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

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Abstract

How regulatory T cells (T$_{reg}$ cell) control lymphocyte homeostasis is not fully understood. Here we identify two T$_{reg}$ cell populations with differing degrees of self-reactivity and distinct regulatory functions. Triple^{hi} (GITR^{hi}PD-1^{hi}CD25^{hi}) T$_{reg}$ cell are highly self-reactive and control lympho-proliferation in peripheral lymph nodes. Triple^{lo} (GITR^{lo}PD-1^{lo}CD25^{lo}) T$_{reg}$ cells are less self-reactive and limit development of colitis by promoting conversion of CD4$^+$ T$_{conv}$ cells into induced T$_{reg}$ cells (iT$_{reg}$ cells). Although Foxp3-deficient (scurfy) mice lack T$_{reg}$ cells, they contain Triple^{hi}-like and Triple^{lo}-like CD4$^+$ T cells with distinct pathological properties. Scurfy

Triple^{hi}CD4$^+$T cells infiltrate the skin whereas scurfy Triple^{lo}CD4$^+$T cells induce colitis and wasting disease. These findings indicate that T cell receptor affinity for self-antigens drives the differentiation of Tregs into distinct subsets with non-overlapping regulatory activities.

The importance of CD4$^+$ regulatory T cells (T$_{reg}$ cell) in maintaining lymphocyte homeostasis is best appreciated in mice and humans lacking these cells. Foxp3-deficient (scurfy) mice[1,2,3] and patients with immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome[4] suffer from excessive lymphocyte activation, lymphocytic infiltration into peripheral organs and colitis, leading to death at an early age. In healthy individuals, T$_{reg}$ cells control homeostatic proliferation of conventional T and B cells and prevent colitis[5,6,7].

T$_{reg}$ cells are comprised of thymic Tregs (tT$_{reg}$ cell) and peripherally-induced T$_{reg}$ cells (pT$_{reg}$ cells or iT$_{reg}$ cells), which originate from different precursor cells and develop in different locations. tT$_{reg}$ cells develop in the thymus and their development requires TCR stimulation with agonist peptide- major histocompatibility complex (MHC)II antigens.[8,9,10] In contrast, iT$_{reg}$ cells are generated in the periphery from naive, mature CD4$^+$ conventional T cells (T$_{conv}$ cells) during T cell activation in the presence of the cytokine TGF-β.[11] Both populations are suppressive and their functional properties have been examined. Several studies suggest that tT$_{reg}$ cells are required to control immune homeostasis and autoimmunity.[5,12,13] On the other hand, iT$_{reg}$ cells have specialized functions depending on the type of inflammation, and have a primary role in controlling mucosal immunity and fetal tolerance.[5,12,13,14] However tT$_{reg}$ cells by themselves are not sufficient to suppress chronic inflammation and autoimmunity in the absence of iT$_{reg}$ cells.[15]

T$_{reg}$ cells have also been characterized for their expression of surface markers and localization in different tissues.[16,17,18] Based on their expression of CD44 and the lymph node homing receptor, CD62L, T$_{reg}$ cells can be broadly divided into CD44^{lo}CD62L$^+$ central T$_{reg}$ (cT$_{reg}$) and CD44^{hi}CD62L$^{lo/-}$ effector T$_{reg}$ (eT$_{reg}$) cells.[16] cT$_{reg}$ cells are quiescent, primarily reside in secondary lymphoid tissues, express high levels of CD25 and are interleukin-2 (IL-2)- dependent. In contrast, eT$_{reg}$ cells, the dominant Treg population in non-lymphoid tissues, are CD25^{lo}, highly proliferative, but prone to apoptosis. It's been suggested that eT$_{reg}$ cell maintenance is driven by TCR and co-stimulatory signals, but not IL-2.[16]

Several studies demonstrated the importance of TCR stimulation to activate cT$_{reg}$ cells in order to generate suppressive eT$_{reg}$ cells.[8,9] Furthermore, studies have provided direct evidence that TCR expression is indispensable for T$_{reg}$ cell survival and suppressive function.[19,20] The T$_{reg}$ cell repertoire contains self-reactive[8,21,22] as well as foreign antigen reactive[23] TCRs. The TCR affinity of T$_{reg}$ cells for self antigen has not yet been fully characterized. Although it's generally accepted that T$_{reg}$ cells and naive CD4$^+$ T$_{conv}$ cells have non-overlapping TCR repertoires, a small percentage of TCRs are found within both CD4$^+$ T cell populations.[24,25] Furthermore, the TCR repertoires of tT$_{reg}$ cells and iT$_{reg}$ cells were shown to be distinct.[26,27] While the tT$_{reg}$ cell TCR repertoire is biased toward self-recognition, TCRs expressed in iT$_{reg}$ cells can recognize foreign antigens with high affinity.[24,26] In line with these findings, it's been shown that activated CD4$^+$ T cells from

TCRβ transgenic (TCRβ-tg) scurfy mice preferentially used TCRs found in the T_{reg} cell TCR repertoire of TCRβ-tg wild type mice.[21] Despite these interesting findings, it's still not clear how a T_{reg} cell's antigen specificity influences its' regulatory properties.

Here report two functionally distinct subgroups of tT_{reg} cells with distinct TCR repertoires and differing TCR affinities for self-antigens. Triple^{lo} (GITR^{lo}PD-1^{lo}CD25^{lo}) T_{reg} cells express TCRs whose affinities for self-antigens are close to the negative selection threshold, whereas Triple^{hi} (GITR^{hi}PD-1^{hi}CD25^{hi}) T_{reg} cells express TCRs with affinities well above this threshold. Functionally, Triple^{lo} T_{reg} cells control colitis by facilitating conversion of CD4^{+} T_{conv} cells into iT_{reg} cells, whereas Triple^{hi} T_{reg} cells maintain lymphocyte homeostasis within peripheral lymph nodes (LNs). Finally, Foxp3-deficient (scurfy) mice contain Triple^{hi}- like and Triple^{lo}- like CD4^{+} T cells with distinct pathological properties. Our results provide evidence that the degree of thymocyte self-reactivity drives the generation of distinct T_{reg} cells subtypes, which control different aspects of lymphocyte homeostasis.

Results

Distinct T_{reg} cell subsets

Foxp3[+] T_{reg} cells express a continuum of GITR and PD-1 (Fig 1a). As GITR[hi]PD-1^{hi} T_{reg} cells express higher levels of CD25 compared to GITR^{lo}PD-1^{lo} T_{reg} cells (Fig. 1a), we refer to these populations as Triple^{hi} (GITR^{hi}PD-1^{hi}CD25^{hi}) and Triple^{lo} (GITR^{lo}PD-1^{lo}CD25^{lo}) T_{reg} cells, respectively. To compare these T_{reg} cell populations to previously described T_{reg} cells subsets[16,17,18], we examined their expression of various homing and chemokine receptors (Fig. 1b) as well as transcription factor Helios and semaphoring receptor Nrp-1(Fig. 1c). Based on their expression of these proteins, Triple[hi] and Triple[lo]T_{reg} cells are distinct from each other and distinct from centralT_{reg} and effectorT_{reg} cells (Table 1;[16,17,18]). This analysis also shows that centralT_{reg} and effectorT_{reg} cells are contained within the Triple intermediate (Triple[int]) gate (Supplementary Fig.1).

Origin of Triple[hi] and Triple[lo] Tregs

Triple[hi] and Triple[lo]T_{reg} cells present in the thymus (Fig. 2a) could represent T_{reg} cells recirculating from the periphery as opposed to *de novo* generated tT_{reg} cells.[28,29] To resolve this, we examined thymic T_{reg} cells in mice expressing Foxp3-RFP and Rag-GFP reporters (Fig. 2b). RFP[+]GFP[+] CD4 single positive (SP) thymocytes are *de novo* generated thymic T_{reg} cells since they are still Rag-GFP[+], while RFP[+]GFP^{−} CD4SP cells in the thymus are recirculating T_{reg} cells from the periphery.[29] The frequency of *de novo* generated (RFP[+]GFP[+]) Triple[hi] and Triple[lo]T_{reg} cells in the thymus is similar to that observed among LN T_{reg} cells. The fact that both Triple[hi] and Triple[lo]T_{reg} cells develop in the thymus argues against the idea that either population are iT_{reg} cells. To address the possibility that Triple[hi] and Triple[lo]T_{reg} cells might be induced by foreign antigens or inflammation, we examined T_{reg} cells in germ-free (GF) and antigen-free (AF) mice. AF mice are offspring of GF mice that were weaned onto and raised on the elemental diet of glucose and amino acids.[30] As these animals lack a microbiome and are not exposed to dietary antigens, they contain exclusively self-antigens. GF and AF mice contain similar frequencies of LN T_{reg} cells

compared to standard SPF animals (Fig. 2c, top). Importantly, SPF, GF and AF mice contain similar frequencies of Triple^{hi} and Triple^{lo}T$_{reg}$ cells (Fig. 2c, bottom), which also express similar levels of Nrp-1 and Helios protein (Supplementary Fig. 2). These data rule out the idea that the Triple^{hi} and Triple^{lo} phenotypes are a response to inflammation. Furthermore, these results strongly suggest that Triple^{hi} and Triple^{lo}T$_{reg}$ cells are exclusively generated through recognition of self-antigens.

