$ mice. After incubation with RPMI containing 0.2 μg rGM-CSF (Peprotech) for 7 days, $CD11c^+$ DC were generated (70–80% purity). DC were incubated with 3 mg/mL OVA for 1 h at 37°C. Then CpG-ODN (5 nmol) was added, followed by an additional 30-min incubation. Cells were washed three times, and 1×10^6 DC were injected s.c. in a total volume of 100 μL PBS.

Determination of *in vitro* antigen uptake, processing and presentation

GM-CSF-derived DC were exposed to FITC-labeled OVA (10 μg/mL) or medium for 30 min at 37°C, washed twice with ice-cold 3% FBS/PBS and stained with APC-labeled anti-CD11c (clone HL3; BD Pharmingen). To analyze the expression of MHC class I-associated SIINFEKL on the surface of OVA-CpG-ODN complex-pretreated (24 h) DC, cells were stained with the $H\text{-}2K^b$/SIINFEKL-specific antibody 25D1.16 (obtained from R. Germain, Bethesda, MD) and an Alexa Fluor 488-coupled anti-mouse IgG (Molecular Probes). Activation of antigen-presenting cells by OVA-CpG-ODN complex or OVA alone was analyzed by incubation of DC with 10 μg/mL of the indicated substances for 24 h, and subsequent staining with anti-CD11c (clone HL3; BD Pharmingen) and anti-CD40 (clone 3/23; BD Pharmingen). In addition, supernatants of stimulated BMDC were analyzed by ELISA for IL-6 and IL-12p40 (R&D Systems) as instructed by the manufacturer.

In vitro stimulation of human PBMC with recombinant CMV protein

Human PBMC from two different CMV-seropositive donors were left untreated or CD4 MACS-depleted (depletion >95%) using anti-CD4 human microbeads and LS-columns (Miltenyi Biotec, Bergisch Gladbach, Germany). For stimulation, 3×10^6 cells per well (24-well plate) for the 'untreated' groups were used, the size of depleted samples was adjusted to equal $CD8^+$ cell numbers in each well. Cells were subsequently stimulated with 20 μL human recombinant pp65 protein (Miltenyi Biotec) together with 5 μg/mL imiquimod (Invivogen, Toulouse, France), and after 3 days IL-2 was added. Four days later the cells were counted and stained with HLA-A2 $pp65_{495-503}$ or HLA-B35 $pp65_{123-131}$ multimers, and IFN-γ production was determined after restimulation with PepMix pp65 (JPT Peptide Technologies, Berlin, Germany).

Statistical analysis

Indicated significance levels were calculated using standard Student's *t*-test.

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Abbreviations: **DEREG:** depletion of Treg by transgenic expression of diphtheria toxin receptor under control of the Foxp3 promoter · **DT:** diphtheria toxin · **LLO:** listeriolysin O · ***L.m.*****:** Listeria monocytogenes · **ODN:** oligodenucleotides

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FoxP3+ regulatory T cells essentially contribute to peripheral CD8+ T cell tolerance induced by steady state dendritic cells

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Abbreviations: CTL (cytotoxic T lymphocyte), DC (dendritic cell), Treg (regulatory T cell), DT (diphtheria toxin), TAM (tamoxifen), lymphocytic choriomeningitis virus (LCMV)

MvdB, AS and TS designed experiments, AS, CB and MvdB performed experiments, MvdB, AS and HCP analyzed data, KL and TS contributed vital new reagents, HCP, AS and MvdB wrote the paper.

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Abstract

Peripheral T cell tolerance is thought to significantly contribute to the prevention of autoimmunity and it was shown that antigen-presenting steady state dendritic cells efficiently induce peripheral tolerance. We previously showed that dendritic cell-induced tolerance is a T cell-intrinsic process and depends on the co-inhibitory molecules cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1). Here we specifically analyze the involvement of FoxP3^{+} regulatory T cells, which are known to be important for maintenance of self-tolerance. We show that antigen presentation by steady state dendritic cells failed to induce peripheral tolerance in the absence of FoxP3^{+} regulatory T cells but induced protective CD8^{+} T cell-mediated immunity instead. Dendritic cells isolated from mice without regulatory T cells had upregulated co-stimulatory molecules suggesting that regulatory T cells contribute to peripheral tolerance by keeping the dendritic cells in an immature state.

Introduction

Most autoreactive T cells are deleted in the thymus by so called negative selection. Although this process is efficient, the presence of autoreactive T cells in every healthy individual demonstrates that it is not complete (1). Peripheral, mature autoreactive T cells are kept in check by peripheral tolerance, which acts through a variety of mechanisms that are not necessarily mutually exclusive and that include unresponsiveness/anergy, regulation/suppression and deletion.

We, and others have recently demonstrated that dendritic cells (DC) play a central role in the induction of peripheral tolerance. Using transgenic mice that allow the inducible expression of viral cytotoxic T lymphocyte (CTL) epitopes selectively by DCs (DIETER mice), we showed that presentation of CTL epitopes by steady state DCs induces robust tolerance in antigen specific CD8$^+$ T cells. We found this tolerance to depend on signaling via the inhibitory receptors PD-1 and CTLA-4 and to follow a recessive mechanism such as induction of anergy in or deletion of CD8$^+$ T cells specific for the antigens presented by the steady state DCs. Using adoptive transfer of naïve T cells into tolerant mice, we did not find any evidence for involvement of a dominant suppressive mechanism such as the induction of antigen specific regulatory T cells (Treg) or the production of immunosuppressive cytokines. We did, however, not formally address the contribution of Treg to peripheral tolerance induced by steady state DCs in DIETER mice.

CD4$^+$CD25$^+$FoxP3$^+$ Treg were first characterized by their immunosuppressive properties (2, 3) and comprise approximately 10-15% of all peripheral CD4$^+$ T cells in mice. The forkhead box transcription factor FoxP3 is crucially involved in the development of Treg and it is the best marker for Treg at present (4-8). That Treg play a critical role in the control of autoimmunity is illustrated by the fact that loss-of-function mutations in the FoxP3 gene, as well as depletion of Tregs (9, 10), results in fatal autoimmune disease in humans and mice (11, 12). Furthermore, naturally occurring FoxP3$^+$ Treg were shown to regulate inflammatory disorders

such as colitis and immune responses to transplants, tumors and infectious agents (3).

