The role of self-reactivity in regulatory T cell development and acquisition of diverse regulatory activities

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to my parents

"Science is not only a disciple of reason but, also, one of romance and passion."

Stephen Hawking

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1 Summary

The importance of regulatory T cells (Tregs) in maintaining lymphocyte homeostasis is best appreciated in mice and humans lacking these cells. FoxP3 KO (scurfy) mice and patients with immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome suffer from excessive lymphocyte activation, lymphocytic infiltration into peripheral organs, and colitis leading to death at an early age. In healthy mice and humans, Tregs control homeostatic proliferation of conventional T and B cells and prevent colitis. Tregs have been classified based on their activation status, expression of surface markers and their ability to migrate to certain tissues. In humans, the proportion of activated and resting Tregs vary with age and the presence immunological disorders. While Tregs have been shown to express self-reactive and foreign-antigen reactive T cell receptors (TCRs), it's not clear how their antigen specificity determines their regulatory function.

In this study, we identified two Treg cell populations, which are differentially selected, based on their affinity for self-antigens; we further investigate their self-reactivity *in vitro* and assessed their different regulatory properties using three disease models *in vivo*.

To investigate Treg cell development in the thymus, we made use of a FoxP3 transgenic (FoxP3tg) mouse. This strain expresses 16 tandem copies of the genomic FoxP3 gene and the transgene is correctly expressed in T lineage cells. The transgene is not expressed in DP thymocytes; while up to 30% of CD4SP thymocytes are $FoxP3^+$. This contrasts B6 mice, which contain 3% $FoxP3^+$ thymocytes among the CD4SP population. In the periphery of FoxP3tg animals, ~ 90% of all $CD4^+$ T cells are $FoxP3^+$ and express some Treg markers.

In contrast to B6 mice where the majority of Treg cells are $Helios^+$, the majority of FoxP3tg Treg cells are Helios negative. Even though the frequencies of $Helios^+$ Treg cells in FoxP3tg and B6 mice are different, the cell numbers of $Helios^+$ Treg cells is similar in both mice. Interestingly, the $Helios^+$ Treg cells of both strains express similar surface markers. In this FoxP3tg strain, positively selected $CD4^+$ T cells are thought to be diverted into the Treg lineage as a consequence of expressing the FoxP3tg; this likely generated the increased numbers of $Helios^-$ Treg cells.

Back-crossing the FoxP3tg to a $Rag^{-/-}$ mouse expressing a monoclonal class II MHC restricted TCR (B3K506), generated mice, where the $Helios^+$ Treg population disappears but the $Helios^-$ Treg population remains. Based on these findings, we

hypothesize that the generation of Helios^{+} Treg cells in B6 requires negative selection. This implies that Helios^{+} Treg cells express a high-affinity self-reactive TCR repertoire.

In co-cultures of CD4^{+} T cells with autologous dendritic cells (autoMLR), Helios^{+} Treg cells expressed high levels of CD25 and proliferated. This required recognition of MHC II antigens on the APCs and IL-2, which is secreted by conventional CD4^{+} T cells in these cultures. Helios^{-} Treg cells express lower amounts of CD25 and proliferated much less extensively, while conventional CD4^{+} T cells do not proliferate under these conditions. The data are consistent with the idea that Helios^{+} Treg cells recognize MHCII-self-antigens presented and therefore upregulate CD25, allowing these cells to "profit" the most from the limiting amount of IL-2 secreted by conventional CD4^{+} T cells.

We then compared the ability of Triple$^{\mathbf{high}}$ (GITR$^{\mathbf{high}}$, PD1$^{\mathbf{high}}$, CD25$^{\mathbf{high}}$) Treg cells, reflecting the Helios^{+} Treg cells, and Triple$^{\mathbf{low}}$ (GITR$^{\mathbf{low}}$, PD1$^{\mathbf{low}}$, CD25$^{\mathbf{low}}$) Treg cells, reflecting the Helios^{-} Treg cells, to suppress the development of scurfy-like disease in FoxP3^{DTR} mice, acutely depleted of Treg cells. Transferring B6.Triple^{high} Treg cells markedly reduced lymphoproliferation observed in acutely Treg cell depleted, FoxP3^{DTR} mice. In contrast, transfer of Triple$^{\mathbf{low}}$ Treg cells only poorly prevents the lymphoproliferation that follows Treg depletion in these hosts. Conversely, Triple$^{\mathbf{low}}$ Treg cells but not Triple$^{\mathbf{high}}$ Treg cells control colitis in lymphopenic hosts by facilitating the conversion of Tconv into peripheral induced Treg cells. Therefore, these two Treg cell subpopulations have distinct functional properties.

In addition, we found Triple^{high} and Triple^{low} Treg like cells in FoxP3 deficient (scurfy) mice. Sorted Triple^{high} and Triple^{low} CD4^{+} T cells from scurfy mice were separately transferred into T cell deficient recipients to compare their homing properties and their ability to transfer the scurfy phenotype. Our results indicate that scurfy Triple^{high} CD4^{+} T cells preferentially home to the peripheral lymph nodes and induces skin lesions, whereas scurfy Triple^{low} CD4^{+} T cells preferentially home to the mesenteric lymph nodes and induce colitis.

Taken together, these data suggests that Treg cells express TCRs with different degrees of self-reactivity. High-affinity self-reactive Treg cells are able to control the homeostatic proliferation of conventional T cells and B cells while low affinity self-reactive Treg cells maintain lymphocyte homeostasis in the colon.

2 Introduction

2.1 Innate and adaptive immunity

The immune system is an extremely complex and potent network consisting of both the innate and the adaptive immune system. The activation of the innate immune system is the first response upon infections and provides immediate but unspecific host defense. The innate immunity is composed of soluble factors (complement, cytokines) and innate immune cells containing neutrophils, macrophages, dendritic cells (DCs) and natural killer (NK) cells. These cells express so-called pattern-recognition receptors (PRRs) on their surface, which allows them to recognize a broad range of highly conserved pathogen-associated-molecular-patterns (PAMPs) expressed by pathogens. This further leads to rapid activation of leukocytes resulting in the secretion of cytokines (e.g. Tumor necrosis factor alpha (TNFα), interferons (INF) and interleukins (IL)) or phagocytosis of the pathogens. The latter mechanism results in the clearance of the pathogen but also links the innate immunity to the adoptive immunity by processing the pathogen derived proteins and presenting peptide fragments on major histocompatibility complex (MHC) molecules to the cells of the adoptive immune system.

In contrast to the innate immune system, the response of the adoptive immune system is much slower and needs several days or weeks to develop. However, the hallmarks of the adoptive immune system are antigen-specificity and the development of an immunological memory providing an enhanced protection to reinfections, which is not covered by the innate immune system. The main components of the adaptive immune system are B and T cells. Both cell types make use of an antigen-specific receptor that is generated by the pairing of distinct variable chains and the random recombination of variable receptor gene segments. B cells develop in the bone marrow and recognize extracellular pathogens by their specific B cell receptor (BCR) on the cell surface. This leads to the pathogen internalization and further processing into peptide fragments. These peptides are then presented on MHC class II molecules to T helper cells. Cytokines, released by the activated T helper cells, result in the stimulation of B cells, helping them to become plasma cells. These cells then secret high amounts of pathogen specific antibodies that promote further clearance of extracellular pathogens, also called humoral response. T cells develop in the thymus (section 2.2) in contrast to B cells, are specialized to recognize intracellular pathogens via their antigen-specific T cell receptor (TCR). T cells can recognize these antigens

when presented by antigen presenting cells (APC) via major histocompatibility complex (MHC) molecules expressed on their surface. The recognition of antigens leads to the activation of T cells. Activated T cells either actively clear infected cell via cytotoxic lysis, support the humoral response described above, or activates innate immune cells by the secretion of various cytokines. Even though the adaptive immune system is highly specialized, only the interplay between the innate and the adaptive immune system ensures effective host defense from infections induced by foreign pathogens. Additionally, a tight control of both systems, but especially the adoptive immune system, is important to prevent the induction of self-antigen driven autoimmune reactions. (reviewed in [1, 2])

2.2 T cell development and establishing tolerance

T cells can be subdivided into two broad populations, which are defined by the expression of their co-receptors leading to the recognition of antigens presented by different MHC molecules on the surface of APCs. One population expresses the cluster of differentiation (CD)8 co-receptor that leads to the interaction with peptides presented on MHC class I molecules. MHC class I molecules are expressed on every nucleated cell. The other population expresses the CD4 co-receptor that leads to the interaction with peptides presented on MHC class II molecules. In contrast to MHC class I, MHC class II molecules are only expressed on specialized APCs, including macrophages, DCs, B cells and some specialized epithelial cells in the thymus. The strength of the adaptive immune system is the generation of T cells with a diverse T cell receptor (TCR) repertoire that is highly antigen-specific but self-tolerant. The development of the highly specialized T cells occurs in the thymus and is also described as the "central tolerance".[3, 4]

2.2.1 The early thymocytes development DN to DP stage

Early T cell precursors are $CD4^-CD8^-$ double negative (DN) thymocytes that can be further divided into four differentiation stages based on cKit (CD117), CD44 and CD25 expression. DN1 cells are defined to be $cKit^+ CD44^+CD25^-$. In this stage cells have the potential to develop into αβ T cells, γδ T cells, NK cells, DCs, macrophages and B cells. The further description will focus on the development of αβ T cells. During the development into the DN2 stage, thymocytes start the expression of CD25 and become $cKit^+ CD44^+CD25^+$. In the transition stage from DN2 into DN3 ($cKit^- CD44^-CD25^+$), thymocytes migrate to the sub-capsular zone of the thymus and start the rearrangements of TCR-β genes, mediated by the recombination-activating genes

(Rag)1 and Rag2. This occurs until a functional β-chain is generated. Due to a negative feedback-loop, further rearrangement of this locus is blocked, Rag genes get down regulated and thymocytes can proceed to the DN3 stage.[5, 6] In this stage, cells express the invariant pre-TCRα and assemble to a functional TCRpre-α/β complex leading to activation and proliferation of these cells. This induces the further progression into the DN4 ($cKit^-CD44^-CD25^-$) stage, followed by the double positive (DP) stage that is characterized by the upregulation of CD4 and CD8 on the surface of the thymocytes. In the DP stage, Rag genes become activated again and induce the rearrangement of the TCR-α until a functional αβTCR is assembled. The rearranged TCR-α replaces the preTCRα from the DN3 stage.[4, 7, 8]

2.2.2 Positive and negative selection, educating the developing thymocytes, DP to SP stage

Once the DP stage is reached, thymocytes have to go through a "quality proof" process before they are allowed to leave the thymus and emigrate as mature T cells into the periphery. About 90% of all developed DP cells, "die by neglect". The TCR expressed on the surface of the neglected cells does not recognize peptide-MHC complexes and fails to receive a survival signal. This means that only about 10% of all DP αβTCR-expressing thymocytes survive this first positive selection process. Only these cells undergo a further positive and negative selection process setting up the central tolerance to ensure that developing T cells are functional (restricted by self MHC) but not self-reactive (self tolerant). The general mechanism underlying the selection of this highly sophisticated T cell repertoire is based on the affinity of the TCR for self-peptide/MHC complexes. Cells expressing TCRs, which bind self-peptide-MHC complexes too strongly, are negatively selected and undergo apoptosis, while cells expressing TCRs that bind self-peptide/MHC complexes specifically, but with low affinity, get positively selected and mature to single positive (SP) CD4 and CD8 cells, able to emigrate into the periphery (Fig.1).[9]

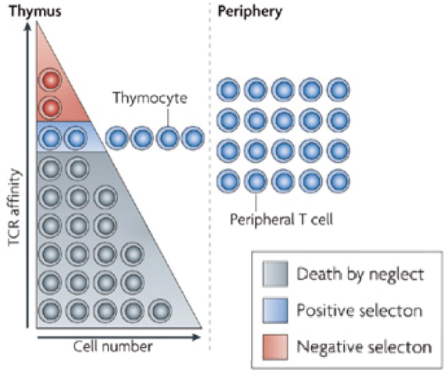


Figure 1: Thymic selection depends on T cell receptor affinity for self peptide-MHC complexes.
Schematic model for the selection of DP in the thymus while only positively selected cells emigrate into the periphery (adapted from [9])

2.2.3 The affinity threshold, how thymocytes make the decision

During the selection process, positively selected DP thymocytes mature to CD4 and CD8 single SP thymocytes. The destiny of whether DP thymocytes become CD4 or CD8 SP cells is based on the restriction of their TCR to either MHC class I (CD8SP) or MHC class II (CD4SP) peptide complexes. The expression of the co-receptor not only determines the MHC restriction, it has an even more important role in the further selection process. Co-receptors bind to the peptide-MHC complexes and influence the TCR affinity for the peptide-MHC complex defined as the "apparent affinity".[9] However, high apparent affinity results in negative selection, and low apparent affinity results in positively selected thymocytes. This does not explain whether a self-antigen induces positive or negative selection. That decision is tightly regulated, and described by a narrow apparent affinity threshold, where minimal differences in affinities can result in either positively or negatively selected thymocytes (Fig.2).[10, 11]

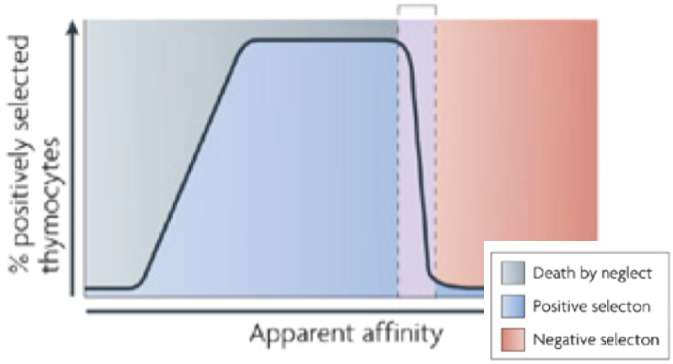


Figure 2: Thymic selection depends on the apparent affinity of peptide-MHC complexes for the TCR-co-receptor pair.
Schematic model for the apparent affinity threshold (purple) between positive and negative selection (adapted from [9])

Our group recently suggested a model of how developing thymocytes can measure self-antigen affinity to initiate negative selection and generate self-tolerance.[12] The affinity threshold for negative selection by peptide-MHC class I[10] and peptide-MHC class II[13] is shown to be similar in the absence of CD8 or CD4 co-receptor expression (defined by the dissociation constant: $K_D=k_{off}/k_{on}$[I]), but different when CD4 or CD8 co-receptor are present (defined by the dwell time $\tau_{½}$[II]). MHC class II restricted thymocytes ($\tau_{½}$= 0.2s) use a shorter dwell time for negative selection than MHC class I restricted thymocytes ($\tau_{½}$= 0.9s) and therefore have a lower affinity threshold for negative selection.[12] The question of why developing CD4 and CD8 thymocytes measure self-antigen affinity differently can be based on the difference of Lck coupling to the respective co-receptor. Lck, a Src family kinase[14], phosphorylates

[I] k_{off}= dissociation rate , k_{on}= association rate
[II] median **dwell time** of the antigen-TCR interaction ($\tau_{½}=\ln 2/k_{off}$)

immunoreceptor tyrosine activation motifs (ITAMs) on the ζ or CD3 molecules of the TCR when the TCR engages with its high affinity ligand (e.g. negative selecting ligand). To induce further downstream signaling e.g. recruitment of Zap70[15], ITAMs need to be doubly phosphorylated. Recruited Zap70, a protein tyrosine kinase (PTK) further phosphorylates downstream molecules inducing determinant outcome. In thymocytes, only a few CD4 and CD8 co-receptors are coupled with Lck. Therefore an antigen-engaged TCR needs to scan multiple co-receptor molecules to find one that is coupled to Lck that can induce the phosphorylation of the ITAMs. The lower threshold dwell time for MHC class II restricted thymocytes, compared to MHC class I restricted thymocytes, can be explained by the higher frequencies of CD4 than CD8 co-receptors that are loaded with Lck, and therefore, have increased chance of ITAM phosphorylation of the peptide-engaged TCR. This means that the basal chance for signal transmission in these cells is higher.[12]

2.2.4 Developmental stages of thymocytes are located within different areas of the thymus and driven by different APCs

Progenitor cells from the bone marrow migrate into the thymus near the cortico-medullary junction. The early development (DN to DP stages) takes place in the cortex, which is arranged by a three dimensional scaffold of cortical thymic epithelial cells (cTECs). During the later transition stage from DP to SP, thymocytes migrte through the outer cortex and scan the cTECs for positively selecting ligands. It is suggested that cTECs have specific mechanisms to present "wobbly" binding self-peptides (generated by β5t-containing thymoproteasomes), leading to a fast TCR off-rate. This induces a signal that leads to CD4 or CD8 committed SP thymocytes being positively selected for peptide-MHC complexes. The SP thymocytes relocate into the medulla, the inner area of the thymus, which is mainly composed by medullary TECs (mTECs), resident (thymic) and migratory DCs (from the periphery). After reaching this area, SP cells stay there for about four to five days before leaving into the periphery, given that they do not get negatively selected. During this time, thymocytes randomly migrate and scan mTECs and DCs for the presentation of peptides. In this stage, mTECs have a profound role in the presentation of tissue-restricted antigens (TRAs). This is regulated by the expression of the autoimmune regulator (AIRE) gene, DCs, especially immigrated from the periphery, can present self- but also peripherally derived non-self antigens to the thymocytes. (reviewed in [16])

2.3 Peripheral tolerance

Even though thymic selection of T cells is a highly efficient and tightly controlled mechanism to prevent the development of auto reactive T cells, it is not completely efficient, since especially threshold antigens can lead to autoimmune diseases.[17] Furthermore, lymphocytes could only encounter their cognate (self)-antigen in the periphery, e.g. food antigens but also antigens, displayed during chronic infections. To control these potentially dangerous cells, several mechanisms are used.

2.3.1 Anergy

Anergy represents a mechanism where cells, which get an activating TCR signal, turn hyporesponsive either due to the lack of an activating, or the presence of an inhibiting co-stimulatory signal. Normally, when a T cell becomes activated in presence of a TCR signal, a second co-stimulatory signal induced via CD28 ligation to a member of the B7 family (CD80/CD86, expressed on the APC) would lead to the secretion of cytokines such as IL2. IL2 subsequently signals through the IL2R complex (section 2.7) and fully activates the PI3K/AKT-mTOR pathway that leads to the proliferation of the cell. In case of a lacking CD28 signal, downstream TCR signaling and IL2 expression get repressed which leads to a non-responding T cell.[19] However, the presence of a negative co-stimulator e.g. Programmed Dead Receptor (PD1) or cytotoxic T-lymphocyte-associated protein 4 (CTLA4) can prevent T cell activation. Both molecules are expressed on T cells after high affinity TCR stimulation and have an important role in development and function of regulatory T cells (section 2.5) PD1 interaction with its ligands PDL1 or PDL2 in the presence of a TCR signal can lead to the inhibition of the PI3K and Akt pathways which results in the silencing of self-reactive T cells.[20] CTLA4 induced anergy is mediated similarly. While PD1 binds to PD ligands, CTLA4 binds with high avidity to B7 family members expressed on APCs and induces a negative signal that prevents the cell cycle progression.[18] One of the key players among APCs are peripheral DCs. Normally, these cells are the inducers of immune responses but can also act as regulators for tolerance induction and maintenance.[21] So called, tolerogenic DCs do not belong to a specific subset, rather they are generated by incomplete maturation or induction via anti-inflammatory or immunosuppressive agents including IL-10, TGFβ1, corticosteroids and rapamycin. Tolerogenic DCs differ from “inducing” DCs in the expression of surface molecules, e.g. B7 family members or PDL1 and PDL2.[22] Another source of tolerogenic APCs is found within the lymph node stromal cells (LNSCs). These cells

are able to express tissue-specific-antigens. Furthermore, a specialized extra thymic AIRE-expressing stroma cell (eTAC), lacking B7 family could support anergy/tolerance induction in the periphery.[23, 24]

2.3.2 Peripheral deletion

Peripheral deletion is mediated by Fas or Bim induced apoptosis of self-reactive T cells. Fas (CD95), also called "death receptor", is expressed on T cells while its ligand FasL (CD178) is only expressed upon repetitive stimulation with cognate antigen and in the presence of IL2. The interaction of Fas with FasL leads to the "death inducing signaling complex" (DISC). The ativated Caspase-8 and effector caspases then promote the activation induced cell death (AICD). Bim, a member of the Bcl2 family, mediates another pathway. This pathway is required to shut down the immune response to an acute infection. Bim directly activates Bax/Bak and causes permeabilisation of the mitochondrial outer membrane to the induction of apoptosis.[25]

2.3.3 Regulatory T cells

Regulatory T (Treg) cells are highly potent cells in the suppression and prevention of autoimmune diseases and maintaining peripheral tolerance. Various subsets of Treg cells including, conventional Treg cells, $CD8^+$ Treg cells, Tr1 regulatory cells, Th3 cells, natural killer like (NKT) cells and even regulatory B cells have been identified (reviewed in [26, 27])

However, within this study we focused only on conventional Treg cells that are defined as $CD4^+FoxP3^+$ Treg cells (section 2.4). Nevertheless, even within this natural Treg population several subpopulations based on their origin e.g. thymic (section 2.5.2) or peripheral (section 2.5.3) derived Treg cells, their suppressive functions, their migratory properties or their activation status respectively e.g. central, memory or effector Treg cells are discussed (section 2.5.6).[28-31] The latter are not well esatblished populations and the terminology can vary, even while describing the same or similar populations. Wherein thymic derived Treg cells (tTreg cells) and peripheral derived Treg cells (pTreg cells) are well established in the literature, for terminolgy, we have chosen the recently suggested recommendations.[32]

2.4 History and characteristics of regulatory T cells

The first evidence of a T cell population with suppressive functions was given more than 40 years ago. In early days, this population was described as suppressor T cells. Nowadays, the term regulatory T cell is used.

In the early 1970s Gershon and Kondo used different experimental setups to describe a population of thymus-derived cells that not only induces immune responses, but also can induce tolerance. [33, 34] In parallel, Nishizuka and Sakakura observed the induction of autoimmune diseases when mice were thymectomized between day 3 and day 7 after birth, but not when thymectomy was performed after day 7. This suggests that tolerogenic thymus-derived cells can accumulate in the periphery between day 3 and day 7 and prevent the induction of autoimmune diseases.[35] This was proved in an experiment during the early 1980s, in which thymectomized neonates were rescued from the induction of autoimmune diseases by the transfer of splenocytes, isolated from adult mice. [36] Following these, different studies investigating various subpopulations of CD4 cells showed that the transfer of some CD4 subpopulations into athymic recipients resulted in multi-organ autoimmunity like gastritis, thyroiditis, diabetes or testicular inflammation.[37, 38] In the beginning of the 1990s the concept of suppressor T cells was revoked. It was assumed that suppressor T cells mediate the inhibition of other immune cells by secreting a soluble factor called “I-J protein”. The I-J locus was mapped to a region between I-A and I-E.[39, 40] However, cloning of the DNA sequence between the I-A and I-E region has shown that the locus did not exist.[41]

However, in the mid 1990s, the concept of suppressor T cells came back with the revolutionary finding that within the $CD4^+$ T cell population, about 5-10% of cells expresses the alpha chain of the IL2 receptor (CD25).[42] When $CD4^+$ T cell depleted from these $CD4^+CD25^+$ T cells were transferred into immune deficient mice (nude mice), recipient mice developed multiple organ autoimmune diseases. The subsequent injection of $CD4^+CD25^+$ T cells could prevent the development of disease. Furthermore, the previously described autoimmune diseases, induced by the thymoctemy of 3-day old neonates, could be prevented by the administration of $CD4^+CD25^+$ T cells. These findings brought the evidence that $CD4^+CD25^+$ T cells appear after day 3 of age and then accumulate within 14 days to the level, observed in adult mice.[42, 43] The full recovery of the concept of suppressive T cells was given by the findings that in an *in vitro* model, $CD4^+CD25^+$ T cells are able to suppress the proliferation and the production of IL2 of activated $CD4^+$ T cells in a contact-

dependent manner.[44] Even though, Treg cells were re-established in the concept of peripheral tolerance, CD25 was not a reliable marker since $CD4^+$ T cells upregulate CD25 expression when stimulated.[45] Therefore, research for additional surface markers for more precise definition of Treg cells was performed and adhesion molecule L-selectin (CD62L)[46], CTLA4[47] and Glucocorticoid-Induced Tumor necrosis factor receptor (GITR) were identified to play a crucial role in the function of $CD4^+CD25^+$ Treg cells. However, all these markers are not exclusively expressed on Treg cells, but also on activated T cells.

The breakthrough discovery for the characterization and the more specific identification of Treg cells was made less than 15 years ago. The human autoimmune disease IPEX (Immune dysregulation Polyendocrinopathy Enteropathy X linked syndrome) was linked with the scurfy mouse, which shows a similar phenotype (section 2.8.1).[48] The underlying defect, causing this severe disease, is a mutation in the transcription factor Forkhead box protein 3 (FoxP3) gene leading to the deficiency or dysfunction of Treg cells. Prove was given by the fact that scurfy mice were rescued from disease development when they were treated with Treg cells within three days of age.[49]

The importance of FoxP3 acting as a master regulator for the regulatory properties of Treg cells was shown by the rescue of scurfy mice by the transgenic (tg) expression of FoxP3, driven by its own promoter.[48] Furthermore, T cells isolated from wild type FoxP3tg mice, including $CD4^+CD25^-$ and $CD8^+$ Tcells, showed suppressive functions *in vitro*.[50] Additionally, retroviral infection of $CD4^+CD25^-$ T cells with FoxP3 resulted in the acquisition of a regulatory phenotype. These cells were potent in suppressing activation of non-transfected $CD4^+CD25^-$ T cells *in vitro* and *in vivo*.[49, 51]

Based on these findings, and that FoxP3 is not upregulated in activated $CD4^+$ T cells, at least in mice, FoxP3 was defined as the key marker for Treg cells. Furthermore, any genetic or environmental modification leading to defective FoxP3 expression or downregulation of FoxP3 resulted in the loss of suppression and the development of inflammation.[52-54]

Due to the importance of learning more about the mechanisms and functions of Treg cells several genetically engineered mice were established and were also used within this study. Mice expressing fluorescent proteins as reporters for FoxP3 are used to study FoxP3 expression *ex vivo*. This enables the sorting of highly pure Treg cells populations. By using these reporter mice it was also confirmed that most

$CD4^+CD25^+$ cells co-express FoxP3 and other previously described markers like CTLA4 and GITR.[55]

Additionally, mice, in which FoxP3 expressing cells can be selectively depleted by the injection of diphtheria toxin (DTx), show a scurfy like phenotype after induced depletion. These mice are an important tool to study e.g. immune regulation in the absence of Treg cells or for *in vivo* suppression assays to asses suppressive functions of different Treg cell populations as we were performing in our study.[52, 53]

2.5 Origin, TCR repertoire and characteristic markers of Treg cells

The undisputed role in the maintenance of immune homeostasis by Treg cells was shown when FoxP3 deficiency was linked with the severe autoimmune diseases IPEX and scurfy in human and mice. From that time on Treg cells were investigated for their characteristic markers, their developmental origin, their side of action and their TCR repertoire.

2.5.1 Characteristic Treg cell markers

Within the last 15 to 20 years, Treg cells and a lot of different markers were investigated. In this section, the characteristics of the markers FoxP3, CD25, GITR and PD1 will be highlighted.

2.5.1.1 FoxP3

FoxP3 is a transcription factor of the forkhead/winged-helix family, located on the X chromosome[48] and highly conserved within mammalian species.[56] FoxP3 acts as a transcriptional regulator mediated by direct or indirect DNA binding and can activate or repress transcription of the target gene.[57] As described above, FoxP3 has a central role in the development and function of Treg cells. The maintenance of FoxP3 expression is required to preserve the Treg phenotype.[58] In this context, FoxP3 was shown to directly regulate the expression of Treg-associated markers like CD25, GITR and CTLA4 but not the entire Treg signature.[59] This was investigated by signature analysis of *in vitro* induced, retroviral induced and naturally FoxP3 expressing $CD4^+$ Treg cells.[59] Similarly, transgenic overexpression of FoxP3 was found to be sufficient to induce suppressive function, but cannot fully induce the phenotype observed in naturally arising Treg cells.[50] Different from acting as an activator, FoxP3 represses IL2 expression by direct interactions with the nuclear factor of activated T cells (NFAT) at the IL2 gene promoter.[60] Structurally, the FoxP3 gene contains different cis-regulatory elements that have a central role in the differentiation and stability of Treg cells. Next to the FoxP3 promoter, which was

shown to have a rather weak transactivation activity, three conserved non-coding sequence (CNS) regions are important for its regulation.

The CNS1 region acts as a binding site for the transcription factor Smad3 and NFAT, and was identified to be critical for the FoxP3 induction during the peripheral differentiation of Treg cells. In contrast, the development of Treg cell in the thymus is not affected. Additionally, it was shown, that binding of Smad3 and NFAT to the CNS1 region is important for the TGFβ mediated conversion *in vitro*.[61] Moreover, CNS1 deficient mice are not able to induce peripheral Treg (pTreg) cells and suffer from allergic type Th2 inflammation at mucosal interfaces like the lung and gut.[62,63,64] The CNS2 region contains a CpG island which is demethylaed in fully committed thymic derived Treg cells and methylated in conventional T cells and peripheral induced Treg cells.[65] CNS2 knockout mice are suffering from a mild lymphoproliferative disorder but not from a severe autoimmune disease. Furthermore, the CNS2 region is not required for the induction of FoxP3 expression but for its maintenance, especially in a proinflammatory environment and after TCR mediated activation.[66,67] The stabilization of FoxP3 expression, especially after TCR activation, is influenced by IL2 mediated signaling and the subsequent Stat5 activation downstream of the IL2 receptor. Stat5 can bind to the CNS2 region and this probably facilitates Stat5 to bind the FoxP3 promoter.[68] Additionally, it was suggested that FoxP3 has the potential to maintain its own expression through binding to the CNS2 region, but this only occurs on the demethylated CNS2 region.[64] The CNS3 region was shown to bind the transcription factor c-Rel after TCR, CD28 and IL2 mediated activation. Using CNS3 deficient mice, it was shown that the CNS3 region has a profound role in the induction of FoxP3 in all Treg cells reflected by an impaired development of thymic and peripheral derived Treg cells.[64]

Therefore, not only the expression of FoxP3 determines the Treg phenotype, but also the molecular modification of the gene impacts the composition, size and maintenance of the Treg cell population.

2.5.1.2 CD25

CD25, the α subunit of the high affinity IL2 receptor (IL2R) was the first marker for Treg cells.[42] The expression of CD25 is induced by an activating TCR signal. Furthermore, signaling through the IL2R also can mediate the expression of CD25 in positive feedback mechanisms. IL2R signaling mediates Stat5 activation, which can further lead to the activation of CD25 transcription. Furthermore, in FoxP3$^+$Treg cells, FoxP3 acts as direct positive activator of the CD25 gene.[69] CD25 expression is

required for the development and function of Treg cells and CD25 deficient mice develop lymphoproliferative disorders and autoimmune diseases.[47] Even though CD25 is constitutively high expressed on Treg cells, CD25 can be upregulated on effector T cells after activation. Furthermore within the FoxP3$^+$ Treg cell population, different levels of CD25 expression were observed.[69] Altogether, CD25 is an important but not exclusive marker for Treg cells.

2.5.1.3 GITR

GITR belongs to the tumor necrosis factor-receptor (TNFR) family and is constitutively expressed on the surface of all Treg cells, but is also upregulated on effector T cells after TCR stimulation[70,71] Even though GITR deficient mice are not suffering from autoimmune diseases[72], the numbers of Treg cells are reduced. In turn, in mice overexpressing GITR ligand on B cells, the numbers of FoxP3$^+$ Treg cells are higher compared to wild type mice. It was shown that the accumulation of Treg cells was due to an increased proliferation.[73] In addition, GITR expressing T cells from FoxP3tg mice are suppressive, independent of their CD25 expression.[50] Interestingly, the adoptive transfer of GITR depleted CD4$^+$ T cells into lymphopenic hosts resulted in death caused by a severe autoimmune inflammation while CD25 depleted CD4$^+$ T cells only caused colitis. Based on these observations, GITR is suggested as a more convenient marker for Treg cells than CD25.[71]

2.5.1.4 PD1

The receptor PD1 (CD279) is an immunoglobulin (Ig) superfamily member and binds to PD ligand1 (PDL1) expressed on hematopoietic and nonhematopoietic cells and tissues, as well as PDL2, which is only expressed on hematopoietic cells. PD1 is expressed in the thymic development in DN thymocytes during the TCRβ rearrangement.[74] In the periphery, PD1 can be expressed on several hematopoietic cells including CD4$^+$ and CD8$^+$ T cells. In T cells, PD1 is induced by high TCR signaling and remains highly expressed in presence of persistent antigen stimulation. In effector T cells, PD1 expression is a marker for non-functional, exhausted, death prone T cells.[75] Furthermore, follicular regulatory T cells, a population derived from the Treg cell pool and specified in the regulation of humoral immunity, express high levels of PD1.[76] Additionally, it was suggested that PD1 is important for the induction of pTreg cells but analysis of PD1 deficient mice revealed that PD1 has no impact on thymic or peripheral Treg cell development.[77] However, a recent study

investigating thymic and peripheral Treg cells addressed a higher PD1 expression to Treg cells of thymic origin.[78]

2.5.2 Development of Treg cells in the thymus

First evidence for the thymic origin of Treg cells was already given with the thymectomy of neonates leading to autoimmune diseases. The development of thymic Treg (tTreg) cells was found to be dependent on several factors like TCR stimulation, antigen presenting cells, cytokines and co-stimulation.

It is thought that Treg cells have TCRs with an increased affinity for self-antigens compared to conventional $CD4^+$ T cells. The importance of TCR stimulation for the development of Treg cells was first given by the finding that transgenic mice, expressing a single TCR (DO11.10) recognizing the foreign antigen chicken ovalbumin, do not develop Treg cells.[79] First hints of a self-reactive TCR repertoire of Treg cells were then given by the higher response of $CD25^+CD4^+$ Treg cells to viral superantigens.[80] Several studies reported that TCR transgenic mice only developed Treg cells when the cognate antigen was also expressed in the thymus. [81-83] Furthermore, Treg cells do not develop when the transgenic TCR has a lower affinity for the transgenic expressed antigen in the thymus.[81, 82] The self-reactivity of Treg TCRs was also assessed in a system where TCRs from Treg or naïve $CD4^+$ T cells were isolated and retrovirally transduced into $Rag^{-/-}$ TCli TCRtg[III] T cells. These cells then were adoptively transferred into lymphopenic hosts. T cells expressing TCRs from Treg cells underwent increased homeostatic proliferation compared to T cells expressing TCRs from naïve T cells, indicating a higher self-reactivity of Treg cells.[85] Recently, a mouse expressing green fluorescence protein (GFP) driven by the promoter of the transcription factor Nur77 was generated ($Nur77^{GFP}$).[86] Nur77 expression can be correlated with the strength of the TCR signal. Thus, the GFP expression correlates with the TCR signal strength in these mice. $FoxP3^+$ Treg cells in $Nur77^{GFP}$ mice were shown to express higher amounts of GFP than naïve $CD4^+$ T cells.[86] Furthermore, tTreg cells express higher levels of CD5 on their surface.[16, 87] CD5 expression was described to quantitatively correlate with the TCR signal intensity.[88] Taken together, these results strongly suggest that Treg cells are skewed towards a TCR repertoire for self-antigens but the distinct antigens have not been identified yet. It was demonstrated that Aire mediated expression of self-antigen in mTECs drives the thymic development of tTreg cells. Mice deficient for Aire

[III] TCli TCRtg: transgenic TCR, specific fort the human invariant chain-derived CLIP peptide (hCLIP) in the context of IA^b.[84]

expression are suffering from severe autoimmune inflammation, comparable to scurfy disease.[89] In addition, not only the affinity of the self-antigen but also the avidity, the amount of expressed and presented self-antigens in the thymus, impacts the development of Treg cells.[90]

In addition, the Treg cell development is also dependent on the local milieu. This includes the presence of cytokines most importantly IL2, but also TGFβ, IL15 and IL7 seem to play a role in a proper development of tTreg cells. As previously described, IL2 signaling through CD25 expressed on Treg cells, induces the expression of FoxP3 and provides survival signals.[68] In addition, co-stimulation via CD28 is important for the efficient generation of fully functional tTreg cells. In CD28 deficient mice, tTreg cells were dramatically reduced. This was only partially observed when CD28 was knocked out specifically on Treg cells. However, CD28 deletion on Treg cells leads to the slow progression of autoimmune driven diseases similar to scurfy disease (section 2.8.1) indicating the importance of co-stimulation for the development of tTreg cells.[91] Taken together, thymic development is dependent on the TCR signal strength, the antigen, the antigen-presenting cell, the cytokine milieu and the co-stimulatory signals.

2.5.3 Development of Treg cells in the periphery

Treg cells can develop in the periphery from naïve $CD4^+$ T cells.[92] Naïve $CD4^+$ T cells transferred into congenic hosts can convert into Treg cells. The conversion seems to be independent from the thymus since the same experiment in thymectomized mice leads to the same induction of pTreg cells.[93] The differentiation of pTreg cells *in vivo* can be induced when naïve $CD4^+$ T cells are stimulated with low doses of their cognate antigen, but not when stimulated with a low affinity peptide.[94] The TCR specificity of pTreg cells is not clearly resolved yet, but it is suggested that these cells express most dominantly TCRs for non-self antigens, although specificity for self-antigens cannot be excluded.[92] However, the best-studied cases of *in vivo* pTreg cell generation are Treg cells induced in the gut with TCR specificities for microbiota-derived antigens since re-colonization of germfree mice with bacteria increases numbers of colonic Treg cells. The induction of pTreg cells was shown to be dependent on TGFβ; administration of TGFβ blocking antibodies prevented the induction of Treg cells. Additionally, these pTreg cells were highly protective against colitis.[95,96] A recent study investigating TCRs specific for colonic bacterial isolates described that naïve $CD4^+$ T cells expressing these colonic bacteria-reactive TCRs converted to pTreg cells, specifically in the lamina propria of the

colon.[97] In the gut, pTreg development was also shown to be dependent on retinoic acid (RA) promoting a TGFβ dependent conversion. Furthermore, specialized $CD103^+$ dendritic cells in the gut and mesenteric lymph nodes (mLN) can facilitate the conversion process.[98] In addition, metabolic products from the microbiota like short-chain-fatty-acids, e.g. butyrate, was shown to promote the conversion from naïve $CD4^+$ T cells to Treg cells *in vivo* and *in vitro*.[99-101] In addition to TGFβ, as for tTreg cells, IL2 is required for the development and function of pTreg cells.[69] Furthermore, for the *in vitro* conversion of naïve $CD4^+$Tconv cells to Treg cells, the presence of IL2 is indispensible.[102] However, the stability of pTreg cells seems to be much lower than the stability of tTreg cells. Especially under lymphopenic conditions, where IL2 availability is probably limited, the stability of FoxP3 expression in pTreg cells was much lower and a greater proportion of pTreg cells lost FoxP3 expression compared to tTregs.[92, 103]

In general, antigen presentation by specialized APCs in the presence of an optimal microenvironment containing soluble factors seems to be crucial for the pTreg development.

