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

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How regulatory T cells (Tregs) control lymphocyte homeostasis is not fully understood. Here we identify two Treg populations with differing degrees of self-reactivity and distinct regulatory functions. Triple^{high} (GITR^{high}, PD1^{high}, CD25^{high}) Tregs are highly self-reactive and control lympho-proliferation in peripheral lymph nodes (LNs). Triple^{low} (GITR^{low}, PD1^{low}, CD25^{low}) Tregs are less self-reactive and limit development of colitis by promoting conversion of CD4 Tconvs into induced Tregs (iTregs). Although FoxP3^{KO} (scurfy) mice lack Tregs, they contain Triple^{high}-like and Triple^{low}-like CD4 T cells with distinct pathological properties. Scurfy Triple^{high} T cells infiltrate the skin while scurfy Triple^{low} cells induce colitis and wasting disease. These findings indicate that TCR affinity for self-antigens drives the differentiation of Tregs into distinct subsets with non-overlapping regulatory activities.

The importance of CD4^{+} regulatory T cells (Tregs) in maintaining lymphocyte homeostasis is best appreciated in mice and humans lacking these cells. FoxP3^{KO} (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, Tregs control homeostatic proliferation of conventional T and B cells and prevent colitis[5,6,7].

Tregs are comprised of thymic Tregs (tTregs) and peripherally-induced Tregs (pTregs or iTregs), which originate from different precursor cells and develop in different locations. tTregs develop in the thymus and their development requires TCR stimulation with agonist peptide-MHCII antigens.[8,9,10] In contrast, iTregs are generated in the periphery from naïve, mature CD4^{+} conventional T cells (Tconv)

during T cell activation in the presence of TGFβ.[11] Both populations are suppresive and their functional properties have been examined. Several studies suggest that tTregs are required to control immune homeostasis and autoimmunity.[5,12,13] On the other hand, iTregs have spezialized functions dependening on the type of inflammation, and have a primary role in controlling mucosal immunity and fetal tolerance.[5,12,13,14] A recent study indicated that tTregs by themselves are not sufficient to suppress chronic inflammation and autoimmunity in the absence of iTregs.[15]

Tregs 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, Tregs can be broadly divided into $CD44^{lo}CD62L^{+}$ central Tregs (cTregs) and $CD44^{hi}CD62L^{lo/-}$ effector Tregs (eTregs).[16] cTregs are quiescent, primarily reside in secondary lymphoid tissues, express high levels of CD25 and are IL-2 dependent. In contrast, eTregs, the dominant Treg population in nonlymphoid tissues, are $CD25^{low}$, highly proliferative, but prone to apoptosis. It's been suggested that eTreg maintenance is driven by TCR and co-stimulatory signals, but not IL-2.[16]

Several studies demonstrated the importance of TCR stimulation to activate cTregs in order to generate suppressive eTregs.[8,9] Furthermore, two very recent studies provided direct evidence that TCR expression is indispensable for Treg survival and suppressive function.[19,20] The Treg repertoire contains self-reactive[8,21,22] as well as foreign antigen reactive[23] TCRs. Considering self-reactive Tregs, their TCR affinity for self-antigens has not yet been fully characterized. While it's generally accepted that Tregs and naïve $CD4^{+}$ Tconvs have non-overlapping TCR repertoires, a small percentage of TCRs are found within both T

cell populations.[24,25] Furthermore, the TCR repertories of tTreg and iTregs were shown to be distinct.[26,27] While the tTreg TCR repertoire is biased toward self-recognition, TCRs expressed in iTregs can recognize foreign antigens with high affinity.[24,26] In line with these findings, it's been shown that activated $CD4^+$ T cells from TCRβtg scurfy mice preferentially used TCRs found in the Treg TCR repertoire of TCRβtg wild type mice.[21] Despite these interesting findings, it's still not clear how a Treg's antigen specificity influences its' regulatory properties.

Here we report two functionally distinct subgroups of thymic Tregs with distinct TCR repertoires and differing TCR affinities for self-antigens. $Triple^{low}$ ($GITR^{low}$, $PD1^{low}$, $CD25^{low}$) Tregs express TCRs whose affinities for self-antigens are close to the threshold for negative selection, while $Triple^{high}$ ($GITR^{high}$, $PD1^{high}$, $CD25^{high}$) Tregs express highly self-reactive TCRs, with affinities well above the negative selection threshold. Functionally, $Triple^{high}$ but not $Triple^{low}$ Tregs control the extensive lymphoproliferation in mice acutely depleted of Tregs. Conversely, $Triple^{low}$ but not $Triple^{high}$ Tregs control colitis by facilitating conversion of CD4 Tconv into induced Tregs (iTregs). Finally, $FoxP3^{KO}$ (scurfy) mice contain $Triple^{high}$- like and $Triple^{low}$- like CD4 T cells with distinct pathological properties. $Triple^{high}$ scurfy T cells infiltrate the skin while scurfy $Triple^{low}$ T cells cause colitis and wasting disease. Our results provide evidence that the degree of thymocyte self-reactivity drives the generation of distinct Treg subtypes, which control different aspects of lymphocyte homeostasis in the host.

1 **Results**

2 **GITR, PD1 and CD25 expression define Treg subsets**

3 FoxP3^{+} regulatory T cells express a continuum of GITR and PD1 (Fig 1a). As
4 GITR^{high} / PD1^{high} Tregs express higher levels of CD25 compared to GITR^{low} /
5 PD1^{low} Tregs (Fig 1a), we refer to these populations as Triple^{high} (GITR^{high},
6 PD1^{high}, CD25^{high}) and Triple^{low} (GITR^{low}, PD1^{low}, CD25^{low}) Tregs, respectively.
7 To compare these Treg populations to previously described Treg subsets[16,17,18],
8 we examined their expression of various homing and chemokine receptors (Fig
9 1b). Based on their expression of these proteins, Triple^{high} and Triple^{low} Tregs are
10 distinct from each other and distinct from central and effector Tregs (Fig S1-
11 table;[16,17,18]). This analysis also shows that central and effector Tregs are
12 contained within the Triple^{intermediate} gate (Fig S2).

13 **Triple^{high} and Triple^{low} Tregs originate in the thymus**

14 Although Triple^{high} and Triple^{low} Tregs are present in the thymus (Fig 2a), they
15 could represent Tregs recirculating from the periphery as opposed to de novo
16 generated thymic Tregs.[28,29] To resolve this, we examined thymic Tregs in mice
17 expressing FoxP3-RFP and Rag-GFP reporters (Fig 2b). RFP^{+}GFP^{+} CD4 SP
18 thymocytes are de novo generated thymic Tregs since they are still Rag-GFP^{+},
19 while RFP^{+}GFP^{-} CD4 SP cells in the thymus are recirculating Tregs from the
20 periphery.[29] The frequency of de novo generated (RFP^{+}GFP^{+}) Triple^{high} and
21 Triple^{low} Tregs in the thymus is similar to what's observed among LN Tregs. The
22 fact that both Triple^{high} and Triple^{low} Tregs develop in the thymus argues against
23 the idea that either population are induced Tregs (iTregs). To address the
24 possibility that Triple^{high} and Triple^{low} Tregs might be induced by foreign antigens
25 or inflammation, we examined Tregs 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 Tregs compared to standard SPF animals (Fig 2c, top row). Importantly, SPF, GF and AF mice contain similar frequencies of Triple^{high} and Triple^{low} Tregs (Fig 2c, bottom row), which also express similar levels of NRP1 and Helios (Fig S3). These data rule out the idea that the Triple^{high} and Triple^{low} phenotypes are a response to inflammation. Furthermore, these results strongly suggest that Triple^{high} and Triple^{low} Tregs are generated through recognition of self-antigens.

Triple^{high} and Triple^{low} Tregs express distinct TCR repertoires

To directly compare the TCR repertoires of Triple^{low} and Triple^{high} Tregs to CD4 Tconvs, all three populations expressing the Vα2 family (Fig 3a), were sorted from a Rag^{+}, single TCR β chain strain (Yae62, Vβ8.2, TCRα$^{+/KO}$, FoxP3^{GFP-KI}; Fig S4) 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 shows that the CD4 Tconv sequences from three independent groups of mice (see Methods) are similar to each other but significantly different from Triple^{low} and Triple^{high} Treg sequences obtained from the same mice (Fig 3b). Triple^{low} sequences isolated from different groups of mice are similar to each other as well, but different from CD4 Tconv and Triple^{high} TCRs (Fig 3c). Interestingly, Triple^{high} TCR sequences are not only different from CD4 Tconv and Triple^{low} sequences, but they are also variable between different groups of mice (Fig 3d). Despite their significant sequence differences, the TCR repertoires of CD4 Tconvs and Triple^{lows} are similarly diverse (Fig 3 e,f). The

repertoire of Triple^{high} Treg TCRs may be less diverse than the CD4 Tconv and Triple^{low} repertoires, at least according to Shannon Entropy analysis. Taken together, deep sequencing analyses showed that Triple^{high} Tregs, Triple^{low} Tregs and CD4 Tconvs have clearly distinct TCR repertoires, implying that TCR specificity is important in selecting these Treg subtypes.

Triple^{high} Tregs are more self-reactive than Triple^{low} Tregs

Up to this point, our findings suggested that Triple^{high} and Triple^{low} Tregs are distinct populations (Fig 1, 3) selected on self-antigens (Fig 2c). To directly test whether Triple^{high} and Triple^{low} Tregs differ in their degree of self-reactivity, we examined CD5 and Nur77 expression in each subset (Fig 4a). The expression of these markers reflects T cell activation and correlates with TCR affinity for its' pMHC ligand.[31,32] The higher expression of Nur77 and CD5 by Triple^{high} Tregs, compared to Triple^{low} Tregs (Fig. 4a) argues that Triple^{high} Tregs are more self-reactive than their Triple^{low} counterparts. To test this idea, we used in vivo BrdU labeling and observed that Triple^{high} Tregs proliferate more frequently *in vivo* compared to Triple^{low} Tregs and CD4 Tconvs (Fig 4b). Furthermore, culturing unsorted CD4 T cells on syngeneic bone marrow DCs, Triple^{high} Tregs proliferate more extensively than Triple^{low} Tregs and CD4 Tconvs (Fig 4c); this proliferation requires expression of MHC II self-antigens on antigen presenting cells (APCs).