In order to investigate the role of FoxP3$^+$ Treg in peripheral tolerance of CD8$^+$ T cells, we crossed DIETER mice to DEREG mice, thus generating a system in which FoxP3$^+$ Treg can be depleted by injection of diphtheria toxin (DT) (10) and in which peripheral tolerance results from induced antigen presentation by steady state DC (13). We found that the induction of peripheral tolerance by steady state DC was severely impaired in the absence of regulatory T cells. Rather than inducing tolerance, antigen presentation by steady state DCs resulted in priming of a functional CTL response.

Results

Transient antigen presentation by steady state DCs primes protective immunity in the absence of FoxP3$^+$ regulatory T cells

DIETER and DEREG/DIETER chimeras were injected with tamoxifen (TAM) to induce presentation of lymphocytic choriomeningitis virus (LCMV) $GP_{33\text{-}41}$ and $\beta\text{-gal}_{497\text{-}505}$ by approximately 5% of CD11c^{high} cells (13). Half of the mice received multiple injections with DT that resulted in absence of GFP$^+$ FoxP3$^+$ cells in DEREG/DIETER mice for at least 8 days. We measured the frequency of LCMV $GP_{33\text{-}41}$- and $\beta\text{-gal}_{497\text{-}505}$-specific T cells 8 days after TAM injection. As expected, we found that antigen presentation by steady state DCs did not induce measurable expansion of endogenous CD8$^+$ T cells specific for LCMV $GP_{33\text{-}41}$ or $\beta\text{-gal}_{497\text{-}505}$ and we had shown previously that this treatment induced robust and antigen-specific peripheral tolerance (13). In contrast, TAM injection into DT-treated DEREG/DIETER mice induced LCMV $GP_{33\text{-}41}$- and $\beta\text{-gal}_{497\text{-}505}$-specific responses (Fig. 1A), suggesting that antigen presentation by steady state DCs results in priming if FoxP3$^+$ Treg are absent. In order to test whether the expanded CD8$^+$ T cells can execute effector function, we challenged the four groups of mice with 200 pfu LCMV strain WE at day 8 and measured splenic viral titers 5 days later. We found no evidence for protective immunity in any of the experimental groups except for DEREG/DIETER mice that were treated with TAM and DT, which had a significantly lower virus load in their spleens (Fig. 1B).

Transient depletion of FoxP3$^+$ regulatory T cells changes the phenotype and number of splenic DCs

We have shown previously that antigen-presenting steady state DCs induce peripheral tolerance of CD8$^+$ T cells. Therefore, mechanisms that impede DC maturation may contribute to self-tolerance and there is experimental evidence that Treg interfere with DC maturation. In

order to test whether the short term specific depletion of FoxP3$^+$ cells resulted in phenotypic changes of endogenous DCs in our model system, we depleted Treg from DEREG mice during 5 days and analyzed the surface expression of molecules that are associated with DC maturation such as CD40, CD70, CD80, CD86 and MHC class I and II molecules on *ex vivo* isolated splenic DCs. We found a significant ($p<0.02$) upregulation of CD40, CD80, CD86 and MHC class II molecules on DCs from DT-treated DEREG mice compared to DT-treated C57BL/6 or untreated DEREG or C57BL/6 mice, whereas the upregulation of CD70 and MHC class I molecules showed the same tendency but was not significant ($p=0.12$ and $p=0.051$, respectively) (Fig. 2A, B). In addition to displaying a more activated phenotype, also the number of CD11c$^+$ cells in the spleen increased significantly in the absence of FoxP3$^+$ Treg (Fig. 2C).

Discussion

We have shown that antigen presentation by steady state DCs in the absence of Tregs resulted in expansion of endogenous LCMV GP_{33-41}-specific $CD8^+$ T cells to 1-2% of total $CD8^+$ T cells and these CTL mediated an approximately 10-fold reduction of virus titers after LCMV challenge. This response, however, is considerably weaker than the response primed when interactions of the inhibitory molecules PD-1 and CTLA-4 were blocked during antigen presentation by steady state DCs, in which case 10-16% LCMV GP_{33-41} specific $CD8^+$ T cells and full protection to subsequent LCMV challenge were observed, or than the response when only PD-1 interactions were blocked, in which case 8-12% LCMV GP_{33-41} specific $CD8^+$ T cells and full protection to subsequent LCMV challenge were observed (14). The response in the absence of Tregs is, however, comparable in magnitude to the response observed when only CTLA-4 interactions were blocked. This is of particular interest in the light of experiments demonstrating a crucial role of CTLA-4 for suppressive function of Tregs (15, 16). The weaker priming upon Treg depletion as compared to blocking of PD-1 might be interpreted such that Treg are required for full induction of peripheral $CD8^+$ T cell tolerance but other inhibitory mechanisms such as signaling via the PD-1 receptor are operative even in the absence of regulatory T cells.

Several mechanisms by which Tregs suppress T cell responses have been proposed (reviewed in (17)): Tregs may outcompete effector T cells for access to antigen presenting DCs, they may directly interact with conventional T cells thus inactivating or eliminating them or they may modulate the activation state and function of DCs. It was shown that, upon short contact with Treg, DCs downregulate co-stimulatory molecules (18, 19), upregulate IL-10 (20) and the co-inhibitory molecule B7-H3, and are compromized in the stimulation of naïve T cells (18). The suppressive impact of Treg on steady state DCs can largely be overruled by inflammatory stimuli such as lipopolysaccharide (LPS) (8, 20, 21), which is consistent with the fact that pathogens usually induce strong immune responses despite the presence of