2.5.4 Helios and NRP1, markers to distinguish tTreg from pTreg cells?

Both, peripheral and thymic derived Treg cells express high levels of CD25, GITR, CTLA4 and FoxP3 and thus are not distinguishable. To learn more about different functional and regulatory properties of these two subsets, extensive research on specific markers was performed. Within the scope of this research, two markers were identified.

The first described marker to define tTreg cells was Helios, a transcription factor of the Ikaros family. Initially, a higher Helios expression in $FoxP3^+$ Treg cells compared to conventional $CD4^+$ T cells (Tconv cells) was described in 2006[104] and shown to be independently from FoxP3 expression.[105] Treg cells from Helios deficient mice do not show essential differences in the suppressive capacity compared to wild type mice.[105] However, Helios was identified to bind to the FoxP3 promoter in human Treg cells and its expression by small interfering RNA (siRNA) oligonucleotides resulted in the downregulation of FoxP3 and the suppressive functions of these cells.[106] Another study reported that all Treg cells in the thymus express Helios, whereas in the periphery, only 70% of Treg positive for Helios.[107] This indicates that in the periphery only 70% of Treg cells have thymic origin whereas 30% are converted from naïve $CD4^+$ T cells in the periphery.[107] Additionally, in germfree mice, the majority of Treg cells in the lamina propria express Helios.[97] Furthermore

the Treg-specific demethylated region (TSDR) is fully demethylated in Helios$^+$ Treg cells but only partially in Helios$^-$ Treg cells.[108] Even though all data indicated that Helios is as a marker for tTreg cells, a further study argued that Helios rather serves as a marker for activated T cells.[109] Initally, *in vitro* and *in vivo* induced Treg cells were reported not to induce Helios expression[107] though, it is possible under certain conditions *in vitro* and transiently *in vivo*.[110, 111] While the reliance on Helios as a tTreg cell marker dropped, neuropilin 1 (Nrp1), a receptor for members of the vascular endothelial growth factor (VEGF) family as well as for the semaphorin family, first detected in 2004[112], was suggested as a tTreg specific marker[78, 113]. Equally to Helios deficient mice, Nrp1 deficient mice do not show defective Treg cell development.[114] Nrp1 was detected to be a high-affinity receptor for LAP, LAP-TGFβ1 and active TGFβ1. In combination, Nrp1 and TGFβ are promoting Treg cell activity by enhancing the TGFβ downstream signaling via Smad2/3.[115] Nrp1$^+$ Treg cells are suggested to be induced in the thymus and Nrp1$^-$ Treg cells in the periphery. However, both have a similar suppressive function.[78, 113] The use of Nrp1 as a marker for tTreg cells seems to be as controversial as Helios, since Nrp1 expression can be induced in activated T cells, similarly to Helios, under certain *in vitro*, although others disclaimed this.[78] Recently, a concomitant analysis of Helios and Nrp1 serving as a marker for tTreg cells in naïve mice suggested Helios as a more reliable marker for tTreg cells than Nrp1.[116] In addition, several studies showed that Helios expressing Treg cells have a higher suppressive function than Helios negative counterparts.[111, 116, 117] However, both markers can be induced under certain conditions and thus, other markers need to be identified to clearly distinguish between tTreg and pTreg cells.

2.5.5 TCR repertoire diversity of tTreg, pTreg and Tconv cells

The affinity of TCR for self-antigens expressed on Treg cells is not yet fully defined. It is widely accepted that TCR diversity plays an important role in the thymic selection and also in the differentiation of Treg cells. Several studies suggested that the TCR repertoire of Treg cells and naïve CD4$^+$ T cells are mostly not overlapping, while a small percentage of overlapping TCRs are found within both T cell populations.[85, 118] Treg cells and naïve CD4$^+$ T cells can be easily separated by flow cytometry using Treg cells co-expressing FoxP3 and GFP, while the separation of tTreg and pTreg cells is more challenging, since specific markers are controversially discussed. Nevertheless, assuming that Treg cells from the intestinal mucosa contain mostly pTreg cells, Treg cells isolated from the gut and from other tissues were

compared. TCR repertoire analysis implied that pTreg cells in the gut have a distinct repertoire from Treg cells derived from other tissues.[97] In another study, Nrp1 was used to distinguish between tTreg ($Nrp1^{high}$) and pTreg ($Nrp1^{low}$) cells. In line with the previous report, the TCR repertoires of tTreg and pTreg cells was different from each other and different from the TCR repertoire of Tconv cells.[92]

The most used technique to study TCR repertoire is complementarity-determining region 3 (CDR3) sequencing. To limit the overall diversity of the repertoire, mice expressing fixed transgenic TCRβ chains are used. From these mice, different cell populations can be isolated and the CDR3 sequences of the TCRα chains can be analyzed. The diversity is normally illustrated by using the Morisita-Horn similarity index, in which values from 0 to 1 represents low to high similarity between two data sets.[92, 97]

2.5.6 Treg cells, different origin, different location, different functional properties?

The development of tTreg and pTreg occurs from different precursor cells at different locations. For both, similar factors are indispensable for their development. First, for the FoxP3 induction and thus the Treg lineage commitment, TCR stimulation is required, while in tTreg and pTreg cells FoxP3 expression is not induced by the same TCR signal strength for self-antigens. Thymic Treg cells require an intermediate to high affinity TCR for self-antigens, while pTreg cells probably have lower TCR affinities for self-antigens. Second, in the thymus, TGFβ has an antiapoptotic effect on tTreg cells but is not required, while in the periphery, TGFβ is required for the development of pTreg cells. TGFβ induces Smad binding to the FoxP3 locus and directly promotes the transcription of FoxP3 in pTreg cells. Third, IL2 is highly required for the generation of both Treg cell populations, while tTreg cells are more stable in the absence of IL2 than pTreg cells. The instability of FoxP3 expression in pTreg was shown in a lineage reporter system where pTreg cells could be distinguished from “ex pTreg cells” because of the distinct expression of the fluorescent reporters YFP and GFP.[119] The decreased stability of the FoxP3 expression in pTreg cells could serve as a mechanism to control ongoing inflammation followed by a decline when the immune responses are terminated. This means that this may allow pTreg cells to revert back to Tconv cells when the infllamation is cleared, and serves as a mechanism of short-term suppression in local inflammation.[92]

The functional properties of tTreg and pTreg cells were studied in different experimental setups.

In general, the deletion of tTreg and pTreg cells, in FoxP3 deficient mice (section 2.8.1) leads to the development of severe autoimmune driven multi-organ inflammation. This disease is, with high evidence, controlled by tTreg cells, while pTreg cells have a minor role. The depletion of Treg cells in the absence of microbiota (germfree FoxP3^{DTR} mice) leads to the same multi organ inflammation as observed when Treg cells are depleted in specific pathogen free (SPF) housed mice. The only difference was reported in a delayed onset of gut inflammation and an acceleration of exocrine pancreatitis when Treg cells were depleted from germfree mice.[120] pTreg cells were described to be efficient in controlling the islet-specific autoimmune response in NOD.CD28$^{-/-}$ mice[78] and microbiota specific pTreg cells can prevent efficiently from colitis.[95, 96] Furthermore, in mice, in which CNS1 of the *foxp3* gene is knocked out, the development of pTreg is inhibited. This deficiency dose not lead to the development of severe immunopathologies as observed in scurfy mice, but CNS1 deficient mice are suffering from allergic type Th2 inflammation at mucosal interfaces like the lung and gut and females display increased embryo abortion in combination with increased immune cell infiltration during allogeneic pregnancy.[62-64, 121] Given the data, it suggests that tTreg cells are important to control the immune homeostasis and autoimmunity, while pTreg cells have spezialized functions dependent on the type of inflammation, but with a primary role in the control of mucosal immunity and fetal tolerance.[92] In contrast to this, a recent study denied that tTreg cells sufficiently suppress chronic inflammation and autoimmunity in the absence of pTreg cells. The adoptive transfer of tTreg cells into FoxP3 deficient neonates was not sufficient to maintain tolerance, but when tTreg cells were co-transferred with naïve CD4$^+$ T cells tolerance could be established because 15% of naïve CD4$^+$ T cells converted into pTreg cells and established tolerance together with the tTreg cells.[122] However, it seems to be difficult to draw clear conclusions out of the used model, since the behavior of effector T cells in scurfy mice is inconsistent.[92]
Even though, an extensive amount of research has been done on the question how tTreg and pTreg cells function and whether they have differences in their behavior due to their different TCR specificity or their origin, the lack of highly specific markers to distinguish between tTreg and pTreg makes it speculative. Because of this, Treg cells have been categorized by the expression of different surface markers and their localization into different tissues independently of their origin (Fig.3).[123] The most promoted categorization at the moment is the separation of Treg cells according to the activation marker CD44 and the lymph node homing receptor CD62L. By

using these two markers, Treg cells can be broadly divided into central Treg (cTreg) cells expressing $CD44^{lo}CD62L^{+}$ and effector Treg (eTreg) cells $CD44^{hi}CD62L^{lo/-}$.[28] cTreg cells are quiescent and mainly located in the secondary lymphoid tissues. They express high levels of CD25 and are dependent on IL2. In contrast, eTreg cells are highly proliferative, prone to apoptosis, the dominant Treg population in nonlymphoid tissues and are lower in CD25 expression. Their maintenance is IL2 independently mediated by TCR and co-stimulatory signals. Additionally, a population called memory Treg (mTreg) cells is suggested. This population is maintained without antigen in the target tissue after resolution of the disease, mTreg cells are able to attenuate subsequent autoimmune reactions are maintained by IL7 secreted by stromal cells.[30, 123]

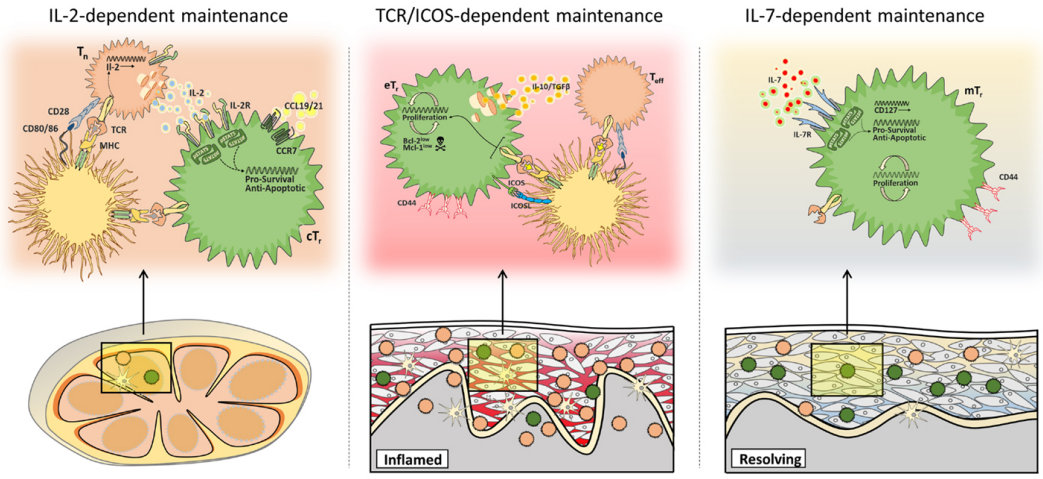


Figure 3: Multiple mechanisms of Treg cell maintenance.
Central Treg cells (cT_r) access paracrine IL-2 in secondary lymphoid tissues (left), maintenance of effector Treg cells (eT_r) in non-lymphoid tissues depends on continued TCR/ICOS signals (middle), and memory Treg cells (mT_r) in the skin are supported by IL-7/IL-7R-mediated survival signals (right). Adapted from [123]

2.6 Suppression mechanisms of Treg cells

The mechanisms of how Treg cells works are still not fully understood. It is widely accepted that Treg cells have an important role in the maintenance of peripheral tolerance and in the prevention of autoimmune diseases. Thus, a profound knowledge of how Treg cells work is of great importance. Several studies indicated the importance of TCR stimulation of Treg cells in peripheral lymphoid organs, allowing them to become effector Treg cells and to obtain suppressive capacity.[81, 82, 124] Two very recent studies gave direct evidence that TCR stimulation of Treg cells is indispensable for the suppressive function *in vivo* and *in vitro*. Levine et al. showed that the suppressive function of Treg cells was diminished after TCR ablation, in a mouse model where the expression of the TCR could be inducible ablated in Treg

cells. In these TCR ablated Treg cells, the Treg cell phenotype was not altered.[125] Schmidt et al. corroborated these results by showing that an impaired TCR downstream signaling resulted in an impaired suppressive function of Treg cells.[126] How TCR stimulation is linked to the suppressive capacity of Treg cells has not been clarified yet. However, several mechanisms mediating suppression were found and extensively investigated. The basic mode of actions can be grouped into four different mechanisms and are described in the following sections (Fig.4)

2.6.1 Suppression mediated by inhibitory cytokines

Treg cells can secrete inhibitory cytokines e.g. IL10, IL35 and TGFβ, which directly inhibit the proliferation of antigen-activated naïve T cells.[127] Even though blocking of IL10 or the deficiency in IL10 production by Treg cells was not affecting their suppressive function *in vitro*[127], Treg cell specific IL10 deficient mice suffered from inflammation of the colon and from increased allergic lung inflammation and hyper-reactivity. This indicates that IL10 has a crucial role in the regulation of inflammation at sites where inflammatory responses are induced by pathogens.[128] Indeed, studies of mouse models for inflammatory bowel disease (IBD) showed that the IL10 secretion of Treg cells was crucial for the prevention of colitis.[129]

The importance of TGFβ for the suppressive function of Treg cells is controversially discussed. Some studies show that the high amounts of soluble and membrane bound TGFβ, produced by Treg cells, impacts the control of autoimmunity[130] while others did not find a significant TGFβ mediated suppression.[131] Treg cells lacking TGFβ or the blocking of TGFβ did not impair the suppressive function *in vitro.* Also $CD4^+$ T conventional (Tconv) cells lacking TGFβ receptor can be normally suppressed.[127] However, several studies showed that TGFβ has a crucial role in the prevention of colitis.[132] Additionally, secreted TGFβ is necessary for the development of peripheral or *in vitro* induced Treg cells and also has a non redundant role in the maintenance of Treg cells in general.[133] A recently discovered cytokine is IL35, which is composed by pairing of Epstein-Barr virus-induced gene3 (Ebi3) and IL12a. Treg cells deficient for either Ebi3 or IL12a do not suffer from autoimmunity but have impaired suppressive functions *in vitro* and failed to resolve IBD *in vivo.*[134]

2.6.2 Suppression mediated by cytolysis

Treg cells were described to have some cytolytic capacities, mediated by the expression and secretion of ganzyme A and B in a perforin-dependent or independent mechanism.[135-137] Apoptosis of effector T cells was also shown in a contact-

dependent mechanism, mediated through a TRAIL-DR5 (tumor-necrosis-factor-related apoptosis-inducing ligand -death receptor 5) pathway.[138]

2.6.3 Suppression by modulating APCs

Another mechanism by which Treg cells can promote suppression is by the direct interaction with APCs leading to the induction of tolerogenic APCs. One mechanism is mediated by the expression of CTLA4 on Treg cells. The importance of CTLA4 mediated tolerance is shown by the observation of the spontaneous development of severe autoimmunity in mice deficient for Treg cell specific CTLA4 expression. Treg cell mediated suppression via CTLA4 can be induced by a process called trans-endocytosis[IV] leading to the downregulation of CD80/CD86 on the APC.[139] Additionally, CTLA4 induced signaling can increase the expression of indoleamine 2,3-dioxygenase (IDO) in certain DC subsets. IDO leads to the starvation of effector T cells and can directly arrest the cell cycle. Furthermore, IDO was shown to have potential to convert effector T cells into Treg cells.[140, 141] Similarly, Treg cells can drive DCs to produce less IL6 but more IL10, resulting in a immunosuppressive cytokine milieu.[142] The adhesion molecule Lymphocyte-activation gene 3 (LAG3), expressed on Treg cells, is discussed to influence the activation of DC cells. Even though blocking or deficiency of LAG3 resulted in less suppressive Treg cells, LAG3 deficient mice are not suffering from signs of autoimmune disease.[131, 143] Furthermore, expression of lymphocyte function-associated antigen 1 (LFA1) on Treg cells, was described to support the formation of stable immunological synapse and as a consequence, physically out-compete naïve T cells from getting activated. Subsequently, LFA1-mediated interaction of Treg and APC cells, leads to the downregulation of CD80 and CD86 on the APCs.[144, 145]

2.6.4 Suppression by metabolic disruption

The fourth mechanism of how Treg cells suppress effector T cells can be summarized as the suppression by metabolic disruption of effector T cells. One possibility of how the metabolism of effector T cells is mediated by adenosine nucleosides, released from Treg cells. Released adenosine activates the adenosine receptor 2A on the effector T cell, leading to the suppression of effector T cell functions, including the inhibition of IL6 secretion, but inducing TGFβ release. This in turn promotes a milieu favorable to convert effector T cells into Treg cells.[146] Additionally, it was shown that Treg cells could directly transfer cyclic AMP (cAMP) into effector T cells through

[IV] A process where particles (e.g. receptors) from one cell are taken up into another cell is called trans-endocytosis. [139]

membrane gap junctions. High levels of cAMP could lead to a dampening of IL2 transcription and less proliferation of suppressed effector T cells.[147] However, these mechanisms are not so extensively examined and need further investigation. Another mechanism, which is broadly discussed and still not fully solved, is the idea that Treg cells can act as a "sink" for IL2 due to their expression of high levels of CD25. This could lead to the deprivation of activated T cells for IL2. IL2 is needed to induce proliferation, subsequent differentiation and survival of T cells.[127]

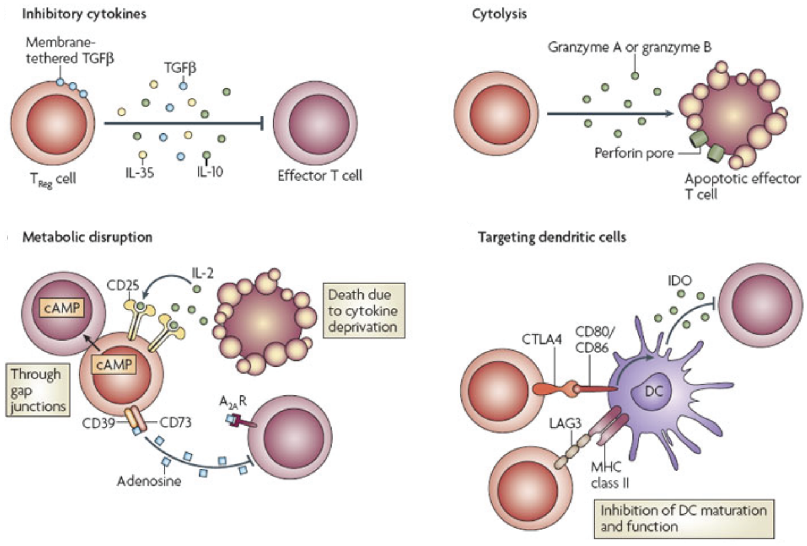


Figure 4: Summary of basic mechanisms used by Treg cells (adapted from[127])

2.7 IL2 and Homeostasis of Treg cells

IL2, a cytokine secreted primarily by activated $CD4^+$ T cells, but also naïve $CD8^+$ T cells, DCs, and thymic cells and was the first molecularly cloned T cell growth factor. IL2 binds to and signals via a receptor complex composed by either three (high affinity receptor) or two (intermediate affinity receptor) distinct subunits. The high affinity receptor is composed of IL2Rα (CD25), IL2Rβ (CD122) and the common γ-chain (γ_c, CD132), while the intermediate affinity receptor is composed of CD122 and γ_c and lacking CD25. Independent from their binding affinity for IL2, both complexes are fully competent to signal.[148] The γ_c is not only important for IL2R formation but also combines with other cytokine receptor chains to form complexes for IL4, IL7, IL9, IL15 and IL21.[149] As previously described, prior to the discovery of FoxP3, CD25 was used as the marker for Treg cells since it is highly expressed on these cells and seemed to be important for their development. However, mice lacking IL2, IL2Rα or IL2Rβ expression were generally immunocompetent and are able to resolve experimental viral infections or can reject cardiac and islet cell allografts.

Within 3-6 weeks of age, all mice start to show massive enlargement of lymph nodes, spleen and gut associated lymphoid tissue caused by the polyclonal expansion of T and B cells, resulting in death due to fatal colitis or severe hemolytic anemia.[150] FoxP3 GFP reporter mice lacking IL2 signaling (IL2 or IL2Rα deficient mice) showed that Treg cells were present in the thymi and in the periphery of these mice but at much lower frequencies compared to unmanipulated mice[151], giving evidence that IL2 signaling is important for the maintenance of Treg numbers in the periphery.[150] The progression of a fatal lymphoproliferative disease in mice lacking IL2 signaling indicates that effector T cells are not dependent on high affinity IL2 signals. Indeed, peripheral proliferation of effector cells is mainly dependent on IL7, constitutively produced by stromal cells.[152] However, Treg cells were shown to have a higher basal proliferation rate compared to Tconv cells. It is not known, whether this high proliferation rate is driven by self-reactivity or by the FoxP3 transcriptional program per se.[152] Regardless of this, the high turn over of Treg cells is counterbalanced by a higher apoptosis rate. The Bim (Bcl-2-interacting mediator of cell death) pathway, induced by FoxP3 dependent phosphorylation, can be rescued by IL2. IL2 signaling leads to the upregulation of pro-survival proteins, antagonizing the pro-apoptotic function of Bim.[152] Since Treg cells are not capable to produce IL2, because FoxP3 and Helios acts as direct repressors of the IL2-locus[153], other cells than Treg cells must secrete IL2. The main producers of IL2 are $CD4^+$ T cells, especially after receiving activating signals. However, a recent study suggests that about 10% of naïve $CD4^+$ T cells have a high baseline of self reactivity, sufficient to drive IL2 transcription but not sufficient to induce proliferation.[154, 155] Thus, under homeostatic conditions, Treg cells are dependent on the (limited) IL2, secreted by $CD4^+$ T cells. The amount of IL2 is enough to balance proliferation and apoptosis of Treg cells and keeping the IL2 production by $CD4^+$ T cells low. Infections can interrupt these homeostatic conditions and results in $CD4^+$ T cells activation, expansion, and therefore higher IL2 secretion. This leads to higher proliferation and reduced apoptosis of Treg cells. Consequently, the enhanced suppressive capacity of the expanded Treg cell population leads to the suppression of the activated $CD4^+$ T cells and therefore, reduced IL2 levels. This in turn results in limited IL2 and therefore in higher apoptosis and lower proliferation of Treg cells. The system returns to homeostasis (Fig.5).[152]

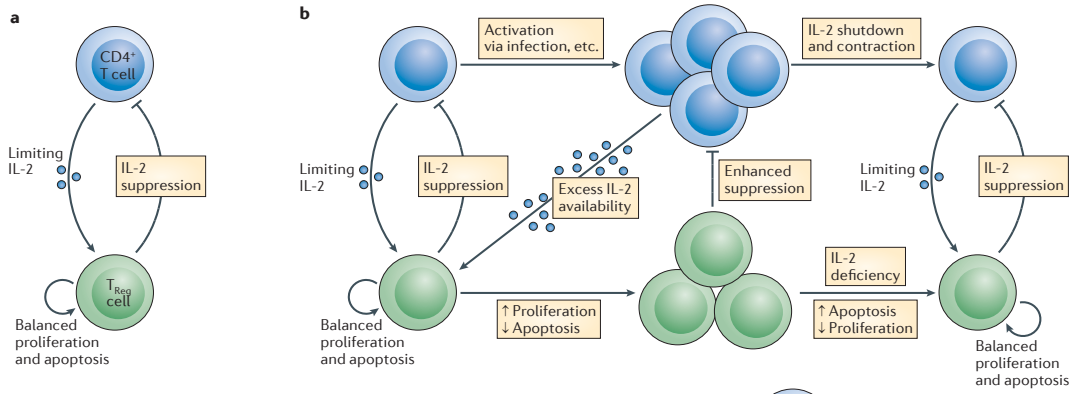


Figure 5: Models for Treg cell homeostasis and disorders triggered by infection/inflammation.
a) Homeostatic conditions **b)** temporarily suspended homeostatic condition by an infection (adapted from[152])

2.8 Treg cells in diseases

Extensive studies on Treg cells have been done and revealed the importance of Treg cells in the prevention of autoimmunity by establishing and maintaining immunological self-tolerance[156], oral tolerance[157], maternal tolerance[158], pathogen induced immunopathology[159], transplantation tolerance[160], suppression of allergy and asthma[161] and inhibition of antitumor immunity[162]. Even though, knowing the importance of Treg cells to control autoimmune diseases, it is not fully understood how and which Treg cells can suppress different diseases. In the further sections I highlight the role of Treg cells in the aspects of first, the pathology of FoxP3 deficiency (section 2.8.1), and second, Treg cells in IBD (section 2.8.2).

2.8.1 FoxP3 deficiency, IPEX and the scurfy mouse

To maintain self-tolerance in mice and men, FoxP3 expressing Treg cells are indispensable.[52, 53] Humans, bearing a mutation in the *foxp3* gene, suffer from severe autoimmune diseases. Because the *foxp3* gene is located at the X chromosome, males are more affected than females. The so-called IPEX (immune disregulation, polyendocrinopathoy, enteropathy, X-linked) syndrome is characterised by the neonatal onset of systemic multi-organ inflammation and patients can suffer from diarrhea, insulin-dependent mellitus, eczematous dermatitis, anemia, thrombocytopenia, tubular nephropathy and neutropenia.[163, 164] IPEX mutations are rare and can affect different positions of the *foxp3* gene and results in different severe effects on Treg cells. The severity of the symptoms can vary within patients and among families an also depends on the environment and age of the patient. The disease is usually lethal in infancy but the survival also depends on the severity of the mutation.[163]

The homologous disease in mice is described as the lethal X-linked lymphoproliferative disorder and naturally arises in the scurfy mouse strain, where a 2 base pair (bp) mutation of the *foxp3* gene leads to a loss-of-function mutation and results in the total absence of Treg cells.[48,165] The critical contribution of lymphocytes in the scurfy multi-organ inflammation was demonstrated by the inhibition of the disease when the scurfy mutation was bred on the $Rag1^{-/-}$ or $Rag2^{-/-}$ genetic background.[166, 167] Furthermore, breeding the scurfy mice onto the $CD4^{-/-}$ or the $\beta2m^{-/-}$ [V] background, demonstrated that $CD4^+$, and not $CD8^+$ T cells, induce the multi-organ inflammation. The disease onset in scurfy mice, lacking $CD4^+$ T cells was delayed, but progressed normally in β2m deficient mice.[169] Additionally, the administration of anti-CD4 mAb also resulted in a milder and delayed multi-organ inflammation.[169]

Even though scurfy mice are deficient in Treg cells what results in uncontrolled expansion of polyclonal $CD4^+$ T cells with a broad TCR repertoire, only eyes, skin, lungs, liver and tail are inflamed.[167] Common autoimmune diseases such as diabetes, arthritis or colitis are not observed. This can be explained with the fact that scurfy mice die within 4 weeks of age. The early death or pre-weaning conditions (probably protection via IgA in mother milk) could prevent from the progression of other diseases.[48, 167] The prolongation of the lifespan (up to 20 weeks) of scurfy mice, by the introduction of the apoptosis-preventing $Fas^{lrp/lrp}$ gene, or by the transfer of scurfy LN cells into $Rag1^{-/-}$ recipients resulted in severe inflammation of skin, lung and liver, but also of stomach, pancreas, small intestine, salivary gland, accessory reproductive organs and colon.[170, 171] This indicates that scurfy mice have a large repertoire of inflammation inducing T cells. Experiments, manipulating the TCR repertoire by breeding a tg foreign Ag-specific TCR into scurfy mice, altered the scurfy phenotype. This indicates that the recognition of antigen is indispensable for the onset of disease. Specifically, TCRtg scurfy mice bred on the $Rag^{-/-}$ background did not develop multi organ inflammation, because only the monoclonal foreign Ag-specific TCR is expressed and the cognate antigen is lacking in the periphery. Scurfy mice on a quasi monoclonal background (TCRtg scurfy Rag^+) had a prolonged lifespan, but developed multi organ inflammation with a delayed onset, compared to polyclonal scurfy mice. Even though a tg TCR reduces the endogenous TCR gene rearrangement, it does not block it. Thus, in quasi monoclonal scurfy mice, a decent fraction of $CD4^+$ T cells expresses a dual TCR allowing them to develop an endogenouse TCR repertorie. These cells where shown to proliferate in the periphery

[V] β2m: β2 microglobulin, a component of MHC class I, mice lacking β2m cannot develop CD8 T cells. [168]

(but not in the thymus) and evoked the multi-organ inflammation. The delayed onset is caused by the quantity of generated cells in the thymus. For the development of the multi organ inflammation, sufficient TCR affinity for endogenouse antigens is required. This means that positively selecting antigens are no sufficient to induce multi organ inflammation, even in the absence of Treg cells. In a study, using the endogenouse viral superantigens (VSAg) as the primary self antigens, they showed that the absence of functional FoxP3 in scurfy mice caused preferential accumulation of autoreactive T cells.[54] Additionally, TCR repertoires analysis from TCRβtg scurfy mice showed that activated scurfy $CD4^+$ T cells preferentially used TCRs found in the TCR repertoire of Treg cells from TCRβtg wild type mice.[172] In two studie where FoxP3 Treg cell precursors were identified by the co-expression of a the mutated FoxP3 and the eGFP reporter in the FoxP3 locus, they suggesed that the scurfy disease is caused by $CD4^+$ T cells from the conventional and not from the Treg cell pool because the scurfy Treg wannabe cells did not proliferate after adoptive transfer into T cell deficient hosts.[173, 174] However, it is not clear which T cell population is promoting the induction of multi organ inflammation and how TCR affinity for self or foreign antigen is linked to the onset of different pathologies observed in scurfy mice. We will address this question in the third part of this thesis.

In scurfy mice, the absence of functional FoxP3 does not lead to the absence of probably highly self -reactive "Treg cell should-bes". Therefore, it seems to be more appropriate to study the absence of Treg cells in $FoxP3^{DTR}$ mice. In these mice, the diptheria toxin receptor (DTR) is controlled under the FoxP3 promoter and enables the specific ablation of FoxP3 expressing cells by the administration of deptheria toxin.[52, 53]

2.8.2 Treg cells in IBD

The intestine mucosal tissues are the largest mucosal surfaces in the body. The maintenance of the immune homeostasis is an ongoing challenge, since these tissues are in direct contact with the external environment and the tolerance to commensal bacteria, food and potential pathogens must be maintained. At the same time, the intestine must be permeable to allow the absorption of nutrition. To cope with this task, a complex immune network, including intestinal epithelial cells, macrophages, DCs, conventional T cells and Treg cells, has developed. Treg cells play a crucial role in the maintenance of this intestinal homeostasis and the protection from diseases.[132, 159] The interruption of this tightly controlled system can induce severe inflammations in the gut, summarized as IBD, and is mediated by activated $CD4^+$ T cells.[159, 175] In

mice, the transfer of naïve $CD4^+$ T cells into T cell deficient hosts can induce a microbiota-driven colitis (mimics human IBD).[159, 175] Evidence for a microbiota-driven disease is given by the observations that the adoptive transfer of naïve $CD4^+$ T cells into germfree $Rag^{-/-}$ mice does not result in the development of colitis.[176]

Treg cells have been identified to prevent the onset of colitis when co-transferred with naïve $CD4^+$ T cells, or even are able to cure the colitis when adoptively transferred after the onset of the colitis.[177] Furthermore, it was shown, that mice with impaired Treg function or development, observed in IL2-, IL10-, or in some reports TGFβ- deficient mice, suffer from severe IBD. IL2- deficient mice develop multi-organ inflammations, including colitis[150] and IL10- deficient mice mainly develop spontaneous colitis[176]. TGFβ- deficient mice do not develop colitis per se, probably due to their early death within 4 weeks of life, but have an increased susceptibility to dextran-sulfate induced colitis[178]. Additionally, transfer of TGFβ receptor deficient $CD4^+$ T cells into lymphopenic mice resulted in colitis.[179] IL10 and TGFβ are crucial for the induction and homeostasis of (microbiota specific) pTreg cells[180], because of that it seems highly evident that the generation of this population impacts the maintenance of the intestinal homeostasis. Although Treg cells were also found in the intestine of germfree mice, the frequency of this population is up to 3 fold lower compared to colonic Treg cells in conventional mice.[97] The Treg population in the intestine of germfree mice seems to be generated in the thymus, since the majority of Treg cells express Helios.[96, 97, 107] Furthermore, colonic Treg cells from conventional mice, but not from germfree mice, can specifically respond to antigens from the gut microbiota.[97] In addition, Treg cells from conventional mice protect better from T cell-mediated colitis than Treg cells isolated from germfree mice.[181] Altogether indicates that the induction of Treg cells with TCRs specific for the microbiota or gut-derived antigens are crucial for the maintenance of the intestinal homeostasis.

3 Material and Methods

3.1 Materials and Reagents

3.1.1 Reagents

β-mercaptoethanol (β-me)	Gibco
RPMI-1640 (phenol red, L-glutamine, 25mM HEPES)	Gibco
Fetal Calf Serum (FCS)	Amimed
Non essential ammino acids (NEAA) (100x)	Gibco
Sodium Pyruvate (100x)	Gibco
L-Glutamine (100x)	Gibco
Penicillin – Streptavidin (100x)	Gibco
Bovine Serum Albumin (BSA)	Sigma Aldrich
NOPIL	Mepha Pharma AG
DMSO	Sigma Aldrich

3.1.2 Material

Polystyrene 96 well round bottom plate, sterile	Corning Incorp
Cell Strainer Nylon 70µm, sterile	Corning Incorp
CellTrics® filter 50µm	Sysmex
Polystyrene tissue culture dish (100 x 20mm), sterile	Corning Incorp
Polystyrene round-bottom tube 5ml	Corning Incorp
Polypropylene conical tubes 15ml, 50ml, sterile	Corning Incorp
Polypropylene round-bottom tube 5ml, sterile	Corning Incorp
Transwell Permeable supports (no. 3470)	Corning Incorp
Polystyrene 24 well flat bottom plate, sterile	Corning Incorp

3.1.3 Instruments

BD FACSCanto™II	BD Bioscience
BD FACSAria™III	BD Bioscience
BD Influx™	BD Bioscience
Corning® LSE™ Mini Microcentrifuge	Corning Incorp
DynaMag™ 15 (1ml-15ml) magnet	Invitrogen
DynaMag™ 50 (1ml-50ml) magnet	Invitrogen
Heraeus™ Megafuge™ 16R	Thermo Scientific
Heraeus™ Multifuge™ 3 S-R	Kendro Laboratory Products
Maxisafe 2020 Class II	Biological Safety Cabinet
HERACell™ 150i CO_2 incubator	Thermo Scientific

Metrohm 691 pH meter	Metrohm Schweiz AG
Microcentrifuge 5417R	Vaudaux-Eppendorf AG
Invertoscope ID03 microscope	Carl Zeiss AG
Vortex-Genie® 2	Faust Laborbedarf
Olympus BX61 Diana	Olympus AG

3.1.4 Solutions and media

1x PBS (pH7.3)	137 mM	NaCl	
	8 mM	$Na_2HPO_4*2H_2O$	
	2.7 mM	KCl	
	1.5 mM	KH_2PO_4	
Culture medium	1x	RPMI-1640	
	10%	FCS	
	1x	NEAA	
	1x	sodium pyruvate	
	1x	penicillin (100U/ml) – streptomycin (100µg/ml)	
	2mM	L-glutamine	
	5mM	ß-me	
BmDC medium		Culture medium	
	10x	GM-CSF hybridoma supernatant	
FACS buffer	1x	PBS	
	3%	FCS	
Sorting buffer	1x	PBS	
	0.1%	BSA	
Wash buffer	1x	RPMI-1640	
	0.1%	BSA	
RBC lysis buffer	155mM	NH_4Cl	9 x
	170mM	Tris-HCL, pH7.65	1 x

3.1.5 Antibodies

CD3ε	145-2c11	BD Bioscience
CD4	RM 4-5	BD Bioscience
CD5	53-7.3	BD Bioscience
CD8	53-5.8	BD Bioscience
CD11b	M1/70	BD Bioscience
CD11c	HL3	BD Bioscience
CD19	ID3	BD Bioscience
CD16/CD32	2.4G2	BD Bioscience
CD25	PC61	BD Bioscience
CD44	IM7	Biolegend
CD45.1	A20	BD Bioscience / Biolegend
CD45.2	104	BD Bioscience
CD62L	Mel14	Biolegend
CD69	H1.2F3	BD Bioscience
CD80	16-10A1	Biolegend
CD86	GL-1	Biolegend
CD279 (PD1)	RMP1-30	Biolegend
	29F.1A12	Biolegend
CD357 (GITR)	DAT-1	eBioscience
	YGITR765	Biolegend
FoxP3	FJK-16s	eBioscience
	150D	Biolegend
Helios	22F6	eBioscience
NRP1	polyclonal	R&D Systems
Vα2	B20.1	BD Bioscience
Vα3.2	RR3-16	BD Bioscience
Vα8.3	B21.14	BD Bioscience
Vβ5	MR 9-4	BD Bioscience
Vβ8	MR5-2	BD Bioscience
α4β7 integrin	DATK32	BD Bioscience / Biolegend
IA/E (blocking)	M5/114.15.2	Biolegend
IA^b (staining)	AF6-120.1	BD Bioscience
pcJUN (S73)	D47G9	CellSignaling Technologies
pERK (P-p44/42 MAPK)	197G2	CellSignaling Technologies
pCD3ζ (pY142)	K25-407.69	BD Bioscience

3.1.6 Kits

LIVE/DEAD Fixable Dead Cell Stain Kit	Life Technologies
Cell Trace™ CFSE Cell Proliferation Kit	Life Technologies
Dynabeads® Untouched™ Mouse CD4 Cells Kit	Life Technologies
FITC BrdU Flow Kit	BD Pharmingen
FoxP3/Transcription Factor Staining Buffer Set	eBioscience

3.1.7 Cytokines and special material

IL2 hybridoma supernatant ($5x10^4$ U/ml)	in house-product
GM-CSF hybridoma supernatant	in house-product
α - CD4 (rat IgM, clone RL172)	K.Hafen&G.Holländer
α - CD8 (rat IgM, clone 31M)	K.Hafen&G.Holländer
α – CDThy1.1 (rat IgM, clone T24)	K.Hafen&G.Holländer
α – CDThy1.2 (rat IgM, clone HO)	K.Hafen&G.Holländer
Recombinant mouse TGFb1	R&D Systems
Diphtheria Toxin (DT)	Calbiochem
Lipopolysaccharide (LPS)	Sigma Aldrich
Low-Tox Rabbit Complement	Cedarlane
AccuCheck Counting Beads	Molecular Probes

3.1.8 Peptides

Peptides were received lyophilized from Eurogentec and were diluted in DMSO to a concentration of 100µM. Stock solutions were kept at -80°C. Working solutions of 2 µM, diluted in full medium, were kept at -20°C.