Distinct Triple^{hi} and Triple^{lo} T$_{reg}$ cell TCR repertoires

To directly compare the TCR repertoires of Triple^{lo} and Triple^{hi}T$_{reg}$ cells to CD4$^+$T$_{convs}$ cells, all three populations expressing the V$_\alpha$2 family (Fig. 3a), were sorted from a Rag$^+$, single TCRβ chain strain (Yae62, Vβ8.2, TCRα$^{+/KO}$, Foxp3$^{GFP–KI–}$ Supplementary Fig. 3) and subjected to deep sequencing. The 500 most frequent clonotypes in each group were analyzed for their similarity (Fig. 3 b-d) and diversity (Fig. 3e,f). Morisita-Horn analysis, which measures sequence similarity shows that the CD4$^+$T$_{conv}$ sequences from three independent groups of mice (see Methods) are similar to each other but significantly different from Triple^{lo} and Triple^{hi} TCR sequences obtained from the same mice (Fig. 3b). Triple^{lo} sequences isolated from different groups of mice are similar to each other as well, but different from CD4$^+$T$_{conv}$ and Triple^{hi} TCRs (Fig. 3c). Triple^{hi} TCR sequences are not only different from CD4$^+$T$_{conv}$ and Triple^{lo} sequences, but they vary between different groups of mice (Fig. 3d). Despite their significant sequence differences, the TCR repertoires of CD4$^+$T$_{conv}$ cells and Triple^{lo}T$_{reg}$ cells are similarly diverse (Fig. 3e, f), while the Triple^{hi}T$_{reg}$ cell TCR repertoire may be less diverse, at least according to Shannon Entropy analysis, which is used to measure sequence diversity. Taken together, deep sequencing showed that Triple^{hi}T$_{reg}$ cells, Triple^{lo}T$_{reg}$ cell and CD4$^+$T$_{conv}$ cells have clearly distinct TCR repertoires, implying that TCR specificity is important in selecting these T$_{reg}$ cell subtypes.

Self-reactivity of Triple^{hi} and Triple^{lo} T$_{reg}$ cells

To directly test whether Triple^{hi} and Triple^{lo} T$_{reg}$ cell differ in their degree of self-reactivity, we examined CD5 and Nur77 protein expression in each subset (Fig. 4a). The expression of these markers reflects T cell activation and correlates with TCR affinity for its peptide-MHC (pMHC) ligand.[31,32] The higher expression of Nur77 and CD5 by Triple^{hi} T$_{reg}$ cells, compared to Triple^{lo} T$_{reg}$ cells (Fig. 4a) argued that Triple^{hi} T$_{reg}$ cells are more self-reactive than their Triple^{lo} T$_{reg}$ cell counterparts. To test this idea, we used *in vivo* BrdU labeling and observed that Triple^{hi}T$_{reg}$ cells proliferate more frequently *in vivo* compared to Triple^{lo}T$_{reg}$ cell and CD4$^+$T$_{conv}$ cells (Fig. 4b). Furthermore, culturing unsorted CD4$^+$T cells on syngeneic bone marrow dendritic cells (BMDCs), Triple^{hi}T$_{reg}$ cells proliferate more extensively than Triple^{lo}T$_{reg}$ cells and CD4$^+$T$_{conv}$ cells (Fig. 4c); this proliferation requires expression of MHCII self-antigens on antigen presenting cells (APCs).

To examine the influence of antigen affinity on the generation of CD4SP thymocytes with a Triple^{hi} or Triple^{lo} phenotype (Fig. 4d), B3K508TCR-tg Rag2$^{–/–}$ thymocytes, expressing the B3K508 TCR and recognizing the 3K, P2A and P-1A peptides presented by I-A^b were cultured on syngeneic BMDCs in the presence of TGF-β and IL-2. Addition of P-1A peptide (high affinity threshold negative selector) induced development of Triple^{lo} CD4SP

thymocytes, while the P2A peptide (intermediate affinity negative selector) induced intermediate (Triple^{int}) CD4SP thymocytes; finally, 3K-peptide (high affinity negative selector) induced only Triple^{hi} CD4SP thymocytes (Fig. 4d, top). Foxp3$^+$T$_{reg}$ cells were also generated in these cultures, but only in the presence of negative selecting peptides (Fig. 4d, middle, Supplementary Fig. 4a). Culturing B3K508TCR-tg *Rag2$^{-/-}$* thymocytes with the negative selecting ligands, P-1A, P2A and 3K generated Foxp3$^+$T$_{reg}$ cells expressing increasing amounts of PD-1 (Fig 4d), CD25 and Helios protein (Supplementary Fig. 4b). Taken together, the data indicate that threshold-, intermediate- and high- affinity negative selecting antigens induce Triple^{lo}, Triple^{int} and Triple^{hi} T$_{reg}$ cells, respectively (Fig 4d, bottom; Supplementary Fig. 4b). That the threshold negative selector induces weaker TCR signals is supported by its decreased ability to induce pCD3ζ, pJun and pErk (Supplementary Fig. 4c,d). These *in vitro* results were confirmed using bone marrow chimeras, where OT-II thymocytes developed in a RIP-OVA host expressing the cognate antigen, ovalbumin in the thymus and the pancreas under the control of the rat insulin promoter (Fig. 4e). These chimeric mice contain Triple^{hi} (and Triple^{int}) but not Triple^{lo} T$_{reg}$ cells in the thymus. Taken together, these data imply that Triple^{hi} and Triple^{lo} T$_{reg}$ cells are likely generated by exposure to negatively selecting antigens; moreover, the resulting T$_{reg}$ cells phenotype is most likely determined by the affinity of its TCR for self-antigen.

Triple^{hi} Treg cells suppress lymphoproliferation

To compare the regulatory properties of these two populations, Foxp3^{DTR} mice received sorted Triple^{hi} or Triple^{lo} T$_{reg}$ cells from B6 mice (Supplementary Fig. 5a), which are unaffected by diphtheria toxin (DTx). Three days later, endogenous T$_{reg}$ cells from the Foxp3^{DTR} host were depleted by injecting DTx every other day. LN lymphocytes then were examined by flow cytometry 11 days following the onset of T$_{reg}$ cells depletion (Supplementary Fig. 5b). Triple^{hi} T$_{reg}$ cells control the extensive proliferation of T cells and B cells in peripheral LNs of mice depleted of their endogenous T$_{reg}$ cells (Fig. 5a), while Triple^{lo} T$_{reg}$ cells function poorly in this respect. As expected, Triple^{hi} T$_{reg}$ cells limit the activation of CD4$^+$ T$_{conv}$ cells (Fig. 5b,c). Taken together, these results show that Triple^{hi} T$_{reg}$ cells regulate lymphocyte homeostasis in peripheral LNs.

Triple^{lo} T$_{reg}$ cells suppress induction of colitis

To examine whether any of these T$_{reg}$ cell subsets control colitis, CD3-deficient (*Cd3e$^{-/-}$*) mice were injected with sorted naive CD4$^+$ T$_{conv}$ cells (Supplementary Fig. 6a), a treatment, which results in weight loss (Fig. 6a) and colitis (Fig. 6b, upper left panel) as previously described7. Co-transfer of Triple^{lo} (Fig. 6a, solid brown line; Fig. 6b, upper middle panel) but not Triple^{hi} (Fig. 6a, solid red line; Fig. 6b, upper right panel) T$_{reg}$ cells prevented weight loss and limited lymphocyte infiltration of the colonic mucosa. Analysis of LN cells from these mice indicated that co-transferred Triple^{lo} T$_{reg}$ cells facilitated the conversion of some CD4$^+$ T$_{conv}$ cells into iT$_{reg}$ cells (Fig, 6c,d). Mice receiving Triple^{lo}T$_{reg}$ cells had the highest percentage of iT$_{reg}$ cells (Fig, 6c,d), very limited infiltration of the colonic mucosa (Fig. 6b, upper middle panel) and maintained their weight (Fig. 6a).

To test whether iT$_{reg}$ cells were required to control colitis15, CD4$^+$ T$_{conv}$ cells isolated from Foxp3^{DTR} mice were transferred into *Cd3e$^{-/-}$*) mice (Supplementary Fig. 6a). These animals

were additionally treated with DTx every third day to deplete any iT_{reg} cells developing from transferred Foxp3^{DTR} CD4$^+$T$_{conv}$ cells. iT_{reg} cells depletion accelerated weight loss and development of colitis (compare solid blue Fig. 6a and dashed blue lines in Fig. 6e). Co-transferred B6 Triple^{lo} T$_{reg}$ cells (unaffected by DTx) were unable to control the development of colitis when iT_{reg} cells were depleted (compare solid brown Fig. 6a and dashed brown lines in Fig. 6e; compare upper middle and lower middle panels in Fig. 6b). The data support the idea that Triple^{lo}T$_{reg}$ cells facilitate conversion of some CD4$^+$T$_{conv}$ cells into Foxp3$^+$ iT_{reg} cells (Fig. 6d), which in aggregate limit development of colitis.