regulatory mechanisms. Most of these studies used *in vitro* co-cultures of DCs and Treg, in which the ratio Treg:DC as well as the duration of contact presumably exceed those found under physiological circumstances. A recent *in vivo* study, however, confirmed the Treg-mediated impact on the phenotype and the function of DCs (18). In addition, a recent study using DT mediated depletion of FoxP3+ cells in a transgenic model similar to the DEREG mice used here, described upregulation of CD40 and CD80 on DC and an increase in numbers of DCs in secondary lymphoid organs in the context of autoimmunity following Treg depletion (9). We show here that depletion of Treg resulted in a significant up-regulation of co-stimulatory molecules such as CD40, CD80, CD86 and MHC class II on DC as well as a two-fold increase of their number in secondary lymphoid organs. It remains to be determined, whether this increase is a direct consequence of the Treg depletion, *i.e.* Tregs being required for the suppression of the intrinsic proliferation of DCs, for the enhanced differentiation of DCs from precursors or for enhanced emigration of tissue resident DCs. Alternatively, the increased DC numbers in secondary lymphoid organs might reflect enhanced immigration in the absence of Treg, which fits the recent observation that Treg inhibit the production of CCR5 ligands, limiting the CCR5-dependent recruitment of DC to the lymph nodes (22), or differentiation of DCs following T cell activation and secretion of proinflammatory cytokines that results from the absence of Treg. The latter view is supported by the observation that DCs have a steady state phenotype and normal numbers in TCR transgenic mice on a RAG deficient background that lack naturally occurring Tregs, suggesting that the absence of Tregs alone is not sufficient to trigger DC activation (9). The observed increase in DC numbers following Treg depletion will presumably result in an increase in the number of antigen presenting DCs upon TAM injection of DEREG/DIETER mice. But it is unlikely that this mere increase in the number of antigen presenting DCs is the reason for priming instead of tolerance induction in the absence of Treg, as injection of higher doses of TAM (up to 6

mg), which also leads to an increase in the number of antigen presenting DCs, does not induce priming (unpublished data).

We had previously shown that peripheral $CD8^+$ T cell tolerance induction following the presentation of $CD8^+$ T cell epitopes on steady state DCs is a T cell-intrinsic phenomenon involving deletion or anergy of the T cells recognizing their antigen on steady state DCs (14). Antigen presentation in the absence of DC activation did not lead to a dominant suppressive tolerance that affected T cells of other specificities, which made the induction of regulatory T cells by the steady state DC unlikely. This is difficult to reconcile with the results presented here at first glance but may be explained by multiple levels or mechanisms of peripheral tolerance induction, which probably operate in concert. DCs constitutively express ligands for co-inhibitory molecules such as CTLA-4 and PD-1, which are expressed to a low level on naïve T cells and the result of the interaction between naïve T cells and DCs under steady state conditions usually is tolerance. Inflammatory stimuli induce DC maturation and the co-stimulatory interactions will overrule the inhibitory signals, resulting in T cell activation. If naïve T cells scan DCs in the steady state, they will contact many resting DCs that transmit inhibitory signals, but also a few activated or mature DCs that are presumably present in every individual. But because the inhibitory signals outnumber the activating ones tolerance ensues, except in situations in which the inhibitory interactions are prevented, as we have shown before (14). Our data suggest that Treg act on a different level, which is keeping the immature status of DCs or increasing the threshold for DC activation. At present it is unclear whether this results from a direct interaction between DCs and Treg or whether absence of Treg allows activation of other cell types, which then modulate the DC phenotype.

Taken together, we have shown that $FoxP3^+$ regulatory T cells are required for efficient induction of $CD8^+$ T cell tolerance upon presentation of $CD8^+$ T cell epitopes on resting DCs. Analysis of DCs in the absence of regulatory T cells revealed an activated phenotype and increased numbers of DCs in secondary lymphoid organs. Thus, Treg seem to be required to

ensure the non-activated phenotype of DCs that is essential for the induction of peripheral tolerance.

Materials and Methods

Mice

C57BL/6 mice were obtained from the Institute of Laboratory Animal Science (University of Zurich). DIETER double transgenic mice were described (13) and allow tamoxifen (TAM)-inducible presentation of three LCMV-derived CTL epitopes ($GP_{33\text{-}41}/D^b$, $GP_{34\text{-}41}/K^b$ and $NP_{396\text{-}404}/D^b$) and one β-galactosidase-derived CTL epitope ($\beta\text{-gal}_{497\text{-}504}/K^b$) by DCs. DIETER mice were bred onto DEREG (10) mice that allow DT-mediated depletion of FoxP3$^+$ Treg. All mice were generated on a C57BL/6 background or were backcrossed with C57BL/6 mice for at least 10 generations and were kept at the University Hospital Zurich (Institute of Laboratory Animal Science). Experiments were carried out in accordance with the Swiss federal and cantonal law on animal protection and performed with age- and sex-matched mice. To increase the number of available DIETER and DEREG/DIETER mice, bone marrow chimeras were generated as described (13).

Lymphocytic choriomeningitis virus (LCMV)

LCMV strain WE (LCMV-WE) was propagated on L929 fibroblast cells at a low multiplicity of infection (m.o.i.). LCMV titers were determined in spleen 5 days after infection as described (23).

Treatment of Mice

Cre recombinase activity resulting in antigen presentation by steady state DCs was induced *in vivo* by injecting DIETER or DEREG/DIETER mice i.p. with 2 mg TAM as described (13). FoxP3$^+$ cells were depleted from DEREG/DIETER mice by i.p. injection of 1 μg diphtheria toxin (DT, Sigma Chemical Co, St Louis, MO, USA) in 100 μl PBS at day -1, +1, +3 and +5

relative to TAM injection or with PBS as control. This regimen depleted GFP$^+$FoxP3$^+$ cells from DEREG/DIETER mice to undetectable levels at least until day 8 (data not shown).

Priming of endogenous, GP_{33-41}/D^b- and β-$gal_{497-504}/K^b$-specific CD8$^+$ T cells was measured 8 days after TAM injection. Functionality of GP_{33-41}/D^b-specific CD8$^+$ T cells was measured by their capacity to protect against a subsequent i.v. infection with 200 plaque forming units (pfu) LCMV-WE.

Peptides

LCMV-derived peptides GP_{33-41} (KAVYNFATC, H-2D^b)) and β-galactosidase-derived peptide β-$gal_{497-504}$ (ICPMYARV) were purchased from NeoMPS (Strasbourg, France) in immunograde quality.

Staining with Tetrameric MHC Class I-Peptide Complexes

Tetrameric peptide-MHC complexes were generated and staining was performed as described (13, 24). Samples were measured with a FACS Calibur, Becton Dickinson, Mountain View, CA and analyzed using FlowJo Analysis Software (Tree Star Inc, Ashland, OR).