To examine the influence of antigen affinity on the generation of CD4SP thymocytes with a Triple^{high} or Triple^{low} phenotype (Fig 4d), B3K508TCRtg Rag^{KO} thymocytes were cultured on syngeneic BmDCs in the presence of TGFβ and IL-2. Addition of P-1A peptide (threshold negative selector) induced development of Triple^{low} CD4 SP thymocytes, while the P2A peptide (intermediate affinity negative selector) induced Triple^{intermediate} CD4SP thymocytes; finally, 3K-peptide (high

1 affinity negative selector) induced only Triple^{high} CD4SP thymocytes. FoxP3^{+}
2 Tregs were also generated in these cultures, but only in the presence of negative
3 selecting peptides (Fig 4d, middle row, Fig S5a). Culturing B3K508 Rag^{KO}
4 thymocytes with the negative selecting ligands, P-1A (threshold negative selector),
5 P2A (intermediate affinity negative selector) and 3K (high affinity negative
6 selector) generated FoxP3^{+} Tregs expressing increasing amounts of PD1 (Fig 4d),
7 CD25 and Helios (Fig S5b). Taken together, the data indicate that threshold-,
8 intermediate- and high- affinity negative selecting antigens induce Triple^{low},
9 Triple^{intermediate} and Triple^{high} Tregs, respectively (Fig 4d, bottom row; Fig S5b).
10 That the threshold negative selector induces weaker TCR signals is supported by
11 its decreased ability to induce pCD3ζ, pcJun and pERK (Fig S5c, d). These in vitro
12 results were confirmed using bone marrow chimeras, where OT-II thymocytes
13 developed in a RIP-OVA host expressing the cognate antigen, ovalbumin (Fig 4e).
14 These chimeric mice contain Triple$^{high\ (and\ intermediate)}$ but not Triple^{low} Tregs in the
15 thymus. Taken together, these data imply that Triple^{high} and Triple^{low} Tregs are
16 likely generated by exposure to negatively selecting antigens; moreover, the
17 resulting Treg phenotype is most likely determined by the affinity of its' TCR for
18 self-antigen.

19 **Triple^{high} Tregs suppress lymphoproliferation**

20 To compare the regulatory properties of these two populations, FoxP3^{DTR} mice
21 received sorted Triple^{high} or Triple^{low} Tregs from B6 mice (Fig S6a), which are
22 unaffected by diphtheria toxin (DTx). Three days later, endogenous Tregs from the
23 FoxP3^{DTR} host were depleted by injecting DTx every other day. LN lymphocytes
24 then were examined by flow cytometry 11 days following the onset of Treg
25 depletion. (Fig 5a). Triple^{high} Tregs control the extensive proliferation of Tcells and

B cells in peripheral LNs of mice depleted of their endogenous Tregs (Fig 5b), while Triple^{low} Tregs function poorly in this respect. As expected, Triple^{high} Tregs limit the activation of CD4 Tconvs (Fig 5c).

Triple^{low} Tregs suppress induction of colitis

To examine whether any of these Treg subsets control colitis, CD3^{KO} mice were injected with sorted naïve CD4 Tconvs (Fig 6a), a treatment, which results in colitis (Fig 6d, upper left panel) and weight loss (Fig 6b, solid blue line) as previously described[7]. Co-transfer of Triple^{low} (Fig 6b, solid brown line; Fig 6d, upper middle panel) but not Triple^{high} (Fig 6b, upper panel, solid red line; Fig 6d, 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-transferred Triple^{low} Tregs facilitated the conversion of some CD4 Tconv into induced Tregs (iTregs) (Fig 6e,f). Mice receiving Triple^{low} Tregs had the highest percentage of iTregs (Fig 6e,f), very limited infiltration of the colonic mucosa (Fig 6d, upper middle panel) and maintained their weight (Fig 6b).

To test whether iTregs were required to control colitis[15], CD4 Tconv cells isolated from FoxP3^{DTR} mice were transferred into CD3^{KO} mice (Fig 6a). These animals were additionally treated with DTx every third day to deplete any iTregs developing from transferred FoxP3^{DTR} CD4 Tconvs. iTreg depletion accelerated weight loss and development of colitis (compare solid blue (Fig 6b) and dashed blue lines in Fig 6c). Co-transferred B6 Triple^{low} Tregs (unaffected by DTx) were unable to control the development of colitis when iTregs were depleted (compare solid brown (Fig 6b) and dashed brown lines in Fig 6c; compare upper middle and lower middle panels in Fig 6d). The data support the idea that Triple^{low} Tregs

1 facilitate conversion of some CD4 Tconv into FoxP3^{+} iTregs, which in aggregate
2 limit development of colitis.
3 Taken together, the data (Fig 5,6) argue for two populations of Tregs: Triple^{highs},
4 which control lymphoproliferation in peripheral LNs and Triple^{lows}, which limit the
5 development of colitis (at least in a lymphopenic setting). It should be noted that
6 the phenotypes of Triple^{highs} are stable over the 11d time course of the experiment
7 in Fig 5 while Triple^{lows} are stable over the 6 week time course of the experiment
8 in Fig 6. (see also Fig S6b). As Triple^{lows} don't suppress lympho-proliferation and
9 Triple^{highs} don't suppress colitis, there is no evidence for a significant degree of
10 trans-differentiation between the two subsets during the time frame of these
11 experiments.
12 **Scurfy Triple^{high} and Triple^{low} CD4 T cells induce different pathologies**
13 While FoxP3^{KO} (scurfy) mice cannot develop Tregs due to the lack of functional
14 FoxP3, they do carry out negative selection.[21] For this reason, we wondered
15 whether FoxP3^{KO} (scurfy) mice contain Triple^{high}- like and Triple^{low}- like CD4 T
16 cells despite their lack of a functional FoxP3 molecule. Flow cytometric analysis
17 shows that these mice contain GITR^{high}, PD1^{high}, CD25^{high} (scurfy Triple^{high}) and
18 GITR^{low}, PD1^{low}, CD25^{low} (scurfy Triple^{low}) CD4 T cells. Scurfy Triple^{high} T cells
19 resembled B6 Triple^{high} Tregs in terms of PD1, GITR, CD25, Helios, CD5 and
20 CD62L expression (Fig 7a). Given their lack of FoxP3 expression and suppressive
21 capacity, scurfy Triple^{highs} may be similar to previously reported Treg
22 "wannabes".[21,33,34] Scurfy Triple^{low} T cells, on the other hand resembled CD4
23 Tconv cells with respect to their expression of these markers (Fig 7a).
24 To investigate their pathological activities, scurfy Triple^{low} and scurfy Triple^{high}
25 CD4 T cells were sorted (Fig S7a) and separately transferred into T cell deficient,

1 CD3^{KO} hosts (Fig 7b). Transferred scurfy Triple^{low} T cells promoted weight loss
2 and colitis (Fig 7c,f). Moreover, they accumulate in mesenteric LNs (Fig 7d,e)
3 where ~35% of these cells express α4β7, an integrin that enables homing to the
4 gut[35] (Fig S7b). In contrast, transferred scurfy Triple^{highs} do not cause weight loss
5 (Fig 7c) and preferentially accumulate in peripheral but not mesenteric LNs (Fig
6 7d,e). Moreover, scurfy Triple^{high} T cells induce massive inflammation in the skin
7 but only minimal inflammation in the colon (Fig 7f). Taken together, these results
8 indicate that the absence of normal Tregs is not the sole cause of scurfy disease;
9 the activity of dysregulated (Treg-like) scurfy Triple^{highs} accounts for some of the
10 pathology observed in these mice.
11
12 **Discussion**
13 We examined the functionality of Treg subsets with distinct TCR repertoires
14 and differing affinities for self-antigens. Our data suggest that Triple^{high} and
15 Triple^{low} Tregs are generated as an offshoot of negative selection. The high affinity
16 self-reactive TCRs expressed by Triple^{high} Tregs likely drives their selection in the
17 thymus as well as their proliferation and suppressive activity in peripheral LNs.[36]
18 On the other hand, thymic precursors expressing lower affinity self-reactive TCRs
19 plausibly differentiate into Triple^{low} Tregs, which facilitate conversion of CD4
20 Tconvs into iTregs. We also demonstrate that FoxP3^{KO} (scurfy) mice contain
21 Triple^{high}- and Triple^{low}- like CD4 T cells, which are presumably derived from
22 negative selection[21] and have distinctive pathological activities: scurfy Triple^{high} T
23 cells preferentially migrate to peripheral LNs and elicit skin pathology (scurfy skin)
24 while scurfy Triple^{low} cells are found in the mesenteric LNs and cause colitis.