Taken together, the data (Fig. 5 and 6) argue for two populations of T$_{reg}$ cells: Triple^{hi} T$_{reg}$ cells, which control lymphoproliferation in peripheral LNs and Triple^{lo} T$_{reg}$ cells, which limit the development of colitis (at least in a lymphopenic setting). It should be noted that the phenotypes of Triple^{hi} T$_{reg}$ cells are stable over the 11d time course of the experiment (Fig. 5) while Triple^{lo} T$_{reg}$ cells are stable over the 6 week time course of the experiment (Fig. 6, Supplementary Fig. 6b). As Triple^{lo} T$_{reg}$ cells did not suppress lympho-proliferation and Triple^{hi} T$_{reg}$ cells did not suppress colitis, there was no evidence for a significant degree of trans-differentiation between the two subsets during the time frame of these experiments.

Triple^{hi} and Triple^{lo} CD4$^+$ T cells in scurfy mice

Although Foxp3-deficient (scurfy) mice cannot develop T$_{reg}$ cells due to the lack of functional Foxp3, they do carry out negative selection.[21] For this reason, we wondered whether scurfy mice contain Triple^{hi}- like and Triple^{lo}- like CD4$^+$ T cells despite their lack of a functional Foxp3 molecule. Flow cytometry analysis shows that these mice contain GITR^{hi}PD-1^{hi}CD25^{hi} (Scurfy Triple^{hi}) and GITR^{lo}PD-1^{lo}CD25^{lo} (Scurfy Triple^{lo}) CD4$^+$ T cells. Scurfy Triple^{hi} CD4$^+$ T cells resembled B6 Triple^{hi} T$_{reg}$ cells in terms of PD-1, GITR, CD25, Helios, CD5 and CD62L protein expression (Fig. 7a). Given their lack of Foxp3 expression and suppressive capacity, Scurfy Triple^{hi}CD4$^+$ T cells may be similar to previously reported Treg 'wannabes'.[21,33,34] Scurfy Triple^{lo} CD4$^+$ T cells, on the other hand resembled CD4$^+$ T$_{conv}$ cells with respect to their expression of these markers (Fig. 7a).

To investigate their pathological activities, Scurfy Triple^{lo} and Scurfy Triple^{hi} CD4$^+$ T cells were sorted (Supplementary Fig. 7a) and separately transferred into T cell deficient, *Cd3e*$^{-/-}$) hosts (Supplementary Fig. 7b). Transferred Scurfy Triple^{lo}CD4$^+$ T cells promoted weight loss (Fig. 7b) and colitis (Fig. 7c). Moreover, they accumulated in mesenteric LNs (Supplementary Fig. 7c) where ~35% of these cells express $\alpha_4\beta_7$, an integrin that enables homing to the gut[35] (Supplementary Fig. 7d). In contrast, transferred Scurfy Triple^{hi}CD4$^+$ T cells do not cause weight loss (Fig. 7b) and preferentially accumulate in peripheral but not mesenteric LNs (Fig. 7d, Supplementary Fig. 7c). Moreover, Scurfy Triple^{hi}CD4$^+$ T cells induce massive inflammation in the skin but only minimal inflammation in the colon (Fig. 7c, Supplementary Fig. 7e). Taken together, these results indicate that the absence of normal T$_{reg}$ cells is not the sole cause of scurfy disease; the activity of dysregulated (T$_{reg}$ cell-like) Scurfy Triple^{hi} CD4$^+$ T cells accounts for some of the pathology observed in these mice.

Discussion

We examined the functionality of T_{reg} cell subsets with distinct TCR repertoires and differing affinities for self-antigens. Our data suggest that Triple^{hi} and Triple^{lo} T_{reg} cells are generated as an offshoot of negative selection. The high affinity self-reactive TCRs expressed by Triple^{hi} T_{reg} cells likely drive their selection in the thymus and their suppressive activity in peripheral LNs.[36] On the other hand, thymic precursors expressing lower affinity self-reactive TCRs plausibly differentiate into Triple^{lo} T_{reg} cells. Triple^{hi} and Triple^{lo} T_{reg} cells are distinct from central and effector T_{reg} cell subsets based on their expression of CD25, CCR7, CD103, Helios and Nrp-1 proteins.[16] Foxp3-RFP Rag-GFP dual reporter mice, clearly show that Triple^{hi} and Triple^{lo} T_{reg} cell are present among *de novo* generated, Rag-GFP$^+$, thymic T_{reg} cells. Antigen free mice contain virtually no foreign antigens (they lack a microbiome and are fed an elemental diet), but express normal frequencies of Triple^{hi} and Triple^{lo} T_{reg} cells; this demonstrates that their differentiation is driven exclusively by self-antigens. Taken together, these data demonstrate that Triple^{hi} and Triple^{lo} T_{reg} cells are generated in a programmed fashion, based on their affinity for self-antigens.

T_{reg} cells and CD4$^+$ T_{convs} cells are differently selected and have dissimilar TCR repertoires. [24,25] A comparison of the TCR repertoires expressed in thymic and peripheral (induced) T_{reg} cells is difficult due to the absence of specific markers for cell sorting.[8,9,14,37,38] However, analysis of peripheral (assumed to be thymus-derived) and colonic (assumed to be peripherally induced) T_{reg} cells revealed different TCR repertories expressed in these two populations.[26] Deep sequencing shows that the TCR repertoires of Triple^{hi}, Triple^{lo} T_{reg} and CD4$^+$ T_{conv} cells indicates are distinct; this is expected if TCR specificity is linked to T_{reg} cell differentiation. The decreased TCR diversity among Triple^{hi} T_{reg} cells may be due oligoclonal expansion; this is consistent with their increased proliferation *in vivo*.

Based on CD5 and Nur77-GFP reporter expression,[31,32] the affinity hierarchy for self-reactivity is likely Triple^{hi} T_{reg} cells > Triple^{lo} T_{reg} cells > CD4$^+$ T_{conv} cells. Exposing MHCII restricted TCR-tg thymocytes to threshold- (weak deleting), intermediate- (moderate deleting) or high- affinity (strong deleting) antigens generates Triple^{lo}, Triple^{int} or Triple^{hi} T_{reg} cells, respectively. The principle that thymocyte affinity for self-antigen determines cell fate also applies to T_{reg} cell development.

Whether different T_{reg} cell populations suppress different aspects of autoimmunity is not fully known.[15] Acute T_{reg} cell ablation in Foxp3^{DTR} mice leads to the activation of T cells specific for "available-antigens" including genome encoded self, environmental and food antigens.[39] We show that the massive expansion of T_{conv} and B cells in T_{reg} cell ablated mice is controlled by transferring Triple^{hi}, but not Triple^{lo} T_{reg} cells. Triple^{hi} T_{reg} cells may suppress lymphoproliferation in peripheral LNs by either modifying DCs towards a tolerogenic phenotype[41] or by directly interacting with T_{conv} cells.[39, 40, 42]

A number of reports show that co-transfer of T_{reg} cells, in particular microbiota-specific T_{reg} cells prevents the onset or even cures mice from colitis.[7, 43, 44] iT_{reg} cells are essential for maintaining immune homeostasis, especially at mucosal interfaces; additionally iT_{reg} cells

contribute to fetal tolerance.5,12,13 In the gut, naive CD4+T cells are converted into iT$_{reg}$ cell following TCR stimulation in the presence of TGF-β and IL-2; other compounds such as retinoic acid (RA) or short-chain-fatty-acids from microbiota mediate conversion as well.7 IL-10 is also a key player in maintaining lymphocyte homeostasis in the gut as IL-10 deficient mice suffer from spontaneous colitis.7 Our results clearly show that Triple^{lo} T$_{reg}$ cells suppress colitis induction. Triple^{lo} T$_{reg}$ cells by themselves do not control colitis induction, but function by promoting the generation of iT$_{reg}$ cells from CD4+ T$_{conv}$ cells. To our knowledge, there is no study, showing that a particular T$_{reg}$ cell population can induce the conversion of CD4+ T$_{conv}$ cells in iT$_{reg}$ cell *in vivo*. M2a macrophages were shown to promote a supportive environment for iT$_{reg}$ cells and directly contribute to immunological homeostasis in the gut.45 Nevertheless, how Triple^{lo} T$_{reg}$ cells facilitate the generation of iT$_{reg}$ cells is still an open question.

T$_{reg}$ cell-like 'wannabe' CD4+ T cells accumulate in scurfy mice.33,34 These T$_{reg}$ cell-like Scurfy CD4+T cells are phenotypically similar to bona fide T$_{reg}$ cells and even express similar TCRs.21 Transfer of T$_{conv}$-like CD4+ T cells from scurfy mice resulted in colitis, but not the other features of scurfy disease.33 Here, we show that Scurfy Triple^{hi} CD4+T cells are similar to bona fide Triple^{hi} T$_{reg}$ cells with respect to PD-1, GITR, CD25 and Helios expression. Transferred Scurfy Triple^{hi} CD4+T cells proliferate extensively in peripheral LNs, infiltrate the skin and cause cutaneous lesions similar to those seen in scurfy mice. Interestingly, IL-2 deficient scurfy mice do not develop skin lesions, while IL-4-, IL-6-, IL-10-, Stat6- or CD103- deficient scurfy mice do46 suggesting that IL-2 acts as an important mediator of skin inflammation in scurfy mice. Scurfy Triple^{hi} CD4+T cells likely require but do not produce their own IL-2, since they express Helios, a repressor of IL-2 transcription.34 This might explain the accumulation of scurfy Triple^{hi} CD4+T cells around IL-2 secreting, skin resident DCs in the dermis.47 In contrast, scurfy Triple^{lo}CD4+T cells induce severe colitis within four weeks when transferred to T cell deficient recipients. It's unclear whether scurfy Triple^{lo} CD4+T cells are the scurfy equivalent to B6 Triple^{lo} T$_{reg}$ cells or to B6 CD4+ T$_{conv}$ cells. A portion of Scurfy Triple^{lo} CD4+T cells are likely to be microbiota specific, since germfree scurfy mice are less prone to develop colitis compared to scurfy mice housed under SPF conditions.26,36 Taken together, these results indicate that scurfy disease is pleiotropic. Although the absence of bona fide T$_{reg}$ cells is the major contributor to the scurfy phenotype, the presence of dysregulated T$_{reg}$ cells-like cells very likely initiates several pathological aspects of this disease.