3K (WT)	NH_2-FEAQKAKANKAVD-COOH
P2A	NH_2-FEAAKAKANKAVD-COOH
P-1A	NH_2-FAAQKAKANKAVD-COOH

3.1.9 Mice

All mice were between 5–12 weeks old and had a C57BL/6 genetic background except for FoxP3KO, which were used at 2 -3 weeks of age. CD45.1 congenic C57BL/6 (B6 Ly5.1), CD45.2 congenic C57BL/6 (B6), RIP-OVA mice expressing a membrane bound form of Ova under the control of the rat insulin promoter (RIP)[17, 182,52] OTII TCRtg mice recognizing $IA^b/OVA_{323-339}$[183], B6.Nur77-GFP[86], H2Ab1(Bm12) and $FoxP3^{KO}$[,174] mice were all obtained from The Jackson Laboratory (Bar Harbor, ME). 3BK506 TCRtg and 3BK508TCRtg mice recognizing IA^b/3K and $Triple^{KO}$ mice deficient for MHC class II, invariant chain and Rag (referred here as

B6.MHCII^{KO}) were provided by P. Marrack and J. Kappler (Denver, USA) and are described elsewhere[184]. FoxP3^{DTR,52} mice were kindly provided by A. Rudensky (New York, USA) FoxP3eGFP and CD3ε$^{-/-}$ were kindly provided by T. Rolink (Basel, Switzerland) and single TCR β chain (OT-I Vβ5) transgenic mice kindly provided by D. Zehn (Lausanne, Switzerland) and are described elsewhere.[185, 186,187] ABM (anti bm12) mice were generated by E.Palmer and described previously[188]. Mice were housed under specific pathogen-free conditions and bred in our colony (University Hospital Basel) in accordance with Cantonal and Federal laws of Switzerland. Animal protocols were approved by the Cantonal Veterinary Office of Basel-Stadt, Switzerland.

3.2 Cell preparation

3.2.1 T cell isolation from lymphoid organs

Mice were euthanized with CO_2 suffocation and spleen, thymus, pooled peripheral lymph nodes (axillary, cervical, brachial and inguinal), pancreatic lymph node and/or gut draining mesenteric lymph nodes were isolated. Single cell suspensions were obtained by disrupting organs through a 70µm nylon cell strainer (BD) in wash buffer. Erythrocytes from spleen and thymus samples were lysed before further processing. Cell pellets were resuspended in 1ml RBC lysis buffer (mixture of 9 parts ACT I (155mM NH4Cl) and 1 part ACT II (170mM Tris-HCL, pH7.65) and incubated for 1min at RT. Reaction was stopped by diluting with medium follow by centrifugation and subsequent washing with medium. All steps were performed on ice and centrifugation of cells was performed at 300xg and 4°C for 10min unless otherwise indicated.

3.2.2 Cell counting

To determine absolute cell numbers, cells were counted either by using a Beckman Coulter Z2 particle counter (Beckman Coulter) or AccuCheck Counting Beads (Invitrogen) according to manufacturers instructions. In brief, cells were resuspended in a defined volume (500µl -1ml) of FACS buffer. In the following, 50 or 100 of Accu Check Counting Beads were mixed with ten times less of cell suspension. Mixed suspension was measured and 5000 to 10000 Counting Beads were acquired. Due to higher SSC of counting beads, beads could be discriminated from the lymphocyte scatter signal. The difference in FSC/SSC allowed direct correlation of lymphocyte event number (LG) and counting bead event number (BG).

$$c_{(Lymphocytes)} = LG/BG \times c_{(counting\ beads)} \times 10\ (dilution\ factor)$$

3.2.3 Magnetic beat selection and fluorescence-activated cell sorting (FACS)

Lymphocytes were isolated as described above. To enrich for CD4 T cells, the Dynabeads® Untouched™ Mouse CD4 Cells Kit (Invitrogen) was used according to manufacturers protocol. In brief, cell suspension was incubated with a cocktail of CD8 T cell, B cell, monocyte/macrophage, NK cell, dendritic cell, erythrocyte and granulocyte specific monoclonal rat IgG antibodies for 20 min at 4°C. Cells were washed with an excess of wash buffer and incubated for 15 min with magnetically labeled Mouse Depletion Dynabeads® before applying to the magnet. Supernatant with the untouched mouse CD4 T cells could be used for further downstream analysis.

To isolate subpopulations of $CD4^+$ T cells, cells were enriched as described above and were further separated with fluorescence-activated cell sorting (FACS). Therefore, cells were labeled with fluorochrome-coupled mAbs. In brief, cell suspension was adjusted to $20x10^6$ cells/ml. To prevent unspecific labeling, Fc receptor blocking was performed using purified CD16/32 antibody (BD) for 10 min at 4°C followed by 10 min incubation with required fluorochrome-coupled mAbs at 4°C. Cells were washed and resuspended in sorting buffer ($20x10^6$ cells/ml). Dead cells were excluded by DAPI staining previous to cell sort. FACS was performed using FACSAriaIII or Influx sorter (BD Bioscience)

3.3 Flow cytometric analysis of lymphocytes

All flow cytometric stainings were performed in 50µl FACS Buffer, at 4°C and cells were washed with 100µl FACS Buffer and centrifuged for either 3 min/350g or 2min/1000g if not other indicated.

3.3.1 Surface molecule staining

To analyse the expression of surface antigens, lymphocytes either from cell cultures or directly from organs, were collected in FACS Buffer or transferred into 96-well-U-shaped microtiter plates (Corning, Germany). Unspecific Fc receptor blocking was performed using s purified CD16/32 antibody (BD) for 10 min. To discriminate live and dead cells, cells were incubated with LIVE/DEAD near-IR dye (Life Technologies) in PBS for 10 min prior to surface antibody staining. Surface staining with required mixture of fluorocrome-labeled antibodies was then performed for 10 min in FACS buffer. Then cells were either analyzed by flow cytometry or further processed for intracellular staining.

3.3.2 Intracellular molecule staining

For detecting intracellular markers e.g. transcription factors, intracellular staining was performed using the FoxP3/Transcription Factor Staining Buffer Set (eBioscience) after the surface staining. Briefly, cells were fixed for 20 min in 100µl fixation buffer (1 part concentrate: 4 parts diluent) and then washed with 100µl permeabilization buffer (1x in H_20). Then cells were incubated with a mixture of fluorocrome-labeled antibodes in permeabilization buffer for either 40min at RT or ≥1h at 4°C. Cells then were washed with permeabilization buffer and resuspended in FACS buffer prior to flow cytometric analysis.

3.3.3 Intracellular staining of Bromodeoxyuridine (BrdU)

Incorporation of BrdU was detected by using the BrdU Flow Kit (BD Bioscience) according to the manufacturers protocol with minor modifications. In brief, surface and intracellular staining was performed on single cell suspension as described before. Then, cells were fixed with 100µlCytofix/Cytoperm buffer for 10 min at 4°C and washed with 100µl BD Perm/Wash buffer. Further, the cells were incubated in 100µl BD Cytoperm Plus buffer for 5min at 4°C, followed by a wash with 100µl BD Perm/Wash buffer and fixed again with 100µl Cytofix/Cytoperm buffer for 5min at 4°C. After washing, cells were resuspended in 100µl DNAse I solution (300µg/ml) and incubated for 1h at 37°C. After washing with BD Perm/Wash, intracellular staining was performed with FITC-anti-BrdU mAb in BD Perm/Wash for 20min at RT. Before analysis by flow cytometry cells were washed in BD Perm/Wash and resuspended in FACS buffer.

3.3.4 CFSE labeling of lymphocytes

To asses the *in vitro* proliferation of different lymphocyte population magnetic bead enriched CD4 cells were adjusted to $2x10^6$ cells/ml in PBS, 1ul/ml of CFSE solution (5mM in DMSE) was added to the cell suspension and incubated for 5-10 min at 37°C. The reaction was quenched by adding 10 fold excess volume of ice cold FCS/Medium (1:1) and incubated for 5min on ice before centrifugation (10min, 300g). After, cells were counted and used for further downstream applications.

3.4 *In vitro* culture and experiments

Cells were cultured in culture medium under sterile conditions at 37°C, 5% CO2. Used fetal calf serum (FCS) was heat inactivated for 30 minutes at 56°C to eliminate complement factors.

3.4.1 Generation of bone marrow derived DCs

To generate mature DCs, femurs from 5-7 week old C57BL/6 or B6.MHCII^{KO} mice were dissected and bone marrow cells were isolated by flushing the bones with medium, using a 10ml syringe with 27Gx¾ needle (Terumo, Belgium). Cells were centrifuged (300g, 8min, 4°C) resuspended in BmDC-medium and filtered to obtain a clump free cell suspension. 2x10^6 cells/plate C57BL/6 or 3x10^6 cells/plate B6.MHCII^{KO} bone marrow cells were plated in 10ml BmDC-medium in 100mm cell culture dishes. Cultured cells were fed every third day with fresh BmDC medium and were cultured at least for 10 days before use. Before downstream application, the maturation of DCs was assesed by flow cytometry for CD11c, CD11b, CD80, CD86 and MHC class II expression.

3.4.2 In vitro autologous mixed lymphocyte reaction (autoMLR)

To investigate the proliferation of CD4 subpopulations upon self-antigen recognition, autoMLR was performed. Two different setups were used:

3.4.2.1 Contact setup

1x10^5 mature syngeneic BmDCs were plated in 96-well-U-shaped microtiter plates (Corning, Germany) one day prior to CD4 cell isolation and incubated over night at 37°C. The following day, CD4 T cells were isolated from pooled LN and mLNs. Magnetic bead purified CD4 cells were CFSE labeled and 3x10^5 cells were co-cultured with BmDCs for up to 5 days at 37°C. Cell numbers, surface/intracellular molecule expression and proliferation were analyzed by flow cytometry.

3.4.2.2 Transwell setup

6x10^5 mature syngeneic BmDCs were plated in the lower well (24 well plate, Corning, Germany) one day prior to CD4 cell isolation and incubated over night at 37°C. The following day, CD4 T cells were isolated from pooled LN and mLNs. Magnetic bead purified total CD4 cells (3x10^5 cells) or sorted Helios$^+$Treg cells (2-3x10^4 cells) were both CFSE labeled and cultured in a 6.5mm insert well with a perforated polyester membrane (0.4µm pore size, Corning, Germany). In some experiments, sorted 1.8x10^6 Tconv (CD4$^+$GFP$^-$) cells were co-cultured in the lower well. Cultures were analyzed after 5 days at 37°C. Cell numbers, surface/intracellular molecule expression and proliferation were analyzed by flow cytometry.

3.5 *In vivo* experiments

All transferred cells were injected intravenously (i.v) into the tail vein in a total volume of 200µl PBS and all other administrated compounds (e.g. BrdU, DTx) were injected intraperitoneally (i.p) in a total volume of 100µl PBS if not other indicated.

3.5.1 In vivo BrdU labeling

To investigate the proliferation of different lymphocyte subsets *in vivo*, mice were injected i.p. with 0.5mg of BrdU in 200µl PBS every 12h for 3 consecutive days. The thymidine analog can incorporate into the newly synthesized DNA of dividing cells and therefore labels proliferating cells. The last injection was performed 12h before mice were sacrificed and required organs were isolated and prepared for further analysis by flow cytometry as described before.

3.5.2 Generation of bone marrow chimeric mice

Protocol was adapted from Koehli et al.[17] Recipient mice (CD45.1/2) were lethally irradiated with 900 rad (GammaCell, Best Theratronics, CA). Bone marrow cells were isolated from femurs of 5-8 week old C57BL/6 mice (CD45.1) and OT-II RagKO (CD45.2). Red blood cells were lysed (RBC lysis Buffer) and bone marrow cells were filtered (70uq cell strainer, BD). Mature T cells were depleted by staining bone marrow cells with anti-CD4, anti-CD8, anti-Thy1.1 and anti-Thy1.2 (obtained from hybridoma supernatants, kindly provided by K.Hafen and G. Holländer) followed by incubation with complement (Low-Tox-M Rabbit Complement, Cedarlane, CA) at 37°C for 45 minutes. A mixture of 9:1 of C57BL/6 and OT-II RagKO bone marrow cells ($4x10^6$ total cells) were injected into irradiated recipient mice. These mice were treated with antibiotics (Nopil, Mepha Pharma AG) in the drinking water until 2 weeks before analysis. Then the mice were analyzed 12-14 weeks after reconstitution. The congenic markers CD45.1 and CD45.2 were used to identify the T cells from the different origins.

3.5.3 Skin transplantation model

For the transplantation of the skin graft, recipient mice were under general anesthesia with isoflurane and received Dafalgan (Bristol-Myers Squibb SA) in their drinking water directly after transplantation. Mice were transplanted with a ca $1cm^2$ piece of tail skin donated from allogeneic Bm12 mice. Therefore a piece of skin from the same size was removed on the back of the recipient mouse and replaced with the tail skin. Grafts were glued using Histoacryl (Aesculap AG) and were covered with finger stripes (Hansaplast, Beiersdorf). To avoid the graft from sticking on the stripe, the

stripes were coated with Vaseline. Bandages were removed after 7 days. To allow complete wound healing, in experiments where mice were challenged with naïve ABM T cells, cells were adoptively transferred (i.v.) earliest 14 days after bandage was removed. The appearance of the graft was observed until rejection and for maximal up to 100 days using following scoring system: 3 (no rejection), short hair, black stripe, shiny greyish appearance; 2 (first signs of inflammation), small red area, loss of hair shine and dryness; 1 (progressed inflammation), large red area, no black stripe and dryness; and 0, rejected.

3.5.4 Treg depletion and rescue of FoxP3DTR mice

To investigate the suppressive ability of different Treg populations in an autoimmune driven disease, FoxP3 DTR mice were depleted from FoxP3 expressing cells by diphtheria toxin (DTx) as these cells co-express the diphtheria toxin receptor (DTR) under the control of FoxP3.

Therefore mice were injected every other day i.p. with 50µg/kg DTx for the first two injections or 25µg/kg for following injections for 10-12 days. In some groups $2.5x10^5$ sorted Treg cells (isolated from pooled LNs and mLNs as described before) were injected i.v. 3 days prior to first DTx injection. One day after last DTx injection, mice were sacrificed and required organ samples were isolated and prepared for further analysis.

3.5.5 Adoptive transfer inflammatory bowel disease (IBD) model

3.5.5.1 Induced Treg competent setup

To asses the suppressive potential of different Treg population in a commensal antigen-driven disease, CD45.1 congenically marked $3.2x10^5$ sorted naïve C57BL/6 CD4 T cells ($CD4^+CD25^-$, isolated from pooled LNs and mLNs as were adoptively transferred into T cell deficient (CD3KO) mice. In some groups, CD45.2 congenically marked 0.8 $x10^5$ sorted Treg cells were co-transferred with the sorted naïve CD4 Tcells (isolated from pooled LNs and mLNs as described before). Weight of the mice was determined weekly. To avoid differences due to daily fluctuations, weight was determined always at the same time of the day. Mice were sacrificed when initial bodyweight droped more than 20% or latest after six weeks. Required organ samples were isolated and prepared for further analysis. The congenic markers CD45.1 and CD45.2 were used to identify the T cells from the different origins.

3.5.5.2 Induced Treg depleted setup

To define the suppressive potential of different Treg population without the help of possible induced Treg cells from the naïve CD4 T cell pool, IBD was induced as described above but instead of naïve C57BL/6 CD4 T cells, $3.2x10^5$ sorted naïve FoxP3 DTR CD4 T cells (CD45.1) were adoptively transferred in presence or absence of $0.8 x10^5$ sorted Treg cells (CD45.2) into CD3KO recipients.

To continuously deplete possible converting Tregs, mice were injected every third day with low dose of DTx (10ug/kg). Mice were weighed weekly at the same time of the day and sacrificed when initial bodyweight droped more than 20% or latest after six weeks. Required organ samples were isolated and prepared for further analysis. The congenic markers CD45.1 and CD45.2 were used to identify the T cells from the different origins.

3.5.6 Scurfy disease transfer model

Newborn hemizygous FoxP3KO male pubs from heterozygous FoxP3KO female mice crossed with CD57BL/6 male mice developed a severe multi organ inflammation within 2 weeks of age and die shortly after onset. To identify potential scurfy disease causing cells, different cell populations from pooled LNs and mLNs of 2-3 week old sick FoxP3KO male mice were sorted and $5x10^5$ cells were adoptively transferred into T cell deficient (CD3KO) mice. Mice were weighed weekly at the same time of the day and were observed for signs of skin-inflammation at the tail and the ears. When the initial bodyweight droped more than 20% or latest after six weeks, mice were sacrificed. The required organ samples were isolated and prepared for further analysis.

3.6 Data analysis

Flow cytometry was performed on a FACScantoII by using the FACSDiva software (BD Bioscience) while FACS analysis was performed with the FlowJo software (Tree Star). Histological pictures were taken on the Olympus BX61 Diana and analyzed with the ImageJ software (NIH). Graphs and statistical analysis (unpaired-t-test, one-way-ANOVA or Kaplan-Meier Log-rank (Mantel Cox) test, where indicated) were done with Prism (GraphPad Software Inc.)

4 Aim of Study

Regulatory T cells are key players in maintaining lymphocyte homeostasis and preventing autoimmune disease. Their indispensability is best appreciated in mice and humans lacking Treg cells. FoxP3 KO (scurfy) mice and patients with IPEX syndrome suffer from excessive lymphocyte activation, multi organ lymphocyte infiltration and colitis, leading to death at an early age. Over the past years, extensive research has been carried out which classified Treg cells based on their origin, activation status and expression of surface markers as well as their ability to migrate into certain tissues. Treg cells, generated in the thymus, have been shown to have a TCR repertoire skewed towards self-reactivity while peripheral induced Treg cells were thought to have a more commensal specific TCR repertoire. However, it is still not clear how their degree of self-reactivity contributes to their development and functional capabilities.

The aim of this study was to identify Treg cell populations with differing degrees of self-reactivity and to understand how these Treg cell subsets maintain lymphocyte homeostasis.

5 Results

5.1 The FoxP3 transgenic (tg) mouse: A model to learn more about regulatory T cells

The FoxP3 transgenic mouse was developed in the laboratory of Steven Ziegler and Fred Ramsdell in 2001 where they generated five different transgenic mouse lines.[48] For this study the FoxP3tg line 2826 on the B6 genetic background was used. The 2826 FoxP3tg mouse line was described to expresses 16 copies of a 30.8kb BAC transgene containing a cosmid construct containing the entire *foxp3* gene along with 18kbp of 5' sequences and 4kbp of 3' sequences. Because the gene is controlled by its own regulatory elements, the expression parallels that of the endogenous gene with respect to tissue distribution.[48, 50] In previous reports, the FoxP3tg mouse was described to be healthy and did not show any signs of disease.

In our hands, FoxP3tg mice on a heterozygous background for the FoxP3 transgene also showed normal development and any progression of disease for more than 20 weeks. Surprisingly, when we crossed the FoxP3tg mouse on a homozygous FoxP3tg background, these mice showed developmental deficiencies and a severe ataxic phenotype, characterized by a shaking gait and general difficulties of hind limb control. Because cells, expressing FoxP3, tightly regulate the immune system, we wondered, whether the observed phenotype was caused by an immunological defect. Neither the adoptive transfer of homozygous FoxP3tg lymphocytes into $Rag2^{-/-}$ hosts nor the reconstitution of lethally irradiated $Rag2^{-/-}$ with FoxP3tg bone marrow induced the ataxic phenotype in recipient mice. We therefore excluded that an immunological defect causes the observed phenotype. Next, we wondered, whether the integration site of the BAC transgene could cause an unexpected knock out and leads to the phenotype in the homozygous mouse. Therefore, we performed extensive thermal asymmetric interlaced (TAIL) PCR, a nested PCR technique to identify unknown integration sites in the genome.[189] Due to the high copy numbers (16 copies) of the transgene, we only were able to find tandem sites of the transgene but not the genomic flanking site. Because of this, we cannot exclude that the ataxic phenotype is the consequence of unlucky integration site in the genome. A further possibility for the ataxic phenotype we addressed to regulatory functions of FoxP3 outside the immune system.[190] In previous reports, FoxP3 was described to act as a negative regulator for the proto-oncogene ErbB2.[191] Mice deficient for ErbB2 showed a similar phenotype as observed in homozygous FoxP3tg mice.[192] The disease

showed in ErbB2 deficient mice was caused by a defect in the muscle spindle formation and lead to the described ataxic phenotype. We performed H&E staining of frozen tissue sections of the soleus, isolated from B6, heterozygous and homozygous FoxP3tg mice. Preliminary data indicated that homozygous FoxP3tg mice have clearly reduced muscle spindle formation compared to B6 mice. Heterozygous FoxP3tg mice had slightly reduced muscle spindle formation. These results indicate that FoxP3 is expressed, to some extend, in muscles and the overexpression of FoxP3 downregulates ErbB2.

Taken together, we conclude that the observed phenotype was either caused by an untoward integration of the transgene or a muscular malfunction and not an immunological problem. Based on these results, we decided to perform further experiments with FoxP3tg mice on a heterozygous background.

5.1.1 Characterization of heterozygous FoxP3tg mice

5.1.1.1 Thymic phenotype of FoxP3tg mice

We first analyzed the thymic development of FoxP3tg and B6 control mice where we could not observe any defect in FoxP3tg mice reflected by comparable thymus cellularity of FoxP3tg and B6 mice (Fig.6a). In flow cytometric analysis of live thymocytes for CD4 and CD8b expression (Fig, 6b,c) we could not find significant differences between frequencies or cell numbers of double negative (DN, CD4-CD8b-), double positive (DP, CD4+CD8+), CD4 single positive (CD4SP, CD4+CD8b-) or CD8SP (CD4-CD8+) cells between FoxP3tg and B6 mice (Fig.6b,c)

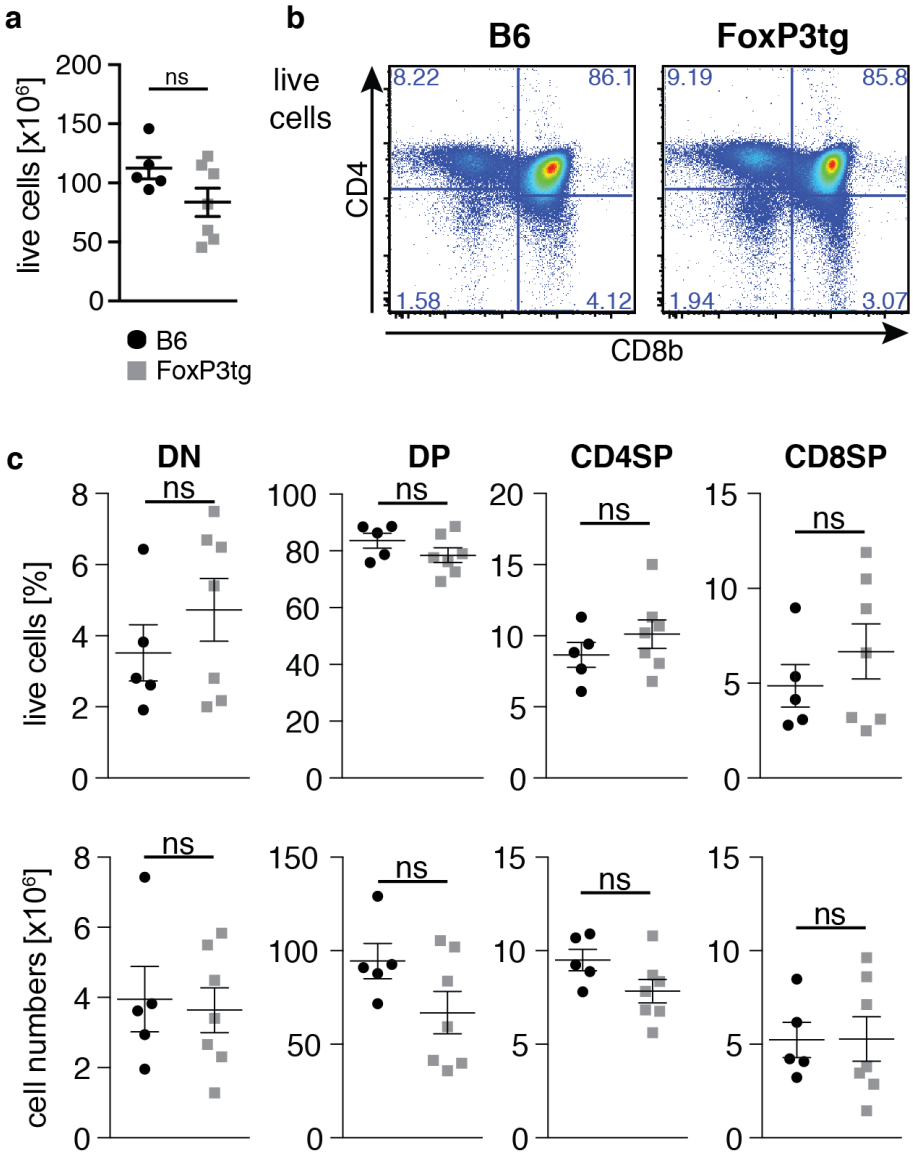


Figure 6: FoxP3tg mice do not have an impaired thymic development.
a) Representative dot plots of live thymocytes from B6 and FoxP3tg mice analyzed by flow cytometer. Thymocytes were stained for CD4 and CD8b that define DN (CD4-CD8b-), DP (CD4+CD8b+), CD4SP (CD4+CD8b-) and CD8SP (CD4-CD8b+) cells. **b)** Cellularity of thymocytes from B6 (black) or FoxP3tg (grey) mice and **c)** frequency (upper row) and cell numbers (lower row) of DN, DP, CD4SP and CD8SP of live thymocytes from B6 (black) or FoxP3tg (grey) mice. Statistical analysis was performed using an unpaired student's t test. Error bars show SEM. ns= not significant.

In a next step, we then analyzed FoxP3 expression in DN, DP, CD4SP and CD8SP thymocytes of B6 and FoxP3tg mice. FoxP3 is not expressed in DN cells (not shown) and only in a small percentage of DP cell in both, FoxP3tg and B6 mice (Fig. 7a,b top panels). In B6 mice we detected about 3% of FoxP3 expressing CD4SP thymocytes whereas in FoxP3tg mice, frequency and cell numbers of this population was increased up to 10 fold (Fig. 7a,b middle panels). As expected, only a small fraction of FoxP3$^+$ CD8SP cells was detected in B6 mice compared to almost 30% in FoxP3tg mice (Fig. 7a,b lower panel).

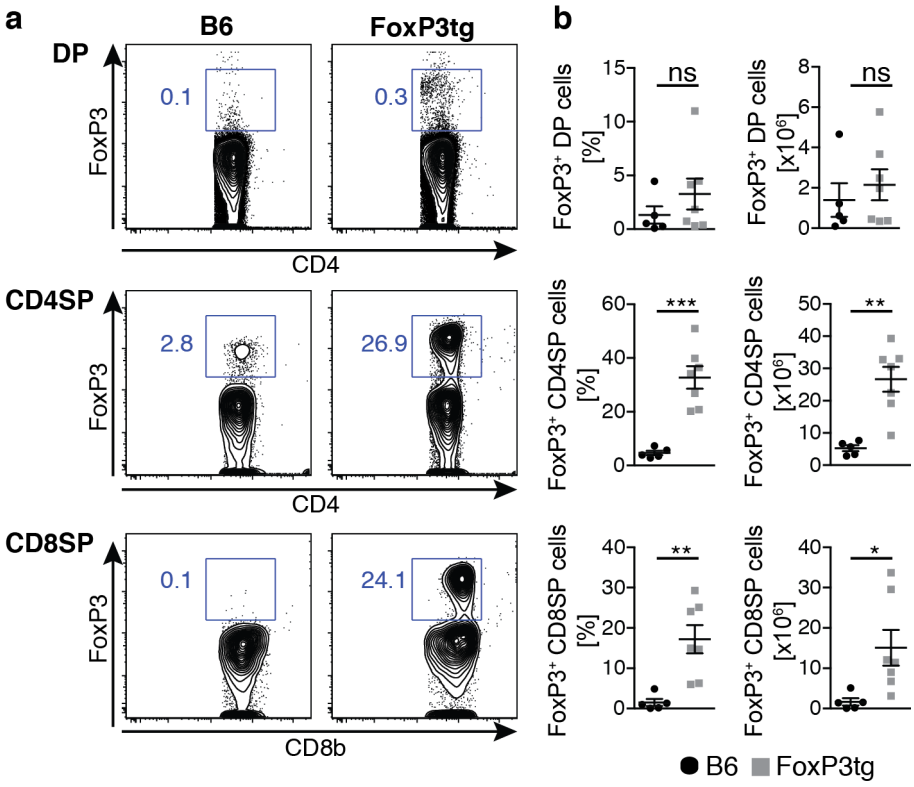


Figure 7: Expression of FoxP3 in B6 and FoxP3tg thymocytes
a) Representative dot plots of live thymocytes from B6 and FoxP3tg mice analyzed by flow cytometry for FoxP3 expression in DP (CD4$^+$CD8b$^+$), CD4SP (CD4$^+$CD8b$^-$) and CD8SP (CD4$^-$CD8b$^+$) cells **b)** Frequency (left) and cell numbers (right) of DP, CD4SP and CD8SP live thymocytes from B6 (black) and FoxP3tg (grey) mice. Statistical analysis was performed using an unpaired student's t test. Error bars show SEM, n=5-7. **, $P < 0.01$; ***, $P < 0.001$, ns= not significant.

This data shows that thymic development in FoxP3tg mice is not impaired. Frequencies as well as cell numbers of CD4SP and CD8SP cells in the thymus of FoxP3tg mice are not different from B6 mice. However, in FoxP3tg mice, a higher frequency of CD4SP and CD8SP cells express FoxP3, compared to B6 mice.

5.1.1.2 Peripheral Phenotype of FoxP3tg mice

Next, we investigated the distribution of adaptive immune cells namely, CD4 T cells, CD8 T cells and CD19$^+$ B cells in B6 and FoxP3tg mice. We isolated lymphocytes from peripheral lymph nodes (axillary, cervical, brachial and inguinal, LN) mesenteric LN (mLN) as well as the spleen and performed flow cytometric analysis. Frequency and absolute cell numbers of CD4 and CD8 T cells in LN, mLN and spleen are markedly reduced in FoxP3tg compared to B6 mice (Fig. 8a,c) whereas frequencies of CD19 positive cells are increased in LN and mLNs (Fig. 8b,c) In the spleen, FoxP3tg and B6 mice express similar frequencies of CD19 positive splenocytes (Fig.8b,c). The absolute number of CD19 positive cells is not different between FoxP3tg and B6 cells isolated from LN, mLN or spleen (Fig. 8c).

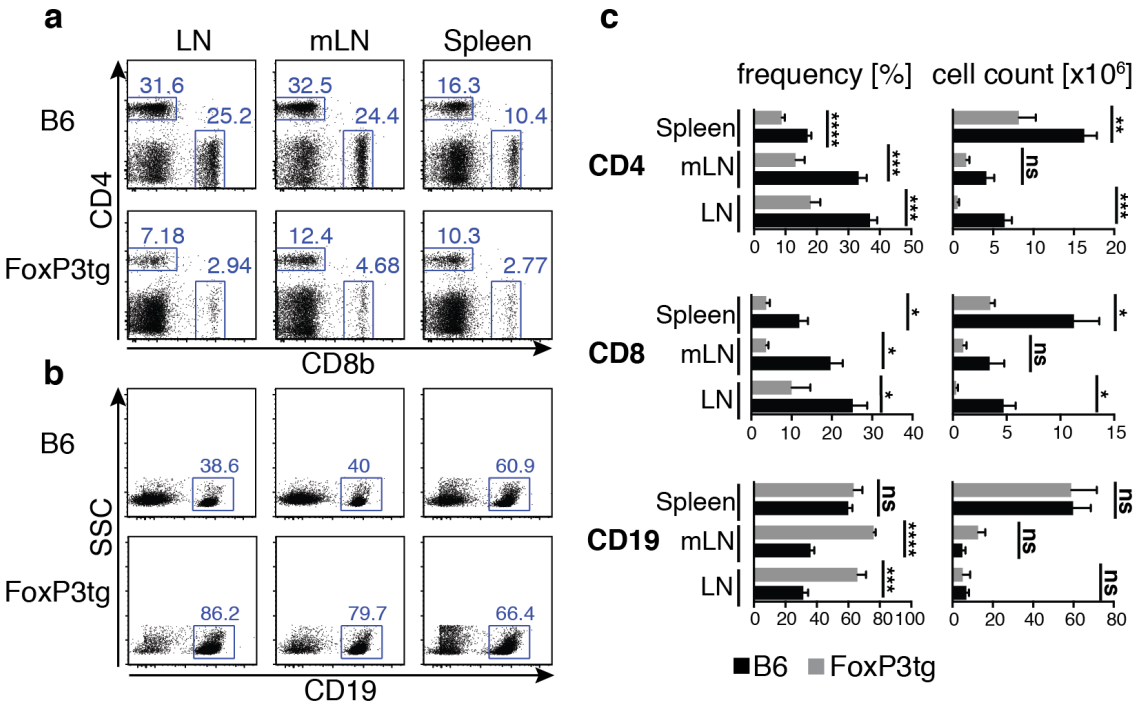


Figure 8: FoxP3tg mice have less T cells than B6 mice.
Representative dot plots of live lymphocytes isolated from peripheral lymph nodes (LN), mesenteric LN (mLN) and spleen from B6 and FoxP3tg mice analyzed by flow cytometry. Lymphocytes were stained for **a)** CD4, CD8b and **b)** CD19. **c)** Frequency (left column) and cell numbers (right column) of CD4$^+$ T cells, CD8$^+$ T cells and CD19$^+$ B cells of live lymphocytes from B6 (black) and FoxP3tg (grey) mice. Statistical analysis was performed using an unpaired student's t test. n=5-7.*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns= not significant.

In previous studies, Kasprowicz et al identified, by confocal microscopy, up to 80% of FoxP3$^+$ CD4$^+$ T cells in the spleen and LN of FoxP3tg mice, compared to 5% of FoxP3$^+$CD4 T cells identified in the spleen and LN of littermate controls. Therefore, in a next step, we analyzed FoxP3 expression in CD4 T cells isolated from LN, mLN and spleen of FoxP3tg and B6 mice by flow cytometry. Our result confirms the previously reported data. Up to 80% of FoxP3tg CD4 T cells isolated from LN, mLN or spleen express FoxP3, but only 10% of B6 CD4 T cells express FoxP3 (Fig. 9a). Even though the frequency of FoxP3 expressing CD4s in FoxP3tg mice is vastly higher than in B6 mice, the difference in absolute cell numbers is diminished (Fig. 9b).