25 Triple^{high} and Triple^{low} Tregs are distinct from central and effector Treg subsets.

With respect to CD44, CD62L and ICOS expression, Triple^{high} Tregs resemble eTregs and Triple^{low} Tregs resemble cTregs; however, expression of CD25, CCR7, CD103, Helios and NRP1 indicates that Triple^{highs} and Triple^{lows} are distinct from these previously described subsets.[16]

Thymic Triple^{low} Tregs might be generated in the thymus or alternatively, are iTregs generated in the periphery, which recirculate back to the thymus. This was clarified using FoxP3-RFP / Rag-GFP dual reporter mice, which clearly show that Triple^{high} and Triple^{low} Tregs are present among de novo generated, Rag-GFP^{+}, thymic Tregs. To address whether Triple^{high} and/or Triple^{low} phenotypes represent an activation state induced by foreign antigens or inflammation, we examined Tregs in germ free (GF) and (foreign) antigen-free (AF) mice. GF and AF mice contain similar frequencies of Triple^{high} and Triple^{low} Tregs as found in SPF mice on a normal diet. Since AF mice contain virtually no foreign antigens (they lack a microbiome and are fed an elemental diet), the differentiation and activation of Triple^{high} and Triple^{low} Tregs has to be driven by self-antigens. Taken together, these data demonstrate that Triple^{high} and Triple^{low} Tregs are generated in a programmed fashion, based on their reactivity to self-antigens.

Several reports show that Tregs and CD4 Tconvs cells are differently selected and have dissimilar TCR repertoires.[24,25] A comparison of the TCR repertoires expressed in thymic and peripheral (induced) Tregs 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) Tregs revealed different TCR repertories expressed in these two populations.[26] Deep sequencing of Triple^{high} and Triple^{low} Tregs as well as CD4 Tconvs indicates

that each of these TCR repertoires is distinct; this is expected if TCR specificity is linked to Treg differentiation. The decreased TCR diversity among Triple^{high} Tregs may be due oligoclonal expansion; this is consistent with their increased proliferation in vivo. It should be pointed out that given their origin, the TCR repertoire expressed on iTregs must be contained within the CD4 Tconv repertoire. That Triple^{lows} and CD4 Tconvs express distinct repertoires argues that Triple^{lows} and iTregs are discrete populations.

Based on CD5 and Nur77-GFP reporter expression,[31,32] the affinity hierarchy for self-reactivity is likely Triple^{high} Tregs > Triple^{low} Tregs > CD4 Tconvs. The increased proliferation of Triple^{high} Tregs in vivo and in vitro also supports this idea. Exposing MHC II restricted TCR transgenic thymocytes to threshold- (weak deleting), intermediate- (moderate deleting) or high- affinity (strong deleting) antigens generate Triple^{low}, Triple^{intermediate} or Triple^{high} Tregs, respectively. These data show that the idea that thymocyte affinity for self-antigen determines its cell fate also applies to Treg development.

Whether different Treg populations suppress different aspects of autoimmunity is not fully known.[15] Acute Treg ablation in FoxP3^{DTR} mice leads to the activation of T cells specific for “available-antigens” including genome encoded self, environmental and food antigens. This is supported by the observation that (foreign reactive) TCRtg T cells do not undergo activation upon Treg ablation.[39] We show that the massive expansion of Tconvs and B cells in Treg ablated mice is controlled by transferring as few as 2.5×10^5 Triple^{high}, but not Triple^{low} Tregs. Within 48h following Treg depletion, DCs upregulate surface MHCII, CD80[39] and undergo a 2-12 fold expansion.[40] Triple^{high} Tregs may suppress

lymphoproliferation in peripheral LNs by either modifying DCs towards a tolerogenic phenotype[41] or by directly interacting with T cells.[42]

Experimental colitis induced by transfer of CD4 Tconvs into T cell deficient hosts is a slow, progressive disease mediated by microbiota-specific $CD4^+$ T cells and characterized by a massive cell infiltration into the colon and weight loss within four to six weeks.[7,43] A number of reports show that co-transfer of Tregs, in particular microbiota-specific Tregs prevents the onset or even cures mice from colitis.[43, 44] iTregs are essential for maintaining immune homeostasis, especially at mucosal interfaces; additionally iTregs contribute to fetal tolerance.[5,12,13] In the gut, naïve $CD4^+$ T cells are converted into iTregs following TCR stimulation in the presence of TGFβ and IL-2; other compounds such as retinoic acid (RA) or short-chain-fatty-acids from microbiota mediate conversion as well.[7] In addition, IL-10 is 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 $Triple^{low}$ but not $Triple^{high}$ Tregs suppress colitis induction. $Triple^{low}$ Tregs by themselves do not control colitis induction, but function by promoting the generation of iTregs from CD4 Tconvs. Mice which received $Triple^{low}$ Tregs, but whose recently generated iTregs were depleted with DTx develop fulminant colitis. To our knowledge, there is no study, showing that a particular Treg cell population can induce the conversion of CD4 Tconvs in iTregs *in vivo*. One recent study indicates that M2a macrophages promote a supportive environment for iTregs and directly contribute to immunological homeostasis in the gut.[45] Nevertheless, how $Triple^{low}$ Tregs facilitate the generation of iTregs is still an open question.

Treg-like “wannabe” CD4 T cells accumulate in scurfy mice.[33,34] These Treg-like scurfy T cells are phenotypically similar to bona fide Tregs and even express similar TCRs.[21] Interestingly, transfer of Tconv-like CD4 T cells from scurfy mice resulted in colitis, but not the other features of scurfy disease.[33]

It’s still not clear which scurfy T cell population promotes multi organ inflammation and how TCR affinity for self or foreign antigen is linked to the various pathologies seen in scurfy mice. Here, we show that scurfy Triple^{high} T cells are similar to bona fide Triple^{high} Tregs with respect to PD1, GITR, CD25 and Helios expression. Transferred scurfy Triple^{highs} 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 do.[46] The authors suggested that IL-2 acts as the main mediator of skin inflammation in scurfy mice. In this context, scurfy Triple^{high} cells cannot likely produce their own IL-2, since they express Helios, a repressor of IL-2 transcription.[34] For this reason, the presence of IL-2 secreting, skin resident DCs might explain the accumulation of scurfy Triple^{highs} in the dermis.[47]

In contrast, scurfy Triple^{low} cells do not initiate cutaneous lesions, but instead induce severe colitis within 4 weeks when transferred to T cell deficient recipients. It’s unclear whether scurfy Triple^{low} cells are the scurfy equivalent to B6 Triple^{low} Tregs or to B6 CD4 Tconv cells. Nevertheless, at least a portion of scurfy Triple^{low} 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 pleotropic. Although the absence of bona fide Tregs is the major contributor to the scurfy phenotype,

1 the presence of dysregulated Treg-like cells very likely initiates several
2 pathological aspects of this disease.

3 In summary, our results show that the extent of self-reactivity underlies the
4 development of two distinct populations of regulatory T cells. The highly self-
5 reactive Triple^{high} Tregs control the homeostatic proliferation of lymphocytes, while
6 the less self-reactive Triple^{low} Tregs facilitate the generation of iTregs in order to
7 maintain lymphocyte homeostasis in the colon. Scurfy mice contain dysregulated
8 Treg-like CD4 T cells, which contribute to the pathology of scurfy disease.
9

Methods

Mice

All mice (female and male) were between 5–12 weeks old and had a C57BL/6 genetic background. Male FoxP3KO mice 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)[48,49,39] OTII TCRtg mice recognizing $IA^b/OVA_{323-339}$[50], B6.Nur77-GFP[31] and FoxP3KO[51] 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^{KO} (referred here as MHCII KO) were provided by P. Marrack and J. Kappler (Denver, USA) and are described elsewhere[52]. $FoxP3^{DTR,39}$ mice were kindly provided by A. Rudensky (New York, USA). FoxP3eGFP and $CD3\varepsilon^{-/-}$ ($CD3^{KO}$) 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] 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 YAe62 TCRβ 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 C57BL/6 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

1 animals and approved by the Regieriungspräsidium Dresden. Antigen free and
2 germ free C57BL/6 mice[30] were bred and maintained at the animal facility of the
3 Pohang University of Science and Technology. This research was approved by
4 the Institutional Animal Care and Use Committees (IACUC) of the Pohang
5 University of Science and Technology (2013-01-0012). Mouse care and
6 experimental procedures were performed in accordance with all institutional
7 guidelines for the ethical use of non-human animals in research and protocols
8 from IACUC of the Pohang University of Science and Technology. FoxP3-RFP /
9 Rag-GFP dual reporter mice[29] on the C57BL/6 background were bred and
10 maintained at the animal facility of the Biomedical Services Unit at the University
11 of Birmingham and all experiments were performed in accordance with local and
12 national Home Office regulations.

13 **Flow Cytometry and cell sorting**

14 Thymocytes and T cell were stained with LIVE/DEAD Fixable near-IR stain Kit
15 (Life Technologies, Invitrogen) and surface antibodies against CD3 (145-2C11),
16 CD4 (RM4-5), CD5 (53-7.3), CD8 (53.58), CD19 (ID3), CD25 (PC61), CD44 (IM7),
17 CD45.1 (A20), CD45.2 (104), CD45R (B220, RA3-6B2), CD62L (MEL-14), CD103
18 (2E7), CD197 (CCR7, 4B12), CD278 (ICOS, 7E.17G9), CD279 (PD1, RMP 1-30),
19 CD357 (GITR, DAT-1/ YGITR765), NRP1 (polyclonal), TCRβ (H57-597) and α4β7
20 (DATK32). Intracellular staining for FoxP3 (FJK-16s/ 150D), Helios (22F6), pcJun
21 (D47G9), pCD3ζ (K25-407.69) and pERK (197G2) was performed using the
22 FoxP3 staining kit (eBioscience). For BrdU experiments, mice were injected with
23 1mg/d BrdU (5-bromodeoxyuridine, BD Bioscience) for 3 days and cells were then
24 stained for incorporated BrdU using a BrdU Flow Kit (BD Bioscience) followed by
25 staining for intracellular markers. All antibodies were purchased from BD

1 Bioscience, BioLegend, eBioscience or CellSignaling Technology. For flow
2 cytometric analysis, a FACS CantoII (BD Bioscience) and FlowJo software
3 (TreeStar) were used. For cell isolation, $CD4^{+}$Tcells were enriched using
4 Dynabeads® Untouched™ Mouse CD4 Cells Kit (Life Technologies, Invitrogen)
5 from cell suspensions from different sources (peripheral LN, mesenteric LN,
6 spleen); subpopulations of enriched CD4 cells were further sorted on a
7 FACSAriaIII or Influx cell sorter (BD Biosciences). Cell numbers were determined
8 using AccuCheck Counting Beads (Life Technologies, Invitrogen) according to
9 manufacturer's instructions.