In summary, our results show that the extent of self-reactivity underlies the development of two distinct populations of regulatory T cells. The highly self-reactive Triple^{hi} T$_{reg}$ cells control the homeostatic proliferation of lymphocytes in peripheral LNs, whereas the less self-reactive Triple^{lo} T$_{reg}$ cells facilitate the generation of iT$_{reg}$ cells in order to maintain lymphocyte homeostasis in the colon. Scurfy mice contain dysregulated T$_{reg}$ cells-like CD4+ T cells, which contribute to the pathology of scurfy disease.

Methods

Mice

CD45.1 congenic C57BL/6 (B6 Ly5.1, strain:002014), CD45.2 congenic C57BL/6J (B6, strain:000664), RIP-OVA mice expressing a membrane bound form of Ova under the control of the rat insulin promoter (RIP, strain: 005433)[48,49] OTII TCR-tg mice recognizing IA[b]-OVA$_{323-339}$ (strain: 004194)[50], B6.Nur77-GFP(strain: 018974)[31] and Foxp3-deficient (scurfy, strain: 019933) mice[51] were all obtained from The Jackson Laboratory (Bar Harbor, ME). 3BK506 TCR-tg and 3BK508TCR-tg mice recognizing IA[b]-3K and mice deficient for MHC class II, invariant chain and *Rag2* gene (referred here as MHCII KO mice) were provided by P. Marrack and J. Kappler (Denver, USA) and are described elsewhere[52]. Foxp3[DTR] mice[39] were kindly provided by A. Rudensky (New York, USA). Foxp3eGFP and *CD3e*^{−/−} mice 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.[53,54,55] Male Foxp3-deficient mice were used at 2-3 weeks of age, for all other strains, male and female mice at the age of 5-12 weeks were used for experiments. 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 Baselstadt, Switzerland. Mice expressing the YAe62TCRβ-chain[56,57] and all mouse sub-lines were maintained in a pathogen-free environment in accordance with institutional guidelines in the Animal Care Facility at the University of Massachusetts Medical School. Foxp3.RFP/GFP mice on the B6 background were bred and maintained at the animal facility of the CRTD (Dresden, Germany) under specific pathogen-free conditions; animal experiments were performed in accordance with the German law on care and use of laboratory animals and approved by the Regierungspräsidium Dresden. Antigen free and germ free B6 mice[30] were bred and maintained at the animal facility of the Pohang University of Science and Technology. This research was approved by the Institutional Animal Care and Use Committees (IACUC) of the Pohang University of Science and Technology (2013-01-0012). Mouse care and experimental procedures were performed in accordance with all institutional guidelines for the ethical use of non-human animals in research and protocols from IACUC of the Pohang University of Science and Technology. Foxp3-RFP Rag-GFP dual reporter mice[29] on the B6 background were bred and maintained at the animal facility of the Biomedical Services Unit at the University of Birmingham and all experiments were performed in accordance with local and national Home Office regulations.

Flow Cytometry and cell sorting

Thymocytes and lymphocytes were stained with LIVE/DEAD Fixable near-IR stain Kit (Life Technologies, Invitrogen) and surface antibodies against CD3 (145-2C11), CD4 (RM4-5), CD5 (53-7.3), CD8 (53.58), CD19 (ID3), CD25 (PC61), CD44 (IM7), CD45.1 (A20), CD45.2 (104), CD45R (B220, RA3-6B2), CD62L (MEL-14), CD103 (2E7), CD197 (CCR7, 4B12), CD278 (ICOS, 7E.17G9), CD279 (PD-1, RMP 1-30), CD357 (GITR, DAT-1/ YGITR765), Nrp-1 (polyclonal), TCRβ (H57-597) and α β (DATK32). Intracellular staining for Foxp3 (FJK-16s/ 150D), Helios (22F6), pcJun (D47G9), pCD3ζ

(K25-407.69) and pErk (197G2) was performed using the Foxp3 staining kit (eBioscience). All antibodies have been validated by their suppliers and references can be found on their website or on the online validation databases Antibodypedia and 1DegreeBio. Antibody dilutions were 1:100 for surface stainings and 1:50 for intracellular stainings. 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, $CD4^+T$ cells were enriched using Dynabeads® Untouched™ Mouse CD4 Cells Kit (Life Technologies, 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 (Life Technologies, Invitrogen) according to manufacturer's instructions. All kits were used according to manufacturer's instructions.

In vitro assays

Bone marrow derived DCs (BMDCs) were generated from bone marrow cells of 5-7 week old B6 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, LUTZ-GMCSF, kindly provided by V.Horejsi). 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, Invitrogen) magnetic bead enriched CD4 cells (Dynabeads, Invitrogen) in 96-well-U-shaped plates for 5 days. For *in vitro*, T_{reg} cell development experiments, $1x10^5$ thymocytes from 3BK508TCR-tg mice were co-cultured with $1x10^5$ B6 BMDCs in the presence of IL-2 (25U/ml, hybridoma X63 supernatant) and recombinant mouse TGF-β1 (10ng/ml, R&D Systems) for 48h with or without 1μM 3K (FEAQKAKANKAV), P2A (FEAAKAKANKAVD) or P-1A (FAAQKAKANKAVD) peptides (all obtained from Eurogentec). Re-aggregated thymic organ cultures were performed as previously described.58 In brief, RTOC were established from B3K508TCR-tg, MHCII KO thymocytes and thymic epithelial cells from B6 mice and were cultured in presence of P-1A (20 μM), P2A (2 μM) or 3K (0.2 μM) peptides for 7 days before analysis. 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.49 was adapted. Recipient mice (CD45.1 CD45.2) were lethally irradiated with 900 rad (GammaCell, Best Theratronics, CA). Bone marrow cells from 5-8 week old B6 mice (CD45.1) and OT-II $Rag2^{-/-}$ mice (CD45.2) were isolated and depleted of mature $CD4^+$ and $CD8^+$ T cells. A mixture of 9:1 of B6 and OT-II $Rag2^{-/-}$ 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

Foxp3^{DTR} mice were injected intra-peritoneal (i.p.) with Diphtheria 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 T$_{reg}$ 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 CD3ε$^{−/−}$ mice received (i.v.) 3.2x10^5 sorted naive CD4$^+$ T cells from pooled LNs of B6Ly5.1 or Foxp3^{DTR} Ly5.1 mice. In some groups, 0.8 x10^5 sorted T$_{reg}$ cells from pooled LN were co-transferred. Recipients of naive Foxp3^{DTR} CD4$^+$ T cells (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 CD3ε$^{−/−}$ were reconstituted with 5x10^5 sorted CD4$^+$ subpopulations from pooled LNs of 2-3 week old sick Foxp3-deficient (scurfy) male mice. Recipient mice were weighed weekly at the same time of day 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.

Histology

Formalin-fixed tissues were processed, stained with hematoxylin and eosin and evaluated blindly.

Clonotype Analysis

Naive CD4$^+$ (CD4$^+$CD25$^−$Foxp3$^−$), Triple^{lo}T$_{reg}$ (CD4$^+$CD25^{lo}Foxp3$^+$GITR^{lo} PD-1^{lo}) or Triple^{hi}T$_{reg}$ (CD4$^+$CD25^{hi}Foxp3$^+$GITR^{hi}PD-1^{hi}) cell populations were sorted from 3 replicate groups (2 mice per group) of single TCRβ-tg (B6.YAe62$\tilde{\beta}$tg$^+$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 V$_α$2 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-multx59 and clonotypes for each population were tabulated using TCRklass60.

Similarity and Diversity analysis 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 Ver9.1.061 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 Entropy62 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 index63 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.