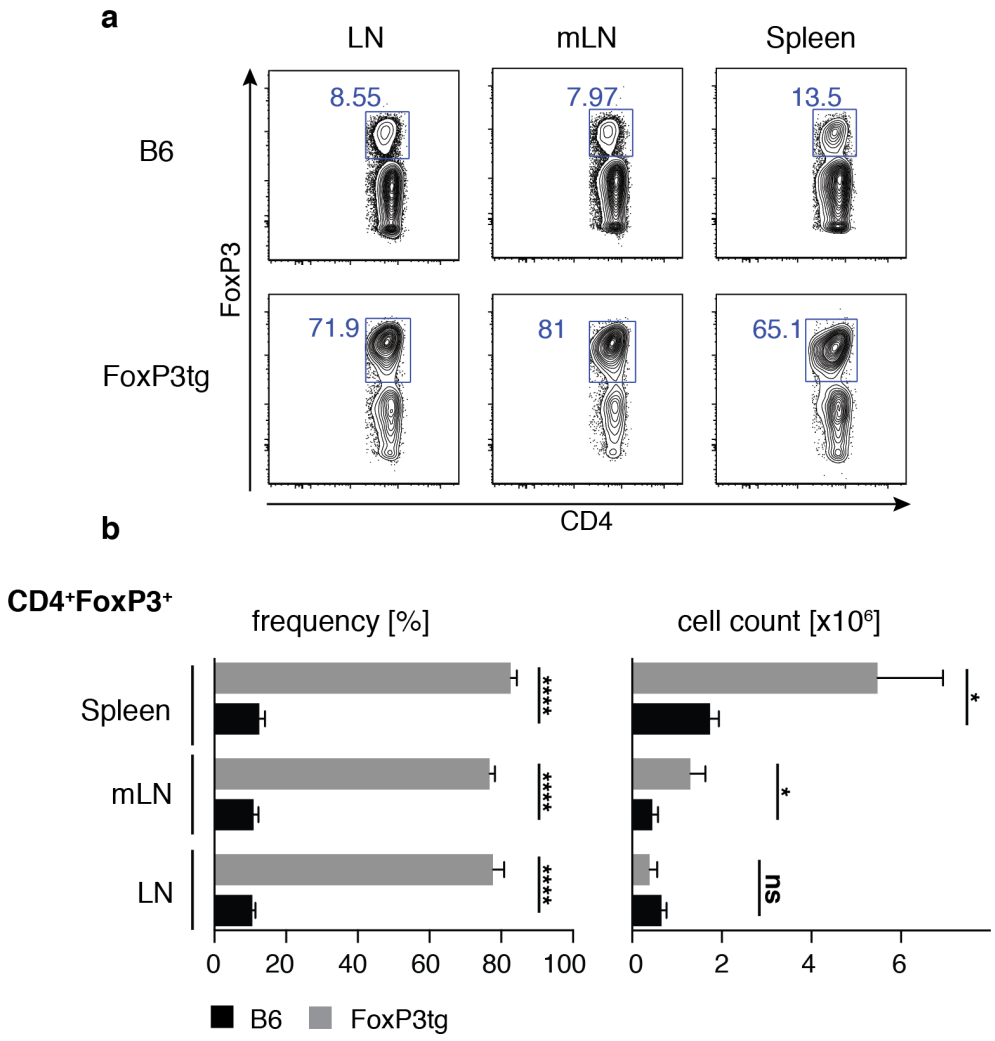


Figure 9: FoxP3tg mice have higher frequencies but similar numbers of FoxP3+ CD4 T cells compared to B6 mice. **a)** Representative dot plots of live lymphocytes isolated from peripheral lymph nodes (LN), mesenteric LN (mLN) and spleen from B6 and FoxP3tg mice analyzed by flow cytometer. CD4+ cells were analyzed for FoxP3 expression. **b)** Frequency (left column) and cell numbers (right column) of FoxP3+ CD4 T cells from B6 (black) and FoxP3tg (grey) mice. Statistical analysis was performed using an unpaired student's t test. n=5-7.*, $P < 0.05$; ****, $P < 0.0001$; ns= not significant.

Taken together, frequencies and cell numbers of peripheral T cells are significantly lower, while frequencies but not numbers of B cells are different in FoxP3tg compared to B6 mice. In turn, frequencies of peripheral $CD4^+$ $FoxP3^+$ T cells are vastly higher in FoxP3tg mice, but absolute cell numbers are not much higher compared to cell numbers found in B6 mice.

5.1.2 Cellular phenotype of FoxP3tg mice is regulated by cell intrinsic mechanisms.

Previous reports indicated that FoxP3 expression is not only limited to lymphocytes.[190] FoxP3 overexpression, in FoxP3tg mice, may influence non-lymphoid cells and indirectly leads to the cellular phenotype observed in FoxP3tg mice. To evaluate whether the reduced frequencies of peripheral T lymphocytes is regulated by cell intrinsic or extrinsic mechanisms we generated bone marrow (BM) chimeras. Lethally irradiated, congenically marked B6 (CD45.1) mice were reconstituted with BM isolated from FoxP3tg mice (CD45.2). Cells isolated from thymus (Fig. 10a) and periphery (LN and Spleen, Fig. 10b) were analyzed 10 to 12 weeks after reconstitution and compared to either unmanipulated B6 thymocytes (Fig. 10a) or FoxP3tg cells isolated from LN or Spleen (Fig. 10b). As expected, the distribution of CD4 and CD8 expressing thymocytes, developed from FoxP3tg BM cells (CD45.2), are comparable to unmanipulated B6 (Fig. 10a top) or FoxP3tg (Fig. 6b) thymocytes. Moreover, in chimeras, FoxP3tg derived CD4SP cells (CD45.2) express about 10 times more FoxP3 (Fig. 10a bottom) than unmanipulated B6 CD4SP (Fig. 10a bottom) and have about the same percentage of $FoxP3^+$CD4SP cells compared to unmanipulated FoxP3tg mice (Fig. 7a). In peripheral lymphocytes isolated from LN or spleen of chimeras, frequencies of CD8b, CD19, CD4 and $CD4^+FoxP3^+$ from FoxP3tg cells (CD45.2) are indistinguishably from frequencies of the same cell subsets found in unmanipulated FoxP3tg mice (Fig. 10b)

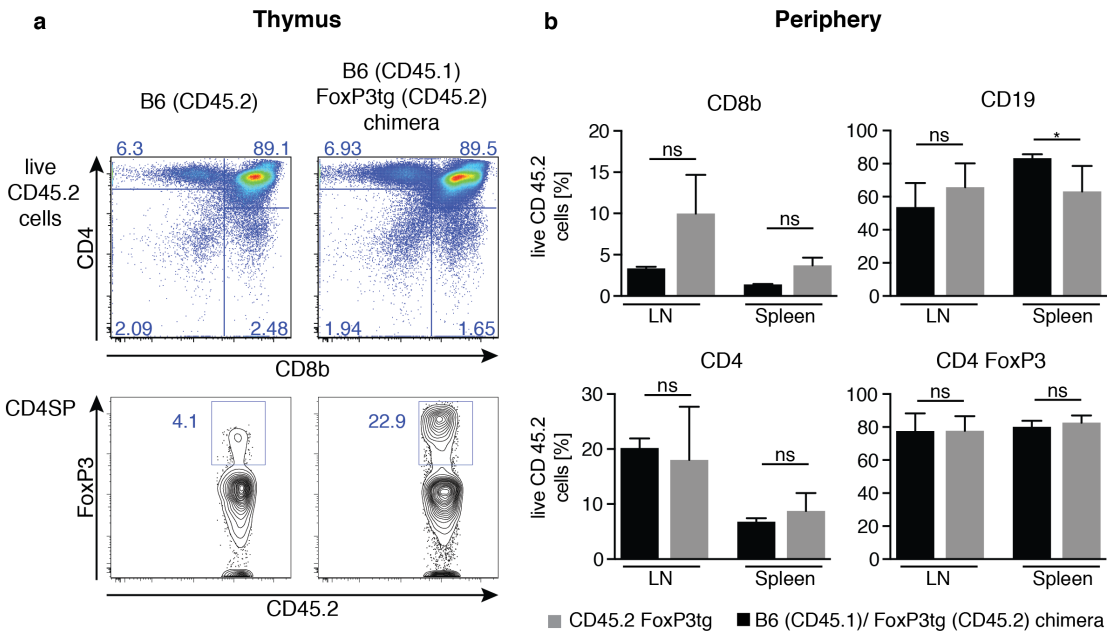


Figure 10: Non-lymphoid cells are not responsible for altered cellular phenotype in FoxP3tg mice.
a) Representative FACS analysis of CD4 and CD8b expression in thymocytes from B6 CD45.2 mice (left) and B6 CD45.1 lethally irradiated hosts reconstituted with BM derived from FoxP3tg mice (right) while bottom panels shows the expression of CD45.2 and FoxP3 on CD4SP thymocytes in respective mice. **b)** Histograms shows frequencies of live CD45.2 CD8b, CD19, CD4 and CD4 FoxP3 cells in LN and Spleen of FoxP3tg (CD45.2, grey) or indicated B6 (CD45.1)/FoxP3tg (CD45.2) chimeric (black) mice. Statistical analysis was performed using an unpaired student's t test. n=4-7, error bars show SEM. *, $P < 0.05$, ns= not significant.

These results show that the observed reduced frequencies of $CD4^+$ and $CD8^+$ T cells, found in FoxP3tg mice, are caused by cell intrinsic and not by cell extrinsic mechanisms. Furthermore, these results indicate that FoxP3 transgene activity influences mainly peripheral lymphocytes rather than thymic development of T cells.

5.1.3 Evaluating the suppressive function of FoxP3tg cells in an allogeneic skin transplantation model

To evaluate the suppressive function of FoxP3tg Treg cells, we used the Bm12 skin graft model. A spontaneous three point mutation in the antigen presenting groove of the IA molecule (IA^{bm12}) was shown to induce an alloimmune response within 14 days when Bm12 skin was transplanted on the back of B6 mice (IA^b)[193, 194] (Fig. 11a, 12b). In T cell deficient mice (e.g. $Rag2^{-/-}$) Bm12 allograft are tolerated for up to 100 days but rejection could be induced within 12 days by the adoptive transfer of $2x10^4$ Bm12 mutation specific TCRtg CD4 T cells (Vα2/Vβ8 TCR, named as ABM cells)[194] (Fig. 11b and 12c)

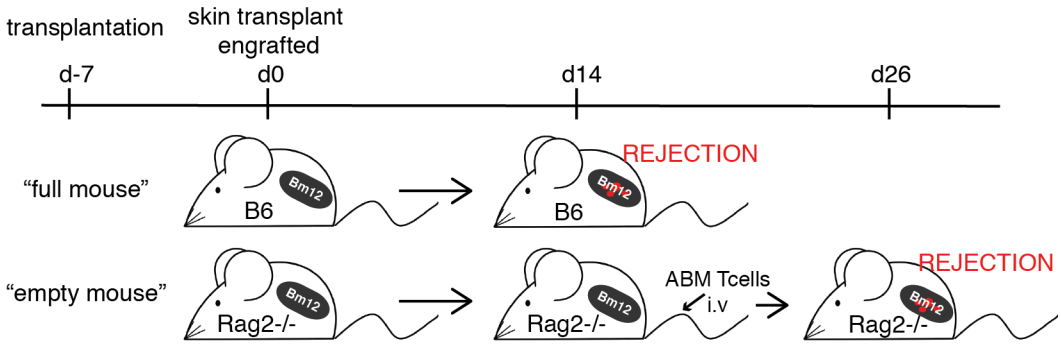


Figure 11: Scheme of B6/Bm12 skin graft model.
Immunosufficient (B6, "full mouse") or immunodefficient (Rag2$^{-/-}$, "empty mouse") mice reacting differently on received skin allograft. "Full mice" reject Bm12 allograft within 14 days whereas "empty mice" are tolerant while rejection of graft can be induced within 12 days by adoptive transfer of ABM cells.

To investigate the suppressive function of FoxP3tg lymphocytes *in vivo*, FoxP3tg or control B6 mice received a Bm12 skin graft at d0. B6 control mice rejected grafts within 14 days whereas FoxP3tg tolerated the grafts for up to 50 days (Fig.12b). We then wondered, whether tolerance was due to the suppressive function of FoxP3$^+$ CD4 or because of too few of FoxP3 negative effector CD4$^+$ T cells in FoxP3tg recipients (Fig.7). To address this, the same FoxP3tg or control Rag2$^{-/-}$ recipients received 2x10^4 ABM cells. Rag2$^{-/-}$ recipients rejected grafts within less than 14 days whereas FoxP3tg recipients did not reject the skin graft for another 30 days (Fig.12c). To rule out that the tolerance of skin grafts was not caused by an insufficient priming of ABM T cells, the same FoxP3tg or control Rag2$^{-/-}$ recipients were challenged with 2x10^4 ABM cells, followed by an injection of 1x10^6 B cells (i.v.) and LPS (25µg i.p.) one day later. FoxP3tg recipients did not reject the grafts for 20 days whereas Rag2$^{-/-}$ recipients rejected grafts within 7 days. (Fig.12d) Regarding the result, that the challenge with primed ABM cells did not mediate graft rejection in FoxP3tg recipients, we considered that the absence of the antigen in the skin graft as a possibility for the robust tolerance. Thus, we re-transplanted the Bm12 skin graft from the FoxP3tg recipients onto Rag2$^{-/-}$ recipients. Rag2$^{-/-}$ recipients with "re-transplants" or "fresh transplants" then were challenged with 2x10^4 ABM cells. Both, "re-transplants" and "fresh transplants" were rejected within 12 days after challenging.

These results strongly indicate that FoxP3tg mice are tolerant, and most probably mediated by the suppressive functions of FoxP3$^+$ CD4 T cells.

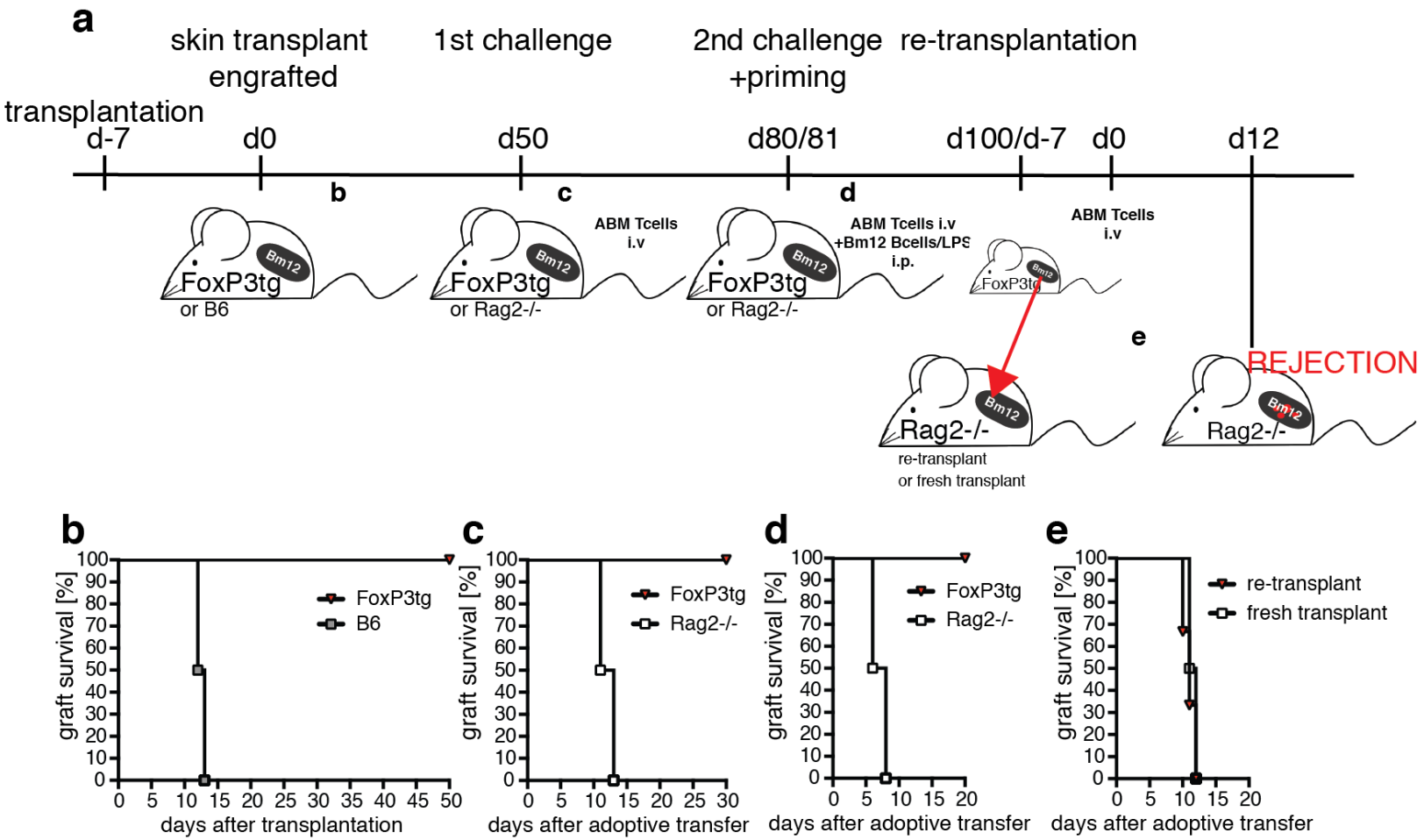
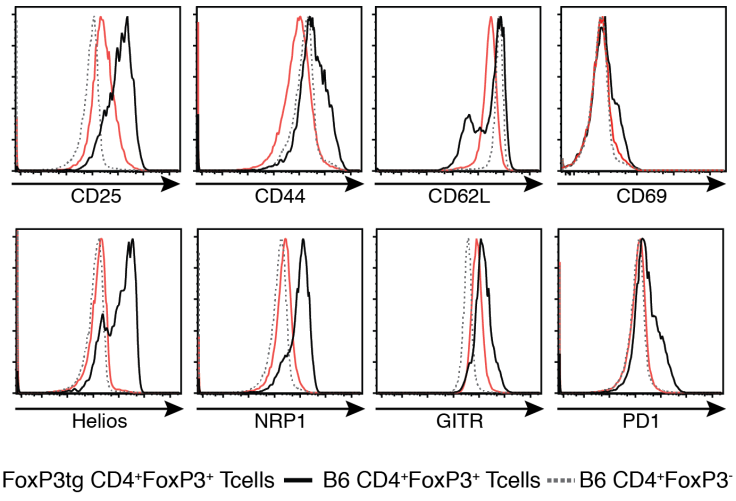


Figure 12: FoxP3tg mice are tolerant and can protect Bm12 skin graft from rejection by primed ABM cells.
a) Schematic overview of experimental setup. **b)** FoxP3tg (red, n=3) or B6 (grey. n=2) mice recipient mice received Bm12 skin allograft on day -7.At day 0 bandage was removed and grafts were controlled daily for rejection. **c)** Same FoxP3tg (red, n=3) mice or Rag2-/- received Bm12 allograft (white, n=2) were adoptively transferred with ABM cells. **d)** Same FoxP3tg (red, n=3) mice or Rag2-/- received Bm12 allograft (white, n=2) were adoptively transferred with ABM cells and Bm12 B cells plus LPS injection the following day. **e)** Rag2-/- recipient mice were either transplanted with Bm12 skin graft removed from FoxP3tg mice indicated before (re-transplant, red, n=3) or fresh Bm12 skin graft (white, n=2). Graft survival is displayed as Kaplan-Meier plot. The difference between the groups in **b,c,d** is statistically significant ($p < 0.05$) and not significant ($p > 0.05$) using Log-rank (Mantel Cox) test.

5.1.4 Phenotype of FoxP3tg Treg cells

Using the Bm12 skin transplantation model, we showed that FoxP3tg mice tolerate the allogeneic skin grafts up to 100 day and even primed, graft specific CD4 effector cells did not induce graft rejection in these mice, whereas B6 mice reject allogeneic skin grafts within two weeks. Peripheral tolerance, including the suppression of auto reactive cells (e.g. ABM cells in the Bm12 model), is mediated by FoxP3 expressing CD4 cells which are phenotypically characterized by the expression of several extra- and intra- cellular molecules e.g. CD25, NRP1, GITR, CD44, CD69, CD62L, PD1 and Helios. To evaluate if FoxP3tg Treg cells exhibit a higher suppressive phenotype than B6 Treg cells, splenocytes from B6 and FoxP3tg mice were evaluated for the expression of these markers. In B6 mice (Fig. 13, B6 Treg cells, solid black line) $CD4^{+}FoxP3^{+}$ Treg cells are characterized, amongst others, by a higher expression of CD25, Helios, NRP1, GITR CD44, CD69, and PD1 and lower expression of CD62L, compared to B6 $CD4^{+}FoxP3^{-}$ (Tconv) (B6 Tconv, dashed gray line) cells. FoxP3tg Treg cells (Fig. 13, solid red line) share to some extend similarities with B6 Treg cells, and show higher CD25 and GITR expression than B6 Tconv cells.



However, the expression of CD25 and GITR on FoxP3tg Treg cells are lower than on B6 Treg cells. Furthermore, CD69, PD1 and CD44 are not upregulated on FoxP3tg Treg cells. Helios and NRP1 are two markers for thymic derived Treg cells, were only marginally expressed on Foxp3tg Treg cells while in B6 mice, the majority of Treg cells express Helios and Nrp1. This indicates that FoxP3tg Treg cells have a different

Figure 13: FoxP3tg Treg cells have "intermediate" Treg phenotype compared to B6 Treg cells.
CD4+ FoxP3+ splenocytes from FoxP3tg (red) or B6 (black) and CD4+FoxP3- (grey dashed) from B6 mice were analyzed for the expression of activation markers CD25, CD44, CD62L and CD69 (upper row) and Treg markers Helios, NRP1, GITR and PD1 (lower row). Histograms show expression of the indicated markers.

origin than B6 Treg cells. Overall, FoxP3tg Treg cells a have an "intermediate" Treg phenotype compared to B6 Treg cells.

We further analyzed Treg cells isolated from thymus, LN, mLN and Spleen from FoxP3tg and B6 mice for the expression of Helios. In the thymus of B6 mice more than 90% of all FoxP3$^+$CD4SP cells express Helios while in peripheral CD4$^+$FoxP3$^+$ cells, only 70% express Helios. In comparison, the vast majority of FoxP3tg Treg cells were Helios negative, irrespective of their origin (Fig. 14a). Even though the frequencies of Helios$^+$ Treg cells are significantly higher in B6 than in FoxP3tg Treg cells, the absolute numbers of total Helios$^+$ Treg cells is comparable in both mouse lines (Fig. 14b). Helios$^+$ Treg cells from FoxP3tg mice express CD44, CD62L, CD69, CD25, NRP1, GITR and PD1 almost equal to Helios+ Treg cells from B6 mice. (Fig. 14c)

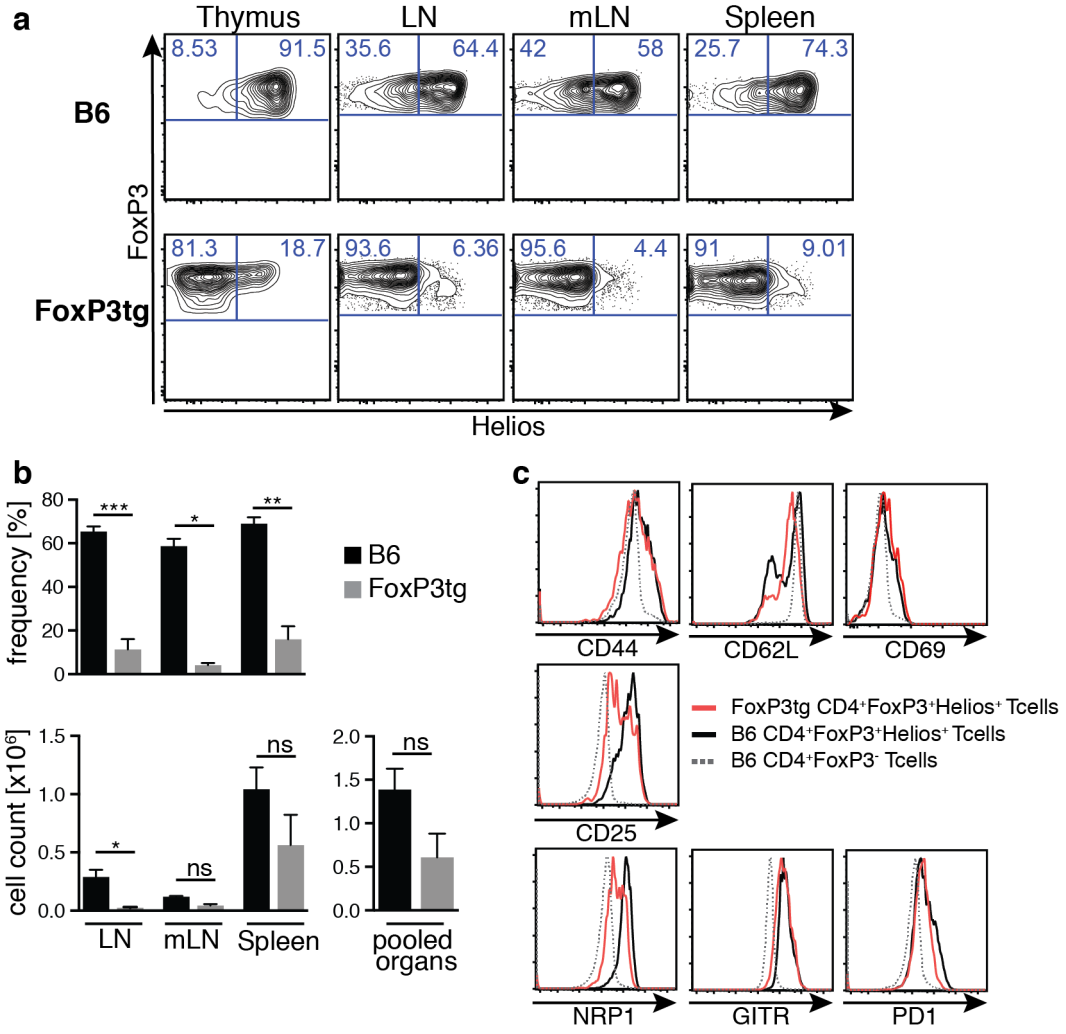


Figure 14: FoxP3tg Helios+ Treg population has a "true" Treg phenotype and similar number as found in B6 mice. CD4$^+$ FoxP3$^+$ cells from FoxP3tg and B6 mice were analyzed **a)** for Helios expression in cells isolated from thymus, lymph nodes (LN), mesenteric lymph nodes (mLN) and spleen. **b)** Graphs display frequency (upper panel) and cell count (lower panel, left) of Helios$^+$ Treg cells from LN, mLN and spleen as well as of pooled organs (lower panel, right) of FoxP3tg (gray) and B6 (black) mice. **c)** Histograms show CD4$^+$FoxP3$^+$Helios$^+$ or CD4$^+$FoxP3$^-$ cells analyzed for CD44, CD62L, CD69, CD25, NRP1, GITR and PD1 from FoxP3tg and B6 mice**.** Statistical analysis was performed using an unpaired student's t test. n=4, error bars show SEM. *, P < 0.05 ; **, P < 0.01; ***, P < 0.001; ns= not significant.

Together, these results show that Helios$^+$ and Helios$^-$ Treg cells from FoxP3tg mice have a Treg phenotype. However, the Helios$^+$ fraction reflects more the Treg population found in B6 mice. Although FoxP3tg mice have much lower frequencies of Helios$^+$ cells, in both mice, about the same number of Helios$^+$ Treg cells are found. This indicates that Helios$^+$ Treg cells from FoxP3tg mice develop in the thymus from negative selection, similarly to Helios$^+$ Treg cells from B6 mice. In contrast, Helios$^-$ Treg cells in the Foxp3tg mouse are positively selected and are driven to express FoxP3 because of the transgene.

5.1.5 Limitation of negative selection limits the development of Helios+Treg cells in FoxP3tg mice.

To investigate the consequences of negative selection limitations on the development of Helios$^+$ Treg cells in FoxP3tg mice, we generated mice where thymocytes are only limited or not negatively selected in the thymus. For these experiments, we used the 3BK506 TCRtg (TCRtg) mouse strain, bearing a transgenic TCR recognizing the 3K peptide in the context of IA^b. The 3K peptide is a variant of the Eα peptide; containing three point mutations were amino acids were substituted with lysine (K).[195] By crossing FoxP3tg with these TCRtg mice, we generated mice with limited negative selection. The CD4SP cells in the thymus have a fixed TCR Vβ chain but because of the active *Rag* gene, the TCR Vα chain still can be rearranged and allows negative selection. Breeding the FoxP3tgTCRtgRag$^+$ mouse on a Rag^{KO} background resulted in mice where negative selection was abolished. This is mediated by the complete deletion of the *Rag* gene and the absence of the cognate antigen in the thymus.

In the thymus of FoxP3tg mice around 25% of all CD4SP cells are FoxP3$^+$ and about 4% co-express Helios. In cells isolated from peripheral lymphoid organs, the majority (80%) of all CD4 cells expresses FoxP3 and between 5-10% co-expressing Helios (Fig.15 and Fig.14b). When negative selection is limited (FoxP3tgTCRtgRag+), or absent (FoxP3tgTCRtgRag^{KO}), the frequency of thymic and peripheral FoxP3 expressing CD4(SP) cells is lower compared to frequencies in FoxP3tg polyclonal mice but still vastly higher compared to B6 mice. (Fig. 7, 9) Helios$^+$ Treg cells seem to be more affected than the Helios$^-$Treg cell population. In FoxP3tgTCRtgRAG$^+$ mice (limited negative selection), the Helios$^+$Treg cell population is tiny, but still detectable within thymic and peripheral CD4 cells, whereas in FoxP3tgTCRtgRAG^{KO} mice Helios$^+$ Treg cells are completely missing.

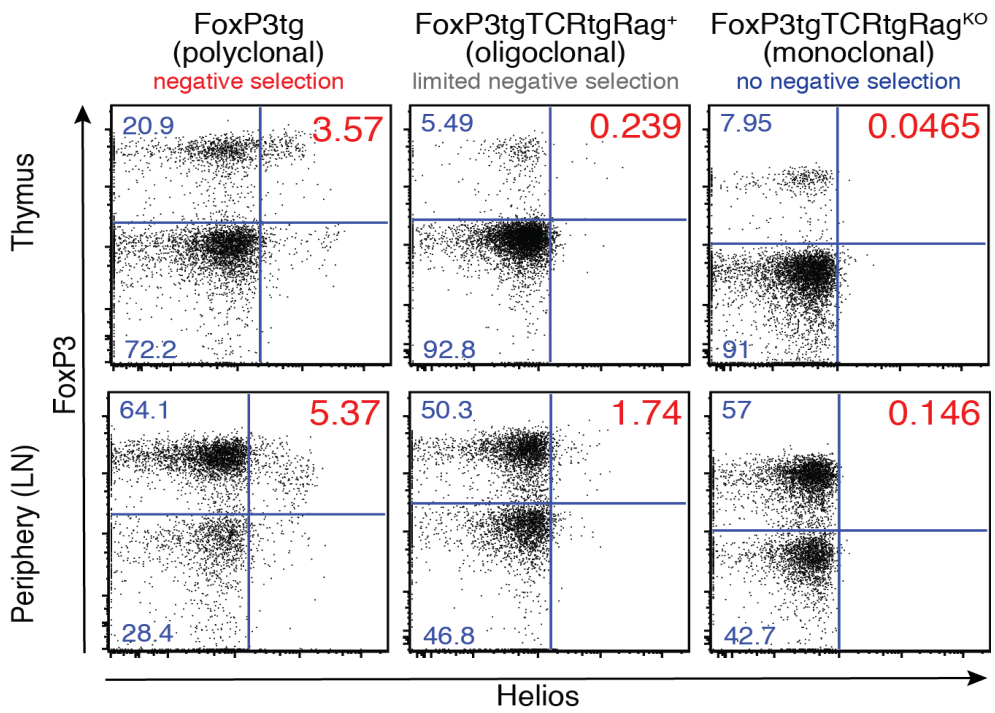


Figure 15: Limiting negative selection limits the development of Helios+Treg cells in FoxP3tg mice. Dot plots shows representative staining of thymocytes (upper row) and lymph node cells (lower row) of FoxP3tg, FoxP3tg 3BK506TCRtgRag+ (FoxP3tgTCRtg Rag+) and FoxP3tg 3BK506TCRtg Rag KO (FoxP3tgTCRtg RagKO) mice for FoxP3 and Helios. For FoxP3tg, numbers represents percentages of live/CD4SP or live/CD4 respectively in the designated gates. For 3BK506TCRtg mice, numbers represents percentages of live/CD4SP/vb8+ or live/CD4/vb8+ respectively in the designated gates.

This data indicates that the development of Helios expressing Treg cells in FoxP3tg mice is driven by negative selection in the thymus and seems not to be influenced by the expression of the FoxP3 transgene. Helios$^-$ Treg cells seem to be less affected by the altered negative selection of thymocytes and are more influenced by the FoxP3 transgene. Thus, we hypothesize that Helios$^+$ Treg cells have a higher affinity for self-antigens than Helios$^-$ Treg cells and Tconv cells in FoxP3tg and B6 mice.

5.2 Studies on the self-reactivity of different Treg cell populations

5.2.1 Autologous Mixed Lymphocyte Reactions (autoMLRs) - an in vitro *model to study self-reactivity -*

To test the hypothesis that Helios$^+$ Treg cells have a higher affinity for self-antigens than Helios$^-$Treg and Tconv cells, we made use of an established *in vitro* model to test self-reactivity of cells. AutoMLR experiments have been described the first time more than 30 years ago[196, 197] and have become an important tool to study self-reactivity *in vitro* [198-201]. The mechanisms, how autoMLRs work are not yet fully understood. In general, it is believed that CD4 T cells respond to self-antigens, presented by MHC class II molecules on syngeneic APCs.[202].

We therefore wondered whether Helios$^+$ Treg, Helios$^-$ Treg and Tconv cells from B6 or FoxP3tg mice respond differently on self-antigens presented by syngeneic APCs. Previous studies suggested DCs as the most potent stimulators in autoMLRs.[201, 203, 204] Based on this, we used an autoMLR model where bone marrow derived DCs (BmDCs) generated from syngeneic (B6 background) BM served as stimulator cells. BmDCs showed a mature phenotype, characterized by the high expression of the surface molecules CD11c, CD80, CD86 and IA^b (MHCII) (data not shown)[205]. As responder cells, CFSE labeled, magnetic bead enriched total CD4 cells, containing Helios$^+$Treg (red), Helios$^-$Treg (brown) and Tconv (blue) cells, isolated from pooled LNs from B6 or FoxP3tg mice were used (Fig. 16b). BmDCs and total CD4 cells were co-cultured in a 1:3 ratio and CFSE dilution of different CD4 T cell populations was analyzed after 5 days by flow cytometry (Fig. 16a).

After 5 days of culture, Helios$^+$ Treg cells from B6 and FoxP3tg mice proliferated the most compared to Helios$^-$Treg and Tconv cells, reflected by the higher CFSE dilution of Helios$^+$Treg cells (red line) compared to Helios$^-$Treg (brown line) and Tconv (blue line) cells (Fig. 16c, lower panels). The analysis of "precursor frequencies going into division" (% dividing cells) shows that Helios$^+$ Treg (red) cells of B6 and FoxP3tg mice proliferate significantly more than Helios$^-$ Treg (brown) and Tconv (blue) cells from B6 or FoxP3tg mice respectively. However, transgenic Helios$^+$ Treg cells respond less than B6 Helios$^+$ Treg cells while Helios$^-$ Treg and Tconv cells do not respond differently (Fig. 16d).

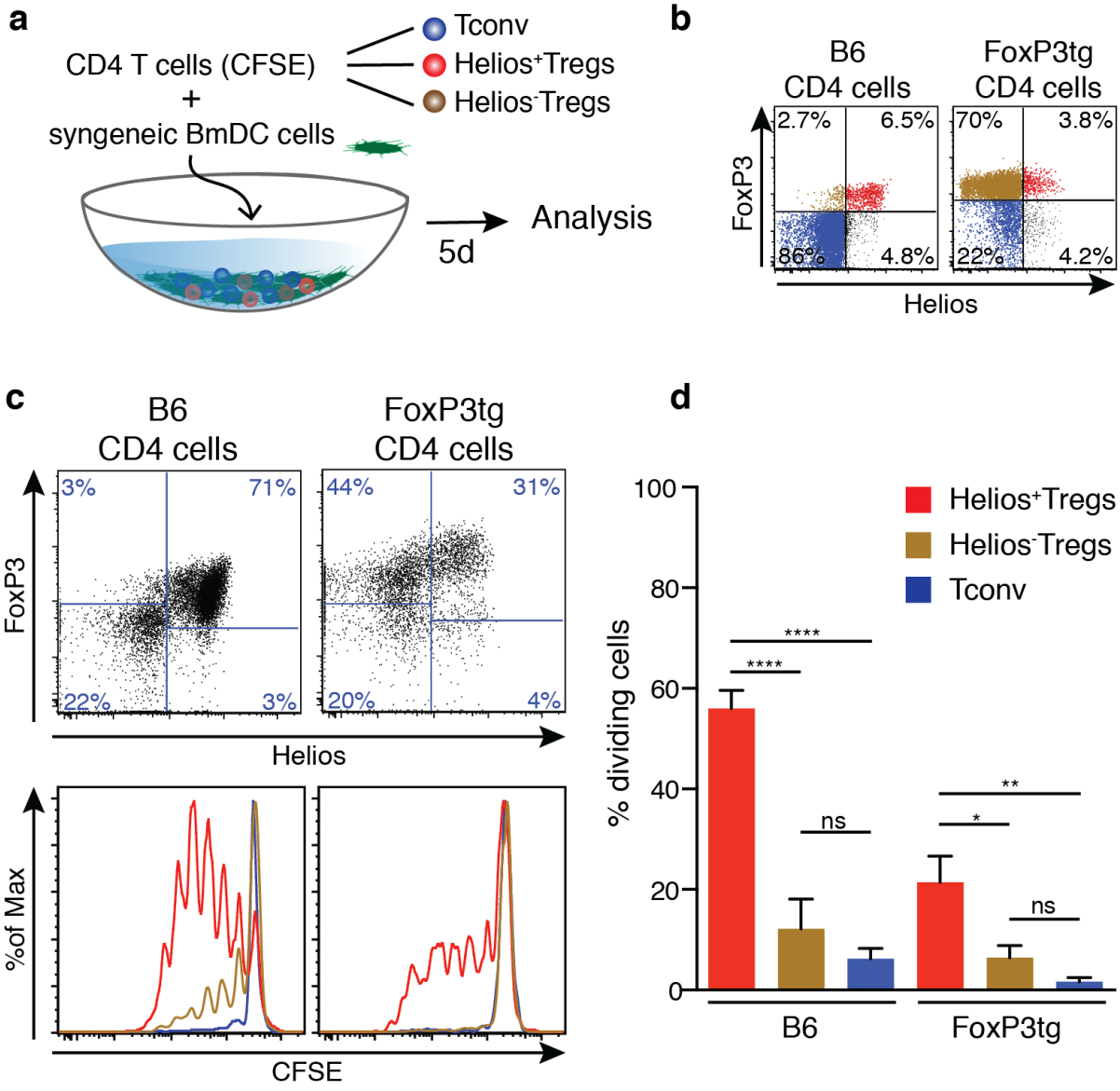


Figure 16: Helios$^+$ Treg cells proliferate almost exclusively in autologous mixed lymphocyte reactions (autoMLR)
General setup of autoMLR experiments **a)** Magnetic beads enriched CFSE labeled CD4 cells isolated from pooled LNs were co cultured with mature syngeneic BmDCs for 5 days before flow cytometric analysis. **b)** Representative dot plots for FoxP3 and Helios expression of freshly isolated CD4 cells from B6 (left) and FoxP3tg (right) mice. **c) ,d)** autoMLR experiment using CD4 cells isolated from B6 or FoxP3tg mice co cultured with B6 BmDCs for 5 days. **c)** Representative dot plots for FoxP3 and Helios expression (upper panels) and histograms (lower panels) for CFSE dilution of Helios$^+$ Treg (Helios$^+$ FoxP3$^+$ CD4$^+$, red), Helios$^-$ Treg (Helios$^-$ FoxP3$^+$ CD4$^+$, brown) and Tconv (Helios$^-$ FoxP3$^-$CD4$^+$, blue) cells of B6 (left) or FoxP3tg (right) CD4 cells. **d)** Graph displays percentage of dividing Helios$^+$ Treg (red), Helios$^-$ Treg (brown) and Tconv (blue) cells. Statistical analysis was performed using an unpaired student's t test. N≥9, error bars show SEM. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$; ns= not significant.