10 **In vitro assays**

11 Bone marrow derived DCs (BmDCs) were generated from bone marrow cells
12 of 5-7 week old B6 or B6.$MHCII^{KO}$ mice. Bone marrow cells were cultured under
13 maturation conditions for 10 days in full medium supplemented with GM-CSF
14 (hybridoma supernatant, LUTZ-GMCSF, kindly provided by V.Horejsi). Autologous
15 mixed lymphocyte reactions (auto-MLRs) were performed by co-culturing $1x10^5$
16 syngeneic (B6 or MHCII KO) BmDCs with $3x10^5$ CFSE labeled (Life Technologies,
17 Invitrogen) magnetic bead enriched CD4 cells (Dynabeads, Invitrogen) in 96-well-
18 U-shaped plates for 5 days. For in vitro, Treg development experiments, $1x10^5$
19 thymocytes from 3BK508tg mice were co-cultured with $1x10^5$ B6 BmDCs in the
20 presence of IL2 (25U/ml, hybridoma X63 supernatant) and recombinant mouse
21 TGFβ1 (10ng/ml, R&D Systems) for 48h with or without 10^{-6}M 3K
22 (FEAQKAKANKAV), P2A (FEAAKAKANKAVD) or P-1A (FAAQKAKANKAVD)
23 peptides (all obtained from Eurogentec). Re-aggregated thymic organ cultures
24 were performed as previously described.[58] In brief, RTOC were established from
25 B3K508, MHC II KO thymocytes and thymic epithelial cells from B6 mice and

1 cultured in presence of P-1A (20 μM), P2A (2 μM) or 3K (0.2 μM) peptides for 7
2 days before analysis. All in vitro assays were performed at 37°C in 5% CO_2 using
3 complete RPMI medium (GIBCO, Life Technologies).

4 **Generation of bone marrow chimeric mice**

5 For generating bone marrow chimeric mice, the protocol from Koehli et al.[49]
6 was adapted. Recipient mice (CD45.1/2) were lethally irradiated with 900 rad
7 (GammaCell, Best Theratronics, CA). Bone marrow cells from 5-8 week old B6
8 mice (CD45.1) and OT-II Rag^{KO} (CD45.2) were isolated and depleted of mature T
9 cells. A mixture of 9:1 of B6 and OT-II Rag^{KO} bone marrow cells ($4x10^6$ total cells)
10 were injected intravenously (i.v.) into irradiated recipient mice. Mice were analyzed
11 12-14 weeks after reconstitution and treated with antibiotics (Nopil, Mepha
12 Pharma AG) in the drinking water until 2 weeks before analysis. The congenic
13 markers CD45.1 and CD45.2 were used to identify T cells derived from different
14 donor bone marrows as well as the host.

15 **In vivo suppression assays**

16 FoxP3DTR mice were injected intra-peritoneally (i.p.) with Diptheria Toxin
17 (DTx) (Calbiochem) every other day for 10-12 days (first and second injection
18 50μg/kg; subsequent injections 25μg/kg). In some groups, $2.5x10^5$ sorted Tregs
19 from pooled LNs were injected i.v. 3 days prior to first DTx injection. Mice were
20 analyzed one day after last their DTx injection. For colitis experiments, 6-10 week
21 old T cell deficient $CD3^{KO}$ mice received (i.v.) $3.2x10^5$ sorted naïve CD4 T cells
22 from pooled LNs of B6Ly5.1 ($CD4^+CD25^-$) or $FoxP3^{DTR}$ Ly5.1 ($CD4^+GFP^-$) mice. In
23 some groups, 0.8 $x10^5$ sorted Tregs from pooled LN were co-transferred.
24 Recipients of naïve $FoxP3^{DTR}$ CD4 Tcells ($CD4^+GFP^-$) were injected every third
25 day with DTx (10μg/kg), i.p.. For adoptive transfer of scurfy CD4 T cells, 6-10

1 week old T cell deficient $CD3^{KO}$ were reconstituted with $5x10^5$ sorted CD4
2 subpopulations from pooled LNs of 2-3 week old sick (scurfy) $FoxP3^{KO}$ male mice.
3 Recipient mice were weighed weekly at the same time of day and sacrificed when
4 initial body weight droped more than 20% or at the latest six weeks after T cell
5 transfer. The congenic markers, Ly5.1 and Ly5.2 were used to identify T cells from
6 the different donors as well the host. Tissue samples were fixed in 4%
7 paraformaldehyde, embedded in parafin, sectioned and stained with hematoxylin
8 and eosin.

9 **Clonotype Analysis of $GITR^{low}$ $PD\text{-}1^{low}$ versus $GITR^{high}$ $PD\text{-}1^{high}$ peripheral**
10 **Tregs**

11 Naïve $CD4^+$ ($CD4^+$ $CD25^-$ $Foxp3^-$), $Triple^{low}$ T_{reg} ($CD4^+$ $CD25^{low}$ $Foxp3^+$
12 $GITR^{low}$ $PD\text{-}1^{low}$) or $Triple^{high}$ T_{reg} ($CD4^+$ $CD25^{high}$ $Foxp3^+$ $GITR^{high}$ $PD\text{-}1^{high}$) T cell
13 populations were sorted from 3 replicate groups (2 mice per group) of single TCRβ
14 chain transgenic (B6.YAe62βtg^+ TCR$\alpha^{+/-}$) mice were sorted to 98% purity (FACS
15 Aria, BD Biosciences). RNA was isolated using Trizol and precipitated with
16 RNase free glycogen (Invitrogen) following the manufactures protocol. cDNA was
17 prepared using oligo-dT's (Promega) and Omniscript RT kit (Qiagen). cDNA was
18 amplified with 20 rounds PCR with generic Vα2 primer (5'-
19 CCCTGGGGAAGGCCCTGCTCTCCTGATA-3') and TCR Cα primer (5'-
20 GGTACACAGCAGGTTCTGGGTTCTGGATG-3'). $1/10^{th}$ volume of the first round
21 PCR was amplified with an additional 20 rounds of PCR using barcoded primers,
22 for post sequence identification of originating T cell population, containing Illunima
23 PE read primer and P5/7 regions, respectively. The resulting 300bp fragment was
24 gel purified (Gene Clean II, MP Biomedicals) and sequenced on a MiSeq using a
25 single read 250bp run (Illumina). Sequence data sets were parsed by barcode

using the program fastq-multx[59] and clonotypes for each population were tabulated using TCRklass[60]. A table of analyzed sequences and their frequencies is shown in Fig S8. All sequences will be made available online.

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[61] 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[62] 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[63] 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 *i*th 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 analysis were performed using Prism 6.0 (Graphpad software). If not other indicated, Students t test (unpaired, two-tailed) 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)

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Author Contributions

L.W. and E.P. conceived and designed the experiments. L.W. performed all experiments except the following: thymic RTOCs carried out by C.G.K; analysis of Tregs in FoxP3.RFP/GFP mice, carried out by S.S. and K.K.; deep sequencing and analysis of TCR clonotypes, carried by B.D.S. and E.S.H; analysis of thymic Tregs in FoxP3-RFP / Rag-GFP dual reporter mice, carried out by N.I.M. and G.A.; analysis of Tregs in GF, AF and SPF mice, carried out by J.Y.L. and C.D.S.; and evaluation of histological sections, carried out by L.M.T. The manuscript was written by L.W. and E.P. All co-authors have read the manuscript.

Author Information

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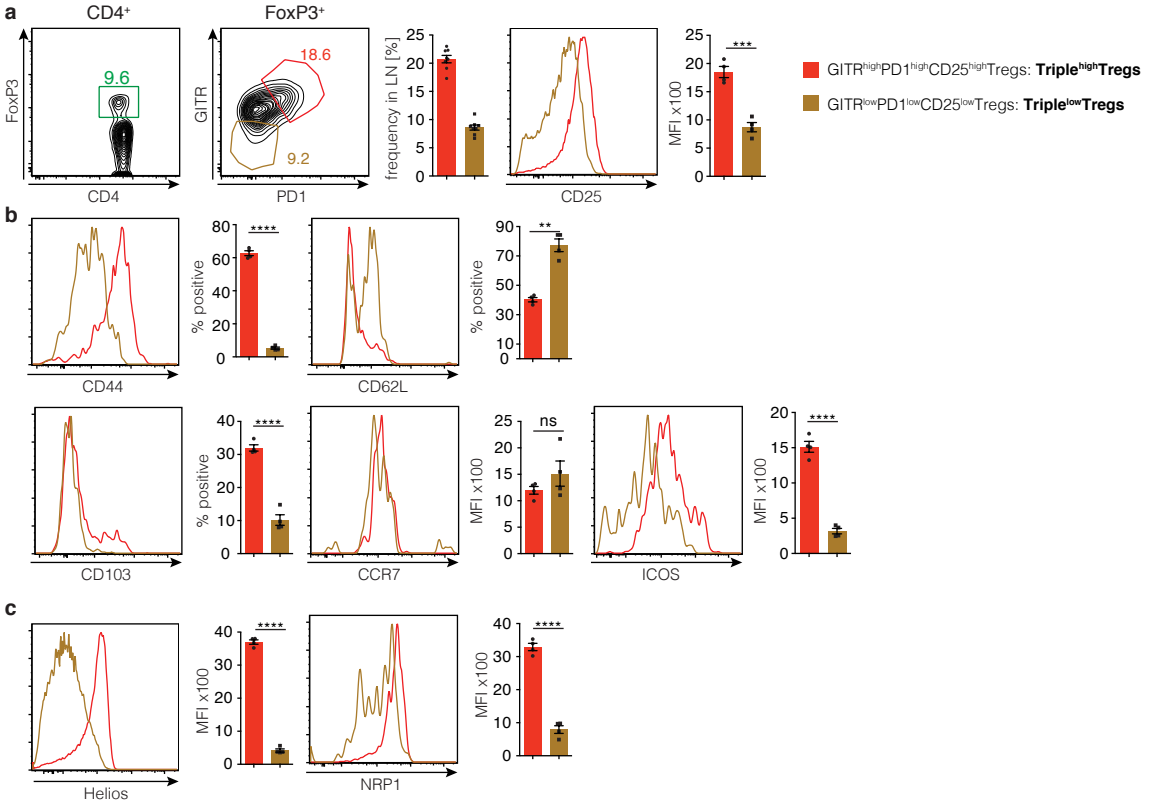
1 **Figure Legends**
2