Statistical analysis

Statistical analyses were performed using Prism 6.0 (Graphpad software). If not other indicated, Students t test (unpaired, two-tailed) was used to assess statistical significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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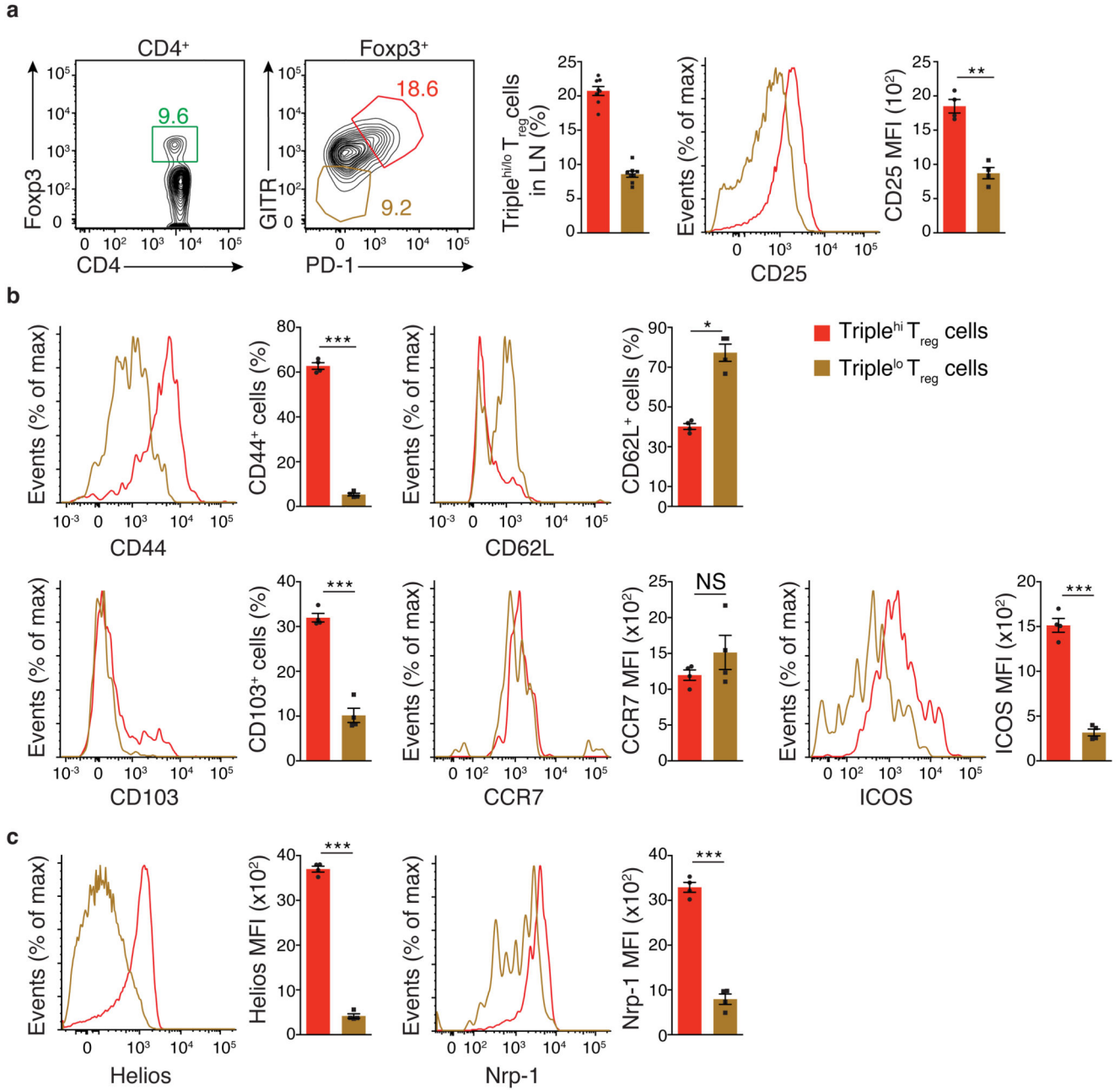
Figure 1.
Characterisation of Triple^{hi} and Triple^{lo} T_{reg} cells. **(a)** Flow cytometry analyzing the expression of Foxp3, GITR and PD-1 and CD25 by CD4^{+} LN cells from 6-12 week old B6 mice. Numbers adjacent to outlined areas indicate percent of Foxp3^{+}CD4^{+} cells (left; green) and frequencies of Triple^{hi}T_{reg} cells (GITR^{hi}PD-1^{hi}CD25^{hi}, middle, red) and Triple^{lo}T_{reg} cells (GITR^{lo}PD-1^{lo}CD25^{lo}, middle, brown) among Foxp3^{+}CD4^{+} cells (n= 6 mice). Middle left panel shows quantification of those results. Histogram (right) shows CD25 expression on Triple^{hi} (red line) or Triple[lo](brown, line) T_{reg} cells (n=4 mice) and quantification of median fluorescence intensity (MFI) of those results. **(b,c)** Flow cytometry analyzing the

expression of **(b)** CD44, CD62L, CD103, CCR7, ICOS and **(c)** Helios and Nrp-1 by Foxp3$^+$CD4$^+$ Triple^{hi} (red lines and bars) and Triple^{lo} (brown lines and bars) T$_{reg}$ cells from LNs of 6-12 week old B6 mice (n= 4 mice) and quantification of those results. Each symbol represents an individual mouse; bars graphs indicate the mean (± s.e.m.) NS = not significant (P> 0.05), *P   0.01, **P   0.001, ***P 0.0001 (unpaired, two-tailed t-test). Data are from three **(a)** or two **(b,c)** independent experiments.
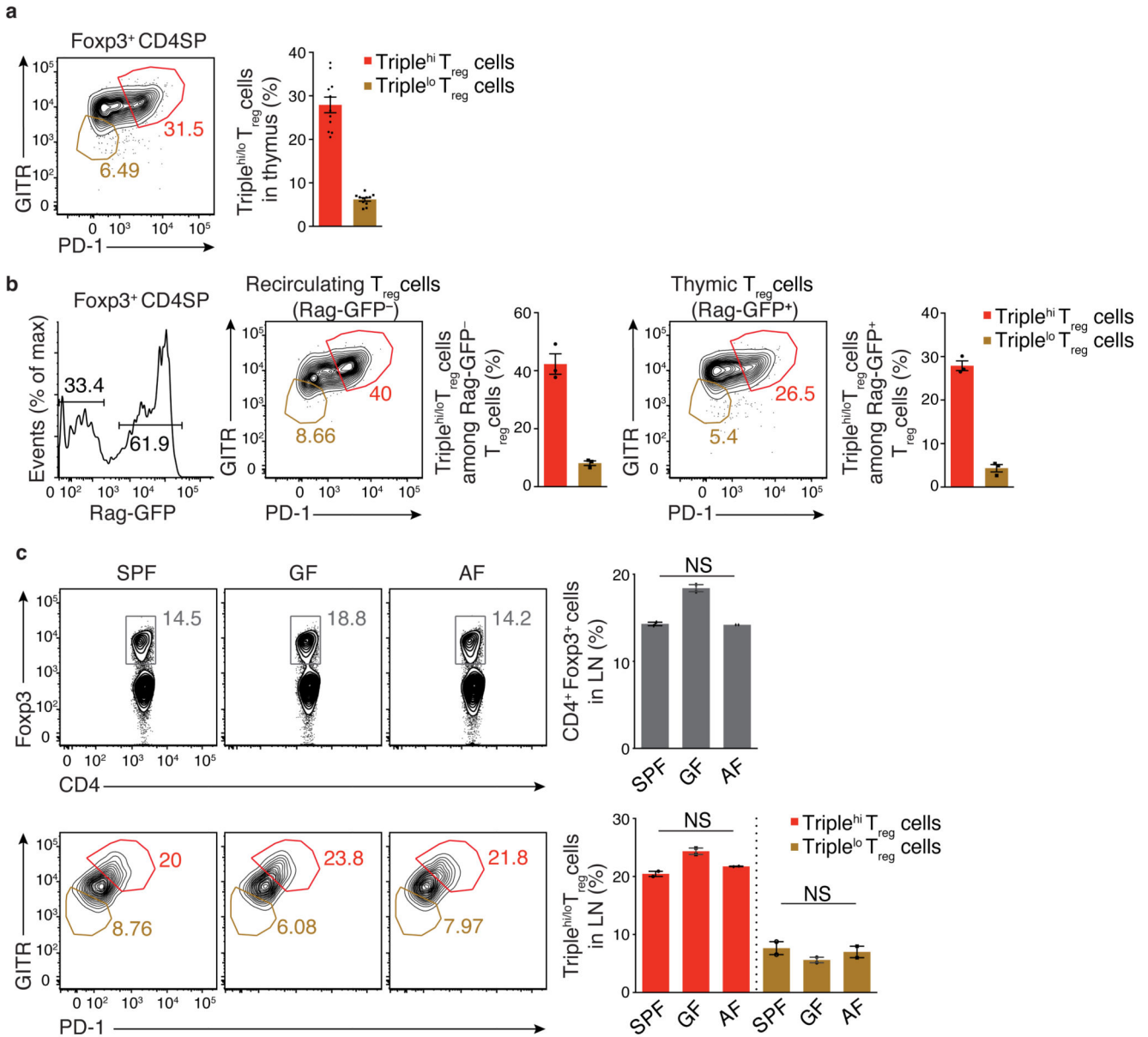
Figure 2.