DC Isolation and Phenotyping

DEREG or C57BL/6 mice were injected i.p. with 1 μg DT or with PBS at day 0 and 2. Spleens were removed at day 5 and were digested with collagenase/DNase. Single cell suspensions were counted and stained for CD11c-APC (clone HL3) plus CD80-FITC (clone 16-10A1), CD86-FITC (clone GL1), CD70-PE (clone FR70), H-2D^b-FITC (clone KH95) or H-2I-A^b-FITC (clone AF6-120.1) in 25 μl FACS buffer for 20 minutes at 4°C. All antibodies were obtained from Pharmingen (Becton Dickinson, Mountain View, CA). Cells were washed twice and

analyzed by flow cytometry (FACS Calibur, Becton Dickinson, Mountain View, CA) using FlowJo Analysis Software (Tree Star Inc, Ashland, OR).

Statistical Analysis

Statistical analysis was performed with the Student's t-test and Prism 4 software (GraphPad Software).

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The authors have no conflicting financial interests.

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Legends to the Figures

Figure 1: Antigen presentation by steady state DCs induces protective immunity instead of tolerance in the absence of FoxP3+ Treg

DIETER (D) and DEREG/DIETER (DD) mice were injected i.p. with 1 μg DT or PBS on day -1, 1, 3 and 5 and with 2 mg TAM on day 0. LCMV GP33-41/D^b and β-Gal497-505/k^b specific $CD8^+$ T cells were quantified in the blood on day 8 by staining with MHC-class I tetramers **(A)**. Each point in the graph represents an individual mouse and a representative experiment out of three is shown. Student's t-test: tetramer GP33-41/D^b: DD vs. DD/DT p=0.0017; β-Gal497-505/K^b: DD vs. DD/DT p=0.0003; all other comparisons: NS

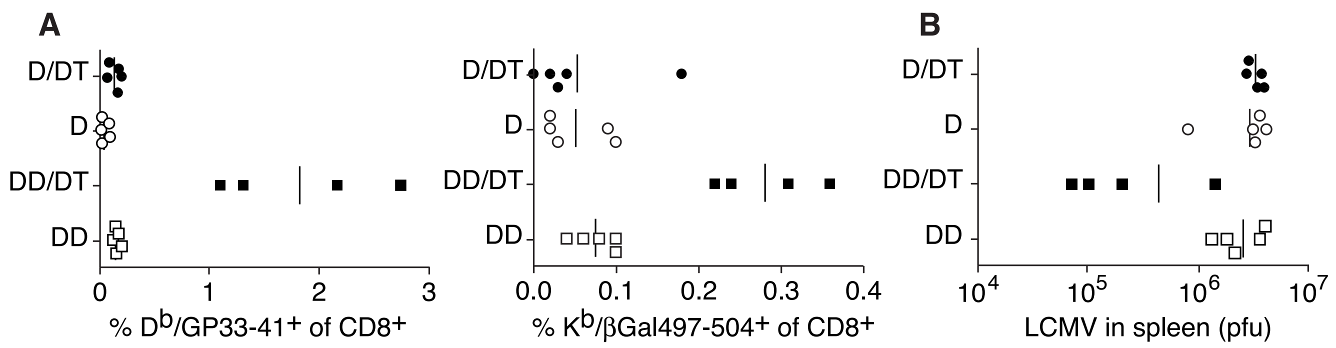
Mice depicted in Figure 1 were challenged on day 8 with 200 pfu LCMV-WE and the splenic virus titers were determined on day 13 using a focus-forming assay **(B)**. Each symbol represents an individual mouse. One representative experiment out of two is shown. Student's t-test: DD vs. DD/DT: p=0.0148; all other comparisons: NS

Figure 2: Depletion of FoxP3+ Treg changes the phenotype and numbers of splenic DCs in vivo