3 **Figure 1: Triple^{high} and Triple^{low} Tregs cells are phenotypically distinct and**
4 **originate in the thymus.**

5 a) CD4 LN T cells were analyzed for FoxP3, GITR, PD1 and CD25 expression by
6 flow cytometry. Gates show frequencies of CD4^{+}FoxP3^{+} cells (left panel, green
7 gate), Triple^{high} Tregs (GITR^{high}PD1^{high}CD25^{high}, second panel, red gate) and
8 Triple^{low} Tregs (GITR^{low}PD1^{low}CD25^{low}, second panel, brown gate). Bar graph (left)
9 shows frequencies of Triple^{high} and Triple^{low} Tregs in LNs of B6 mice (n=6 mice).
10 Histogram (right panel) shows CD25 expression (MFI) on Triple^{high} and Triple^{low}
11 Tregs (n=4 mice) b and c) Expression of homing and activation markers on
12 Triple^{high} (red) and Triple^{low} Tregs (brown), obtained from B6 LNs and analyzed by
13 flow cytometry b) CD44 and CD62L,CD103, CCR7 and ICOS c) Helios and NRP1
14 (n= 4 mice). *p≤ 0.05, **p≤ 0.01, ***p≤0.001, ****p≤ 0.0001 (Student's t test). Bar
15 graphs show mean ± SEM. Data is taken from 2-3 independent experiments.

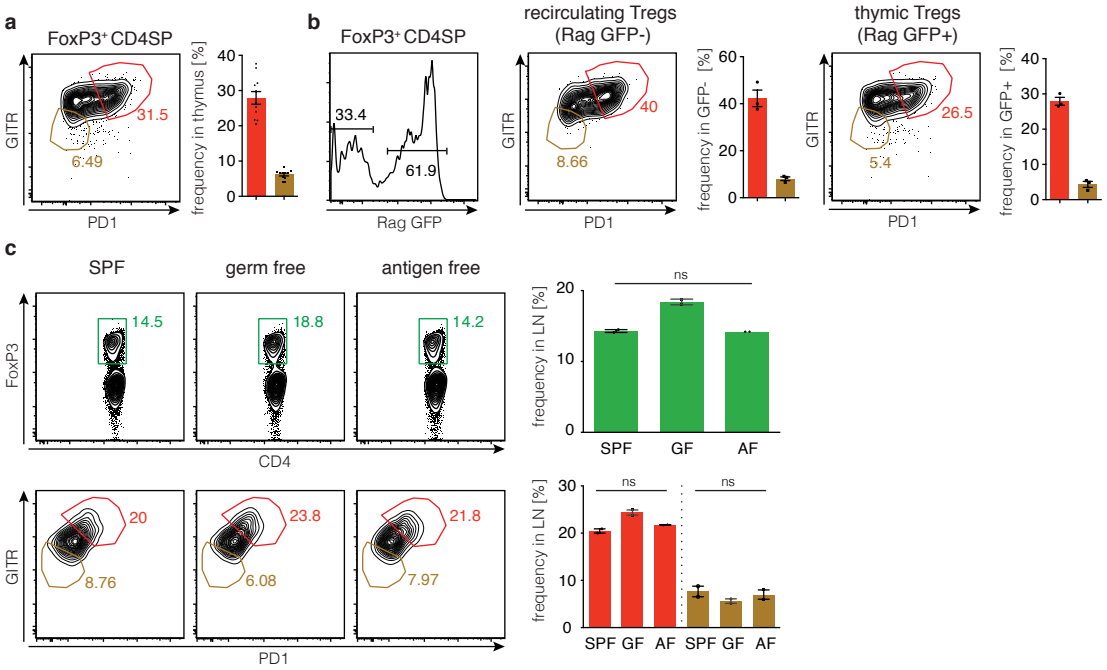
Figure 1


a
CD4+
FoxP3+
FoxP3
CD4
9.6
GITR
PD1
18.6
9.2
frequency in LN [%]
CD25
MFI x100
***
GITRhighPD1highCD25highTregs: Triplehigh Tregs
GITRlowPD1lowCD25lowTregs: Triplelow Tregs
b
CD44
% positive
****
CD62L
**
CD103
CCR7
ns
ICOS
c
Helios
NRP1

1 **Figure 2: Triple^{high} and Triple^{low} Tregs cells have thymic origin.**

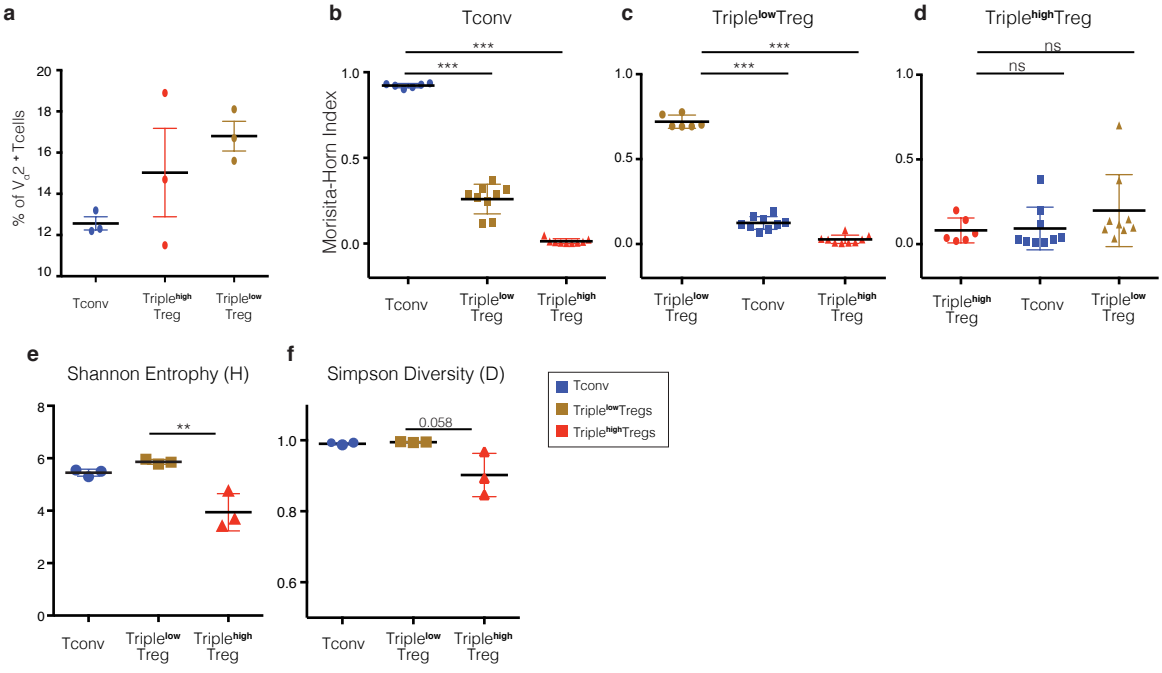
2 a) B6 thymocytes mice were analyzed for FoxP3, GITR and PD1 expression by
3 flow cytometry. Gates show frequencies of Triple^{high} (red) and Triple^{low} Tregs
4 (brown). Bar graph shows mean frequency of Triple^{high} and Triple^{low} Tregs in thymi
5 (n=6 mice, data is taken from 3 independent experiments.) b) CD4SP FoxP3^{+}
6 thymocytes from FoxP3RFP/Rag2GFP dual reporter mice were analyzed by flow
7 cytometry for RagGFP expression (left histogram). Frequencies of Triple^{high} and
8 Triple^{low} Tregs among recirculating thymocytes RagGFP^{-} (left contour plot) and de
9 novo generated RagGFP^{+} (right contour plot) are indicated. Bar graphs depict
10 mean frequencies of Triple^{high} (red) and Triple^{low} (brown) Tregs among
11 recirculating (RagGFP^{-}) and de novo generated (RagGFP^{+}) thymocyte
12 populations. c) Lymph node cells from SPF, germ free (GF) and antigen free (AF)
13 B6 mice were analyzed for CD4, FoxP3, GITR and PD1 expression by flow
14 cytometry. Frequencies of FoxP3^{+} CD4 T cells are shown (top row, green gates).
15 Bar graphs show mean frequency of FoxP3^{+} CD4 T cells. Frequencies of Triple^{high}
16 (red gates) and Triple^{low} Tregs (brown gates) are shown (bottom row). Bar graph
17 show mean frequencies of Triple^{high} (red bar) and Triple^{low} (brown bar) Tregs. (n=2
18 mice) ns= not significant (Kruskal-Wallis Test). Bar graphs indicate mean ± SEM.