Analysis of Triple[hi] and Triple[lo] T_{reg} cell origin **(a)** Flow cytometry analyzing the expression of GITR and PD-1 on Foxp3^{+}CD4 single positive (SP) thymocytes of 6-12 week old B6 mice. Numbers adjacent to outlined areas (left) indicate frequencies of Triple[hi] (red) and Triple[lo] (brown) T_{reg} cells among Foxp3^{+}CD4SP thymocytes. Bar graph (right) shows quantification of those results. (n=10 mice) **(b)** Flow cytometry analyzing expression of Rag-GFP reporter, GITR and PD-1 in Foxp3^{+}CD4SP thymocytes from Foxp3-RFP Rag2-GFP dual-reporter mice. Numbers near bracketed lines indicate percent Rag-GFP[−] (left) and Rag-GPF[+] (right) among Foxp3^{+}CD4SP thymocytes. Numbers adjacent to outlined areas indicate frequencies of Triple[hi] (red) and Triple[lo] (brown) T_{reg} cells among recirculating (Rag-GPF[−], middle) and *de novo* generated thymic (Rag-GPF[+], right) T_{reg} cells. Bar graphs

(middle right and far right) show quantification of those results. (n= 3 mice, data taken from 1 experiment) **(c)** Flow cytometry analyzing Foxp3, CD4, GITR and PD-1 expression in LN cells from specific pathogen free (SPF), germ free (GF) and antigen free (AF) B6 mice. Numbers adjacent to outlined areas indicates frequencies of Foxp3^{+}CD4^{+} T cells (top row, gray) and Triple^{hi} (red) and Triple^{lo} (brown) T_{reg} cells in these mice (bottom row). Bar graphs (right) show quantification of those results. (n=2 mice per group) Each symbol represents an individual mouse; bar graphs indicate the mean (± s.e.m.) NS = P> 0.05 (Kruskal-Wallis Test). Data are from five independent **(a)** or one **(b,c)** experiments.
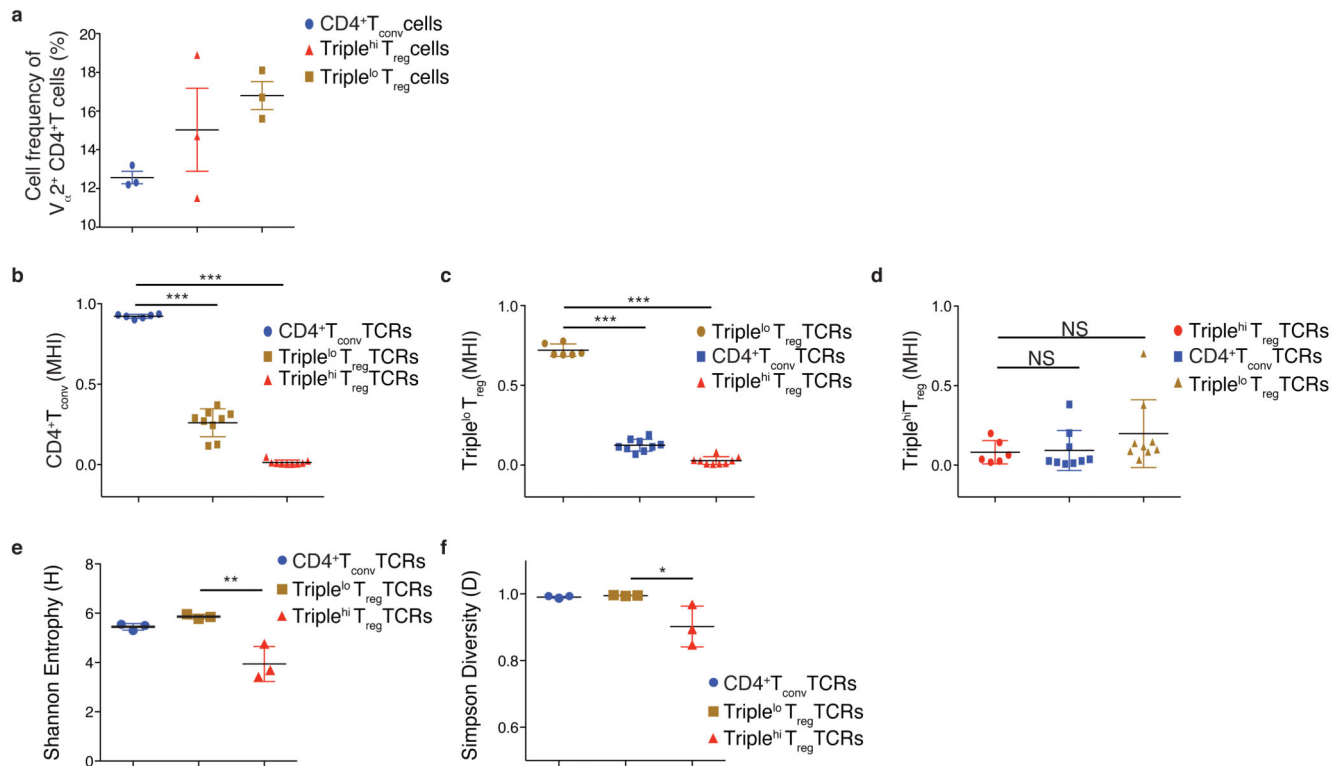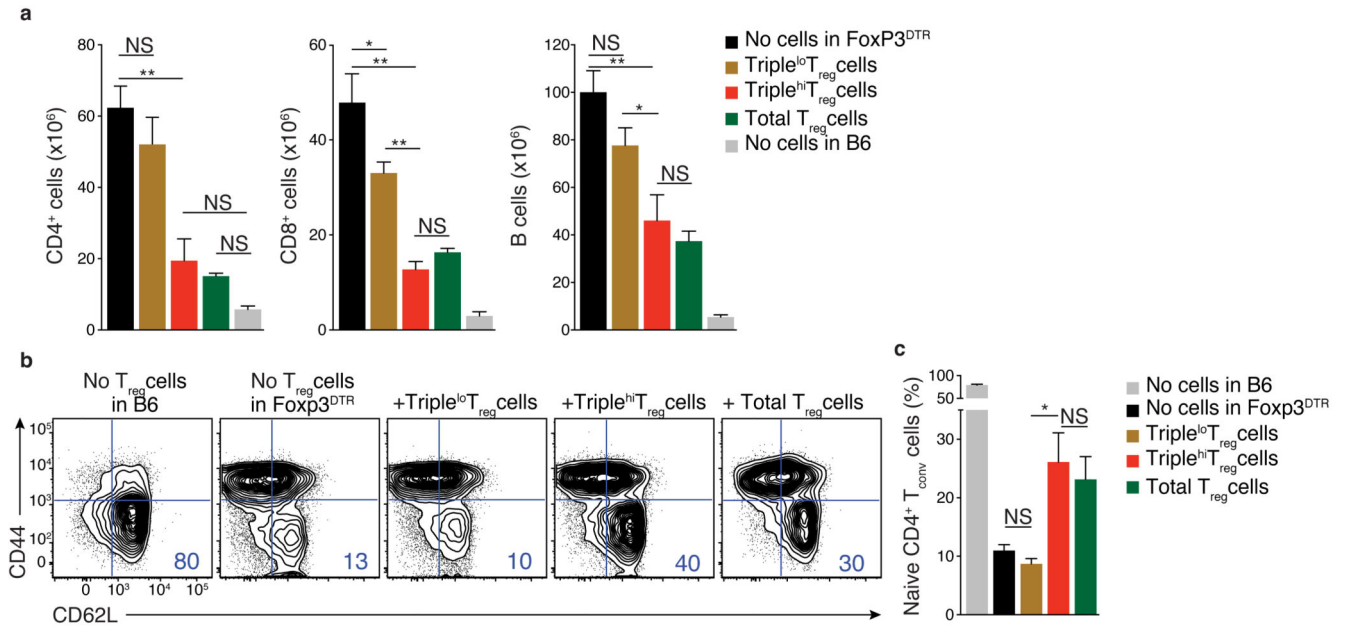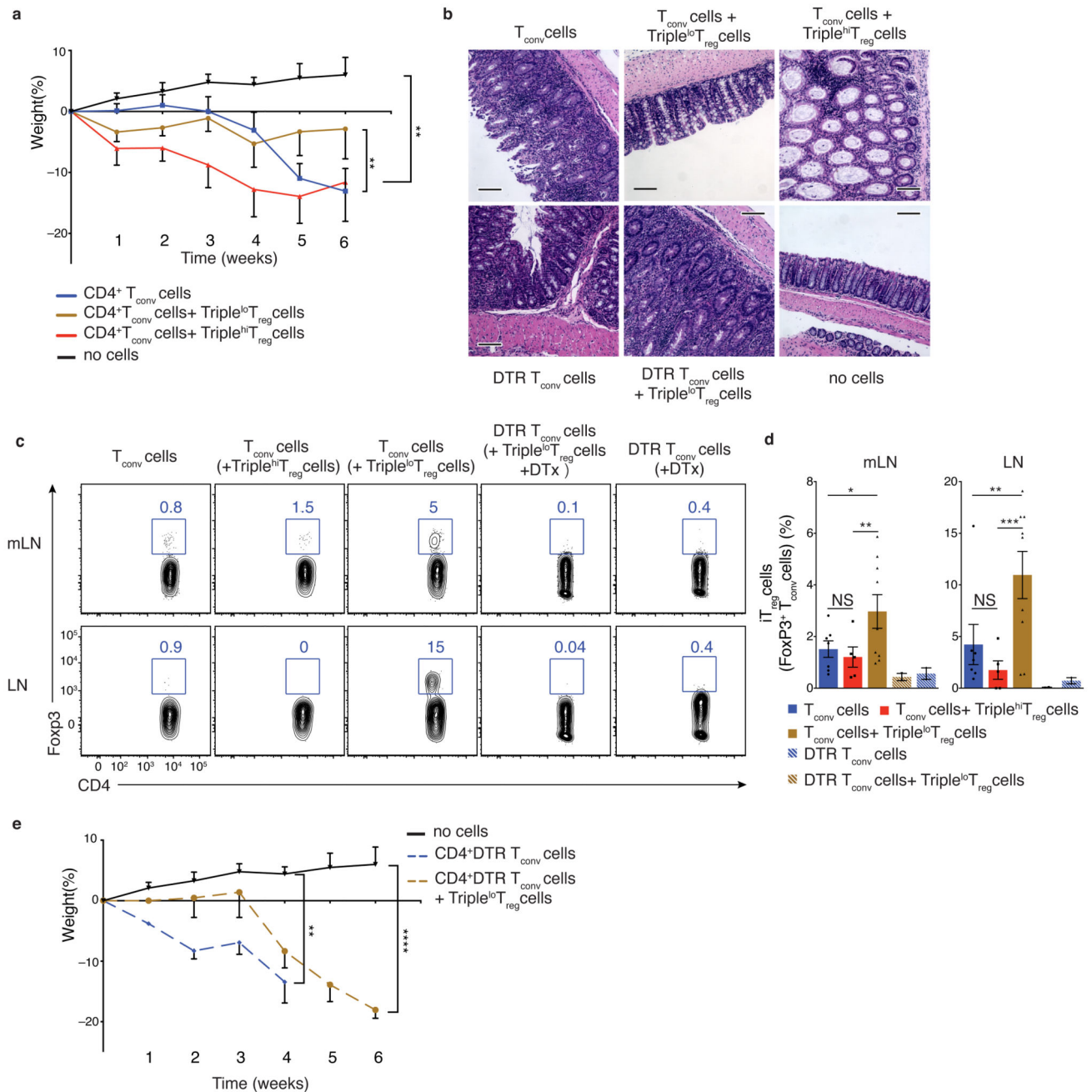
Figure 3.
Sequence similarity analysis between Triple^{hi} T$_{reg}$, Triple^{lo} T$_{reg}$ and CD4$^+$T$_{conv}$ TCRs.
V$_\alpha$2$^+$ TCRα sequences generated from Triple^{hi} T$_{reg}$, Triple^{lo} T$_{reg}$ and CD4$^+$T$_{conv}$ LN cells
from Yae62 Vβ8.2 (single TCRβ chain), TCR$\alpha^{+/KO}$ Foxp3-GFP^{KI} mice. Sequences from
each subset within each group of mice were individually compared to all subsets from all
groups of mice. (**a**) Frequency of V$_\alpha$2 expressing cells among CD4$^+$ T$_{conv}$ cells (blue),
Triple^{hi}T$_{reg}$ cells (red) and Triple^{lo}T$_{reg}$ cells (brown). Evaluation of TCR sequence similarity
based on the Morisita-Horn similarity index (MHI) (**b,c,d**) MHI comparison of V$_\alpha$2$^+$ TCR
sequences (**b**) CD4$^+$ T$_{conv}$ clonotypes (blue) were compared to each other and to Triple^{lo}
(brown) T$_{reg}$ and Triple^{hi} (red) T$_{reg}$ clonotypes (**c**) Triple^{lo}T$_{reg}$ clonotypes (brown) were
compared to each other and to CD4$^+$T$_{conv}$ (blue) and Triple^{hi}T$_{reg}$ (red) clonotypes and (**d**)
Triple^{hi} T$_{reg}$ clonotypes (red) were compared to each other and to CD4 T$_{conv}$ (blue) and
Triple^{lo} T$_{reg}$ (brown) clonotypes (see Methods for full description). Evaluation of TCR
sequences diversity based on Shannon Entropy (**e**) and Simpson Diversity (**f**) scores (see
Methods for full description). Each symbol represents the value from a group of mice (**a**) or
sequences (**e,f**); in (**b,c,d**) each symbol represents a MHI comparison between clonotypes
from two individual groups; small horizontal lines indicate the mean (± s.e.m.) NS = not
significant (P> 0.058), *P=0.058, *P 0.01, ***P 0.001 (Mann-Whittney U test, **b-d** and
unpaired, two-tailed t-test, **e,f**). Data are from one experiment with three independent groups
of TCRβ chain mice (2 mice per group).