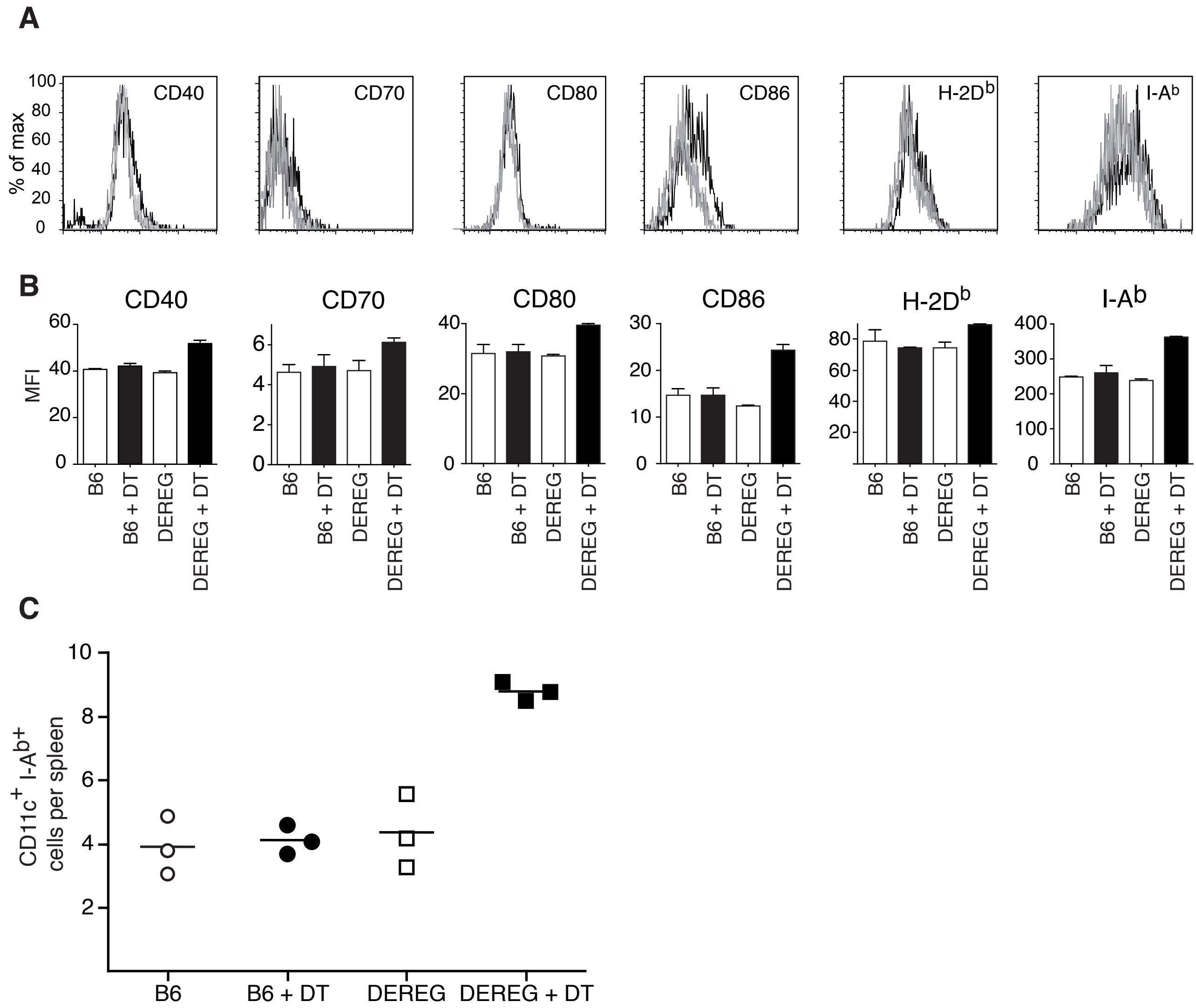
Age- and sex-matched C57BL/6 and DEREG mice were injected i.p. with 1 μg DT or with PBS on day 0 and 2 and spleens were removed on day 5, digested with collagenase and DNase, stained with appropriate antibodies and analyzed by FACS. Samples were gated on I-A^b+ $CD11c^+$ cells. **(A)** FACS plots of one representative mouse per group are shown (light grey line: C57BL/6 + DT; dark grey line; DEREG; black line: DEREG + DT). **(B)** Bars represent the mean fluorescence intensity (MFI) +/- SD of three mice per group for individual activation markers. Student's t-test: D vs. D/DT CD40 p=0.02; CD70 p=0.12 (NS); CD80 p=0.0076; CD86 p=0.015; I-Ab p=0.002; Db p=0.051 (NS); all other comparisons NS **(C)** The total number of I-A^{b+} $CD11c^+$ per spleen was determined; every data point represents one

mouse. The total number of splenocytes was similar in all four experimental groups with a range of 3.6 – 4.2 x 10^7. Student's t-test: D/DT vs. D: $p=0.003$; all other comparisons: NS

Schildknecht et al. Fig1



Schildknecht et al. Fig. 2



Depletion of Foxp3$^+$ cells leads to induction of autoimmunity by specific ablation of regulatory T cells in genetically targeted mice

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Abbreviations used in this paper: Treg, regulatory T cell; IPEX: immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome; DEREG: depletion of regulatory T cells; DTR: diphtheria toxin receptor

Abstract:

We have recently described two independent mouse models in which administration of diphtheria toxin (DT) leads to specific depletion of regulatory T cells (Tregs) due to expression of DTReGFP under the control of the Foxp3 promoter. Both mouse models develop severe autoimmune disorders when Foxp3$^+$ Tregs are absent. Those findings were challenged in a study by Liu's group published in this journal suggesting expression of Foxp3 in epithelial cells as the cause for disease development. By using genetic, cellular and immunohistochemical approaches, we do not find evidence for Foxp3-expression in non-haematopoietic cells.
DT-injection does not lead to loss of epithelial integrity in our FoxP3-DTR models. Instead, Foxp3 expression is Treg-specific and ablation of Foxp3$^+$ Tregs leads to induction of fatal autoimmune disorders. Autoimmunity can be reversed by adoptive transfer of Tregs into depleted hosts and transfer of Foxp3-deficient bone-marrow into T cell deficient irradiated recipients leads to full disease development.

Introduction

The existence of a dedicated population of suppressor T cells has long been surmised. However, the immunology community en large treated this notion with contempt because the markers and other molecular features for the presumed suppressor T cell population were not known. The identification of $CD25^{+}CD4^{+}$ T cells followed by the discovery of the transcription factor Foxp3 as a faithful marker for regulatory T cells allowed for a large body of published reports demonstrating the suppressive activity of Foxp3-expressing T cells (1-5). A fatal early-onset autoimmune syndrome in mice or humans harboring a non-functional Foxp3 allele is manifest to the vital significance of regulatory T cell-mediated suppression. Collectively, the general consensus in the field is that the high level of Foxp3 expressed by regulatory T cells is essential for their suppressive activity. In accordance with this idea, the loss of Foxp3 expression in hematopoietic cells, more specifically in T cells (1), or the loss of regulatory T cells induces an autoimmune syndrome (6, 7), similar to mice with germ-line mutations in Foxp3 (8) (see below). However, this model was recently challenged by Liu and colleagues (9, 10). According to their studies, Foxp3 expression is not restricted to Treg cells, let alone hematopoietic cells, but is widespread in variety of epithelial tissues including thymic, mammary gland, lung, and prostate epithelial cells (9-12). These investigators argued that the loss of epithelial-specific Foxp3 expression is the cause of life-threatening autoimmunity in IPEX patients, Foxp3 deficient mice, or mice engineered to eliminate Foxp3-expressing cells, while Foxp3 expression in hematopoietic cells, including Treg cells, is not essential (10).