Figure 2


a
FoxP3+ CD4SP
GITR
PD1
31.5
6.49
frequency in thymus [%]
40
30
20
10
0
b
FoxP3+ CD4SP
33.4
61.9
Rag GFP
recirculating Tregs
(Rag GFP-)
GITR
PD1
40
8.66
frequency in GFP- [%]
60
40
20
0
thymic Tregs
(Rag GFP+)
GITR
PD1
26.5
5.4
frequency in GFP+ [%]
40
30
20
10
0
c
SPF
germ free
antigen free
FoxP3
CD4
14.5
18.8
14.2
frequency in LN [%]
20
10
0
ns
SPF
GF
AF
GITR
PD1
20
8.76
23.8
6.08
21.8
7.97
frequency in LN [%]
30
20
10
0
ns
ns
SPF GF AF SPF GF AF

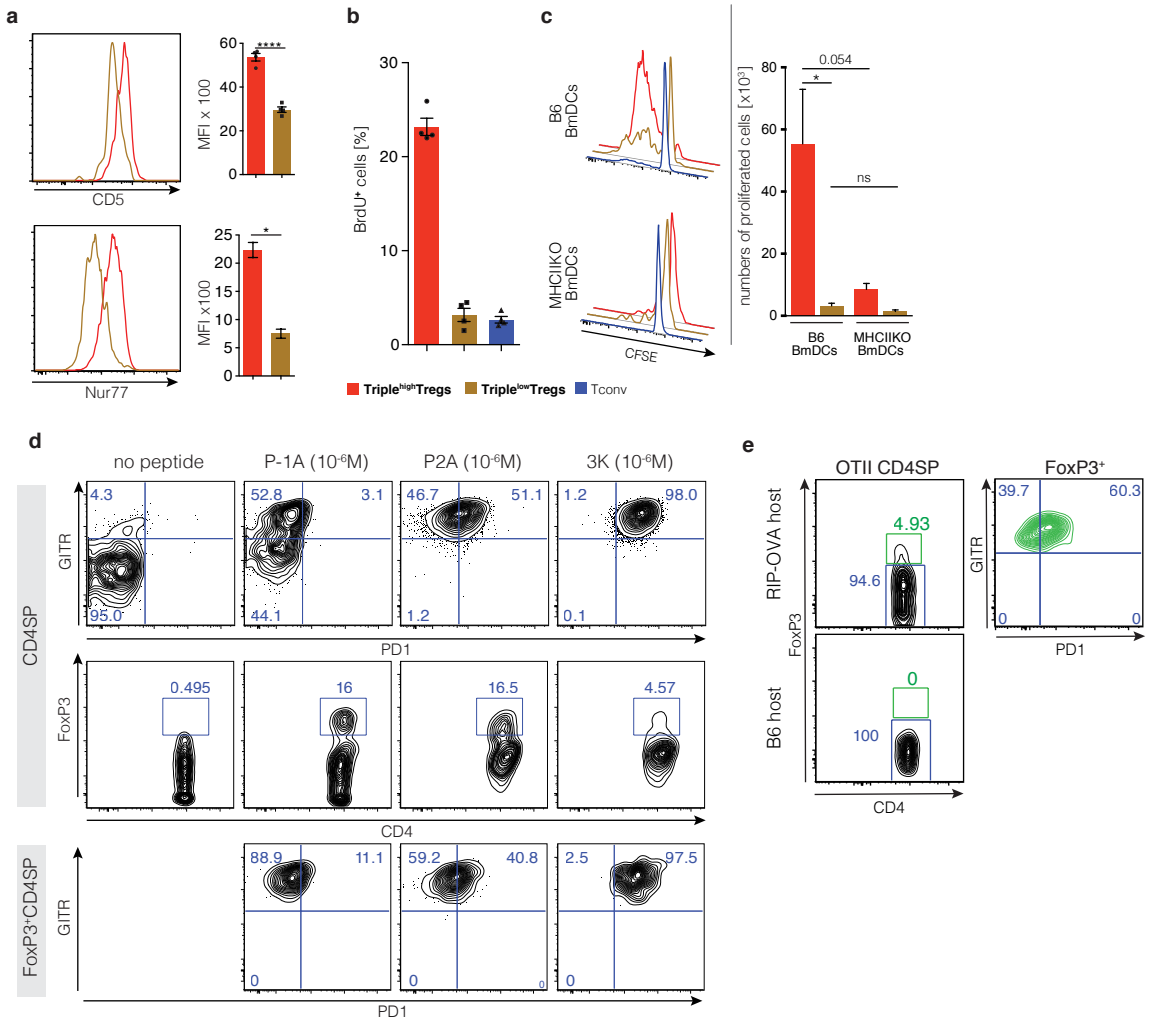
1 **Figure 3: Triple^{high} and Triple^{low} Tregs express distinct TCR repertoires.**
2 Analysis TCR clonotypes in Treg subsets from Yae62 Vβ8.2, TCRα$^{+/KO}$ FoxP3-
3 GFP^{KI} (single TCR β chain) mice. a) Vα2 expression among CD4 Tconvs (blue),
4 Triple^{high} Tregs (red) and Triple^{low} Tregs (brown). b) Morisita-Horn similarity
5 analysis of Vα2^{+} TCR clonotypes from CD4 Tconvs (blue) compared to Triple^{low}
6 (brown) Treg and Triple^{high} (red) Treg clonotypes from three independent groups
7 of single TCRβ chain (YAe62tg, TCRα$^{+/KO}$, Rag^{+}) mice. For the Morisita-Horn
8 Index, values of 0 and 1 represent minimal and maximal similarity, respectively
9 (see Methods for full description). c) Morisita-Horn similarity analysis comparing
10 Vα2^{+} TCR clonotypes from Triple^{low} Tregs (brown) to CD4 Tconvs (blue) and
11 Triple^{high} Treg (red) clonotypes. d) Morisita-Horn similarity analysis comparing
12 Vα2^{+} TCR clonotypes from Triple^{high} Tregs (red) to CD4 Tconvs (blue) and
13 Triple^{low} Treg (brown) clonotypes. TCR repertoire diversity was analyzed by
14 calculating Shannon Entropy (e) and Simpson Diversity (f) scores. For Shannon
15 Entropy analysis, higher H values indicated higher diversity; for Simpson Diversity
16 analysis, the index ranges from 0 to 1 with 1 indicating high diversity (see Methods
17 for full description).
18

Figure 3


a
% of Vγ2+Tcells
20
18
16
14
12
10
Tconv
Triplehigh Treg
Triplelow Treg
b
Tconv
Morisita-Horn Index
1.0
0.5
0.0
***
***
Tconv
Triplelow Treg
Triplehigh Treg
c
Triplelow Treg
***
***
Triplelow Treg
Tconv
Triplehigh Treg
d
Triplehigh Treg
ns
ns
Triplehigh Treg
Tconv
Triplelow Treg
e
Shannon Entrophy (H)
8
6
4
2
0
**
Tconv
Triplelow Treg
Triplehigh Treg
f
Simpson Diversity (D)
1.0
0.8
0.6
0.058
Tconv
Triplelow Treg
Triplehigh Treg
Tconv
Triplelow Tregs
Triplehigh Tregs

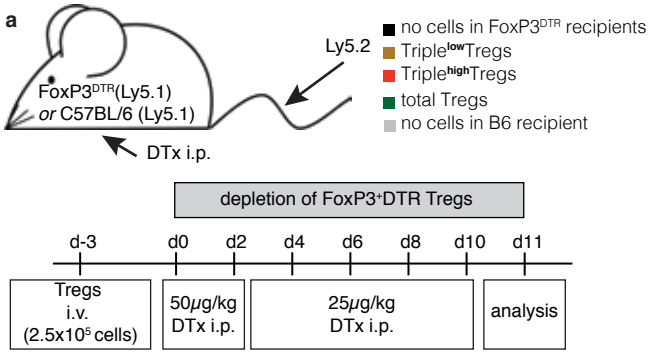
1 **Figure 4: Triple^{high} and Triple^{low} Tregs have different self-reactivity.**
2 a) Triple^{high} (red) and Triple^{low} (brown) Tregs from B6 LNs were analyzed for CD5
3 (n=4 mice) and Nur77-GFP (n=2 mice) expression by flow cytometry. Bar graphs
4 indicate mean MFI. b) In vivo proliferation of B6 LN derived Triple^{high} (red),
5 Triple^{low} (brown) Tregs and Tconvs (blue). Mean percentages of proliferating
6 (BrdU^{+}) cells are shown (n=4 mice). c) Representative histograms show in vitro
7 proliferation (CFSE dilution) of LN-derived Triple^{high} Tregs (red) Triple^{low} Tregs
8 (brown) and CD4 Tconvs (blue) from cultures of purified CD4 LN Tcells and B6 or
9 B6.MHCII^{KO} BmDCs. Bar graph shows mean numbers of proliferating cells (n=6
10 samples from 2 independent experiments). ns= not significant, *p≤ 0.05 (Student's
11 t test). Bar graphs indicate mean ± SEM. d) Representative flow cytometric
12 analysis of GITR and PD1 expression on 3BK508tg CD4SP (top row) or 3BK508tg
13 CD4SP FoxP3^{+} thymocytes (bottom row) 48h after stimulation with P-1A, P2A, 3K
14 or no peptide presented on mature B6 BmDCs in the presence of IL2 and TGFβ
15 (n=3 independent experiments). Middle row shows FoxP3 expression among
16 CD4SP cells in these cultures as indicated e) Left panels: Flow cytometric
17 analysis of FoxP3 expression in OT-II derived, CD4SP thymocytes, of lethally
18 irradiated RIP-mOVA (upper panel) and B6 (lower panel) bone marrow chimeras
19 reconstituted with bone marrow cells from Ly5.1^{+} B6 and Ly5.2^{+} OT-II, Rag^{KO} mice.
20 Numbers indicate percentage of cells within gates. Right panel: Flow cytometric
21 analysis of thymic OTII derived CD4SP FoxP3^{+} thymocytes for GITR and PD1
22 expression in RIP-mOVA hosts. Contour plots are representative of 4 individual
23 chimeric mice.