5.2.2 Absence or blocking of MHC class II on BmDCs abolished proliferation of Helios$^+$Tregs

In order to confirm that the observed proliferation of B6 and FoxP3tg Helios$^+$Treg cells, described above, is driven by TCR:MHCII interactions and not by an interaction with co-stimulatory molecules expressed on mature BmDCs e.g. CD80 and CD86,[18] we used three different experimental setups to test this.

First, we performed the autoMLR as described above (and see Fig.16a) but used BmDCs from B6 mice lacking MHCII expression (B6.MHCII^{KO}). The lack of MHCII on BmDCs diminished the proliferation of Helios$^+$ Treg cells, Helios$^-$ Treg and Tconv cells from both, B6 and FoxP3tg CD4$^+$ cells (Fig.17a,b).

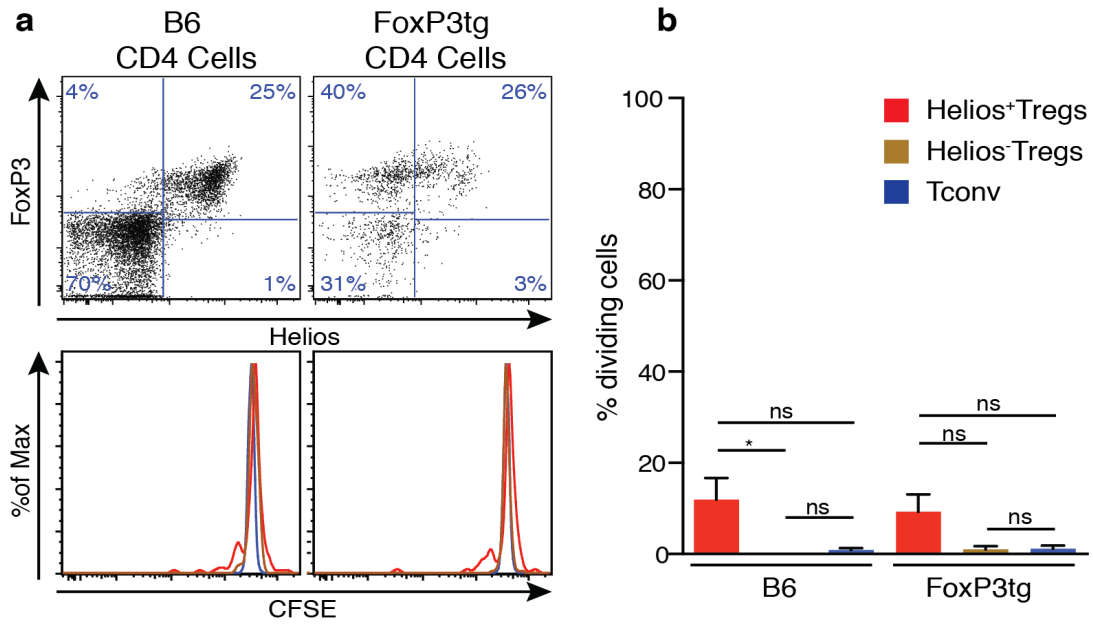


Figure 17: Absence of MHC class II on BmDCs diminishes proliferation of Treg cells.
AutoMLR experiment using CD4 cells isolated from B6 or FoxP3tg mice co-cultured with B6.MHCII^{KO}BmDCs for 5 days. **a)** Representative dot plots for FoxP3 and Helios expression (upper panels) and histograms (lower panels) for CFSE dilution of Helios$^+$ Treg (Helios$^+$FoxP3$^+$CD4$^+$, red), Helios$^-$ Treg (Helios$^-$FoxP3$^+$CD4$^+$, brown) and Tconv (Helios$^-$FoxP3$^-$CD4$^+$, blue) cells of B6 (left) or FoxP3tg (right) CD4 cells. **b)** Graph displays percentage of dividing Helios$^+$ Treg (red), Helios$^-$ Treg (brown) and Tconv (blue) cells. Statistical analysis was performed using an unpaired student's t test. N≥9, error bars show SEM. *, $P < 0.05$; ns= not significant.

Even though B6.MHCII^{KO} BmDCs showed comparable expression of co-stimulatory molecules (e.g. CD80 and CD86, not shown) we wanted to rule out that the knock out of MHCII leads to unexpected functional alterations in these BmDCs. Therefore, we performed the autoMLR as described before (and see Fig.16a) with B6.BmDCs in presence of an MHCII (IA^b) blocking antibody (5ug/ml). Blocking of MHCII on B6.BmDCs resulted in the complete blocking of proliferation in all CD4$^+$ T cells from both, B6 and FoxP3tg mice (Fig.18).

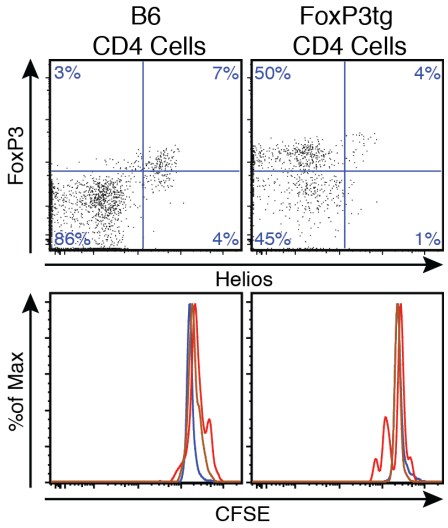


Figure 18: Blocking of MHC class II on BmDCs diminishes the proliferation of all CD4 T cell populations.
AutoMLR experiment using CFSE labeled CD4 cells isolated from B6 or FoxP3tg mice, co-cultured with B6.BmDCs in presence of IA^b blocking antibody (5µg/ml) for 5 days. Representative dot plots for FoxP3 and Helios expression (upper panels) and histograms (lower panels) for CFSE dilution of $Helios^+$ Treg ($Helios^+FoxP3^+CD4^+$, red), $Helios^-$ Treg ($Helios^-FoxP3^+CD4^+$, brown) and Tconv ($Helios^-FoxP3^-CD4^+$, blue) cells of B6 (left) or FoxP3tg (right) CD4 cells.

Although autoMLR experiments using MHCII deficient BmDCs or blocking of MHCII on B6 BmDCs clearly influenced the proliferative response of all $CD4^+$ T cells, we wanted to rule out that soluble factors (e.g cytokines), produced by BmDCs, influences the proliferation. Therefore, we established a "transwell autoMLR" (Fig.19a). In this setting, the insert and bottom well were separated by a perforated membrane with 0.4µm pore size that effects that soluble factors can pass, but cells remains separated. The magnetic beads enriched CFSE labeled CD4 cells isolated from pooled LNs of B6 or FoxP3tg mice were cultured in the insert well, while B6.BmDCs were cultured in the bottom well for 5 days before analysis by flow cytometry. To maintain the "1:3" ratio of BmDC:$CD4^+$ T cells used in the previous experiments, DC numbers had to be scaled up by six fold because the bottom well was six fold bigger than the insert well. The separation of B6 or FoxP3tg CD4 cells from B6.BmDCs abolished the proliferation of all $CD4^+$ T cells cultured in the insert well. This is reflected by the loss of $Helios^+$Treg cells (Fig.19b, upper panels) compared to freshly isolated $CD4^+$ T cells (Fig.16b), the lacking CFSE dilution and the very low frequencies of dividing cells (Fig.19c) compared to autoMLRs where $CD4^+$ T cells could interact with B6.BmDCs (Fig.16c,d).

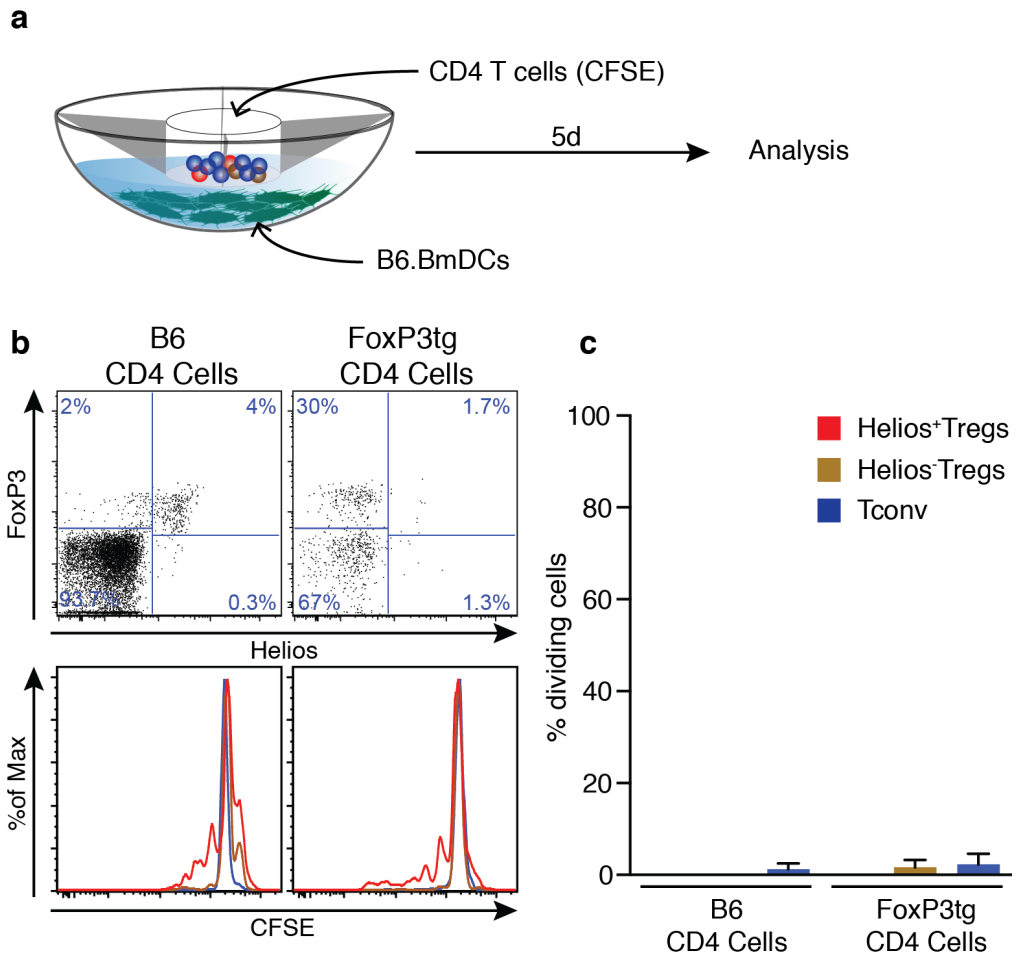


Figure 19: CD4 T cells do not proliferate without direct B6.BmDCs contact.
Setup of transwell autoMLR experiments **a).** Magnetic beads enriched CFSE labeled CD4 cells isolated from pooled LNs were cultured in the insert of a transwell system while B6.BmDCs were cultured in the bottom well for 5 days before flow cytometirc analysis. Insert and bottom wells are separated by a perforated (⦸0.4µm) membrane. **b),c)** Transwell autoMLR experiment using CD4 cells isolated from B6 or FoxP3tg mice (insert) cultured with B6 BmDCs (bottom well) for 5 days. **b)** Representative dot plots for FoxP3 and Helios expression (upper panels) and histograms (lower panels) for CFSE dilution of Helios$^+$ Treg (Helios$^+$FoxP3$^+$CD4$^+$, red), Helios$^-$ Treg (Helios$^-$FoxP3$^+$CD4$^+$, brown) and Tconv (Helios$^-$FoxP3$^-$CD4$^+$, blue) cells of B6 (left) or FoxP3tg (right) CD4 cells. **c)** Graph displays percentage of dividing Helios$^+$ Treg (red), Helios$^-$ Treg (brown) and Tconv (blue) cells. N=5.

Taken together, our data shows that from enriched total CD4$^+$ T cells, cultured on syngeneic B6.BmDCs Helios$^+$ Treg cells highly proliferate whereas only a small fraction of Helios$^-$Treg and Tconv cells are proliferating. The proliferation of all populations is abolished when first, MHCII is knocked out on BmDCs, second, MHCII is blocked on B6.BmDCs or third when we abolished direct contact to B6.BmDC by using a transwell culture system. These results are true for CD4$^+$ T cells isolated from B6 and FoxP3tg mice. Because we observed similar results with CD4$^+$ T cells from B6 and FoxP3tg mice, we decided to perform further experiments with CD4$^+$ T cells from the unmanipulated B6 mouse only.

5.2.3 Helios$^+$ Treg cells need "support" from Tconv cells to proliferate

The Helios$^+$ Treg cell population was the most proliferative population in the previously performed experiments; using total CD4 cells cultured on B6.BmDCs. This supports the hypothesis that Helios$^+$Treg cells have a higher TCR affinity for self-antigens than Helios$^-$Treg or Tconv cells. To further confirm these observations, we performed autoMLR experiments with sorted Helios$^+$Treg cells. Sorting for intracellular markers e.g. Helios and FoxP3 would require the perforation of cells, what kills the cell. Therefore, we had to develop a sorting strategy, which does not require intracellular staining. Using B6.FoxP3eGFP mice where all FoxP3 expressing cells are labeled with GFP in combination with the surface markers GITR and PD1[55] we were able to sort a Treg population with a purity of >85% for Helios expression. To obtain this enriched Helios$^+$ Treg cell population, magnetic bead enriched CD4$^+$ T cells from pooled LNs from several B6.FoxP3eGFP mice were sorted for CD4$^+$, GFP$^+$, GITR^{high} and PD1^{high} (Fig. 20a).

To asses their proliferative capacity induced by self-antigens, CFSE labeled sorted Helios$^+$ Treg cells were co-cultured with either B6.BmDCs or B6.MHCII^{KO} BmDCs in a 1:5 ratio (Tregs:DCs) for 5 days. A small fraction of sorted Helios$^+$ Treg cells divided when cultured on B6.BmDCs (Fig. 20b) but not different from sorted Helios$^+$ Treg cells that were cultured on B6.MHCII^{KO}BmDCs (Fig. 20c). The analysis of frequencies of dividing cells did not show differences between the two groups (Fig. 20c).

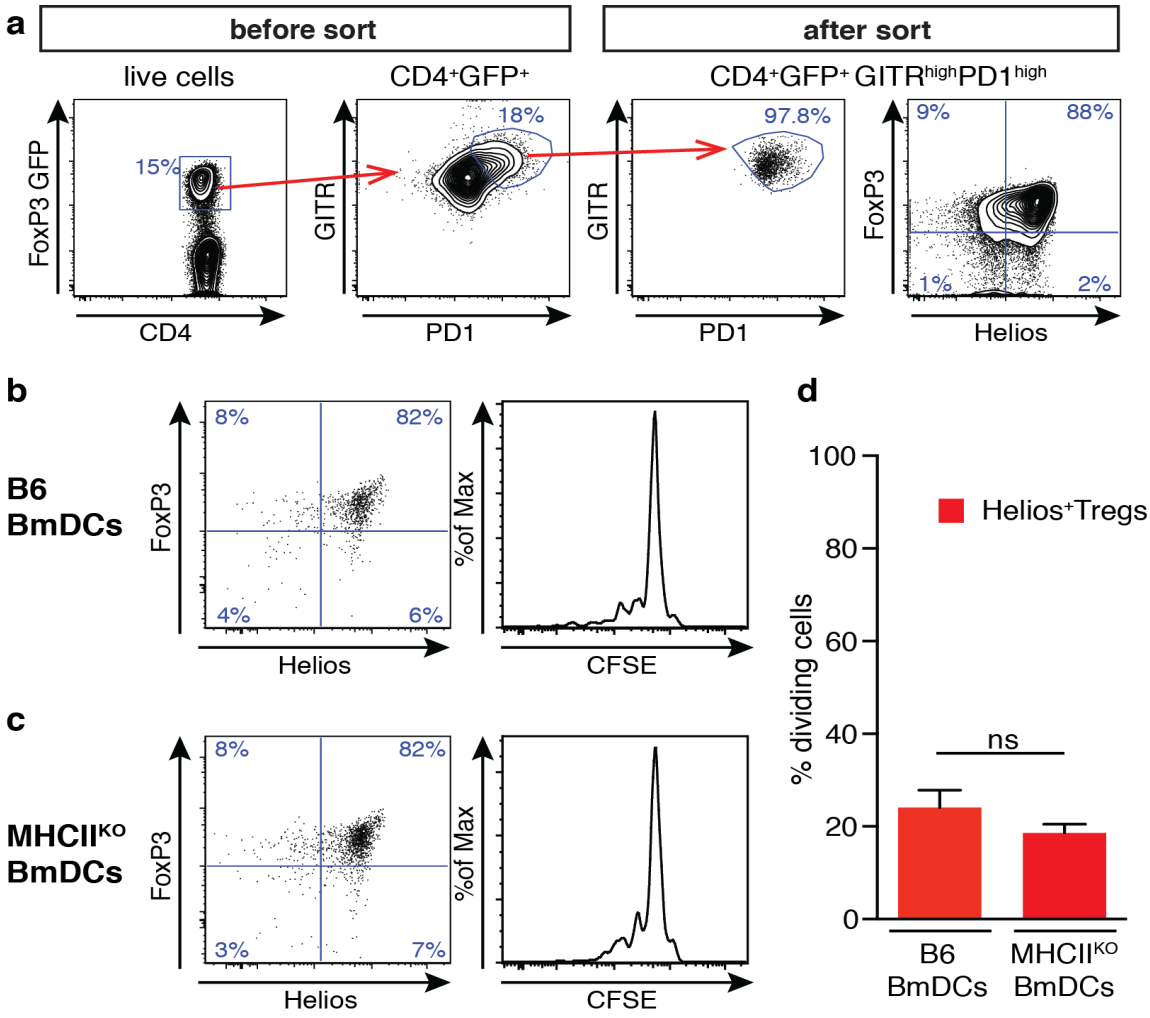


Figure 20: Sorted Helios$^+$ Tregs do not proliferate, independently from MHC class II expression on B6.BmDCs.

a) Sorting strategy of Helios$^+$ Tregs from B6.FoxP3eGFP mice. To obtain highly enriched Helios$^+$Treg cells, magnetic bead enriched CD4 Tcells from pooled LNs from several B6.FoxP3eGFP mice were sorted for CD4$^+$GFP$^+$GITR^{high}PD1^{high} (Helios$^+$Treg cells). Purity of population for Helios expression was > 85%.
AutoMLR experiment using sorted B6 FoxP3eGFP Helios$^+$Treg cells co-cultured with B6 **b)** or B6.MHCII^{KO}BmDCs **c)** for 5 days. Representative dot plots for FoxP3 and Helios expression (left panels) and histogram (right panels) for CFSE dilution of sorted Helios$^+$Treg cells. **d)** Graph displays percentage of dividing Helios$^+$ Treg cells (red). Statistical analysis was performed using an unpaired student's t test. N≥9, error bars show SEM. ns= not significant.

We then wondered whether Helios$^+$ Treg cell proliferation is dependent on the presence of Tconv cells. To assess this, we reconstituted CFSE labeled sorted Helios$^+$ Treg cells with sorted unlabeled Tconv cells (CD4$^+$GFP$^-$ from B6.FoxP3eGFP mice) in a 1:10 ratio. Mixed CD4$^+$ cells were then co-cultured with B6.BmDCs or B6.MHCII^{KO} BmDCs in a 1:3 ratio (CD4 cells:DCs) for 5 days (Fig. 21a). In the presence of Tconv cells, Helios$^+$ Treg cells strongly proliferated in co-cultures with B6.BmDCs (Fig. 21b, left panels) but not with B6.MHCII^{KO} BmDCs (Fig. 21b, right panels). The analysis of frequencies of dividing cells (Fig. 21c) confirms a significant increase of proliferation in co-cultures with B6.BmDCs.

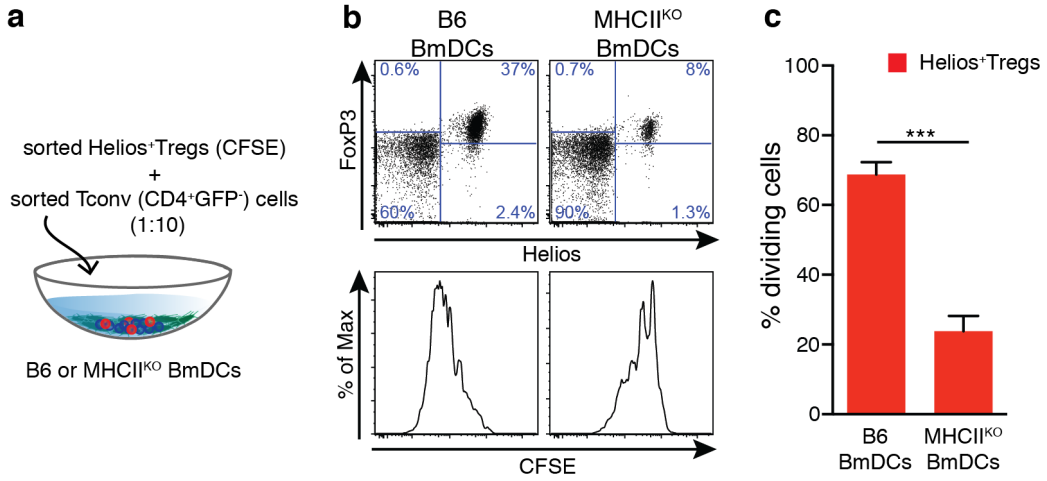


Figure 21: Sorted Helios$^+$ Tregs do proliferate in presence of MHC class II expressing B6 BmDCs when co-cultured with sorted Tconv cells.
a) Setup of experiment. 1:10 mixture of CFSE labeled sorted Helios$^+$ Treg (CD4$^+$GFP$^+$GITR^{high}PD1^{high}) and sorted Tconv (CD4$^+$GFP$^-$, unlabeled) cells isolated from B6.FoxP3eGFP mice were co-cultured with B6.BmDCs or B6.MHCII^{KO}BmDCs for 5 days. **b)** Representative dot plots for FoxP3 and Helios expression (upper panels) and histograms (lower panels) for CFSE dilution of Helios$^+$ Treg (Helios$^+$FoxP3$^+$CD4$^+$) cells. (**c)** Graph displays percentage of dividing Helios$^+$ Treg cells (red). Statistical analysis was performed using an unpaired student's t test. N=5, error bars show SEM. ***, P < 0.001.

The presence of Tconv cells restores the proliferation of sorted Helios^{+} Treg cells when cultured on B6.BmDCs, but not when cultured on B6.MHCII^{KO}BmDCs. Therefore, we wondered whether MHCII dependent stimulation of Tconv induces the secretion of a soluble factor initiating the proliferation of Helios^{+} Treg cells. To test this, we used the transwell autoMLR described before (see Fig. 19a). In contrast to the previous transwell autoMLR, sorted CFSE labeled Helios^{+} Treg cells were cultured in the insert well while sorted Tconv cells were cultured together with either B6.BmDCs or B6.MHC2^{KO}BmDCs in the bottom well for 5 days. To maintain the "1:3" ratio of BmDC:CD4^{+} T cells used in the previous experiments, DC and Tconv cell numbers had to be scaled up by six fold as the bottom well is six fold bigger than the insert well (Fig. 22a). In the presence of Tconv cells and B6.BmDCs, Helios^{+} Treg cells proliferate (Fig.22b, left panels and c) but do not when BmDCs were deficient in MHCII expression (Fig. 22b, right panels and c). This finding supports our assumption that Tconv stimulated by B6.BmDCs secrete a soluble factor that induces the proliferation of the sorted Helios^{+} Treg cells.

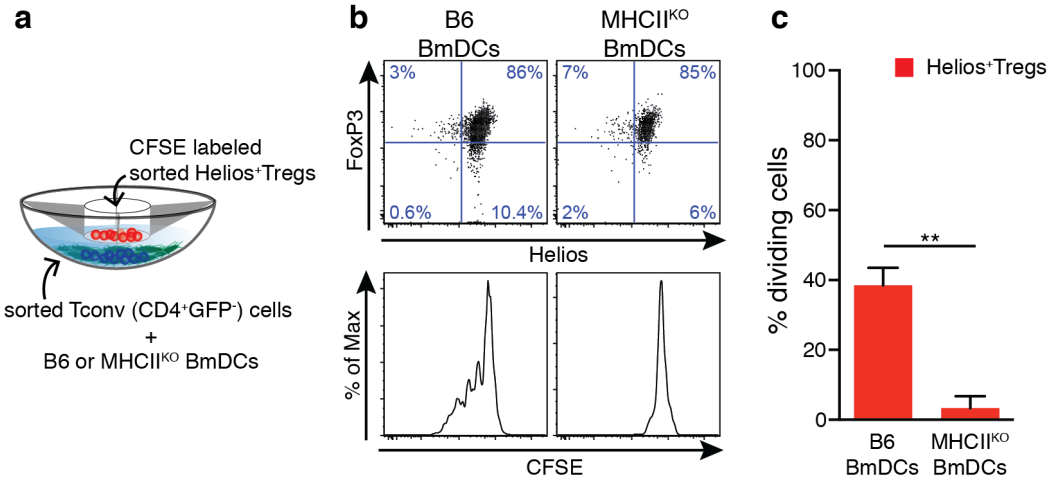


Figure 22: Sorted Helios^{+} Tregs proliferation is induced by (a) soluble factor(s) produced by Tconv cells.
a) Setup of transwell autoMLR experiments. CFSE labeled, sorted Helios^{+} Treg cells (CD4^{+}GFP^{+}GITR^{high}PD1^{high}) from B6.FoxP3eGFP mice were cultured in the insert while in the bottom well sorted Tconv (CD4^{+}GFP^{-}, unlabeled) from B6.FoxP3eGFP mice were co-cultured with B6.BmDCs or B6.MHCII^{KO}BmDCs for 5 days. **b)** Representative dot plots for FoxP3 and Helios expression (upper panels) and histograms (lower panels) for CFSE dilution of Helios^{+} Treg (Helios^{+}FoxP3^{+}CD4^{+}) cells cultured either on top of sorted Tconv cells in presence of B6.BmDCs (left) or B6.MHCII^{KO}BmDC (right). **c)** Graph displays percentage of dividing Helios^{+}Treg cells (red). Statistical analysis was performed using an unpaired student's t test. N=3, error bars show SEM. **, $P < 0.01$.

As a proof of principle, we performed autoMLR experiments where CFSE labeled magnetic bead enriched $CD4^+$ T cells from B6 mice were co-cultured for 5 days with B6.$MHC2^{KO}$BmDCs with different dilutions of supernatant from 5 day co-cultures of sorted Tconv cells and B6.BmDCs (Fig. 23a). $Helios^+$ Treg cells but neither $Helios^-$ Treg nor Tconv cells proliferated. Additionally, $Helios^+$ Treg proliferated in a dose dependent manner (Fig. 23b).

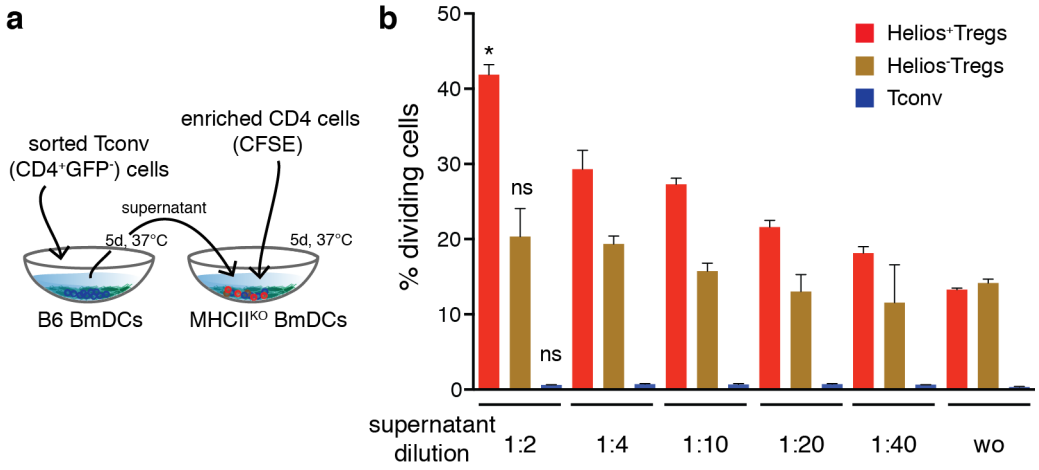


Figure 23: Mainly $Helios^+$Treg cell proliferation is induced by (a) secreted factor(s) produced by Tconv cells. **(a)** Setup of autoMLR experiments. Supernatant of cultures of sorted Tconv cells ($CD4^+GFP^-$, unlabeled) from B6.FoxP3eGFP mice with B6.BmDCs for 5 days was transferred in different dilutions to magnetic bead enriched CFSE labeled B6.CD4 T cells co-cultured with B6.$MHCII^{KO}$BmDCs. CFSE dilution was analyzed by flow cytometry at day 5. **(b)** Graph displays percentage of dividing $Helios^+$ Treg (red), $Helios^-$ Treg (brown) and Tconv (blue) cells. Statistical analysis was performed using a one-way ANOVA test. N=2, error bars show SEM. *, $P < 0.05$, ns= not significant)

Taken together, the proliferation of sorted $Helios^+$ Treg cells is, most probably induced by (a) soluble factor(s) e.g. cytokine(s) secreted by Tconv cells given that MHCII is expressed on B6.BmDCs.

5.2.4 Tconv cells secrete IL2 when stimulated with syngeneic BmDCs

The most prominent cytokine that could promote the proliferation of $Helios^+$ Treg cells is IL2. Normally, high levels of IL2 are secreted from lymphocytes after TCR signal induced activation. To evaluate if Tconv cells produce IL2 in autoMLR experiments, we cultured sorted Tconv cells with either B6.BmDCs or B6.$MHC2^{KO}$BmDCs for 1-4 days and the IL2 concentration of the supernatant was determined. We detected IL2 in the supernatant of three-day cultures and even higher levels in the supernatant of four-day cultures. IL2 secretion was only detected when Tconv cells were cultured with MHCII expressing BmDCs (Fig. 24)

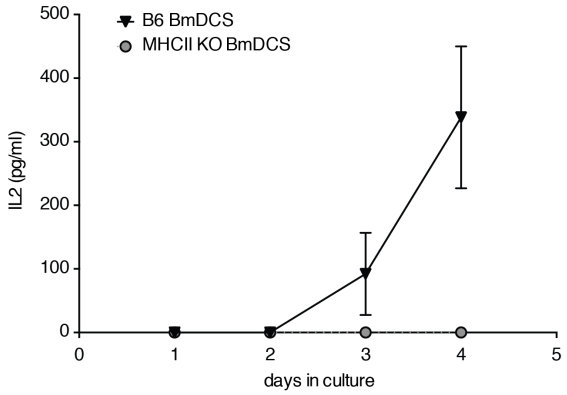


Figure 24: Tconv cells produce IL2 when co-cultured with MHCII expressing B6.BmDCs.
Graph displays concentration of IL2 in the supernatant from co-cultures of sorted Tconv cells ($CD4^+GFP^-$) from B6.FoxP3eGFP mice and B6.BmDCs (black) or B6.$MHCII^{KO}$BmDCs for 1,2,3 or 4 days. Supernatant from different time points were harvested and analyzed for IL2 by BD™ Cytometric Bead Array (CBA). N=2, error bars show SEM.

In experimental setups using B6.$MHC2^{KO}$BmDCs (Fig. 17), B6.BmDCs + MHCI blocking antibodies (Fig. 18) or the transwell system (Fig. 19), Treg cells do not proliferate. Thus, we wondered whether in these conditions, the administration of exogenous IL2 restores the proliferation seen from Treg cells. In all conditions, the high proliferation of $Helios^+$ Treg cells and the lower proliferation of $Helios^-$ Treg cells is restored by the administration of exogenous IL2 (50U/ml, Fig. 25).

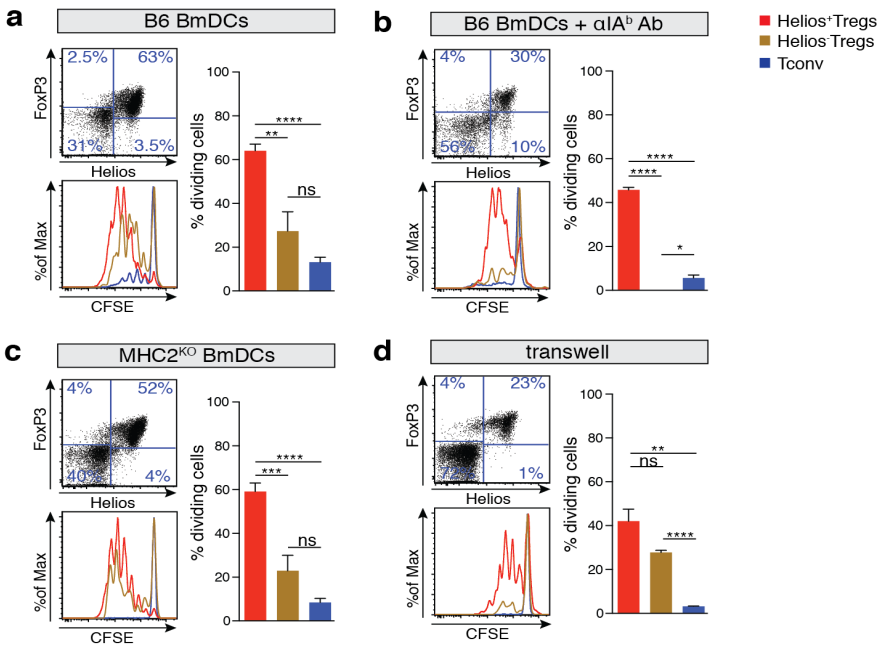


Figure 25: Exogenous IL2 can restore the proliferation of Treg cells when MHCII interaction is missing.
Magnetic beads enriched CFSE labeled CD4 cells isolated from pooled LNs from B6 mice were cultured in presence of 50U/ml IL2 and either **(a)** B6.BmDCs, **(b)** B6.BmDCs + αIA^b Ab (5µg/ml), **(c)** B6.$MHC2^{KO}$BmDCs or **(d)** separated from B6 BmDCs in a transwell system for 5 days. Dot plots shows representative staining for FoxP3 and Helios expression (upper panels) and histograms (lower panels) shows CFSE dilution of $Helios^+$Treg ($Helios^+FoxP3^+CD4^+$, red), $Helios^-$Treg ($Helios^-FoxP3^+CD4^+$, brown) and Tconv ($Helios^-FoxP3^-CD4^+$, blue) cells of B6 CD4 cells. Graphs displays percentage of dividing $Helios^+$Treg (red), $Helios^-$Treg (brown) and Tconv (blue) cells. Statistical analysis was performed using an unpaired student's t test. N≥5, error bars show SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ ****, $P < 0.0001$; ns= not significant.

5.2.5 TCR affinity for self-antigens matters but Il2 rules proliferation

Considering the result, that sorted Tconv cells can produce IL2 when stimulated with B6.BmDCs (Fig. 24) we can assume that in autoMLR experiments with unsorted total CD4 cells, Tconv cells serves as a source of IL2. Thus, IL2, and not a higher-affinity TCR signals drives the proliferation of Helios^{+} Treg cells in these settings (Fig. 16). However, it is striking that Helios^{+} Treg cells almost exclusively proliferate while Helios^{-} Treg and Tconv cells do not (or less). The same is observed, when a high amount of exogenous IL2 is added to co-cultures with B6.BmDCs (Fig. 25a). This means that a competition for IL2 is excluded.

Due to these observations, we wondered whether a different affinity for self-antigens of Helios^{+} Treg, Helios^{-} Treg and Tconv cells results in e.g. better survival or upregulation of the activation marker CD25.

To address this, we performed autoMLR experiments with CFSE labeled magnetic beads enriched CD4 cells from B6 mice, co-cultured with B6.BmDCs or B6.MHC2^{KO}BmDCs for 1-4 days in the presence or absence of a high dose of exogenous IL2 (50U/ml). Cells were harvested at d1, 2, 3 and 4 of culture and were analyzed by flow cytometry for frequencies of dividing cells (Fig.26, left panels), cell numbers of live cells (Fig. 26, middle panels) and geometric mean fluorescence intensity (MFI) of CD25 (Fig. 26, right panels).

In the absence of IL2 (Fig. 26a) about 20% of Helios^{+} Treg (red) and Helios^{-} Treg (brown) cells proliferate at day 2 of culture. The observed proliferation is independent of the presence (B6.BmDCS, solid lines) or absence of MHCII (B6.MHC2^{KO}BmDCs, dashed lines) on BmDCs. At later time points, only Helios^{+} Treg cells proliferate more and only when cultured with B6.BmDCs. Tconv cells (blue) do not proliferate at all (left panel). Furthermore, only absolute numbers of live cells of Helios^{+} Treg cells increase but not of Helios^{-} Treg cells and only when MHCII present (middle panel). Helios^{+} Treg cells highly upregulate CD25 expression when cultured with B6.BmDCs. This effect is diminished when cultured with B6.MHC2^{KO}BmDCs. An upregulation of CD25 is also seen in Helios^{-} Treg cells in the presence of B6.BmDCs but at a much lower level as Helios^{+} Treg cells do. CD25 expression on Tconv cells stays low in all conditions and at all time points of culture (right panel).