Figure 4.
Self-reactivity of Triple^{hi} and Triple^{lo} T$_{reg}$ cells. (**a**) Flow cytometry analyzing Triple^{hi} (red) and Triple^{lo} (brown) LN T$_{reg}$ cells from 6-10 week old B6 mice for CD5 (n=4 mice) and Nur77-GFP (n=2 mice) expression and quantification of MFI of those results. (**b**) Proliferation of LN-derived Triple^{hi} T$_{reg}$ (red), Triple^{lo} T$_{reg}$ (brown) and T$_{conv}$ (blue) cells *in vivo* after intraperitoneal injection of BrdU into B6 mice (n=4 mice). Frequencies of BrdU incorporating (BrdU$^+$) cells are shown. (**c**) Flow cytometry analyzing *in vitro* proliferation (CFSE dilution) of LN-derived Triple^{hi} T$_{reg}$ (red) Triple^{lo} T$_{reg}$ (brown) and CD4$^+$T$_{conv}$ (blue) cells from purified CFSE labeled CD4$^+$LN-derived T cells cultured on B6 or B6.MHCII KO BMDCs. Representative histogram (left) and quantification of those results (numbers of proliferated cells, right) are shown (n= 5 biological replicates). (**d**) Flow cytometry analyzing GITR, PD-1, Foxp3 and CD4 expression by 3BK508TCR-tg CD4SP (top and middle row) or GITR and PD-1 expression by 3BK508TCR-tg Foxp3$^+$CD4SP