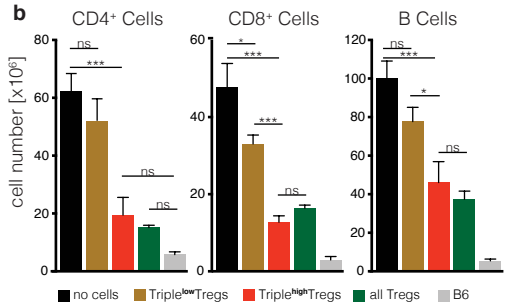
Figure 4

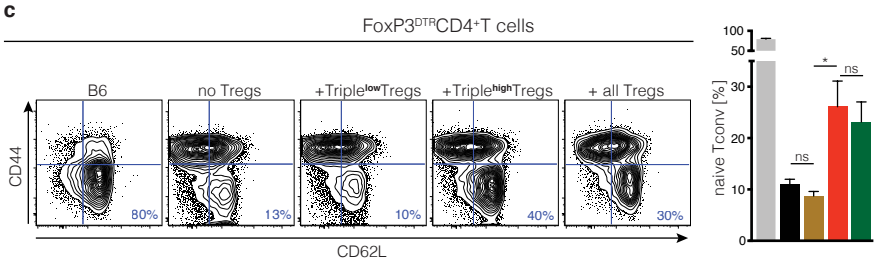

a
CD5
MFI x 100
****
Nur77
MFI x100
*
b
BrdU+ cells [%]
Triplehigh Tregs
Triplelow Tregs
Tconv
c
B6 BmDCs
MHCIIKO BmDCs
CFSE
numbers of proliferated cells [x10³]
0.054
*
ns
B6 BmDCs
MHCIIKO BmDCs
d
no peptide
P-1A (10-6M)
P2A (10-6M)
3K (10-6M)
CD4SP
GITR
PD1
FoxP3
CD4
FoxP3+CD4SP
4.3
95.0
52.8
3.1
44.1
46.7
51.1
1.2
1.2
98.0
0.1
0.495
16
16.5
4.57
88.9
11.1
0
59.2
40.8
0
2.5
97.5
0
e
OTII CD4SP
FoxP3+
RIP-OVA host
B6 host
FoxP3
CD4
GITR
PD1
4.93
94.6
0
100
39.7
60.3
0
0

1 **Figure 5: Triple^{high} but not Triple^{low} Tregs suppress *in vivo***
2 **lymphoproliferation.**
3 a) 2.5x10^{5} sorted B6 Tregs cells, (unaffected by DTx) from pooled B6 LNs were
4 injected intravenously (i.v.) into 6-10 week old FoxP3^{DTR} mice. Three days later,
5 host FoxP3^{+} cells were depleted intra peritoneal (i.p.) injection of DTx every other
6 day for 10 days. Proliferation of host lymphocytes in Ly5.1 FoxP3^{DTR} mice
7 previously injected i.v. with either no cells (black, n=10 mice), B6 Triple^{low} Tregs
8 (brown, n=4 mice) B6 Triple^{high} Tregs (red, n=6 mice), or total B6 Tregs (green,
9 n=3 mice). FoxP3^{DTR} recipients (see above) and B6 control mice (gray, n=4 mice)
10 were treated every other day with diphtheria toxin (DTx) and analyzed at d11-13
11 after cell transfer. Bar graphs show numbers of host-derived, live, CD4^{+}, CD8^{+} and
12 B cells in peripheral LNs. c) Flow cytometric contour plots and bar graph show the
13 percentage of naïve endogenous CD4 Tconvs (Ly5.1^{+} CD44^{low} CD62L^{high}) in mice
14 described in b) n≥4.*p≤ 0.05, **p≤ 0.01, ***p≤0.001, (Student's t test). Bar graphs
15 show mean ± SEM. Data is taken from 2-4 independent experiments.
16

Figure 5


a
FoxP3DTR(Ly5.1)
or C57BL/6 (Ly5.1)
Ly5.2
DTx i.p.
no cells in FoxP3DTR recipients
Triplelow Tregs
Triplehigh Tregs
total Tregs
no cells in B6 recipient
depletion of FoxP3+DTR Tregs
d-3
d0
d2
d4
d6
d8
d10
d11
Tregs i.v. (2.5x10^5 cells)
50µg/kg DTx i.p.
25µg/kg DTx i.p.
analysis


b
CD4+ Cells
CD8+ Cells
B Cells
cell number [x10^6]
ns
***
*
no cells
Triplelow Tregs
Triplehigh Tregs
all Tregs
B6


c
FoxP3DTRCD4+T cells
B6
no Tregs
+Triplelow Tregs
+Triplehigh Tregs
+ all Tregs
CD44
CD62L
80%
13%
10%
40%
30%
naive Tconv [%]
ns
*

1 **Figure 6: Triple^{low} but not Triple^{high} Tregs suppress colitis.**

2 a) To induce colitis, 6-10 week old T cell deficient CD3^{KO} recipients received
3 3.2x10^{5} sorted naïve CD4 Tconvs (CD4^{+}CD25^{-}) isolated from B6 Ly5.1^{+} mice (B6
4 Tconv). In some groups, 0.8 x10^{5} sorted, Ly5.2^{+} Triple^{high} or Triple^{low} Tregs from
5 pooled B6 LNs were co-transferred along with Tconvs. In experiments where iTreg
6 generation was inhibited during colitis induction, 6-10 week old T cell deficient
7 CD3^{KO} recipients were first injected i.v. with 3.2x10^{5} sorted, naïve CD4 Tconvs
8 isolated from FoxP3^{DTR}, Ly5.1^{+} mice (FoxP3^{DTR} Tconv). In some groups, 0.8 x10^{5}
9 sorted B6 Triple^{low} Tregs cells (unaffected by DTx) were co-transferred along with
10 FoxP3^{DTR} Tconvs. To deplete Tconv-derived iTregs, recipients were injected i.p.
11 every third day with DTx (10µg/kg). Mice were weighed weekly at the same time of
12 day and were sacrificed when they lost > 20% of their initial body weight or at six
13 weeks following adoptive transfer. b) Graph shows percentage of weight change
14 of CD3^{KO} mice injected with either no cells (black, n=5), Ly5.1 B6 CD4 Tconvs
15 (blue, n=9 mice) or Ly5.1 B6 CD4 Tconvs co-transferred with either Ly5.2 Triple^{low}
16 Tregs (brown, n=9 mice) or Triple^{high} Tregs (red, n=6 mice). c) Graph shows
17 percentage of weight change in CD3^{KO} mice, which received either no cells (black,
18 n=5 mice), Ly5.1 FoxP3^{DTR} CD4 Tconvs alone (dashed blue, n=3 mice) or Ly5.1
19 FoxP3^{DTR} CD4 Tconvs co-transferred with B6 Triple^{low} Tregs (dashed brown, n=3
20 mice) and injected every third day with DTx. Statistical analysis compares
21 difference of weight change at 6 weeks following cell transfer. *p≤ 0.05, **p≤ 0.01,
22 ***p≤0.001 (Student's t test). d) Representative H&E staining of colon sections
23 from CD3^{KO} mice adoptively transferred with cell populations indicated in a-c). e)
24 Flow cytometric analysis of Ly5.1 B6 CD4 Tconvs or Ly5.1 FoxP3^{DTR} CD4 Tconvs
25 six weeks after transfer from mice described in c). Contour plots show CD4/FoxP3

staining of transferred CD4 Tconvs isolated from mesenteric or peripheral LNs. f) Bar graphs show percentage of CD4 Tconv-derived induced Tregs (iTregs) found in mesenteric or peripheral LNs. *$p \leq 0.05$, **$p \leq 0.01$ (Student's t test) ns = not significant. Bar graphs show mean ± SEM. Data is taken from 2-4 independent experiments.