The supplementation with high amounts of exogenous IL2 (Fig. 26b) fully restores the proliferation of Treg cells in co-cultures with B6.MHC2^{KO}BmDCs. Even though, Helios^{-} Treg cells also proliferate (36%), the frequency of proliferating Helios^{+} Treg cells is clearly higher (up to 60% of dividing cells) while Tconv cells do not divide

(left panel). Although, exogenous IL2 fully restores proliferation in cultures with B6.MHC2^{KO}BmDCs, the high amount of IL2 do not support a better survival of either population in the presence or absence of MHCII expression on BmDCs. It looks like that Helios^{+} Treg cells from cultures with B6.BmDCs survive better than in cultures with B6.MHC2^{KO}BmDCs. The cell numbers of viable Helios^{-} Treg cells are not different in either condition (middle panel). Exogenous IL2 cannot restore the CD25 upregulation on Helios^{+} Treg cells in cell cultures where MHCII is not expressed on BmDCs. The same is seen for Helios^{-} Treg cells, which also express CD25, but at a much lower level than Helios^{+} Treg cells. Exogenous IL2 is not influencing the expression of CD25 on Tconv cells (right panel).

The analysis for all conditions at day four of culture is summarized in Fig. 26c. The bar graphs reflect the analysis for the percentage of dividing cells (left panel), cell numbers (middle panel) and gMFI of CD25 (right panel) of Helios^{+} Treg (red), Helios^{-} Treg (brown) and Tconv (blue) cells in dependency of MHCII on BmDCs and in presence or absence of exogenous IL2 in the culture.

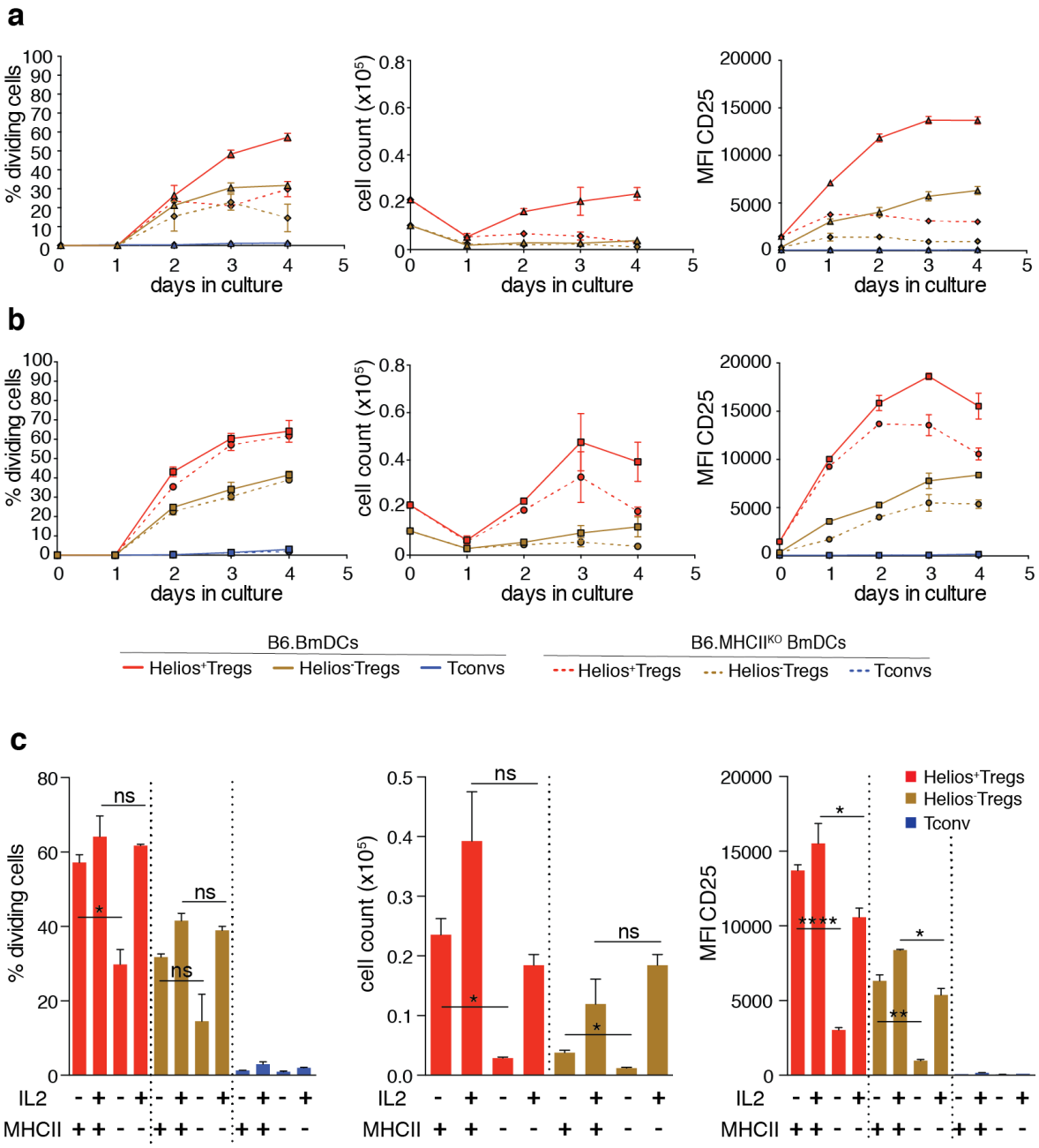


Figure 26: MHC class II expression on BmDCs seems to influence CD25 expression and survival of Treg cells. Magnetic beads enriched CFSE labeled CD4 cells isolated from pooled LNs from B6 mice were cultured in presence (b) or absence (a) of 50U/ml IL2 and either B6.BmDCs (solid line) or B6.MHC2^{KO}BmDCs (dashed line) for 1, 2,3, or 4 days. Helios^{+} Treg (red), Helios^{-} Treg (brown) and Tconv (blue) cells were analyzed for the percentage of dividing cells (left panels), cell numbers (middle panels) and geometric mean fluorescence intensity (MFI) of CD25. (c) Graphs displays quantification of Helios^{+} Treg (red), Helios^{-} Treg (brown) and Tconv (blue) cells for the percentage of dividing cells (left panel), cell numbers (middle panel) or MFI of CD25 (right panel) in absence or presence of MHCII or IL2 respectively. Statistical analysis was performed using an unpaired student's t test. N=3, error bars show SEM. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$; ns= not significant.

5.3 Manuscript

Affinity for self-antigen selects regulatory T cells with distinct functional properties

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Contribution:

B.S. and E.H. performed TCR sequencing and clonotype analysis of $GITR^{low}PD1^{low}$versus $GITR^{high}PD1^{high}$ peripheral Tregs (Figure 3, e-j)

C.G.K. performed reaggregated thymic organ cultures and analysis (Figure S1)

S.S. and K.K. performed lymphocyte staining and analysis of B6.FoxP3-GFP-Cre x FoxP3-IRES-RFP mice (Figure S2)

L.T. performed tissue sectioning and H&E staining of skin and colon samples (Figure 30d, 31d)

Abstract

The importance of regulatory T cells (Tregs) in maintaining lymphocyte homeostasis is best appreciated in mice and humans lacking these cells. FoxP3 KO (scurfy) mice[48, 49] and patients with immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome[206] suffer from excessive lymphocyte activation, lymphocytic infiltration into peripheral organs, and colitis leading to death at an early age. In healthy mice and humans, Tregs control homeostatic proliferation of conventional T and B cells and prevent colitis.[52,79,122,175,207,208] Tregs have been classified based on their activation status, expression of surface markers and their ability to migrate to certain tissues[28-31]. $CCR7^{+}$ Tregs localize to T cell zones in LNs[28], while Tregs expressing the integrin αEβ7 migrate into inflamed tissues[29]. In humans, the proportion of activated and resting Tregs vary with age and the presence immunological disorders.[31] While Tregs have been shown to express self-reactive[81,172,209] and foreign antigen reactive[210] T cell receptors (TCRs), it's not clear how their antigen specificity determines their regulatory function. Here we report two functionally distinct subgroups of Tregs with differing TCR affinities for self-antigens. $Triple^{high}$ ($GITR^{high}$, $PD1^{high}$, $CD25^{high}$) Tregs are generated through

negative selection and express higher affinity self-reactive TCRs. This population also proliferates in response to MHC II encoded self-antigens. In contrast, Triple^{low} (GITR^{low}, PD1^{low}, CD25^{low}) Tregs express lower affinity (but above the threshold for negative selection) self-reactive TCRs and are unable to proliferate on syngeneic antigen presenting cells (APCs). The TCR repertoires of Triple^{high} Tregs, Triple^{low} Tregs and CD4 Tconvs are clearly distinct. Functionally, Triple^{high} but not Triple^{low} Tregs control the extensive lympho-proliferation in mice acutely depleted of Tregs. Conversely, Triple^{low} but not Triple^{high} Tregs control colitis by facilitating conversion of Tconv into induced Tregs (iTregs). The differing TCR affinities for self-antigens likely induce divergent developmental programs, generating two Treg populations with distinct regulatory properties. Finally, FoxP3 deficient (scurfy) mice contain Triple^{high} and Triple^{low} CD4 T cells with distinct pathological properties. Triple^{high} scurfy T cells proliferate extensively in peripheral LNs and infiltrate the skin while scurfy Triple^{low} T cells cause colitis and wasting disease.

Results & Discussion

As previous reports showed that the majority of thymic Tregs are Helios^{+} [106, 107] we examined this subset in polyclonal (B6), oligoclonal (MHC II restricted TCR transgenic, Rag^{+}) and monoclonal (MHC II restricted TCR transgenic, Rag$^{-/-}$) mice. The percentage of Helios^{+} Tregs is clearly decreased in strains with a limited TCR repertoire (Fig.27 a,b). In fact, Helios is expressed in thymocytes undergoing clonal deletion[211] and the development of thymic (Helios^{+}) Tregs is completely impaired in monoclonal mice lacking negative selection. To test this idea, bone marrow chimeras were generated where OT-II thymocytes developed in hosts expressing (RIP-mOVA) or lacking (B6) the cognate antigen, ovalbumin. OT-II thymocytes surviving negative selection in RIP-mOVA mice almost exclusively develop into Helios^{+} Tregs (Fig.27c upper row). In contrast, OT-II thymocytes developing in B6 hosts only generate CD4 Tconv cells (Fig.27c, lower row). Along this line, Tregs develop in re-aggregate thymic organ cultures (RTOCs) only in the presence of negatively selecting cognate antigens (Fig.S1), consistent with results in Fig.27c. Finally, analysis of mice where thymus-derived Tregs are genetically marked, showed that >80% of thymus derived peripheral Tregs are Helios^{+} (Fig.S2)[212].

Taken together, these data imply that most thymic-derived, i.e. Helios^{+} Tregs are survivors of negative selection and for this reason express high affinity self-reactive TCRs (i.e. above the affinity threshold for negative selection[12]). This idea is further

supported by the observation that Helios^{+} Tregs cells proliferate more extensively than Helios^{-} Tregs and CD4 Tconvs, both in vivo (Fig.27d) and in vitro (Fig.27e). Proliferation and survival of Helios^{+} Tregs is dependent on MHC II expression on APCs, confirming their self-reactivity (Fig.27e).

Since Helios expression is not limited to thymus derived Tregs[109,110,213] we looked for another set of markers to identify Treg subpopulations. Tregs express different levels of GITR and PD1 (Fig.27f). As GITR$^{\mathbf{high}}$, PD1$^{\mathbf{high}}$ Tregs express more CD25 than GITR$^{\mathbf{low}}$, PD1$^{\mathbf{low}}$ Tregs, we refer to these populations as Triple$^{\mathbf{high}}$ (GITR$^{\mathbf{high}}$, PD1$^{\mathbf{high}}$, CD25$^{\mathbf{high}}$) and Triple$^{\mathbf{low}}$ (GITR$^{\mathbf{low}}$, PD1$^{\mathbf{low}}$, CD25$^{\mathbf{low}}$) Tregs, respectively. Compared to Triple$^{\mathbf{low}}$ Tregs, their Triple$^{\mathbf{high}}$ counterparts express more Helios, CD5 and Nur77-GFP[86]; higher expression of the latter two markers indicates a high degree of self-reactivity as a consequence of expressing high affinity TCRs (Fig.27f). Finally, similar to Helios^{+} Tregs, Triple$^{\mathbf{high}}$ Tregs preferentially proliferate and survive on syngeneic MHC II expressing BM-DCs, consistent with a high degree of self-reactivity (Fig.27f).[86]

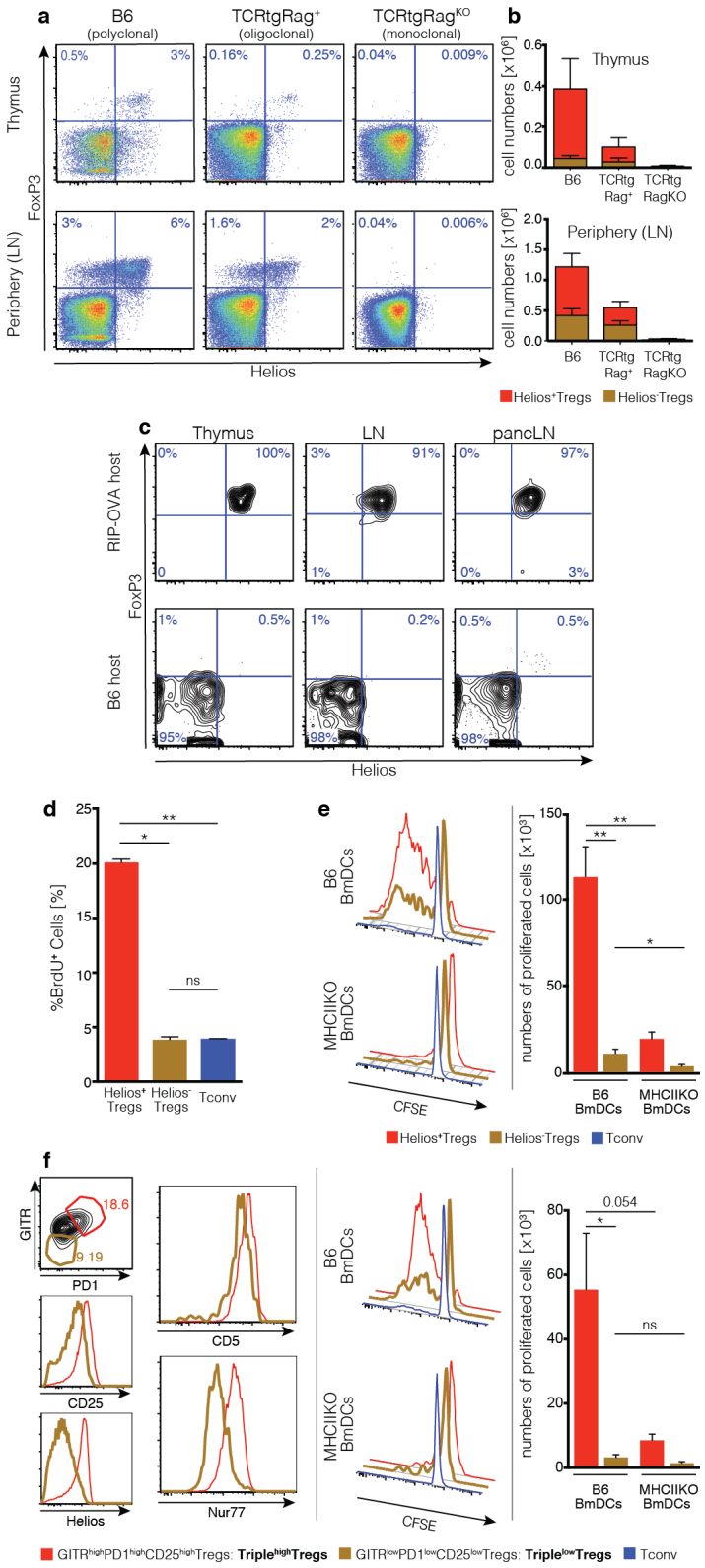

Figure 27: Majority of Tregs are self-reactive.
a) Flow cytometric analysis of FoxP3 and Helios expression on CD4SP thymocytes (upper row) and $CD4^+$ LN T cells (lower row) from C57BL6, 3BK506$TCR^{tg}Rag^+$ ($TCR^{tg}Rag^+$) and 3BK506$TCR^{tg}Rag^{KO}$ ($TCR^{tg}Rag^{KO}$) mice. **b)** Numbers of $CD4^+FoxP3^+Helios^+$ (red) and $CD4^+FoxP3^+Helios^-$ (brown) thymocytes (upper graph) and LN T cells (lower graph) from C57BL/6, $TCR^{tg}Rag^+$ and $TCR^{tg}Rag^{KO}$ mice, n=4. **c)**. Flow cytometric analysis of FoxP3 and Helios expression in OT-II derived, CD4SP thymocytes, peripheral LN and pancreatic LN cells of lethally irradiated RIP-OVA (upper row) and C57BL6 (lower row) recipients reconstituted with bone marrow cells from B6Ly5.1 and Ly5.2 OT-II mice (9:1) **d)** In vivo proliferation of $CD4^+$ Tcells in C57BL6 mice. Percentages of proliferating ($BrdU^+$) cells are shown, n=2. **e)** In vitro proliferation of $CD4^+$ T cells on syngeneic BmDCs. Histograms (left) shows representative data for CFSE dilution of $Helios^+$ Treg (red) $Helios^-$ Treg (brown) and $CD4^+$ Tconv (blue) cells. Bar graph (right) shows numbers of proliferating cells, n=6. **f)** Left panel: Flow cytometric analysis of GITR and PD1 expression on $CD4^+$/FoxP3GFP$^+$ cells. Histograms show Helios, CD25, CD5 and Nur77-GFP expression on $GITR^{high}$ $PD1^{high}$ Tregs (red, $Triple^{high}$ Tregs) and $GITR^{low}$ $PD1^{low}$ Tregs (brown, $Triple^{low}$ Tregs). Middle panel: Histograms show representative data of CFSE dilution of $Triple^{high}$ Tregs (red) $Triple^{low}$ Tregs (brown) and $CD4^+$ Tconvs (blue) cultured on B6 or B6.MHCIIKO BmDCs, n=6. Right Panel: Bar graph (right panel) shows numbers of proliferating cells, n=6. *p≤ 0.05, **p≤ 0.01, ***p≤0.001, ****p≤ 0.0001 (Student's t test). Error bars indicate means ± SEM.

Peptide affinity influences the development of Triple^{high} and Triple^{low} Tregs (Fig.28). When B3K508 Rag$^{-/-}$ thymocytes were cultured on BmDCs in presence of TGFβ and IL-2, addition of the threshold ligand, P-1A, induced development of Triple^{low} CD4 SP thymocytes and Tregs, while the higher affinity P2A induced a mixture of Triple^{low} (PD1^{high}, GITR^{low}) and Triple^{high} (PD1^{high}, GITR^{high}) CD4SP thymocytes and Tregs; finally the highest affinity peptide, 3K induced only Triple^{high} (PD1^{high}, GITR^{high}) CD4SP thymocytes and Tregs. These data indicate that antigens with affinities just over the negative selection threshold induce the Triple^{low} phenotype, while antigens with affinities significantly higher than the negative selection threshold induce the Triple^{high} phenotype (Fig.28a). Interestingly, antigens inducing Triple^{high} development induce both ERK and cJun phosphorylation, while cells undergoing Triple^{low} development contain pERK but not pcJun (Fig.28b,c).

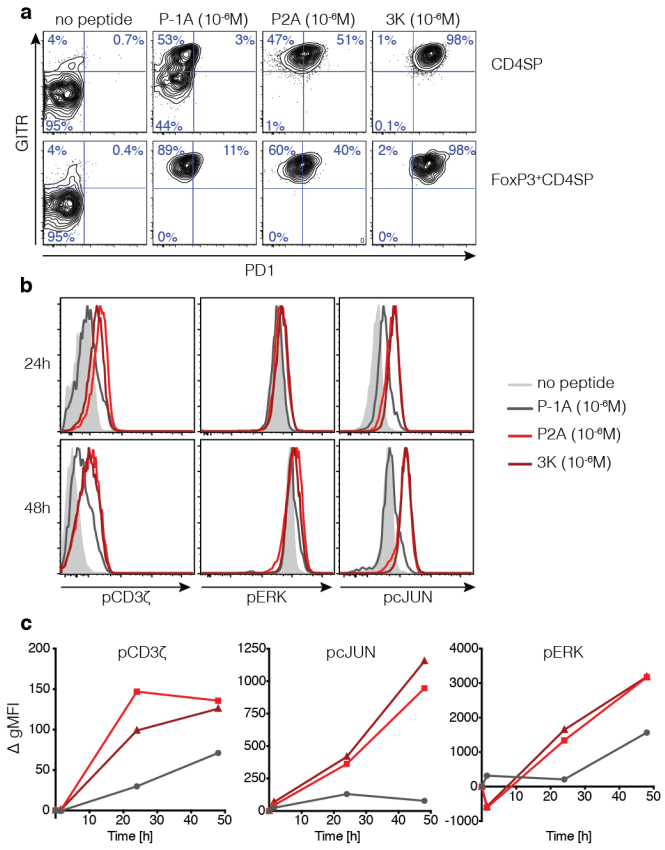

Figure 28: Development of Triple^{high} and Triple^{low} Tregs is dependent on TCR affinity.
a) Representative flow cytometric analysis of GITR and PD1 expression on 3BK508tg CD4SP (upper row) or 3BK508tg CD4SP FoxP3^{+} thymocytes 48h after stimulation with P-1A, P2A, 3K or no peptide presented on mature B6 BmDCs in presence of IL2 and TGFβ. (n=3). **b,c)** Phosphorylation of CD3ζ, ERK and cJUN in 3BK508tg CD4SP stimulated with P-1A (dark grey line), P2A (bright red line), 3K (dark red line) or no peptide (light grey filled) was determined after 90min, 24 and 48h by flow cytometry. **b)** Representative histograms of fluorescence intensity in thymocytes after 24 and 48h of antigen stimulation and **c)** geometric mean fluorescence intensity after 90min, 24h and 48h. (n=1-2)

Given their differing expression of CD5 and Nur77-GFP (Fig.27f), Triple^{high} and Triple^{low} Tregs likely express different TCR repertoires. The frequencies of Triple^{high} and Triple^{low} Tregs in polyclonal and oligoclonal (single TCR β chain transgenic) mice were compared. Among thymic Tregs, there is a preponderance of Triple^{high} Tregs in both strains (Fig.29 a-c). However, among peripheral Tregs, the frequency of Triple^{low} Tregs was increased in polyclonal B6 mice, but not in oligoclonal, single TCR β chain (OT-I Vβ5) transgenic mice (Fig.29 a-c). Constraining TCR repertoire diversity clearly affects the frequencies of Triple^{high} and Triple^{low} Tregs. Furthermore, analysis of Triple^{high} Tregs, Triple^{low} Tregs and Tconvs in single TCR β chain transgenic mice shows a distinct pattern of Vα expression.

The Vα2 and Vα3 families, but not the Vα8 family were expressed at different frequencies within these three subsets of CD4 T cells (Fig.29d), indicating a difference in their TCR repertoires. More subtle differences of Vα expression between the three CD4 subsets were observed in polyclonal B6 mice (Fig.29d). To directly compare the TCR repertoires of Tconv and both types of Tregs, all three populations expressing the Vα2 family (Fig.29e), were sorted from a second, independent single TCR β chain strain (Yae62, Vβ8.1, TCRα$^{+/KO}$**)** and subjected to deep sequencing. The 500 most frequent clonotypes in each group were analyzed for their similarity (Fig.29f-h) and diversity (Fig.29i-j). Morisita-Horn analysis shows that the Tconv sequences from 3 individual mice are similar to each other but significantly different from Triple^{low} and Triple^{high} Treg sequences (Fig.29f). This was observed with Triple^{low} sequences as well (Fig.29g). In contrast, the Triple^{high} TCR sequences from three individual mice are different from each other and different from Tconv and Triple^{low} sequences (Fig.29h). Despite their significant sequence differences, the TCR repertoires of Tconvs and Triple^{lows} are similarly diverse (Fig.29 i,j). The repertoire of Triple^{high} Treg TCRs may be less diverse, according to Shannon Entropy analysis. Deep sequencing showed that Triple^{high} Tregs, Triple^{low} Tregs and Tconvs have clearly distinct TCR repertoires, implying that TCR specificity is important in selecting these Treg subtypes.

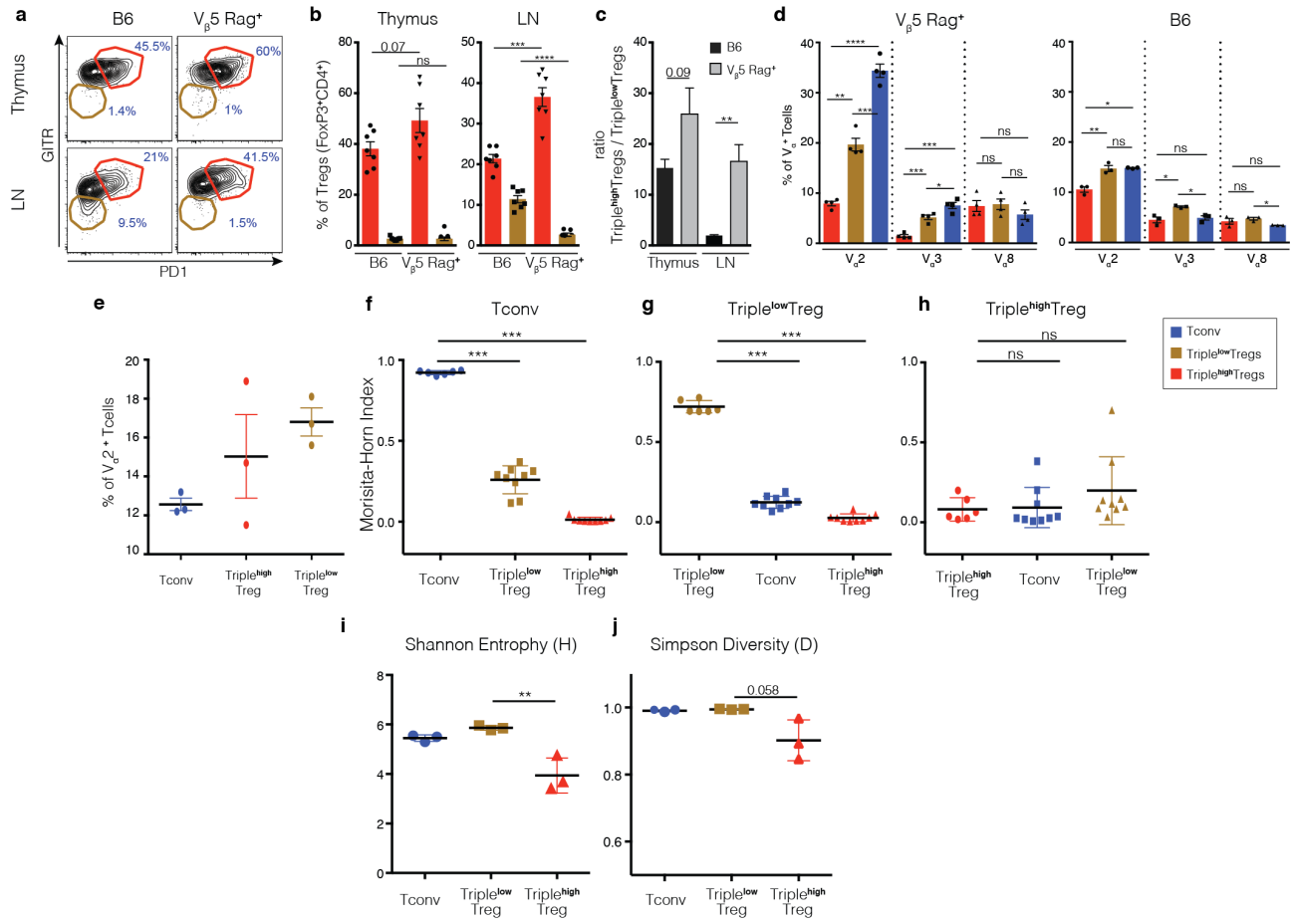

Figure 29: Triple^{high} and Triple^{low} Tregs express different TCR repertoires.
Flow cytometric analysis of GITR and PD1 expression in FoxP3^{+}CD4SP thymocytes and FoxP3^{+}CD4^{+} LN cells of B6 and single TCRβ chain (OT-I Vβ5^{tg}, Rag^{+}) mice. **a)** Representative contour plots, **b)** percentage of Triple^{high} (red) and Triple^{low} (brown) Treg cells and **c)** ratio between Triple^{high} and Triple^{low} Treg cells (n=7) **d)** Vα expression in Triple^{high} Tregs (red), Triple^{low} Tregs (brown) and CD4^{+} Tconvs (blue) from isolated from splenocytes, n=4. **p≤ 0.01, ***p≤ 0.001, ****p≤ 0.0001 (Student's t test) ns = not significant. Error bars show mean ± SEM. e) Vα2 expression in CD4 Tconv (blue), Triple^{high} (red) and Triple^{low} (brown) Tregs. **f)** Morisita-Horn similarity analysis of Vα2^{+} TCR clonotypes from CD4 Tconvs (blue) compared to Triple^{low} (brown) Treg and Triple^{high} (red) Treg clonotypes from three individual, single TCRβ chain (YAe62tg, TCRα$^{+/KO}$, Rag^{+}) mice. For the Morisita-Horn Index, values of 0 and 1 represent minimal and maximal similarity, respectively (see Methods for full description). **g)** Morisita-Horn similarity analysis comparing Vα2^{+} TCR clonotypes from Triple^{low} Tregs (brown) to CD4 Tconvs (blue) and Triple^{high} Treg (red). **h)** Morisita-Horn similarity analysis comparing Vα2^{+} TCR clonotypes from Triple^{high} Tregs (red) to CD4 Tconvs (blue) and Triple^{low} Treg (brown). TCR diversity was analyzed by calculating Shannon Entropy **(i)** and Simpson Diversity **(j)** scores. For Shannon Entropy analysis, higher H values indicated higher diversity; for Simpson Diversity analysis, the index ranges from 0 to 1 with 1 indicating high diversity (see Methods for full description). *p≤ 0.05, **p≤ 0.01, (Mann-Whittney U Test) ns = not significant. Error bars show mean ± SEM.

To compare the regulatory properties of these two populations, FoxP3-DTR mice were acutely depleted of Tregs by injecting DTx; sorted Triple^{high} or Triple^{low} Tregs from B6 mice, which are unaffected by DTx were then transferred to these recipients (Fig.S3). Triple^{high} Tregs control the extensive proliferation of Tcells and B cells in peripheral LNs of mice depleted of their endogenous Tregs (Fig.30a), while Triple^{low} Tregs function poorly in this respect. In addition to controlling lymphocyte proliferation, Triple^{high} Tregs limit the activation of Tconvs (Fig.30b). Taken together, these data show that Triple^{high} Tregs, a subset expressing highly self-reactive TCRs is effective in regulating the homeostatic activity of lymphocytes in peripheral LNs.

To examine the ability of these Treg subsets to control colitis, CD3^{KO} mice were injected with sorted CD4 Tconvs (Fig.S4), which as previously described[175, 207, 208] lost weight (Fig.30c, upper panel, solid blue line) and developed colitis (Fig.30d, upper left panel). Co-transfer of Triple^{low} (Fig.30c, upper panel, solid brown line; Fig.30d, upper middle panel) but not Triple^{high} (Fig.30c, upper panel, solid red line; Fig.30d, upper right panel) Tregs prevented weight loss and limited lymphocyte infiltration of the colonic mucosa. Analysis of LN cells from these mice indicated that co-transfer of Triple^{low} Tregs facilitated the conversion of some Tconv into induced Treg cells (iTregs) (Fig.30e,f). Mice receiving Triple^{low} Tregs had the highest percentage of iTregs (Fig.30e,f), only limited infiltration in the colonic mucosa (Fig.30d, upper middle panel) and maintained their weight (Fig.30c).

To test whether iTregs were required to control colitis, CD4 Tconv cells isolated from FoxP3-DTR mice were transferred into CD3 KO mice (Fig.S6). These animals were treated with DTx to deplete any iTregs developing from Tconv. iTreg depletion accelerated weight loss and development of colitis (compare solid blue (upper panel) and dashed blue (lower panel) lines in Fig.30c). Co-transferred B6 Triple^{low} Tregs (unaffected by DTx) were unable to control the development of colitis when iTregs were depleted (compare solid brown (upper panel) and dashed brown (lower panel) lines in Fig.30c; compare upper middle and lower middle panels in Fig.30d). The data support the idea that Triple^{low} Tregs facilitate conversion of some CD4 Tconv into FoxP3^{+} iTregs, which in aggregate limit development of colitis. Taken together, the data argue for two populations of Tregs: Triple^{highs}, which control lymphoproliferation in peripheral LNs and Triple^{lows}, which limit the development of colitis (at least in a lymphopenic setting).

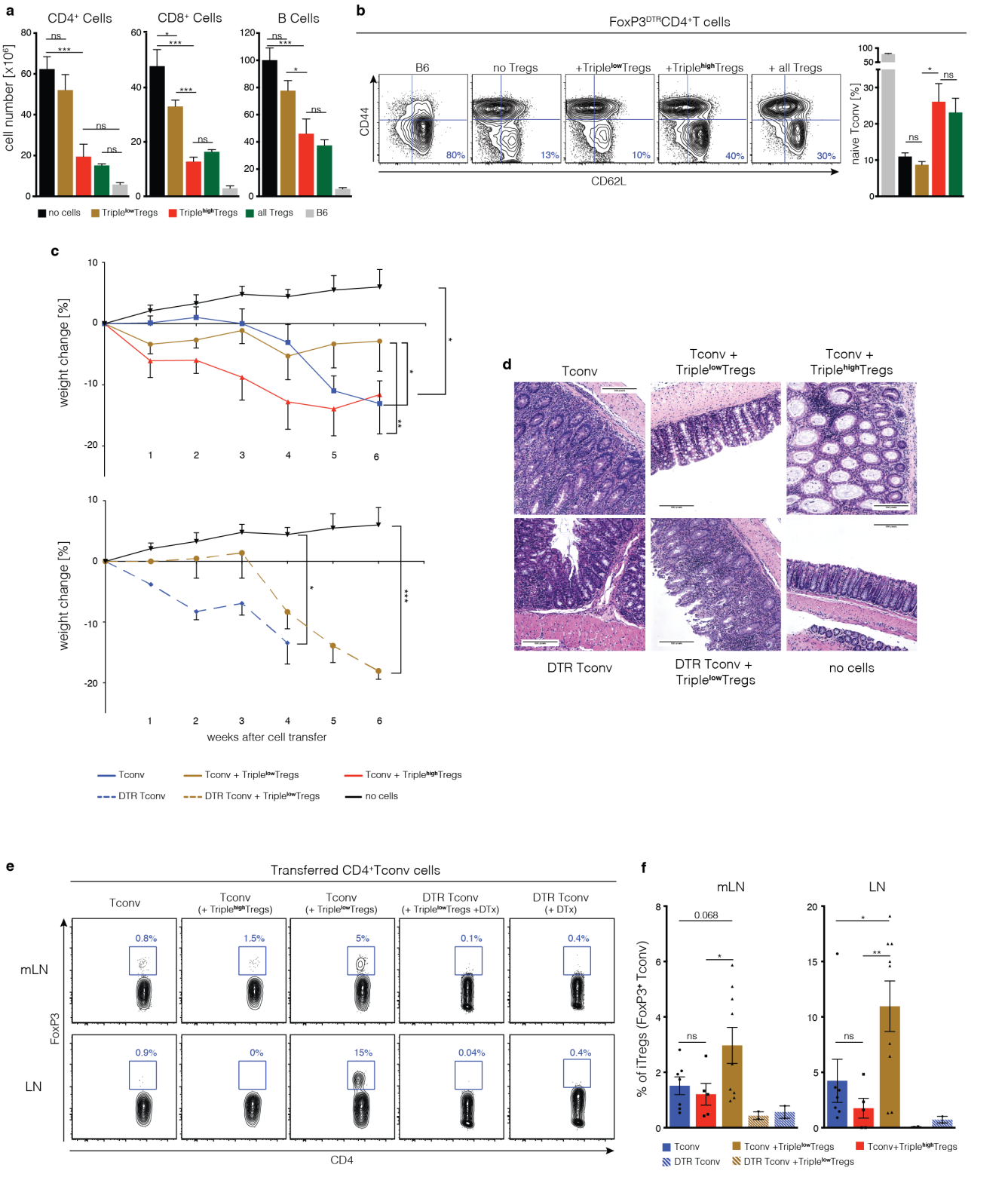

Figure 30: Triple^{high}Tregs and Triple^{low}Tregs have different suppressive functions *in vivo*

a) Ly5.1 FoxP3 DTR host mice were injected i.v. with no cells (black, n=10), B6 Triple^{low} Tregs (brown, n=4), B6 Triple^{high} Tregs (red, n=6), or total B6 Tregs (green, n=3). FoxP3-DTR recipients and B6 control mice (gray, n=4) were treated every other day with diphtheria toxin (DTx) and analyzed at d11-13 after cell transfer. Bar graphs show numbers of host-derived, live, CD4$^+$, CD8$^+$ and B cells in peripheral LNs. *p≤ 0.05, **p≤ 0.01, ***p≤0.001, ****p≤ 0.0001 (Student's t test). Error bars indicate means ± SEM. **b)** Flow cytometric contour plots and bar graph show the % of naïve CD4 Tconvs (CD44) in mice described in a). n≥4 **c)** Upper graph shows percentage of weight change of CD3^{KO} mice injected with no cells (black, n=5), Ly5.1 B6 CD4$^+$ Tconvs (blue, n=9) or Ly5.1 B6 CD4$^+$ Tconvs co-transferred with Ly5.2 Triple^{low} Tregs (brown, n=9) or Triple^{high} Tregs (red, n=6). Lower graph, Two additional groups received either Ly5.1 FoxP3DTR CD4 Tconvs alone (dashed blue, n=3) or Ly5.1 FoxP3DTR CD4 Tconvs co-transferred with B6 Triple^{low} Tregs (dashed brown, n=3) and no cells (black, n=5). Statistical analysis compares difference of weight change at 6 weeks following cell transfer. *p≤ 0.05, **p≤ 0.01(Student's t test). **d)** Representative H&E staining of the colon from CD3^{KO} mice adoptively transferred with cell populations indicated in **c). e)** Flow cytometric analysis of Ly5.1 B6 Tconv or Ly5.1 DTR Tconv cells six weeks after transfer from mice described in **c).** Contour plots show CD4/FoxP3 staining of transferred Tconv cells isolated from mesenteric or peripheral LNs. **f)** Bar graph shows percentage of Tconv-derived induced Tregs (iTregs) found in mesenteric or peripheral LNs. *p≤ 0.05, **p≤ 0.01, ****p≤ 0.0001 (Student's t test) ns = not significant. Error bars show mean ± SEM.