Materials and Methods

Immunohistochemistry

In brief, sections from formalin-fixed paraffin-embedded samples were subjected to a heat-induced epitope retrieval step before incubation with primary antibodies for 30 minutes. For detection of Foxp3, the rat antibody clone FJK-16s (eBioscience, San Diego, CA, USA) was applied at a dilution of 1:10 or 1:100 and slides were blocked using a commercial peroxidase-blocking reagent (Dako, Glostrup, Denmark) followed by a secondary rabbit anti-rat antibody (Dako) and the EnVision peroxidase kit against rabbit antibodies (#K4003, Dako). For Ki-67 (TEC-3, Dako, 1:500), cleaved caspase 3 (Asp175, Cell Signaling, Danvers, MA, USA, 1:200), androgen receptor (ab47563, Abcam, Cambridge, United Kingdom, 1:100) and estrogen receptor (ab21232, Abcam, 1:20) labeling biotinylated rabbit anti-rat (Dako) or donkey anti-rabbit (Dianova, Hamburg, Germany) secondary antibodies were used followed by the streptavidin alkaline phosphatase kit (K5005, Dako). Alkaline phosphatase was revealed by Fast Red as chromogen and peroxidase was developed with a highly sensitive diaminobenzidine (DAB) chromogenic substrate for 10 minutes.

Treg cell transfer

Treg cells were eliminated in 5-6 week old *Foxp3*^{DTR} recipient mice (6). In brief, 50μg/kg DT (Sigma-Aldrich or Calbiochem) was injected intraperitoneally into *Foxp3*^{DTR} mice on two consecutive days, day 1 and 2. $5x10^5$ $CD4^+$ $Foxp3^{GFP+}$ cells isolated from *Foxp3*^{GFP} reporter mice were injected intravenously on day 3 of experiment (13). DT was injected on days 4, 6, and 8, and mice were euthanized on day 9 of experiment.

Results and Discussion

Central to their model of Foxp3 function in epithelial cells, Liu and colleagues reported Foxp3 protein expression in epithelial cells by immunohistochemical staining (10). According to their arguments, eliminating Foxp3 expressing cells in *Foxp3*^{DTR} is expected to dramatically destroy the epithelial tissue architecture (10).To investigate whether tissue destruction was a consequence of diphtheria toxin mediated depletion of Foxp3^{+} cells, we injected Foxp3^{DTR} (DEREG) mice with DT twice before the analysis of several organs by H&E staining. No tissue destruction could be observed in any of the organs (Fig. 1). In sharp contrast to the aforementioned observations, high levels of Foxp3 protein were detected in cells with lymphoid but not epithelial morphology (Fig. 1) in all tissues where epithelial Foxp3 expression was reported. By using the commercially available monoclonal antibody against Foxp3 (clone FJK-16s, eBioscience), we did not detect any Foxp3 protein expression in epithelial cells of the lung, prostate, or thymic cortex (Fig. 1). The latter was in full agreement with a previous report (14) and staining was also not detectable when using the antibody in a ten times higher concentration (data not shown). Furthermore, eliminating Foxp3-expressing cells in *Foxp3*^{DTR} (DEREG (7)) mice resulted in the loss of Foxp3 signal among lymphoid cells whereas very weak background levels of non-specific cytoplasmic Foxp3 staining in epithelial cells remained unchanged. Importantly, the tissue architecture that is supported by epithelial cells was unperturbed in the DT-treated *Foxp3*^{DTR} (DEREG) mice.

Since all studies by Liu *et al*. were performed in BALB/c mice, we crossed our C57Bl/6 *Foxp3*^{DTR} mice onto the BALB/c background for ten generations to exclude strain-specific differences (9-12). Identical to our results on a C57Bl/6 background, non-hematopoietic tissues were not affected by DT administration in BALB/c *Foxp3*^{DTR} (DEREG) mice (Fig. 1). This was further confirmed by cleaved caspase 3 labeling of prostate tissue showing very little ongoing apoptosis and a low rate of regular epithelial proliferation according to a Ki-67 staining both of which remained unchanged in the different mouse genotypes with or without DT injection (Fig. 1).

Additionally, in contrast to another study that reported broad Foxp3 expression by breast epithelial cells and its role as a cancer suppressor (12), we did not detect Foxp3 expression in normal breast tissue (Fig. 2). Again, tissue morphology was not altered following depletion of Foxp3^{+} cells in this organ.

Together, we have been unable to independently confirm the assertion that Foxp3 is highly expressed by epithelial cells. Therefore, we conclude that autoimmunity induced by elimination of Foxp3 expressing cells is not mediated by epithelial cells. To account for these discordant results, it is possible that the polyclonal rabbit anti-Foxp3 antibody utilized in previous studies of Foxp3 expression in epithelial cells non-specifically cross-reacts with an unidentified antigen instead of Foxp3.

As arguably the strongest argument for their contention that Foxp3 expression in epithelail cells is biologically important came from earlier observation that the transfer of Foxp3 deficient bone-marrow into Foxp3-sufficient Rag-deficient recipient mice did not induce autoimmunity (11). This

result suggested that Foxp3 deficiency in bone marrow-derived cells was not primary to autoimmune disease instigation, challenging current dogma. In a stark contrast to these findings, we and other groups have reported that bone marrow transfer from *Foxp3*-deficient mice into *Rag*$^{-/-}$ recipients leads to autoimmune lymphoproliferative disease (2, 15, 16). Additionally wild type bone marrow transferred into *Foxp3*$^{-}$ *Rag*$^{-/-}$ mice did not cause a disease (17). The disease was not caused by mature pathogenic T cells possibly contaminated within the *Foxp3*$^{-}$ donor bone marrow cells because transfer of fetal liver cells from *Foxp3*$^{-}$ mice or bone marrow cells from Foxp3^{-} *nude* mice, both of which do not contain any mature pathogenic T cells, also caused identical disease in irradiated *Rag*$^{-/-}$ recipients (14, 17). The usage of recipient mice with a genetic deficiency in T cell generation is essential for assessing the contribution of Foxp3 expression in hematopoietic cells (17). As we demonstrated, the utilization of lymphocyte-deficient recipients is necessary because radiation-resistant Treg cells in wild-type recipients rapidly reconstitute the Treg cell compartment after irradiation (17). Thus, expanded host-derived Treg cells spare irradiated recipient mice from lethal autoimmunity, irrespective of the donor bone marrow genotype. Notably, an early bone-marrow transfer study used as an additional independently obtained evidence in support of a model for Foxp3 action in non-hematopoietic tissues utilized irradiated wild-type mice as recipients of Foxp3-deficient bone marrow (18). Therefore, the result obtained by Liu and colleagues has not been independently reproduced thus far. It should also be pointed out that the bone marrow transfers in a study, that championed a role for Foxp3 expression in non-hematopoietic cells, lacked a positive control, i.e. bone marrow transfer from *Foxp3*$^{-}$ mice into *Foxp3*$^{-}$ *Rag*$^{-/-}$ mice, obfuscating the interpretation of the key finding (11). In contrast, our studies tested all the combinations of donor and host Foxp3 genotype and demonstrated no contribution of Foxp3-deficiency in non-hematopoietic tissues to the disease development(17). Finally, bone marrow transplantation serves as an effective treatment for IPEX patients in agreement with our bone marrow chimera studies in mice (19).