Figure 6

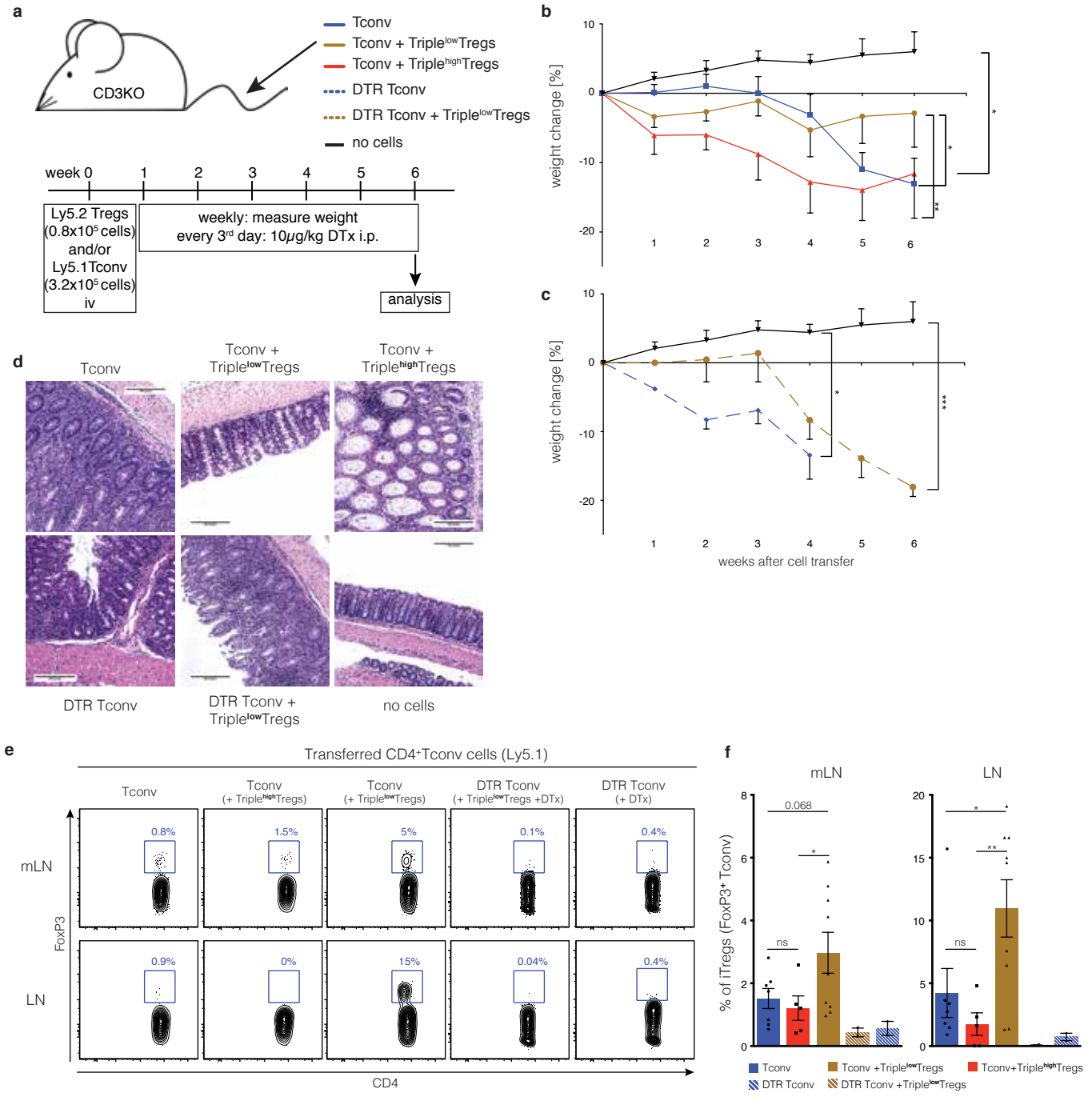
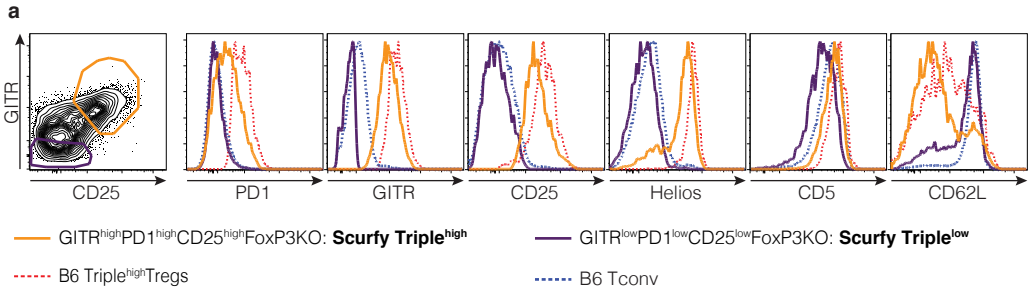
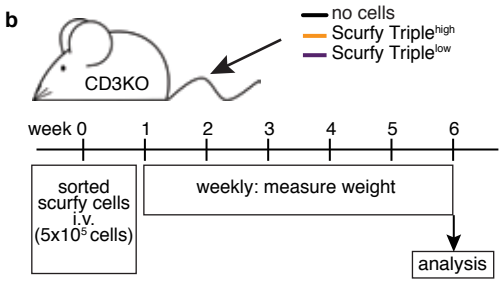

a
CD3KO
Tconv
Tconv + Triple^low Tregs
Tconv + Triple^high Tregs
DTR Tconv
DTR Tconv + Triple^low Tregs
no cells
week 0 1 2 3 4 5 6
Ly5.2 Tregs (0.8x10^5 cells) and/or Ly5.1Tconv (3.2x10^5 cells) iv
weekly: measure weight every 3rd day: 10µg/kg DTx i.p.
analysis
b
weight change [%]
c
weight change [%]
weeks after cell transfer
d
Tconv
Tconv + Triple^low Tregs
Tconv + Triple^high Tregs
DTR Tconv
DTR Tconv + Triple^low Tregs
no cells
e
Transferred CD4+Tconv cells (Ly5.1)
Tconv
Tconv (+ Triple^high Tregs)
Tconv (+ Triple^low Tregs)
DTR Tconv (+ Triple^low Tregs +DTx)
DTR Tconv (+ DTx)
mLN
LN
FoxP3
CD4
0.8%
1.5%
5%
0.1%
0.4%
0.9%
0%
15%
0.04%
0.4%
f
mLN
LN
% of iTregs (FoxP3+ Tconv)
0.068
ns
Tconv
Tconv +Triple^low Tregs
Tconv+Triple^high Tregs
DTR Tconv
DTR Tconv +Triple^low Tregs

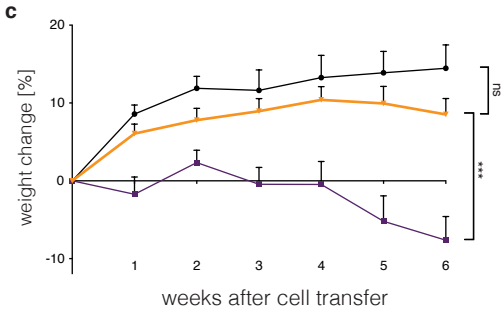
Figure 7: Scurfy Triple^{high} and scurfy Triple^{low} CD4 T cells induce different pathologies.

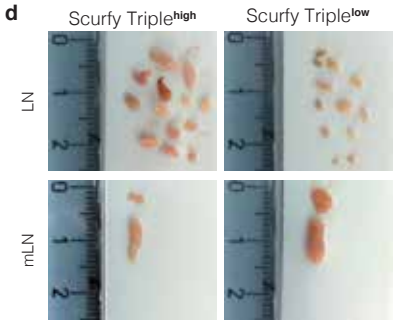
a) FoxP3^{KO} (Scurfy) mice contain Treg like cells. Left panel: Flow cytometric analysis of CD4 T cells showing scurfy Triple^{high} (PD1^{high} GITR^{high} CD25^{high}; orange gate) and scurfy Triple^{low} (PD1^{neg} GITR^{neg} CD25^{neg}; purple gate) CD4^{+} T cells. Additional panels: PD1, GITR, CD25, Helios, CD5 and CD62L 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) Sorted scurfy Triple^{high} or scurfy Triple^{low} T cells were transferred to T cell deficient CD3^{KO} hosts. Host mice were weekly monitored for weight change and development of other pathologies. c) Graph shows mean percentage of weight change ± SEM of CD3^{KO} recipients following adoptive transfer of no (black, n=3 mice), scurfy Triple^{high} (orange, n=8 mice) or scurfy Triple^{low} (purple, n=8 mice) CD4^{+} T cells. ***$p \leq 0.001$ (Student's t test) ns = not significant. d) Photographs of peripheral and mesenteric LNs from CD3^{KO} mice transferred with scurfy Triple^{high} or scurfy Triple^{low} CD4 T cells e) Absolute number of scurfy CD4 cells ± SEM in peripheral LNs and mLNs six week after cell transfer, (orange, scurfy Triple^{highs}; violet, scurfy Triple^{lows}) (n=8 mice) , *$p \leq 0.05$. f) Representative photographs and H&E staining of tail skin and colons sections of CD3^{KO} recipient six weeks after adoptive cell transfer. B6 control mice are shown as well. Data is taken from 5 independent experiments.

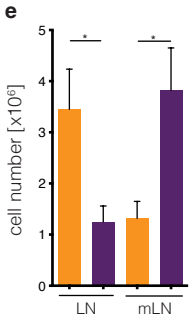
Figure 7

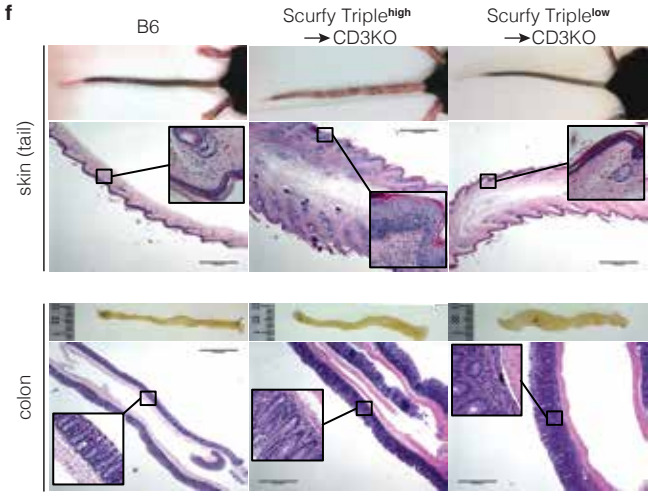

a
GITR
CD25
PD1
GITR
CD25
Helios
CD5
CD62L
GITRhighPD1highCD25highFoxP3KO: Scurfy Triplehigh
GITRlowPD1lowCD25lowFoxP3KO: Scurfy Triplelow
B6 Triplehigh Tregs
B6 Tconv


b
CD3KO
no cells
Scurfy Triplehigh
Scurfy Triplelow
week 0
1
2
3
4
5
6
sorted scurfy cells i.v. (5x10^5 cells)
weekly: measure weight
analysis


c
weight change [%]
20
10
0
-10
1
2
3
4
5
6
weeks after cell transfer
ns
***


d
Scurfy Triplehigh
Scurfy Triplelow
LN
mLN


e
cell number [x10^6]
5
4
3
2
1
0
*
*
LN
mLN


f
B6
Scurfy Triplehigh → CD3KO
Scurfy Triplelow → CD3KO
skin (tail)
colon