FoxP3 KO (scurfy) mice contained analogous populations of CD4 T cells despite their lack of a functional FoxP3 molecule. These mice contain GITR^{high}, PD1^{high}, CD25^{high} (scurfy Triple^{high}) and GITR^{low}, PD1^{low}, CD25^{low} (scurfy Triple^{low}) CD4 T cells. Scurfy Triple^{high} T cells resembled B6 Triple^{high} Tregs in terms of PD1, GITR, CD25, Helios, CD5 and CD62L expression (Fig.31a). Given their lack of FoxP3 expression and suppressive capacity, these cells can be described as Triple^{high} Treg "wannabes". On the other hand, scurfy Triple^{low} T cells resembled B6 CD4 Tconv cells with respect to their expression of these markers (Fig.31a).

To investigate their pathological activities, scurfy Triple^{low} and scurfy Triple^{high} CD4 T cells were separately transferred into T cell deficient, CD3 KO hosts (Fig.S7). Scurfy Triple^{low} T cells promoted weight loss and colitis (Fig.31b,e). Moreover, they accumulate in mesenteric LNs (Fig.31c,d) where ~35% express α4β7, an integrin that enables homing to the gut (Fig.S8). In contrast, scurfy Triple^{high} do not cause weight loss (Fig.31b) and preferentially accumulate in peripheral but not mesenteric LNs (Fig.31c,d). Moreover, scurfy Triple^{high} T cells induce massive inflammation in the skin but only minimal inflammation in the colon (Fig.31e). The behavior of Triple^{high} Treg wannabes is reminiscent of bona fide Triple^{high} Tregs. Both cell types are CD5 ^{high}, Helios ^{high}, are likely survivors of negative selection, express high affinity self-reactive TCRs and preferentially accumulate in peripheral LNs. However, in the absence of functional FoxP3, scurfy Triple^{high} cells expand and induce skin inflammation (Fig.31e). These results suggest that the autoimmune pathology in scurfy mice may be a combination of two diseases. Lymphocyte infiltration into the skin is likely caused by scurfy Triple^{high} cells, while colitis is induced primarily by scurfy Triple^{lows}. A Treg-like population similar to scurfy Triple^{high} cells has been observed[173, 174], but here we demonstrated their ability to cause skin pathology. Scurfy Triple^{lows} may be related to Triple^{low} Tregs or may be the equivalent of Tconv, which can also cause colitis.[175, 208]

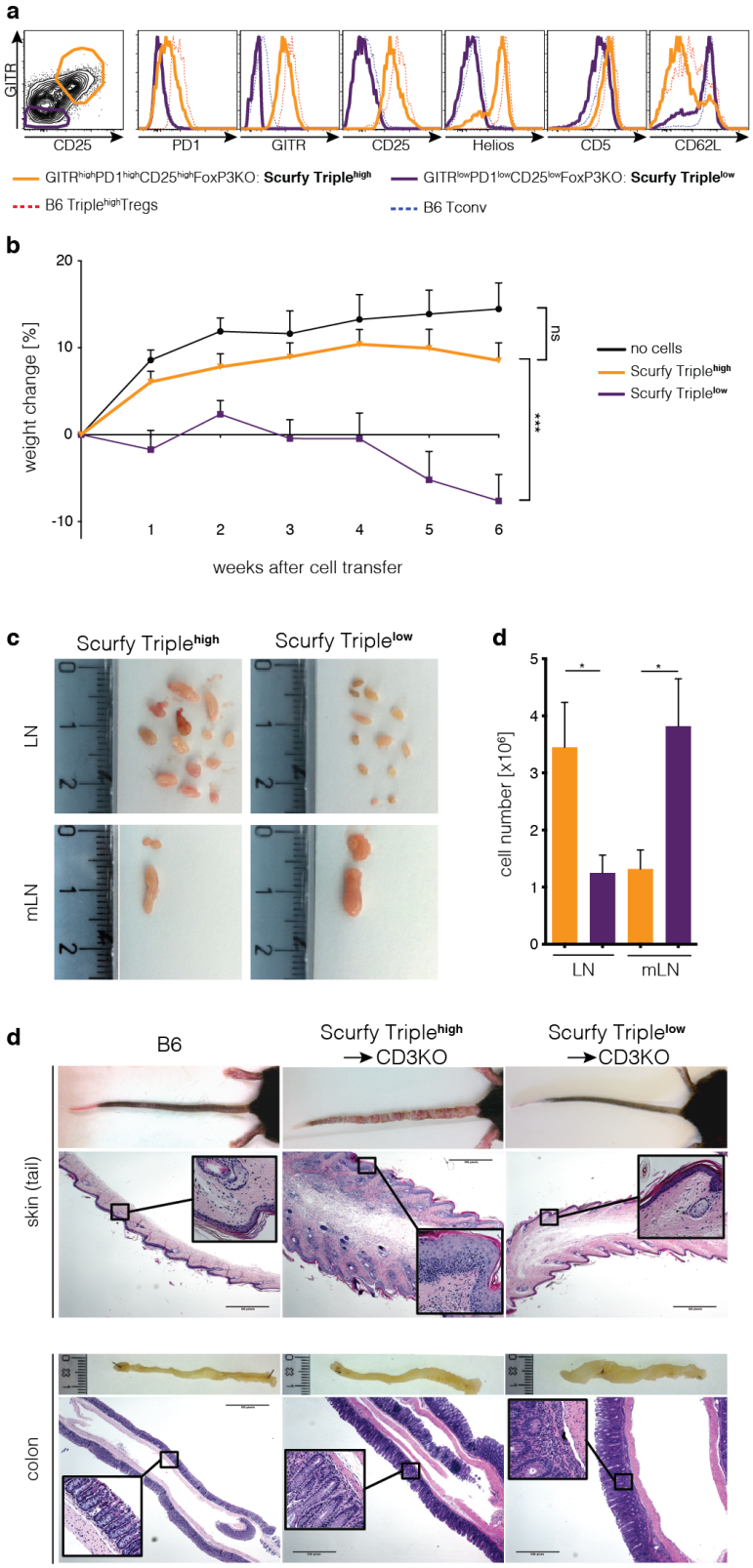

Figure 31: Scurfy Triple^{high} and scurfy Triple^{low} CD4 T cells induce different pathologies.
a) FoxP3^{KO} (Scurfy) mice contain Treg like cells. Left panel: Flow cytometric analysis of CD4 T cells showing scurfy Triple^{high} (PD1^{high} GITR^{high} CD25^{high}; orange gate) and scurfy Triple^{low} (PD1^{neg} GITR^{neg} CD25^{neg}; purple gate) CD4 T cells. Additional panels: PD1, GITR, CD25, Helios, CD5, CD62L and CD44 expression in scurfy Triple^{high} (orange solid), scurfy Triple^{low}, (purple solid), B6 Triple^{high} Tregs (dotted red) and B6 CD4 Tconv (dotted blue) cells. **b)** Graph shows percentage of weight change of CD3^{KO} recipients following adoptive transfer of no (black, n=3), scurfy Triple^{high} (orange, n=8) or scurfy Triple^{low} (purple, n=8) CD4 T cells. **c)** Photographs of peripheral and mesenteric LNs from CD3^{KO} mice transferred with scurfy Triple^{high} (orange, n=8) or scurfy Triple^{low} CD4 T cells **d)** absolute number of scurfy CD4 cells in peripheral LNs and mLNs six week after cell transfer. **e)** Representative photographs and H&E staining of tail skin and colons of CD3^{KO} recipient six weeks after adoptive cell transfer. B6 control mice are shown as well. Results are representative of 3 replicate experiments. *p≤ 0.05, ***p≤ 0.001 (Student's t test) ns = not significant. Error bars show mean ± SEM.

In summary, these data suggest that Triple^{high} and Triple^{low} Tregs are generated as an offshoot of negative selection, but their different TCR affinities for self-antigens induce distinct differentiation programs generating these two Treg populations. The high affinity self-reactive TCRs expressed by Triple^{high} Tregs likely drives their selection in the thymus and their proliferation and suppressive activity in peripheral LNs.[30, 214] On the other hand, the lower (but above threshold) affinity self-reactive TCR repertoire expressed by Triple^{low} Tregs allows them to develop the capacity to maintain immunological homeostasis in the colon by facilitating the generation of iTregs from Tconvs. It's not clear if the lower affinity repertoire of Triple^{low} Tregs is already enriched for commensal specificities or more plausibly, commensal specific Triple^{low} Tregs expand upon exposure to commensal antigen in the gut.[215]

Methods

Mice

All mice were between 5–12 weeks old and had a C57BL/6 genetic background except for FoxP3KO, which were used at 2 -3 weeks of age. CD45.1 congenic C57BL/6 (B6 Ly5.1), CD45.2 congenic C57BL/6 (B6), RIP-OVA mice expressing a membrane bound form of Ova under the control of the rat insulin promoter (RIP)[17,182,52] OTII TCRtg mice recognizing $IA^b/OVA_{323-339}$[183], B6 .Nur77-GFP[86] and FoxP3KO[174] were all obtained from The Jackson Laboratory (Bar Harbor, ME). 3BK506 TCRtg and 3BK508 TCRtg mice recognizing IA^b/3K and Triple KO mice deficient for MHC class II, invariant chain and Rag (referred here as MHCII KO) were provided by P. Marrack and J. Kappler (Denver, USA) and are described elsewhere[184]. FoxP3DTR[52] mice were kindly provided by A. Rudensky (New York, USA) FoxP3eGFP and $CD3\varepsilon^{-/-}$ were kindly provided by T. Rolink (Basel, Switzerland) and single TCR β chain (OT-I Vβ5) transgenic mice kindly provided by D. Zehn (Lausanne, Switzerland) and are described elsewhere.[185, 186,187] Mice were housed under specific pathogen-free conditions and bred in our colony (University Hospital Basel) in accordance with Cantonal and Federal laws of Switzerland. Animal protocols were approved by the Cantonal Veterinary Office of Basel-Stadt, Switzerland.

Flow Cytometry and cell sorting

Thymocytes and T cell were stained with LIVE/DEAD Fixable near-IR stain Kit (Invitrogen) and surface antibodies against CD3, CD4, CD5, CD8, CD19, CD25, CD44, CD45.1, CD45.2, CD62L, CD279 (PD1), CD357 (GITR), $V_\alpha2$, $V_\alpha3$, $V_\alpha8$, $V_\beta5$, $V_\beta8$ and α4β7. Intracellular staining for FoxP3, Helios, pcJun (S73), pCD3ζ (pY142) and pERK(P-p44/42 MAPK) was performed using the FoxP3 staining kit (eBioscience). For BrdU experiments, mice were injected with 1mg/d BrdU (5-bromodeoxyuridine, BD Bioscience) for 3 days and cells were then stained for incorporated BrdU using a BrdU Flow Kit (BD Bioscience) followed by staining for intracellular markers. All antibodies were purchased from BD Bioscience, BioLegend, eBioscience or CellSignaling Technology. For flow cytometric analysis, a FACS CantoII (BD Bioscience) and FlowJo software (TreeStar) were used. For cell isolation, $CD4^+$Tcells were enriched using Dynabeads® Untouched™ Mouse CD4 Cells Kit (Invitrogen) from cell suspensions from different sources (peripheral LN, mesenteric LN, spleen); subpopulations of enriched CD4 cells were further sorted on a FACSAriaIII or Influx cell sorter (BD Biosciences). Cell numbers were determined using AccuCheck Counting Beads (Invitrogen) according to manufacturer's instructions.

In vitro assays

Bone marrow derived DCs (BmDCs) were generated from bone marrow cells of 5-7 week old C57BL/6 or B6.MHCII KO mice. Bone marrow cells were cultured under maturation conditions for 10 days in full medium supplemented with GM-CSF (hybridoma supernatant). Autologous mixed lymphocyte reactions (auto-MLRs) were performed by co-culturing $1x10^5$ syngeneic (B6 or MHCII KO) BmDCs with $3x10^5$ CFSE labeled (Life Technologies) magnetic bead enriched CD4 cells (Dynabeads,

Invitrogen) in 96-well-U-shaped plates for 5 days. For in vitro, Treg development experiments, $1x10^5$ thymocytes from 3BK508tg mice were co-cultured with $1x10^5$ B6 BmDCs in the presence of IL2 (25U/ml, hybridoma supernatant) and recombinant mouse TGFβ1 (10ng/ml, R&D Systems) for 48h with or without 10^{-6}M 3K (FEAQKAKANKAV), P2A (FEAAKAKANKAVD) or P-1A (FAAQKAKANKAVD) peptides (all obtained from Eurogentec). All in vitro assays were performed at 37°C in 5% CO_2 using complete RPMI medium (GIBCO, Life Technologies).

Generation of bone marrow chimeric mice

For generating bone marrow chimeric mice, the protocol from Koehli et al.[17] was adapted. Recipient mice (CD45.1/2) were lethally irradiated with 900 rad (GammaCell, Best Theratronics, CA). Bone marrow cells from 5-8 week old C57BL/6 mice (CD45.1) and OT-II RagKO (CD45.2) were isolated and depleted of mature T cells. A mixture of 9:1 of C57BL/6 and OT-II RagKO bone marrow cells ($4x10^6$ total cells) were injected intravenously (i.v.) into irradiated recipient mice. Mice were analyzed 12-14 weeks after reconstitution and treated with antibiotics (Nopil, Mepha Pharma AG) in the drinking water until 2 weeks before analysis. The congenic markers CD45.1 and CD45.2 were used to identify T cells derived from different donor bone marrows as well as the host.

In vivo suppression assays

FoxP3DTR mice were injected intra-peritoneally (i.p.) with Diptheria Toxin (DTx) (Calbiochem) every other day for 10-12 days (first and second injection 50μg/kg; subsequent injections 25μg/kg). In some groups, $2.5x10^5$ sorted Treg cells from pooled LNs were injected i.v. 3 days prior to first DTx injection. Mice were analyzed one day after last their DTx injection. For colitis experiments, 6-10 week old T cell deficient $CD3\varepsilon^{-/-}$ mice received (i.v.) $3.2x10^5$ sorted naïve CD4 T cells from pooled LNs of B6Ly5.1 ($CD4^+CD25^-$) or FoxP3DTR Ly5.1 ($CD4^+GFP^-$) mice. In some groups, $0.8\ x10^5$ sorted Treg cells from pooled LN were co-transferred. Recipients of naïve FoxP3DTR CD4 Tcells ($CD4^+GFP^-$) were injected every third day with DTx (10μg/kg), i.p.. For adoptive transfer of scurfy CD4 T cells, 6-10 week old T cell deficient $CD3\varepsilon^{-/-}$ were reconstituted with $5x10^5$ sorted CD4 subpopulations from pooled LNs of 2-3 week old sick (scurfy) FoxP3KO male mice. Recipient mice were weighed weekly at the same daytime and sacrificed when initial body weight droped more than 20% or at the latest six weeks after T cell transfer. The congenic markers, Ly5.1 and Ly5.2 were used to identify T cells from the different donors as well the host. Tissue samples were fixed in 4% paraformaldehyde, embedded in parafin, sectioned and stained with hematoxylin and eosin.

Reaggregate thymic organ cultures

Reaggregate thymic organ cultures were established from B3K508, MHC II KO thymocytes, which are arrested at the $CD4^+$ $CD8^+$ DP stage and thymic epithelial cells from B6 mice. Peptides were added at the following concentrations: 20 μM P-1A, 2 μM P2A and 0.2 μM 3K. After 7d in culture, thymocytes were stained for CD4 and FoxP3 and analyzed by flow cytometry.

Clonotype Analysis of GITR^{low} PD-1^{low} versus GITR^{high} PD-1^{high} peripheral Tregs.

Naïve CD4^{+} (CD4^{+} CD25^{-} Foxp3^{-}), Triple^{low} T_{reg} (CD4^{+} CD25^{low} Foxp3^{+} GITR^{low} PD-1^{low}) or Triple^{high} T_{reg} (CD4^{+} CD25^{high} Foxp3^{+} GITR^{high} PD-1^{high}) T cell populations were sorted from 3 replicate groups (2 mice per group) of single TCRβ chain transgenic (B6.YAe62βtg^{+} TCRα$^{+/-}$) mice were sorted to 98% purity (FACS Aria, BD Biosciences). RNA was isolated using Trizol and precipitated with RNase free glycogen (Invitrogen) following the manufactures protocol. cDNA was prepared using oligo-dT's (Promega) and Omniscript RT kit (Qiagen). cDNA was amplified with 20 rounds PCR with generic Vα2 primer (5'-CCCTGGGGAAGGCCCTGCTCTCCTGATA-3') and TCR Cα primer (5'-GGTACACAGCAGGTTCTGGGTTCTGGATG-3'). 1/10th volume of the first round PCR was amplified with an additional 20 rounds of PCR using barcoded primers, for post sequence identification of originating T cell population, containing Illunima PE read primer and P5/7 regions, respectively. The resulting 300bp fragment was gel purified (Gene Clean II, MP Biomedicals) and sequenced on a MiSeq using a single read 250bp run (Illumina). Sequence data sets were parsed by barcode using the program fastq-multx[216] and clonotypes for each population were tabulated using TCRklass[217].

Similarity and Diversity of TCR clonotypes

The similarity of TCRs utilized within each population was quantified using the Morisita-Horn similarity index, 0 (minimal similarity) and 1 (maximal similarity). The Morisita-Horn (M-H) similarity indexes were calculated by tabulating the frequency in which the top 500 clonotypes of an individual population from one replicate sample was found in all other populations, using EstimateS Ver9.1.0[218] software. Statistical significance for M-H index values was assessed using a Mann-Whittney U test, GraphPad Prism version 6.04. The diversity of TCR repertoire for each population was measured using the top 500 most frequent clonotypes. The Shannon Entropy[219] value for each sample was calculated as $H = -\Sigma p_i \log_2 p_i$, where p_i is the frequency of the clonotype within the top 500 clonotypes. Lower H values indicate lower diversity. Additionally, the Simpson's diversity index[220] using the formula $D_s = 1 - \Sigma[n_i(n_i - 1)]/[N(N - 1)]$, where n_i is the TCR clone size of the ith clonotype and N is the total number of the top 500 clonotypes sampled. The index ranges from 0 to 1 with 1 indicating high diversity.

Further Statistical analysis

Statistical analysis were performed using Prism 6.0 (Graphpad software). Students t test was used to asses statistical significiance. P-values ≤ 0.05 were considered significant (*p≤ 0.05, **p≤ 0.01, ***p≤0.001, ****p≤ 0.0001) P values >0.05; non-significant (ns)

Supplementary Data

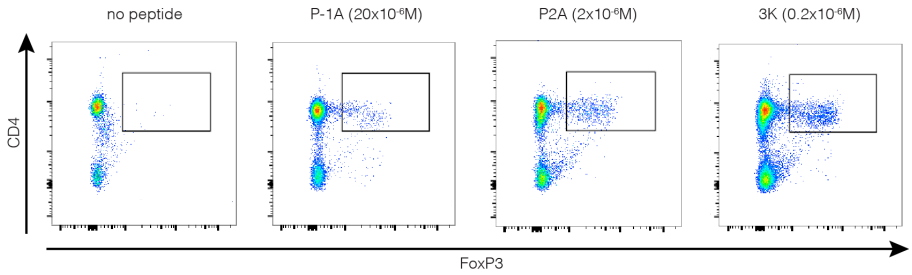

Figure S1: Thymic Tregs are developing in presence of negative selecting peptides but not with endogenous positive selecting peptides. Reaggregate thymic organ cultures were established from B3K508, MHC II KO thymocytes, which are arrested at the $CD4^+$ $CD8^+$ DP stage and thymic epithelial cells from B6 mice. Peptides were added at the following concentrations: 20 µM P-1A, 2 µM P2A and 0.2 µM 3K. After 7d in culture, thymocytes were stained for CD4 and FoxP3 and analyzed by flow cytometry.

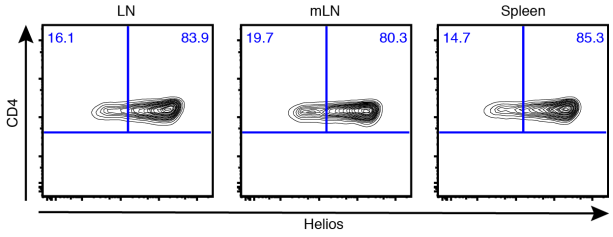

Figure S2: Majority of thymic derived Tregs expresses Helios
Representative contour plots of thymus derived, RFP^+GFP^+ Tregs sorted from lymph nodes (LN), mesenteric lymph nodes (mLN) or splenocytes. Each source of cells was pooled from four B6.FoxP3-GFP-Cre x FoxP3-IRES-RFP mice.[212] After sorting, cells were stained and analyzed for CD4 and Helios expression.

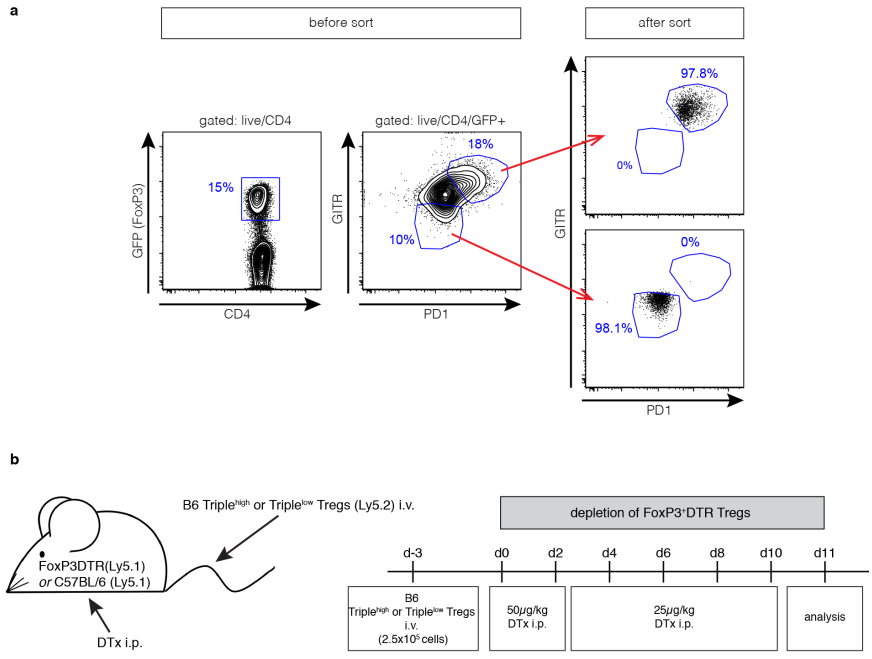

Figure S3: Sorting strategy to obtain $Triple^{high}$ and $Triple^{low}$ Tregs and experimental setup for Treg depletion and adoptive transfer into FoxP3DTR mice.
a) Magnetic bead enriched CD4 cells isolated from pooled lymph nodes from 6-10 week old B6.FoxP3eGFP mice were sorted for $CD4^+$ GFP^+ $GITR^{high}$ $PD1^{high}$ ($Triple^{high}$ Tregs) and $CD4^+$ GFP^+ $GITR^{low}$ $PD1^{low}$ ($Triple^{low}$ Tregs). Purity of both populations was > 95%. **b)** To induce lymphoproliferation, $FoxP3^+$ cells were depleted from 6-10 week old FoxP3DTR mice by intra peritoneal (i.p.) injection of DTx every other day for 10 days (first two injections, 50µg/kg; subsequent injections, 25µg/kg) In some groups, $2.5x10^5$ sorted B6 $Triple^{high}$ or B6 $Triple^{low}$ Tregs cells (unaffected by DTx) from pooled B6 LNs were injected intravenously (i.v.) 3 days prior to first DTx injection. Mice were analyzed one day after last DTx injection.

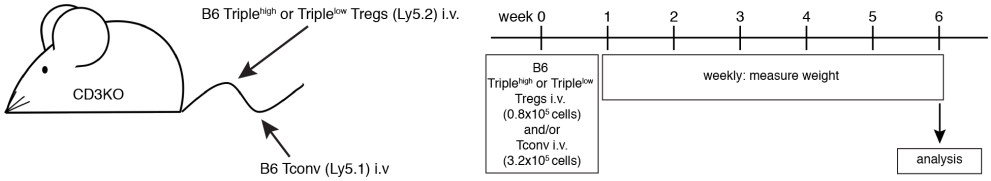

Figure S4: Experimental setup for colitis induction and prevention in lymphopenic CD3^{KO} mice.
For colitis experiments, 6-10 week old T cell deficient CD3^{KO} recipients received 3.2x10^5 sorted naïve CD4 Tconvs (CD4$^+$CD25$^-$) isolated from pooled LNs of several B6 Ly5.1 mice (B6 Tconv). In some groups, 0.8 x10^5 sorted Triple^{high} or Triple^{low} Tregs from pooled B6 LNs were co-transferred along with Tconvs. Mice were weighed weekly at the same time of day and were sacrificed when they lost > 20% of their initial body weight or at six weeks following adoptive transfer.

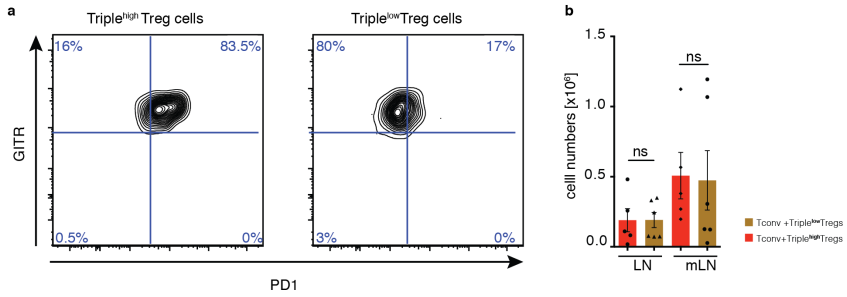

Figure S5: Triple^{high} and Triple^{low} Tregs keep their phenotype in inflammatory conditions
Analysis of Ly5.2 congenially labeled Triple^{high} and Triple^{low} Tregs isolated from CD3^{KO} mice, which were co-transferred with Ly5.1 congenially labeled CD4 Tconvs (CD4$^+$CD25$^-$) six weeks before. **a)** Representative contour plots of Triple^{high} (left panel) and Triple^{low} (right panel) Tregs isolated from LN, analyzed for GITR and PD1 expression. **b**) Cell numbers of Triple^{high} (red) and Triple^{low} (brown) Tregs isolated from LN and mLN n≥5. ns = not significant (Student's t test). Error bars show mean ± SEM.

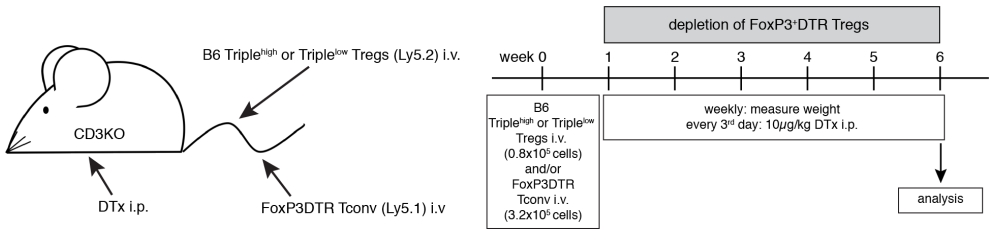

Figure S6: Experimental setup for depletion of iTregs generated during colitis induction.
To inhibit iTreg generation during colitis induction, 6-10 week old T cell deficient CD3^{KO} recipients were reconstituted with 3.2x10^5 sorted naïve CD4 Tconvs isolated from pooled LNs of several FoxP3DTR, Ly5.1 mice (FoxP3DTR Tconv). In some groups, 0.8 x10^5 sorted B6 Triple^{low} Tregs cells (unaffected by DTx) were co-transferred along with Tconvs. To deplete Tconv-derived iTregs, recipients were injected i.p. every third day with DTx (10µg/kg). Mice were weighed weekly at the same time of day and were sacrificed when they lost > 20% of their initial body weight or at six weeks following adoptive transfer.

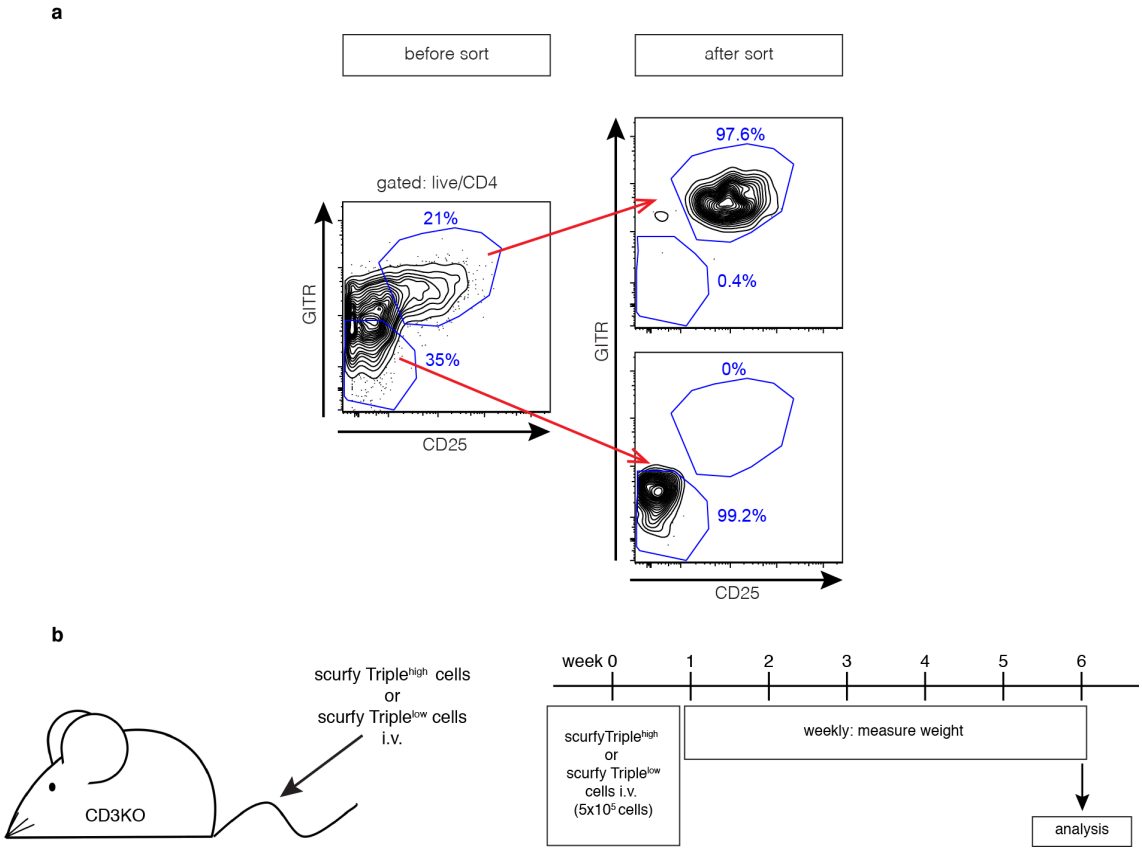

Figure S7: Sorting strategy and experimental setup for adoptive transfer of scurfy disease
a) Sorting strategy to obtain scurfy Triple^{high} and scurfy Triple^{low} CD4 T cells. Magnetic bead enriched CD4 T cells isolated from pooled lymph nodes from 2 week old, male FoxP3^{KO} mice were sorted for CD4^{+} GITR$^{\mathbf{high}}$ CD25$^{\mathbf{high}}$ (scurfy Triple$^{\mathbf{high}}$) and CD4^{+} GITR$^{\mathbf{low}}$ CD25$^{\mathbf{low}}$ (scurfy Triple$^{\mathbf{low}}$) CD4 T cells. Purity of each population was > 95%. **b)** To transfer scurfy disease, 5x10^{5} sorted scurfy Triple$^{\mathbf{high}}$ or scurfy Triple$^{\mathbf{low}}$ cells were transferred i.v. into 6-10 week old CD3$^{\mathbf{KO}}$ recipient mice. Mice were weighed weekly at the same time of day and were sacrificed when they lost > 20% of their initial body weight or at six weeks following adoptive transfer.

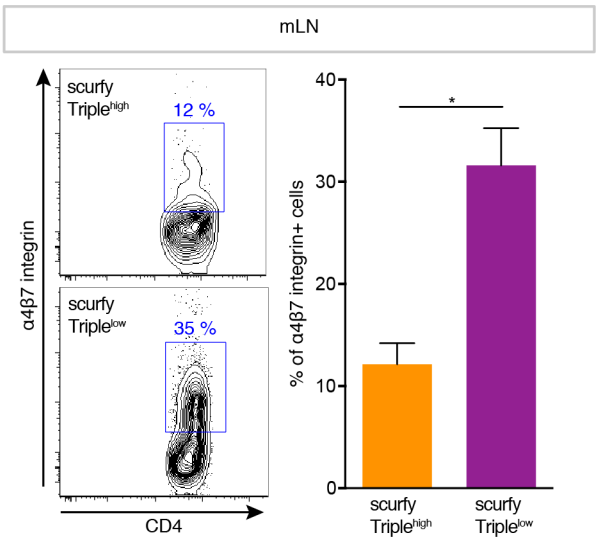

Figure S8: Scurfy Triple$^{\mathbf{low}}$ cells expressing high levels of α4β7 integrin preferentially home to mLNs.
Flow cytometric analysis of α4β7 integrin expression on live scurfy CD4 cells isolated from mesenteric lymph nodes (mLN) of CD3$^{\mathbf{KO}}$ mice six weeks after receiving either scurfy Triple^{high} (top panel, orange bar) or Triple^{low} (lower panel, purple bar) T cells, n=3. *p≤ 0.05 (Student's t test). Error bars show mean ± SEM.

6 Discussion

6.1 The FoxP3 transgenic (tg) mouse: A model to learn more about the development of different Treg cell subsets

In the fist part of the thesis, we re-evaluated and further characterized Treg cells from the FoxP3tg mouse (line 2826), which was generated in 2001 in the laboratory of Ziegler and Ramsdell, at the same time when they and others linked the scurfy disease to the severe autoimmune disease "IPEX" in humans.[48] In their initial study, they showed that the scurfy disease was rescued by crossing in the FoxP3tg. Even though these mice carried the scurfy mutation, they were healthy. This, result, among others, gave proved evidence that FoxP3 expressing cells are crucial for the function and development of suppressive regulatory T cells.[48] In studies where FoxP3tg mice were used, these mice were reported to be healthy, and no pathologies were reported.[48,50, 221-224] Surprisingly, when we bred FoxP3tg mice in our colony to a homozygous background we observed developmental deficiencies and an ataxic phenotype, which was detectable by a shaking gait and general difficulties of hind limb control. However, the pathology was most probably caused by either an integration of the transgene leading to an unexpected knockout when bred to a homozygous background or a muscular malfunction due to a disregulation of ErbB2 caused by the overexpression of FoxP3 in muscle cells. For both hypotheses we found some indication but not full evidence. Anyhow, by the reconstitution of lethally irradiated $Rag2^{-/-}$ with BM from FoxP3tg mice we excluded that the ataxic phenotype was caused by defect in the hematopoietic compartment. Furthermore, the adoptive transfer of $CD4^+$ T cells from sick FoxP3tg mice also did not induce the observed phenotype.

The generation and characterization of the FoxP3tg mouse provided further evidence that FoxP3 acts as a master regulator for Treg cells.[48] At that time, the analysis for Treg cells was based on the expression of CD25. Presently, FoxP3 is the most definitive marker for Treg cells. Since almost all studies, using the FoxP3tg mouse, were performed before the development of FoxP3 antibodies for flow cytometric analysis, we re-analysed the cellular phenotype of the heterozygous FoxP3tg mouse in the thymus and in secondary lymphoid organs. Our data confirms that FoxP3tg mice do not have obvious defects in thymocyte development. As reported previously, frequencies and numbers of CD4SP and CD8SP cells in the thymus of FoxP3tg mice are not altered. It was suggested that the transgene in these mice acts primarily on

peripheral T cells since they could not observe alterations in the thymi of these mice. We detected an almost ten-fold higher frequency of FoxP3 expressing CD4SP and CD8SP cells in the thymi of FoxP3tg, compared to B6 mice, indicating that the FoxP3tg is already active in thymocytes.