Besides bone marrow transfer studies, a large body of genetic evidence established that Foxp3 expression in T cells and more specifically in Treg cells is required to prevent catastrophic autoimmunity. Mice with a Foxp3 deficiency restricted to the T cell lineage through CD4-Cre mediated deletion of a conditional Foxp3 allele are indistinguishable from mice with the germ-line ablation of the *Foxp3* gene (1, 14). In contrast, near complete deletion of the *Foxp3* in thymic epithelial cells was inconsequential, i.e. no changes in thymocyte development and no signs of autoimmunity were observed (14). To accommodate these findings, Liu and colleagues suggested that CD4-Cre is expressed and mediates *Foxp3* deletion in thymic epithelial cells, and that the latter, not Foxp3 deficiency in T cells causes the autoimmune syndrome in these mice (11). However, extensive genetic analyses of Cre-mediated recombination in CD4-Cre transgenic mice using a highly sensitive reporter allele failed to detect Cre expression in thymic epithelial cells and genetically controlled immunhistochemical analysis failed to detect Foxp3 protein expression in thymic epithelial cells (14). Lastly, we have demonstrated that Treg cell-specific Foxp3

deletion via retroviral Cre delivery to purified Treg cells isolated from conditional Foxp3 knockout mice abrogates Treg cell suppression activity, causing fulminating autoimmunity when transferred into lymphopenic recipients either alone or together with T cells from *Foxp3*$^{-}$ mice (20). Together, catastrophic autoimmunity in mice and humans with Foxp3 mutations or engineered mice to inducibly eliminate Foxp3-expressing cells is ascribed to the essential role of Foxp3 in Treg cells.

If autoimmunity in Foxp3 deficient mice and humans is indeed caused by the lack of Treg cells, restoration of the Treg cell compartment is predicted to cure disease. In agreement with this notion, we and five other groups have demonstrated that injecting purified Treg cells into Foxp3 deficient mice is sufficient to prevent life-threatening autoimmunity (1, 16, 17, 21-23). Additionally, transfer of Treg cells into *Foxp3*^{DTR} mice treated with diphtheria toxin to eliminate Foxp3-expressing cells, inhibits tissue pathology in the liver, lung, and skin (Fig. 3). In contrast to these results, Liu and colleagues recently demonstrated that injecting sorted Treg cells into *scurfy* mice did not alleviate morbidity (9). Thus, our results and the published data from several groups are at odds with those generated in the Liu laboratory.

In summary, a significant body of genetic, immunohistochemical, and genetically controlled functional studies by us and others is irreconcilable with the view that Foxp3 function in epithelial cells, including thymic epithelium, is a key factor in prevention of severe autoimmune lesions associated with Foxp3 mutations.

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Figures:

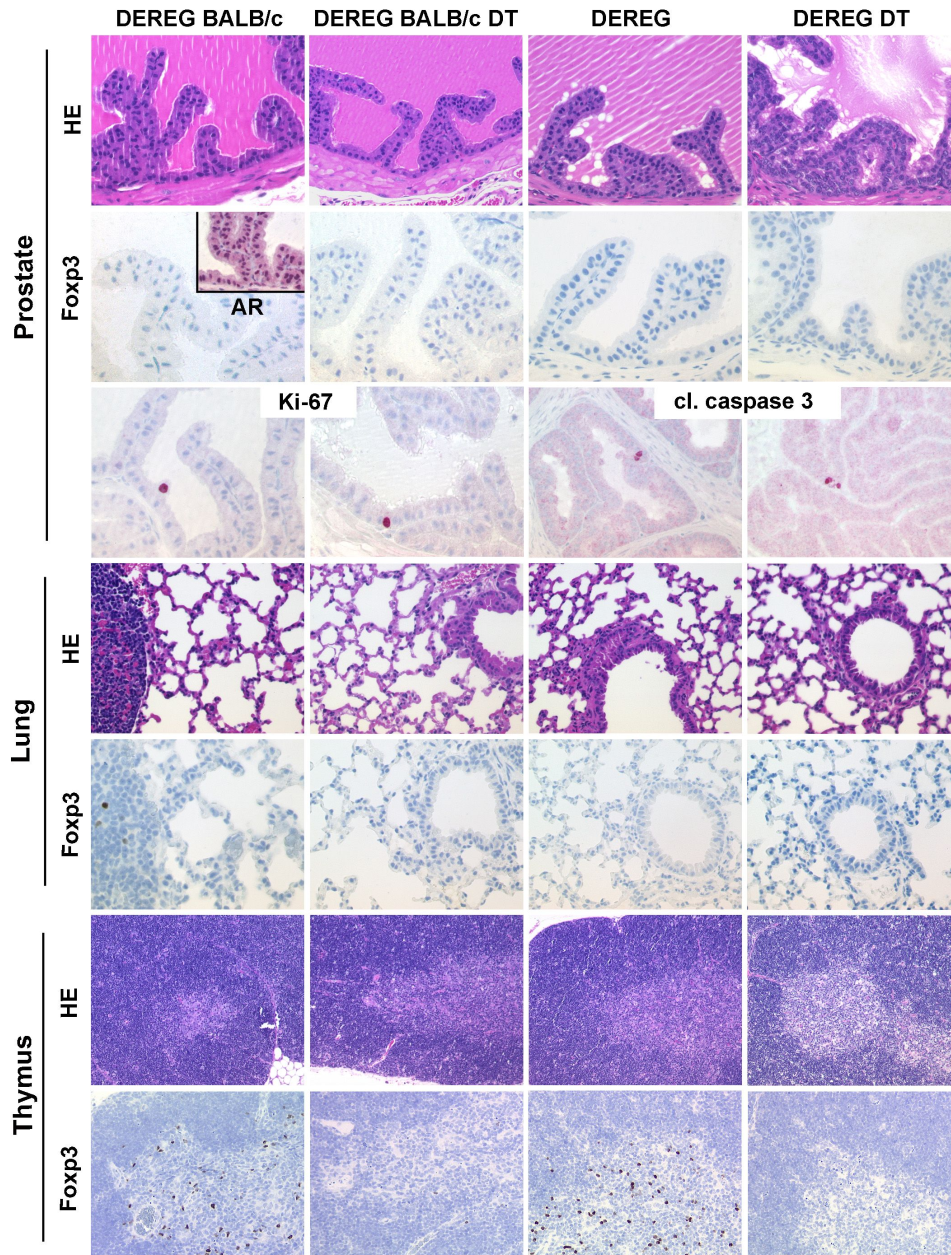

Figure 1. Lack of Foxp3 expression in epithelial cells.
Prostate, lung and thymus from DEREG mice with or without DT administration on either BALB/c or C57/Bl6 background showing no expression of Foxp3 in

epithelial cells of prostatic glands, respiratory epithelium of the bronchi or alveolar lining cells (intraparenchymal lymph node in the first column with scattered Foxp3+ Tregs) or in the thymic cortex (Foxp3^{+} Tregs in the medulla serve as positive intrinsic control). The rate of proliferation (Ki-67) and apoptosis (cleaved caspase-3) was unaltered by DT injection. Ki-67 labeling also serves as a positive control for nuclear antigens, similar to the expression of the androgen receptor (AR) in the prostate epithelial cells (inset, representative for all mouse groups depicted).

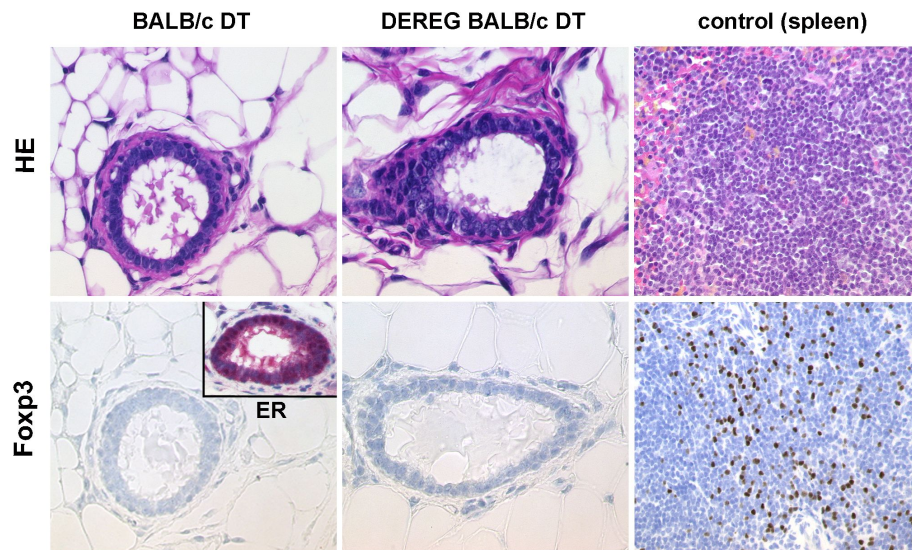

Figure 2. Absence of Foxp3 expression in normal epithelial cells of mammary glands in breast tissue from DT treated BALB/c and DEREG BALB/c mice; splenic parenchyma served as positive control. Expression of the estrogen receptor (ER) in the epithelium of mammary glands served as a control for the labeling of nuclear antigens (inset).

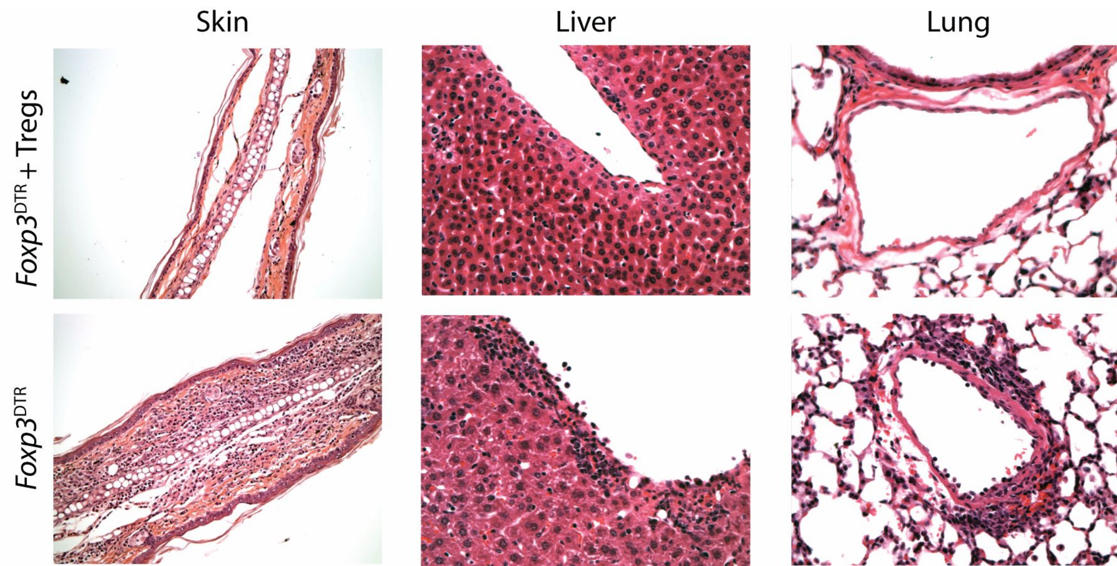

Figure 3. Reconstituting *Foxp3*^{DTR} mice with DT-insensitive Treg cells rescues autoimmunity. Treg cell-depleted *Foxp3*^{DTR} mice were either untreated (n=3) or reconstituted (n=3) with DT insensitive Treg cells. Representative hematoxylin and eosin staining of skin (ear), liver, and lung sections is shown mice were euthanized on 9 days after Treg cell elimination. Data are representative of two independent experiments.