thymocytes (bottom row) after 48h of culture in the presence of P-1A, P2A, 3K or no peptide, mature B6 BMDCs as APCs, IL-2 and TGF-β (**e**) Flow cytometry analyzing expression of Foxp3, CD4 on OTII CD4SP thymocytes (left) and GITR and PD-1 on Foxp3^{+}OTII CD4SP thymocytes obtained from chimeras generated by reconstitution of RIP-OVA (top) or B6 (bottom) hosts with a mixture of bone marrow cells from OTII *Rag2*^{−/−} and B6 mice (n=4 mice each group). Numbers in quadrants indicate percent cells; numbers adjacent to outlined areas indicate (**d**, middle) percent Foxp3^{+} among 3BK508TCR-tg CD4SP and (**e**, left) percent of Foxp3^{+} among OTII CD4SP. Each symbol represents an individual chimera (**a,b**). Bar graphs indicate the mean (± s.e.m.). NS = not significant (P>0.054), *P 0.054, **P 0.01, ***P 0.0001 (unpaired, two-tailed t-test). Data are from one (**a**, Nur77), two (**a**, CD5; **b,c**) independent experiments or from one representative experiment from at total of three (**d**) or two (**e**) independent experiments with similar results.
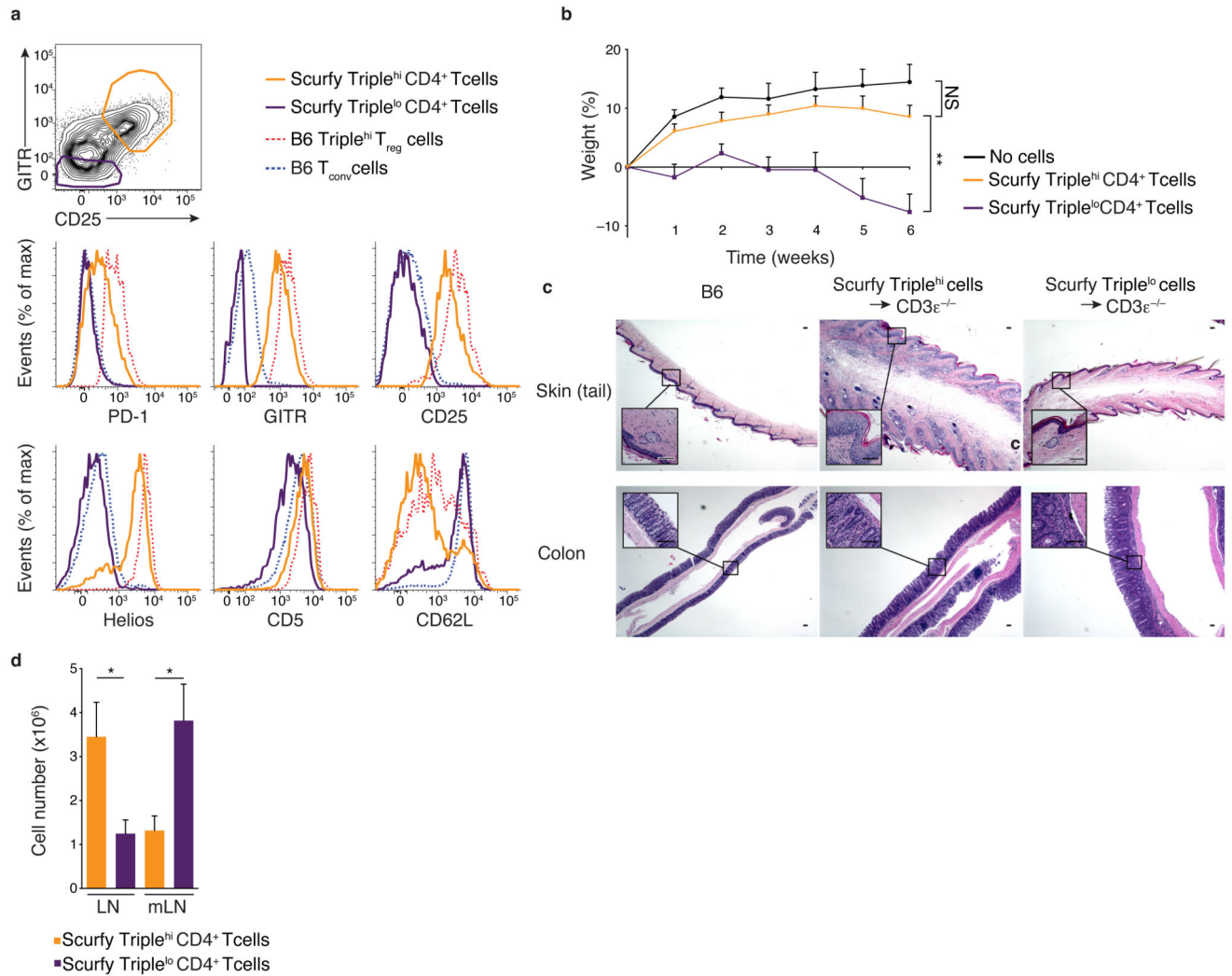
Figure 5.
Triple^{hi} but not Triple^{lo} T$_{reg}$ cells suppress *in vivo* lymphoproliferation.
Analysis of *in vivo* suppressive function of sorted Ly5.2 Triple^{hi} T$_{reg}$, Ly5.2 Triple^{lo} T$_{reg}$ cells or total Ly5.2 T$_{reg}$ cells transferred into 6-10 week old Ly5.1 Foxp3^{DTR} mice treated every other day with diphtheria toxin (DTx). (**a**) Expansion of endogenous CD4$^+$-(left), CD8$^+$-(middle) or B- (right) cells of DTx-treated Ly5.1 Foxp3^{DTR} mice, previously injected (intravenously) with Triple^{lo} T$_{reg}$ (brown, n=4 mice), Triple^{hi} T$_{reg}$ (red, n=6 mice) or total T$_{reg}$ (green, n=3 mice) cells was analyzed at d11-13 after cell transfer. DTx-treated Ly5.1 Foxp3^{DTR} mice receiving no cells (black, n=10 mice) or DTx-treated B6 mice (gray, n=4 mice) were used as controls. (**b**) Representative flow cytometry analyzing CD44 and CD62L expression on endogenous CD4$^+$ T cells, isolated from LNs of DTx treated Ly5.1 Foxp3^{DTR} or B6 mice (described in **a**) (**c**) quantification of endogenous naive (CD44$^-$ CD62L$^+$) CD4$^+$ T$_{conv}$ cells of the results in (**b**). Numbers in quadrants indicate percent cells in each throughout. Bar graphs indicate the mean ($\pm$ s.e.m.). NS = not significant (P>0.05), *P 0.05, **P 0.001 (unpaired, two-tailed t-test). Data are from 2-4 independent experiments (**a**,**b**).

Figure 6.
Triple[lo] but not Triple[hi] T_{reg} cells suppress colitis. Analysis of *in vivo* suppressive function of sorted Ly5.2 Triple[hi] T_{reg} and Ly5.2 Triple[lo] T_{reg} cells co-transferred with sorted, naive ($CD4^+CD25^-$) $CD4^+$ T_{conv} cells from B6 Ly5.1 (**a,b,c,d** $CD4^+$ B6 T_{conv} cells) or Ly5.1 Foxp3[DTR] mice (**b,c,d,e** $CD4^+$ Foxp3[DTR] T_{conv} cells) into 6-10 week old T cell-deficient CD3ε^{−/−} mice. (**a**) Weight change in CD3ε^{−/−} mice following intravenous adoptive transfer of $CD4^+$ T_{conv} cells alone (blue, n=9 mice), $CD4^+$ B6 T_{conv} + Triple[lo]T_{reg} cells (brown, n=9 mice) or $CD4^+$ B6 T_{conv} + Triple[hi]T_{reg} (red, n=6 mice) cells. CD3ε^{−/−} mice injected with no

cells (black, n=5) were used as controls. **b**) Hematoxylin-and-eosin staining of colon sections from CD3$\varepsilon^{-/-}$ mice adoptively transferred with cell populations indicated in **a** and **e**. Scale bar, 100μm. **c**) Flow cytometry analyzing of Foxp3 and CD4 expression by CD4$^+$ B6 T$_{conv}$ cells or CD4$^+$ Foxp3^{DTR} T$_{conv}$ cells isolated from LNs and mesenteric LNs (mLN) from mice described in **a** and **e** six weeks after transfer. Numbers adjacent to outlined areas indicate frequencies of Foxp3$^+$CD4$^+$ (iT$_{reg}$ cells) among those cells and **d**) quantification of those results. **e**) Weight change in CD3$\varepsilon^{-/-}$ mice following intravenous adoptive transfer of CD4$^+$ FoxP3^{DTR} T$_{conv}$ cells alone (dashed blue, n=3 mice) or CD4$^+$ FoxP3^{DTR} T$_{conv}$ + Triple^{lo}T$_{reg}$ cells (dashed brown, n=3 mice). CD3$\varepsilon^{-/-}$ mice not receiving cells (black, n=5) were used as controls. All mice were treated with DTx. NS = not significant (P>0.068), *P=0.068, **P 0.05, ***P 0.01, ****P 0.001(unpaired, two-tailed t-test). Data are from 2-4 independent experiments. Each symbol represents an individual mouse (**d**); mean ± s.e.m in (**a,d,e**).

Figure 7.

Foxp3-deficient (scurfy) mice contain B6 T_{reg} cell-like cells with distinct pathogenicities. **a**) Flow cytometry analyzing the expression of GITR and CD25 on LN CD4$^+$ T cells from Foxp3-deficient (scurfy) mice (top) and PD-1, GITR, CD25 (middle), Helios, CD5 and CD62L (bottom) expression by Scurfy Triple^{hi} (PD-1^{hi}GITR^{hi}CD25^{hi}; orange solid line) CD4$^+$T cells, Scurfy Triple^{lo} (PD-1^{neg}GITR^{neg}CD25^{neg}; purple solid line) CD4$^+$ T cells, B6 CD4$^+$ Triple^{hi} T_{reg} cells (dotted red line) and B6 CD4$^+$ T_{conv} cells (dotted blue line) from 2-3 week old mice (n=4 mice each group). (**b-d**) Analysis of *in vivo* pathogenicity induced by sorted Scurfy Triple^{hi} and Scurfy Triple^{lo} CD4$^+$ T cells isolated from sick, 2-3 week old FoxP3-deficient mice and adoptively transferred into 6-10 week old CD3ε$^{-/-}$ mice. (**b**) Weight change over time following intravenous adoptive transfer of Scurfy Triple^{hi} (orange) or Scurfy Triple^{lo} (purple) CD4$^+$T cells into 6-10 week old CD3ε$^{-/-}$ mice (n= 14 mice each group). CD3ε$^{-/-}$ mice receiving no cells (black, n=7 mice) were used as controls. (**c**) Representative hematoxylin-and-eosin staining of colon and tail skin sections from CD3ε$^{-/-}$ mice adoptively transferred with cell populations indicated in (**b**) and B6 control mice. Scale

bar, 100μm. (**d**) Numbers of Scurfy Triple^{hi} (orange) and Scurfy Triple^{lo} (purple) CD4$^+$ T cells recovered from peripheral LNs and mLNs six weeks after cell transfer (n=10 mice each group). NS = not significant (P>0.05), *P   0.05, **P   0.001 (unpaired, two-tailed t-test). Data are from seven (**b**) or five (**d**) independent experiments or one experiment representative of two (**a**) or five (**c**) independent experiments with similar results; mean ± s.e.m in (**b**).

Table 1

Comparing surface and intracellular marker expression of Triple^{hi}, Triple^{lo}, effector and central T$_{reg}$ cells.

	CD44	CD62L	CD25	ICOS	CCR7	CD103	Helios	Nrp-1
eT$_{reg}$ cells	high	low	low	high	low	high	high (similar to cTreg cells)	high (similar to cTreg cells)
Triple^{hi}T$_{reg}$ cells	high	low	high	high	int (similar to Triple^{lo} T$_{reg}$ cells)	~70% neg	high	high
cT$_{reg}$ cells	low	high	high	low	high	low	high (similar to eTreg cells)	high (similar to eTreg cells)
Triple^{lo} T$_{reg}$ cells	low	high	low	low	int (similar to Triple^{hi} T$_{reg}$ cells)	~90% neg	low	low