In line with previous reports, the frequencies and cell numbers of peripheral T cells are significantly lower. In secondary lymphoid organs, up to 80% of all $CD4^+$ T cells express FoxP3.[222-225]

Interestingly, although the majority of all $CD4^+$ T cells are $FoxP3^+$, we observed that the total numbers of $FoxP3^+CD4^+$ T cells are not much higher between FoxP3tg and B6 mice. Kasprowicz et al showed, that the overexpression of FoxP3 in the Foxp3tg mouse leads to an increased susceptibility to apoptosis following cell activation *in vitro*. The cell death was rescued by the administration of exogenous IL2.[222] The low T cell numbers in FoxP3tg mice could be explained by a higher death rate in addition with a lower IL2 availability in these mice. FoxP3 was shown to be a suppressor of IL2 transcription and in line with that, FoxP3tg $CD4^+$ T cells only secrete little IL2 after TCR stimulation.[50] According to the small numbers of Tconv cells in this strain, IL2 could be the limiting factor for the population size *in vivo* under homeostatic conditions. Evidence for that is given by two reports. First, it was suggested that IL2 producing Tconv cells can sense and response to the IL2 levels and with that coordinate the homeostasis of activated Tconv cells and Treg cells.[154] Second, Treg cells in the periphery can alter their proliferative and apoptotic rate dependent on the availability of IL2 under homeostatic conditions.[226] In addition, we observed a higher frequency of B cells in the lymph nodes of FoxP3tg mice. The total B cell number was not significantly different between FoxP3tg and B6 mice and the higher frequency was only “virtually”, caused by the lower numbers of T cells in FoxP3tg mice. We showed that the observed reduced frequencies of CD4 and CD8 T cells found in FoxP3tg mice are caused by cell intrinsic and not by cell extrinsic mechanisms. We considered the idea that the overexpression of FoxP3 could influence cells other than lymphocytes based on the observation made with homozygous FoxP3tg mice. Additionally, another group suggested that FoxP3 is expressed on several epithelial cells and impacts the thymocyte development.[190, 191] However, studies from the Rudensky group but also our results from the BM chimeras of $Rag2^{-/-}$ mice reconstituted with FoxP3tg BM revealed this idea and showed that in T cells, FoxP3 acts cell intrinsically.[227]

Functional analysis of FoxP3tg T cells demonstrated that the overexpression of FoxP3 is sufficient to induce suppressive function in $CD4^+$ and to some extend in $CD8^+$ T cells *in vitro*. Furthermore, breeding the scurfy or $IL2R\beta^{-/-}$ to the FoxP3tg genetic background rescue these mice from the development of multi organ inflammation *in vivo*.[48, 50, 221, 225] The rescue of these mice does not fully imply the suppressive capacities of FoxP3tg cells *in vivo*. T cells of FoxP3tg mice are functionally impaired and reduced in numbers. Thus, the suppression of the multi organ inflammation could be caused by the reduced numbers of mature T cells and/ or their functional impaired phenotype and not by the suppressive function of FoxP3tg Treg cells per se.[221] Therefore, suppressive function of FoxP3tg cells was not fully assessed yet *in vivo*. Our results from an allogeneic skin graft transplantation model showed that FoxP3tg cells are highly suppressive *in vivo* and can mediate long term tolerance to an allogeneic skin graft for up to 100 days even after challenging with *in vivo* primed, graft specific CD4 effector T cells.

Phenotypically, Treg cells from FoxP3tg mice are somewhat in between Tconv and Treg cells from B6 mice. In unmanipulated mice, $CD4^+FoxP3^+$ Treg cells are characterized by the expression of $CD25^{high}$, $GITR^{high}$, $Helios^{high}$, $NRP1^{high}$, $PD1^{high}$ and have a more activated phenotype compared to B6.Tconv cells. In FoxP3tg Treg cells, the expression of CD25 and GITR is only slightly upregulated. This was surprising because FoxP3 directly induces the expression of CD25 and GITR.[59] Also the expression of Helios and NRP1 in FoxP3tg Treg cells resembles more B6.Tconv than B6.Treg cells. Helios and Nrp1 are two controversially discussed markers to identify Treg cells that have developed in the thymus.[78, 107, 113] After a long fight, Helios is suggested to be a more reliable marker for tTreg cells.[116] Anyhow, in line with previous studies,[78, 107, 113] the majority of B6 Treg cells are generated in the thymus and express Helios and Nrp1 while in FoxP3tg we detected a much lower frequency expressing Helios or NRP1. In contrast to that, we observed about the same number of $Helios^+$ Treg cells in the secondary lymphoid organs of both mice. On top of that, other than the Foxp3tg.$Helios^-$ Treg cells, the phenotype of $Helios^+$ Treg cells from FoxP3tg mice is very similar to the phenotype of B6.Treg cells. This indicated that the $Helios^-$ Treg cell subsets in FoxP3tg probably develop differently than B6.$Helios^-$ Treg cells, while $Helios^+$ Treg cells probably have the same origin. Here we show that in FoxP3tg and B6 mice the restriction of the TCR repertoire and thus, negative selection, lead to a clear reduction of the $Helios^+$ Treg cell population while the $Helios^-$ Treg population mostly was unaffected. In monoclonal FoxP3tg mice, all

thymocytes get positively selected; about 50% of peripheral CD4 cells still express FoxP3 (but not Helios). In contrast, B6 mice with a monoclonal TCR repertoire do not have Treg cells, caused by the absence of the cognate antigen in the thymus.[79, 81-83]

Thus, we hypothesised that Helios$^+$ Treg cells seem to have a higher TCR affinity for self-antigens than Helios$^-$ Treg cells. Helios$^-$ Treg cells in turn, seem to have a lower TCR affinity for self-antigens than Helios$^+$ Treg cells but probably still a higher than Tconv cells and Tconv cells have low-affinity TCRs for self-antigens.

Here we showed in reaggregated thymic organ cultures (RTOCs) with B3K508tg MHCII^{KO} thymocytes and B6 TECs that Treg cells only developed in the presence of negative selecting peptides but not with endogenous positive selection peptides. In line with that, we showed *in vivo* by generating OTII/B6 mixed bone marrow chimeras with RIP-OVA or B6 recipients that OTII Treg cells only developed when their cognate antigen, OVA, was expressed in the thymus. In the absence of OVA expression in the thymus, all cells were positively selected and cells did not develop into Treg lineage. This is in line with previous reports where TCRtg Treg cells failed to develop when their cognate antigen was not expressed or when the TCR rearrangement was blocked.[81-83] Furthermore, polyclonal Treg cells do not develop when AIRE mediated expression of self-antigen in mTECs is not provided. [89] Moreover, we showed that all OII Treg cells generated in the chimeras, expressed Helios. This indicates that in the thymus, Treg selection in presence of a high-affinity ligand, results in Helios expression. Further evidence for that is given by a study of Daley et al. showing that the expression of Helios qualitatively differentiates CD4SP thymocytes making a positive versus a negative selection response *in vivo*. Helios expression is induced during negative selection but decreased during positive selection.[211] In addition to that, both FoxP3tg and B6 mice have comparable Helios$^+$ Treg numbers in secondary lymphoid organs, independent from the expression of the FoxP3 transgene in FoxP3tg mice. This is in agreement with the finding that Helios is independently expressed from FoxP3.[105] The hypothesis of the TCR specificity of Helios$^+$ Treg cells seems to be quite likely; meanwhile, defining the specificity of Helios$^-$ Treg cells is rather vague than clear. In FoxP3tg and B6 mice, a Helios$^-$ Treg population is detectable. In both mice, this population is low (compared to the periphery) in the thymus (B6~0.3%, FoxP3tg~25% among all CD4SP) and increased in the periphery (~10x in B6, ~3-4x in FoxP3tg).

In the FoxP3tg mouse, the high frequency (compared to B6 mice) of Helios$^-$ Treg cells is almost certainly induced by the overexpression of FoxP3. However, it does not explain why only a fraction of CD4SP thymocytes express FoxP3 in these mice. Since the FoxP3 transgene is controlled under its own regulatory elements[48], it is possible that FoxP3$^+$CD4SP cells have TCR affinities for self-antigens around/ close to the negative selection threshold, but induce TCR signals which are still strong enough to induce some FoxP3 expression. Because the FoxP3tg mouse carries a transgene of 16 copies of the entire FoxP3 gene, including all regulatory elements,[48] a less strong TCR signal, than in a B6 mouse, could lead to the induction of FoxP3. It is known that FoxP3 can induce and stabilize its own expression, mediated by a positive feedback loop.[64] In the periphery, the extremely high frequency of FoxP3$^+$ CD4$^+$ T cells (90% among CD4$^+$ T cells) is probably caused by an increased conversion of conventional T cells. This could be mediated by the low-affinity response to self-peptide-MHC ligands[228] or non-self-antigens leading to the induction of the multiple FoxP3 genes from the transgene.

In B6 mice, a small population of Helios$^-$ Treg cells among CD4SP cells in the thymus is present. Whether these cells are from thymic or peripheral origin is controversially discussed. Because Helios was suggested as a marker for thymic derived Treg cells[107] it is possible that Helios$^-$ Treg cells are pTreg cells, recirculating into the thymus. A recent study addressed this question and showed by using GFP labelled recent thymic emigrants that within the Treg cell pool in the thymus, recirculating GFP negative Treg cells exists.[229] Unfortunately, they did not stain for Helios but in contrast to Helios$^-$ Treg cells, the recirculating cells were described to be Nrp1^{high}, CD25^{high} and CD44^{high}. Nevertheless, these results indicate that Helios$^-$ Treg cells most probably do not belong to the recirculating population and possibly can develop in the thymus. However, this does not tell more about their TCR specificity. We assume that Helios$^-$ Treg cells, in the thymus, have TCR affinities for self-peptides at or just above the affinity threshold for negative selection. Evidence for that is provided by the circumstance that first, RTOCs with threshold ligands but not endogenous, positive selecting peptides induce Treg cells, second, FoxP3 induction does not depend on Helios expression,[105] and third, Helios expression needs strong TCR activation.[211]

However, in the periphery of B6 mice the frequency of Helios$^-$ Treg cells increases up to 10 fold compared to the thymus. It was shown that Treg cells could develop from naïve CD4$^+$ T cells expressing most dominantly TCRs for non-self antigens in the

presence of an optimal microenvironment containing IL2 and TGBβ.[92] These cells were mostly Helios negative *in vivo*.[97, 107] More and more evidence indicates that within the positively selected naïve T cells, the strength of the interaction of a T cell with self-peptide-MHC complexes is not uniform.[228] This could mean, that within positively selected cells, the affinity for self can vary from very low self to just below negative selection threshold. We could assume that the latter ones could preferentially commit to the pTreg population in presence of the optimal microenvironment.

Considering our results, we suggest that Helios expression correlates with the strength of the interaction of a Treg cell with self-peptide MHC complexes and hypothesis that in the thymus of FoxP3tg mice (Fig. 32a), Helios$^-$ FoxP3$^-$ (blue) or FoxP3$^+$ CD4SP (brown) are positively selected and low-affinity anti-self, while Helios$^+$ FoxP3$^+$ CD4SP (red) are high-affinity anti-self and can escape negative selection. After emigration of maturated transgenic thymocytes into the periphery, majority of naïve CD4$^+$ T cells turn into FoxP3 expressing CD4$^+$ T cells (brown) (forced by the expression of the FoxP3 transgene) while Helios$^+$ FoxP3$^+$ CD4 T cells were determined in the thymus.

In B6 mice (Fig. 32b) the thymic selection processes results in low-affinity anti-self FoxP3$^-$ CD4SP (blue, positively selected) and self-reactive Helios$^+$FoxP3$^+$ CD4SP cells (red, escaping negative selection). A small population of intermediate-affinity anti-self Helios$^-$FoxP3$^+$ CD4SP cells (brown, threshold, escape negative selection?) can develop in the thymus, but these Helios$^-$FoxP3$^+$CD4$^+$ T cells can also be generated from the conversion of naïve CD4$^+$ T cells in the periphery, either induced by foreign or self-antigens in presence of IL2 and TGFβ.

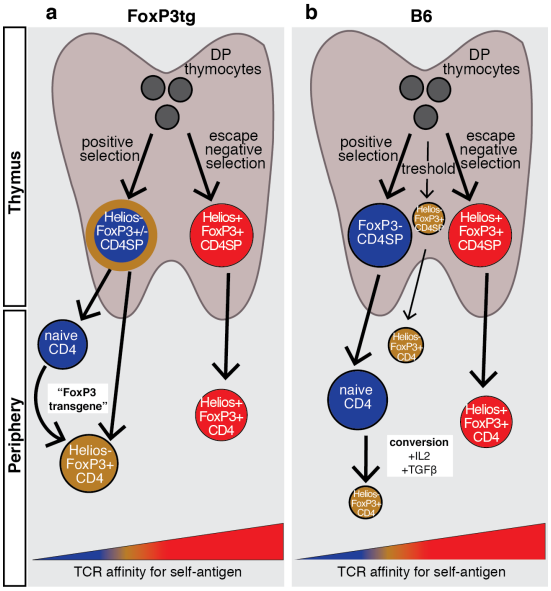

Figure 32: Treg development model in FoxP3tg and B6 mice.
Development of Helios$^+$ Treg (Helios$^+$FoxP3$^+$CD4$^+$, red), Helios$^-$ Treg (Helios$^-$FoxP3$^+$CD4$^+$, brown) and Tconv (Helios$^-$FoxP3$^-$CD4$^+$, blue) cells in **(a)** FoxP3tg and **(b)** B6 mice.

6.2 *In vitro* studies on the self-reactivity of different Treg cell populations; TCR affinity for self-antigens matters but Il2 rules it all

In the second part of the thesis, we studied the self-reactivity of Helios$^+$, Helios$^-$ Treg and Tconv cells from FoxP3tg and B6 mice. The investigations and results from the first part of this thesis led us to the hypothesis that Helios separates Treg cells with higher self-reactive TCRs from Treg cells with lower self-reactive TCRs. To test this, we re-established autoMLR experiments, an *in vitro* model to test self-reactivity, which has been described the first time more than 30 years ago. Syngeneic or autologous mixed lymphocyte reactions (autoMLRs) are defined as the proliferative response of lymphocytes, cultured with syngeneic non-T lymphocytes. Nussenzweig et al. demonstrate that DCs have the unique ability to induce the proliferation of autologous T cells in the absence of exogenous antigen.[201] Furthermore, it is suggested that the presentation of self-antigen by MHC class II molecules on syngeneic DCs is responsible for the proliferation of autologous CD4$^+$ T cells.[202]. Regarding this, we expected that Helios$^+$ Treg cells should proliferate the most since we assumed them to express TCRs with high-affinities for self-antigens. Helios$^-$ Treg cells should proliferate less (than Helios$^+$ Treg) or not while Tconv should not respond to self-antigens.

As predicted, from enriched total CD4$^+$ T cells, cultured on syngeneic B6.BmDCs, Helios$^+$ Treg cells highly proliferated whereas only a small fraction of Helios$^-$ Treg and Tconv cells divided. These results were somewhat amazing. Our results were in line with the hypothesis that Helios$^+$ Treg cells have a high-affinity for self-antigens and even the overexpression of FoxP3 seems not to influence the development of this population. However FoxP3tg cells divided less, frequencies of both, FoxP3tg and B6 Helios$^+$ Treg cells increased 10 fold compared to pre-culture conditions and proliferated significantly more than their co-cultured Helios$^-$ Treg and Tconv cells.

We confirmed in three different experimental setups that the proliferation of Helios$^+$ Treg cell, but also Helios$^-$ Treg cell from B6 and FoxP3tg mice, is highly dependent on the interaction with MHC class II, expressed on BmDCs and excluded that co-stimulatory signals, e.g. CD28:B7 ligand interactions, induced the proliferation of Treg cells[230]. Furthermore, we showed in a transwell culture system that the proliferation of Treg cells was not mediated by IL2[151, 154, 226, 231] or other cytokines produced by BmDCs [148,232] First, Treg cells were described to be anergic *in vitro* after TCR stimulation.[231] In all experiments, we used CD4$^+$ T cells from B6 and FoxP3tg mice and observed very similar behaviour of Helios$^+$ Treg, Helios$^-$ Treg and

Tconv cells. For reasons of simplicity, and more physiologically we decided to continue with B6 $CD4^+$ T cells only.

We were somewhat surprised that sorted, Helios enriched Treg cells did not proliferate in co-cultures with B6.BmDCs, equally to co-cultures with $MHCII^{KO}$.BmDCs. The previous experiments using total $CD4^+$ T cells nicely suggested that the observed proliferation of Treg cells (especially $Helios^+$ Treg cells) is induced by TCR-self-peptide-MHCII interactions independent of co-stimulation or cytokines secreted by BmDCs. However, previous reports showed that Treg cells do not divide after TCR stimulation but can be induced by exogenous administration of IL2.[44, 79]

Indeed, we identified Tconv cells as the source of IL2 producing cells when co-cultured on B6.BmDCs. First, we showed that the reconstitution of sorted, Helios enriched Treg cells regained their proliferative capacity when reconstituted with sorted, Tconv cells, co-cultured with B6.BmDCs. Second, sorted, Helios enriched Treg cells, cultured in the insert of a transwell system, without contact to the APCs, proliferated when purified Tconv cells were cultured with B6.BmDCs in the lower well of the transwell system. Third, from total $CD4^+$ T cells, cultured on B6.$MHCII^{KO}$BmDCs, in presence of supernatant from sorted Tconv: B6.BmDCs co-cultures, $Helios^+$ Treg cells divided. $Helios^-$Treg cells also divided but less than $Helios^+$Treg cells while Tconv cells did not divide in all conditions.

All together strongly indicated that in this *in vitro* model, the proliferation of $Helios^+$ and $Helios^-$ Treg cells is dependent and most probably mediated by IL2, secreted by Tconv cells and not by a TCR interaction with self-peptides presented by MHCII molecules on BmDCs. Interestingly, Tconv cells secreted IL2 only, when MHCII was expressed on BmDCs. Therefore IL2 secretion was probably mediated by a low-affinity TCR self-peptide MHCII interaction.

As mentioned above, the concept that Treg cells proliferate *in vitro* in presence of IL2 is not new.[44, 79] Furthermore, studies from the 1980s already hinted that IL2 can be produced in autoMLRs.[233] However, our findings mirror some recent findings made *in vivo*. They suggested the principle that about 10% of Tconv cells can produce IL2 from weak self-antigen recognition, sufficient to drive the transcription of IL2 but not to induce their proliferation.[154, 155] Thus, this little amount of IL2 is sufficient to induce strong proliferation in Treg cells especially $Helios^+$Treg cells. This mechanism most probably also explains the high proliferation of $CD25^+CD4^+$ Treg cells when adoptively transferred into lymphopenic hosts.[234] Very similarly to our in vitro

autoMLR experiments, it was reported that the proliferation of $CD25^+CD4^+$ T cells in lymphopenic hosts is dependent on MHCII expressed by the host cells.[234, 235] We saw the same when we transferred total $CD4^+$ T cells were into B6.$Rag2^{-/-}$MHCII^{KO} recipients or B6.$Rag2^{-/-}$ (data not shown).

Despite the importance of IL2 for the proliferation of Treg cells it is still not fully resolved whether the proliferation is just a consequence of IL2 consumption mediated by the constitutive expression of CD25, the α subunit of the high affinity IL2 receptor, or whether self-reactivity driven influences this mechanism.

It is striking that Helios$^+$ Treg cells almost exclusively proliferate while Helios$^-$ Treg cells do not or less, even though both cell populations express FoxP3 similarly. We observed that *ex vivo*, Helios$^+$ Treg cells have a higher CD25 expression than Helios$^-$ Treg cells. It is suggested that in Treg cells, FoxP3 directly mediates CD25 expression.[59] Indeed, our data shows, that $FoxP3^+CD4^+$ T cells from FoxP3tg mice express more CD25 than naïve $CD4^+$ T cells from B6 mice, however, the overexpression of FoxP3 did not result in the same CD25 expression as recorded from B6 $FoxP3^+CD4^+$ T cells. This indicates that FoxP3 expression per se is not enough to induce Treg like CD25 expression. Given that CD25 is normally upregulated in activated T cells, after encounter their cognate antigen, one can assume that this is also true for Treg cells.[69] Thus, the CD25 expression on Treg cells is probably a consequence of TCR stimulation and FoxP3 driven expression what could mean that Treg cells with higher affinity for self-antigens express higher levels of CD25. To make a complicated story even more complicated, IL2 also influences CD25 in a positive feedback loop.[69] The administration of exogenous IL2 or Tconv cells re-established the proliferative capacity of Helios$^+$ Treg and, to some extent, of Helios$^-$ Treg cells in all conditions where proliferation was abolished while Tconv cells did not proliferate. However, the upregulation of CD25 on Helios$^+$ Treg and, to a smaller extent, on Helios$^-$ Treg cells, which is induced in co-cultures with B6.BmDCs, could not be restored in co-cultures with B6.MHCII^{KO}BmDCs by the administration of exogenous IL2. This implies that IL2 is not enough to compensate for a missing TCR signal that induce high levels of CD25 in Treg cells. Furthermore, Treg cells, in particular Helios$^+$ Treg cells, cultured with B6.BmDCs, seems to survive better than in co-cultures with B6.MHCII^{KO}BmDCs.

Considering all data, we suggest following model: (Fig. 33) Within the Treg pool, Treg cells with different affinities for self-antigens exists, Treg cells with the highest

affinities for self-antigens express FoxP3 and Helios while Treg cells with lower affinity for self-antigens or non-self antigens only express FoxP3. As a consequence of their affinity, these cells express different amount of CD25. In homeostatic conditions, these cells can response differently to IL2, secreted by Tconv cells, probably mediated by a low-affinity TCR self-peptide MHCII interaction. Even though, the proliferation in this system is driven by IL2, the TCR affinity for self-antigens lays the basis for the response.

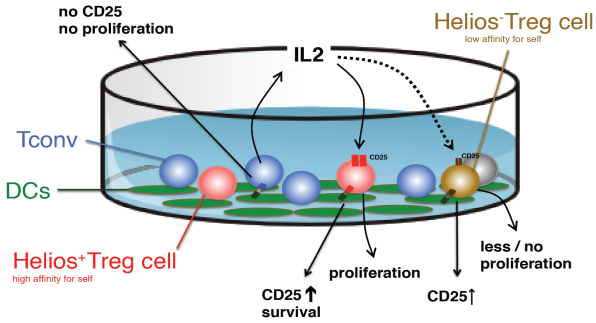

Figure 33: A model how Treg cells with different TCR affinities for self-antigens could behave differently in homeostatic conditions. autoMLR as an *in vitro* model for homeostatic conditions, syngeneic DCs (green), Tconv cells (weak-self TCR, blue), Helios^{+}Treg cells (high-self TCR, red), Helios^{-} Treg cells (intermediate/low-self TCR, brown)

6.3 Affinity for self-antigen selects regulatory T cells with distinct functional properties

In the third part of this thesis, we investigated the functional properties of Treg cells with different TCR affinities for self-antigens. We identified Helios enriched Triple^{high} (GITR^{high}, PD1^{high}, CD25^{high}) Treg cells, expressing higher affinity self-reactive TCRs and Helios negative Triple^{low} (GITR^{low}, PD1^{low}, CD25^{low}) Treg cells expressing lower affinity self-reactive TCRs.

The idea, comparing different Treg subtypes for their TCR repertoire and their different functional properties is not new. It is widely accepted that Treg cells are differently selected and have a different TCR repertoire than Tconv cells.[85, 118] Furthermore extensive studies showed that Treg cells can have different origins e.g. thymus or periphery.[78, 81-83, 93, 107, 113] Due to that, several groups tried to compare the TCR repertoire of these two populations. However, due to the lack of specific marker for tTreg and pTreg cells they assumed that pTreg cells are highly enriched in the colon. Repertoire analysis of colonic and peripheral Treg cells were performed and revealed different TCR repertories between these two populations.[97] A recent study separating tTreg from pTreg cells by the expression of Nrp1, parallels our findings. They showed that the TCR repertoire of Nrp1^{high} and Nrp1^{low} are different from each

other and different from the TCR repertoire of Tconv cells.[92] However, the main source of Nrp1 negative Treg cells are mucosa-generated, from Tconv cells, induced Treg cells.[113]

In contrast to the previous reports, we assume that from Triple^{high} and Triple^{low} Treg cells TCR affinities for self-antigens are different but still higher than from Tconv cells.

Based on the results from the previous parts of this thesis, where we suggest that Helios expressing Treg cells have TCRs with high self-reactivity while Helios negative Treg express lower self-reactivity, but most probably above threshold or threshold, affinity self-reactive TCRs. Even though, Helios could serve as a marker for these two populations, it is not applicable since Helios is expressed intracellular and the identification and FACS sorting of Helios expressing cells would need perforation of the cells. This is not suitable for subsequent functional studies. We therefore established a sorting strategy to identify two Treg cells populations expressing distinct self-reactive TCR repertories. We identified Helios enriched Triple^{high} (GITR^{high}, PD1^{high}, CD25^{high}) Treg cells, expressing higher affinity self-reactive TCRs and Helios negative Triple^{low} (GITR^{low}, PD1^{low}, CD25^{low}) Treg cells expressing lower affinity self-reactive TCRs. We determined the degree of self-reactivity by the expression of CD5 and Nur77-GFP. The expression of Nur77 expression correlates with the strength of the TCR signal[86] and CD5 expression was described to quantitatively correlate with the TCR signal intensity.[16, 87, 88] Both, CD5 and Nur77-GFP are higher expressed in Triple^{high} than in Triple^{low} Treg cells. In addition to that, similar to Helios^{+}Treg cells, Triple^{high} Treg cells proliferated and survived on syngeneic B6.BmDCs while Triple^{low} Treg cells poorly proliferated and did not survive. Furthermore, we showed, that the TCR repertories between Triple^{high}, Triple^{low} and Tconv cells are clearly different from each other. This implied, that the TCR specificity is important for the selection of these Treg subtypes and it indicates that Triple ^{low} Treg are not just converted Tconv cells.

The question how different population of Treg cells function and whether they have differences in their behaviour because to their different TCR specificity or their origin it is still not clear. Furthermore it is disputed whether different population of Treg cells specifically can prevent from different diseases.[120, 122]

Here we report that Triple^{high} but not Triple^{low} Treg cells control the extensive lymphoproliferation in mice acutely depleted of Treg cells. Conversely, Triple^{low} but

not Triple^{high} Treg cells control colitis by facilitating conversion of Tconv cells into induced Treg cells (iTregs).

The acute ablation of Treg cells in FoxP3^{DTR} mice by the administration of diphtheria toxin in adult, healthy mice results in massive lymphoproliferation. It is suggested that the depletion of Treg cells in these mice leads to the activation of T cells specific for "self-antigens" including genome encoded self, environmental and food antigen. Evidence for that was given by the fact that (foreign) TCRtg T cells did not undergo activation upon Treg cell ablation.[52]We observed a massive expansion and activation of CD4$^+$, CD8$^+$ T cells and B Cells in the, lymph nodes and spleens, but not so much in the mesenteric lymph nodes (not shown), of affected mice. Subsequent treatment with DTx resulted in death within 14 days. The transfer of as little as 2.5x10^5 Triple^{high} Treg cells, but not Triple^{low} Treg cells, controlled the lymphoproliferation and activation of CD4$^+$, CD8$^+$ T cells and B Cells. It was reported that within less than 48h after Treg cell depletion, CD4$^+$ T cells and DCs showed sings of activation.[52] DC activation resulted in a 2-12 fold expansion, the upregulation of surface MHCII expression and the upregulation of the co-stimulatory molecules CD80 and CD86. This massive expansion and activation of DCs likely supports the expansion of T and B cells.[236] Unfortunately we were not able to examine the homing receptor expression of Triple^{high} Treg cells in this experimental setup. Anyhow, one could assume that Triple^{high} Treg cells preferentially home to peripheral lymph nodes since this was the site where we saw the main suppression. We suggest three different mechanisms how Triple^{high}, and not Triple^{low}, Treg cells could suppress acute autoimmune driven lypmphoproliferation. First, due to their expression of a high affinity self-antigen TCR, Triple^{high} Treg cells may form stable contacts between Treg cells and antigen-bearing DCs, preventing from activation of Tconv cells.[144, 145] Second, Triple^{high} Treg cells express high levels of CD25 and therefore may dampen the proliferation of activated Tconv cells by "stealing" IL2. Third, The ablation of Treg cells leads to the activation of DCs suggested to be mediated by the local production of Fms-like tyrosine kinase 3 (Flt3) Ligand by a not yet determined cell type.[236] This activation may include the upregulation of PDL1 or PDL2 on DCs. Triple^{high} Treg cells express high levels of PD1. Direct Treg:DC interaction via PD1:PDL1 or PDL2 may regulates DC activation turning them into "tolerogenic" DCs.[22] Furthermore, Flt3L mediated increase of DC numbers effects the homeostasis of Treg cells in combination with IL2 and an not yet described contact-dependent DC-mediated signal.[237] However this mechanism was described to be TCR signal

independent, but as we could show, the maintenance of high CD25 levels still need strong TCR stimulation. All three mechanisms clearly favours Triple^{high} Treg cells and Triple^{low} Treg cells would fail in either mechanism. However, all three mechanisms are possible, neither must be right. To resolve how Triple^{high} Treg cells suppress acute autoimmune lymphophroliferation, further investigation has to be done.

In contrast to the acute ablation of Treg cell, adoptive transfer induced colitis is a slow progressing disease; mediated by microbiota-specific CD4$^+$ T cells. [159, 175]

The transfer of naïve CD4$^+$ T cells into T cell deficient hosts induces colitis and weight loss within four to six weeks and was characterized by a massive cell infiltration into the colon. Various reports showed that the adoptive transfer of Treg cells, in particular microbiota-specific, prevents the onset or even cures from colitis.[95, 96, 177]

Here we show that Triple^{low} Treg cells but not Triple^{high} Treg cells controlled colitis by facilitating the conversion of Tconv into peripheral induced Treg cells. We only can make an assumption how Triple^{low} but not Triple^{high} Treg cells can provide help to convert Tconv cells and prevent from colitis. To control colitis in this model, the conversion of Tconv to induced Treg cells is from great importance. We showed that the consecutive ablation of induced Treg cells resulted in earlier onset and more severe colitis even in the presence of Triple^{low} Treg cells.

It is widely accepted that Treg cells can be induced from naïve CD4$^+$ T cells and is from great importance in maintaining immune homeostasis especially at mucosal interfaces like lung and gut but also in maintaining fetal tolerance.[62-64, 121] In the gut, naïve CD4$^+$ T cells can be converted upon TCR stimulation in the presence of TGFβ and IL2 but also other compounds like RA or metabolic products from the microbiota like short-chain-fatty-acids, e.g. butyrate can mediate conversion.[99-101] In addition to that and IL10 is one of the key players in maintaining homeostasis of the gut. IL10 deficient mice are suffering from spontaneous colitis.[176] To our knowledge, there is no study, showing that a particular Treg cell population can induce the conversion of Treg cells *in vivo*. It was shown that Treg cells support the conversion of naïve CD4$^+$ T cells *in vitro* by secreting IL10 and TGFβ. Therefore, one mechanism how Triple^{low} Treg cells could support *in vivo* conversion could be mediated by secreting tolerogenic and converting cytokines like IL10, TGFβ but also IL35 [134, 238]. This would create a cytokine milieu, favouring the conversion of microbiota activated CD4$^+$ T cells. Another possibility to promote conversion is indirectly induced via DC

modification. Triple^{low} Treg cells could interact with DCs, possible via CTLA4 and CD80/86 interactions, what could lead to the secretion of IDO or IL10 by DCs, mediating the conversion of activated CD4^{+} T cells into induced Treg cells.[140, 141]
It is quite difficult to assume a specific suppression mechanism for Triple^{low} Treg cells. In contrast to Triple^{high} Treg cells, Triple^{low} Treg cells are defined by what they do not expressed. It is also difficult to answer, why Triple^{high} Treg cells do not mediate conversion. Therefore, to answer these questions, further investigation of these cells is indispensable.

The high importance of Treg cells in maintaining immunological tolerance and homeostasis is probably reflected the best in FoxP3 deficient (scurfy) mice. That scurfy mice contain self-reactive T cells was first shown in a study, using the endogenous viral superantigens (VSAg) as the primary self-antigens. The absence of functional FoxP3 in scurfy mice caused preferential accumulation of autoreactive T cells.[54] This is supported by two studies, where co-expression of the mutated FoxP3 gene and the reporter eGFP in the FoxP3 locus identified FoxP3 Treg precursors. Both showed that GFP expressing cells share phonotypical similarities to Treg cells.[173, 174] However, by transferring GFP expressing cells into T cell deficient hosts; they could not induce the scurfy disease. The transfer of GFP^{-} cells resulted in colitis but not the scurfy disease.[173] It is still not clear which T cell population is promoting the multi organ inflammation and how TCR affinity for self or foreign antigen is linked to the onset of different pathologies seen in the scurfy mouse. In this study, we show that FoxP3 deficient (scurfy) mice contain Triple^{high} and Triple^{low} CD4 T cells with distinct pathological properties. Triple^{high} scurfy T cells are very similar to Triple^{high} Treg cells in expressing PD1, GITR, CD25, Helios, CD62L and also CD5 what strongly indicates that Triple^{high} scurfy T cells and Triple^{high} Tregs cells share similar genetic identity and most probably also a self-reactive TCR repertoire. A previous report, analysing the TCR repertoires from TCRβtg scurfy mice revealed that activated scurfy CD4^{+} T cells preferentially uses TCRs found in the TCR repertoire of Treg cells from TCRβtg wild type mice.[172] In contrast to previous reports, after transferring scurfy "Triple^{high} Treg cell wannabes" into T cell deficient hosts, cells proliferate extensively in peripheral LNs and infiltrated in the skin, causing severe skin lesions and inflammation. Interestingly, the Shyr-Te group generated several scurfy mice with deficiencies for different interleukins. IL4-, IL6-, IL10-, Stat6- or Itgae (CD103)- deficient scurfy mice showed inflammation in the

skin, lung and liver whereas IL2 deficient scurfy mice were only suffering from liver inflammations but did not develop skin lesions.[239] The absence of skin inflammation in these mice was not caused by a lack of INFγ or other inflammatory cytokines. They therefore suggested that IL2 acts as the main mediator in skin inflammations observed in scurfy mice. Triple^{high} scurfy cells express high levels of CD25 and are probably dependent on IL2 secretion by other cells. Triple^{high} scurfy cells are highly expressing Helios, a repressor for IL2 and cannot produce their own IL2.[174] Their preference to home to the skin could may be explained with the secretion of IL2 which was reported for several skin resident DCs.[232] In contrast to Triple^{high} scurfy cells, Triple^{low} scurfy cells do not mediate skin lesions. Triple^{low} scurfy cells are low in CD25 and probably not dependent on IL2. However, Triple^{low} scurfy cells induce colitis within 4 weeks when transferred to T cell deficient recipients, very similar to the colitis progression observed after the transfer of naïve B6 CD4^{+} T cells into T cell deficient recipients. It is difficult to say whether these cells are more like B6 Triple^{low} Treg cells or B6 Tconv cells. However, they are with great certainty microbiota specific. Germfree scurfy mice are less prone to develop colitis compared to mice hosed in specific pathogen conditons.[214]

6.4 Conclusion and Outlook

Within this thesis, we were able to define two different B6.Treg populations that seem to have different degrees of self-antigens. Our data suggests that high-affinity Treg cells are able to control the homeostatic proliferation of lymphocytes while low-affinity self-reactive Treg cells support peripheral Treg conversion and maintain lymphocyte homeostasis in the colon. It would be very interesting to investigate the mechanism behind. Resolving the mechanism would help to understand how Treg cells with different origin and different TCR affinity can contribute to maintain lymphocyte homeostasis.

We could show that different T cell populations induce the multi-organ-inflammation in scurfy mice. Our data suggests that uncontrolled highly self-reactive T cells induce sever skin pathology while low-self-reactive T cells induce colitis. Further investigation of inflammatory sites, other than the skin, would help to understand how different T cell population could induce different pathologies.

It would be interesting to combine both models and to see whether B6.Triple^{high} Treg cells are better suppressors for scurfy Triple^{high} CD4^{+} T cells than B6.Triple^{low} Treg cells and vice versa.

7 Appendix

7.1 References

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7.2 Figure legend

7.2.1 Main Figures

7.2.2 Supplementary Figures

7.3 List of Abbreviations

AIRE	autoimmune regulator
APC	antigen presenting cell
β-me	β-mercaptoethanol
BAC	bacterial artificial chromosome
Bcl	B cell lymphoma
BCR	B cell receptor
BmDC	bone-marrow derived dendritic cells
BrdU	bromodeoxyuridine
BSA	Bovine Serum Albumin
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
CDR3	complementarity-determining region 3
CNS	conserved non-coding sequence
cTEC	cortical thymic epithelial cells
cTreg	central Treg cells
CTLA4	cytotoxic T-lymphocyte-associated protein 4
CO_2	carbon dioxide
DC	dendritic cells
DN	double negative
DNA	deoxyribonucleic acid
DP	double positive
DTR	diphtheria toxin recptor
DTx	diphtheria toxin
Ebi3	Epstein-Barr virus-induced gene 3
eGFP	enhanced green fluorescent protein
eTAC	extra thymic AIRE-expressing stroma cell
eTreg	effector Treg cells
FACS	fluorescence activated cell sorting
FoxP3	forkhead box protein P3
FCS	Fetal Calf Serum
GITR	Glucocorticoid-Induced Tumor necrosis factor receptor
GM-CSF	granulocyte macrophage colony-stimulating factor
gMFI	geometric mean fluorescence intensity
H&E	hematoxilin& eosin
i.p.	intraperitoneal
i.v.	intravenous
IA^b	MHC-class II (see MHC)
IBD	inflammatory bowel disease
ICOS	inducible costimulator
IDO	indoleamine 2.3-dioxygenase
Ig	immunoglobulin

IL	interleukin
IL2R	interleukin 2 receptor
INFγ	interferon-γ
IPEX	Immune dysregulatrion Polyendocrinopathy Enteropathy x linked syndrome
Itgea	Integrin α E (CD103)
ITAMs	immunoreceptor tyrosine-based activation motifs
K_D	dissociation constant
LAG	lymphocyte-activation gene 3
LAT	linker for activation of T cells
LFA1	lymphocyte function-associated antigen 1
LN	lymph node
LNSCs	lymph node stromal cells
LPS	lipopolysaccharide
MHC	major histocompatibility complex
mLN	mesenteric lymph node
mTEC	medullary thymic epithelial cells
mTreg	memory Treg cells
NEAA	non-essential ammino acids
NFAT	nuclear factor of activated T cells
NK	natural killer cells
NRP1	neuropilin 1
PAMPs	pathogen associated recognition patterns
PBS	phosphate buffered saline
PD1	programmed death 1
PDL	programmed death ligand
PRRs	pattern recognition receptors
pTreg	peripheral regulatory T cell
Rag	recombination activating gene
RBC	red blood cell
RIP	rat insulin promoter
siRNA	small interfering ribonucleic acid
SP	single positive
STAT	signal transducer and activator of transcription
TAIL	thermal asymmetric interlaced
TCR	T cell receptor
Tconv	conventional T cells
tg	transgene
TGFβ	transforming growth factor-β
Th	helper T cell
TLR	toll-like receptors
TNF	tumor necrosis factor

TNFR	tumor necrosis factor-receptor
TRAs	tissue-restricted antigens
Treg	regulatory T cell
tTreg	thymic regulatory T cells
Vα	Vα chain
Vβ	Vβ chain
VEGF	vascular endothelial growth factor
VSAg	viral superantigens
ZAP70	Zeta-chain-associated protein kinase 70

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