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

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

13  
14 The importance of CD4<sup>+</sup> regulatory T cells (Tregs) in maintaining lymphocyte  
15 homeostasis is best appreciated in mice and humans lacking these cells. FoxP3<sup>KO</sup>  
16 (scurfy) mice<sup>1,2,3</sup> and patients with immunodysregulation polyendocrinopathy  
17 enteropathy X-linked (IPEX) syndrome<sup>4</sup> suffer from excessive lymphocyte  
18 activation, lymphocytic infiltration into peripheral organs and colitis, leading to  
19 death at an early age. In healthy individuals, Tregs control homeostatic  
20 proliferation of conventional T and B cells and prevent colitis<sup>5,6,7</sup>.

21 Tregs are comprised of thymic Tregs (tTregs) and peripherally-induced Tregs  
22 (pTregs or iTregs), which originate from different precursor cells and develop in  
23 different locations. tTregs develop in the thymus and their development requires  
24 TCR stimulation with agonist peptide-MHCII antigens.<sup>8,9,10</sup> In contrast, iTregs are  
25 generated in the periphery from naïve, mature CD4<sup>+</sup> conventional T cells (Tconv)

1 during T cell activation in the presence of TGF $\beta$ .<sup>11</sup> Both populations are  
2 suppressive and their functional properties have been examined. Several studies  
3 suggest that tTregs are required to control immune homeostasis and  
4 autoimmunity.<sup>5,12,13</sup> On the other hand, iTregs have specialized functions  
5 depending on the type of inflammation, and have a primary role in controlling  
6 mucosal immunity and fetal tolerance.<sup>5,12,13,14</sup> A recent study indicated that tTregs  
7 by themselves are not sufficient to suppress chronic inflammation and  
8 autoimmunity in the absence of iTregs.<sup>15</sup>

9 Tregs have also been characterized for their expression of surface markers  
10 and localization in different tissues.<sup>16,17,18</sup> Based on their expression of CD44 and  
11 the lymph node homing receptor, CD62L, Tregs can be broadly divided into  
12 CD44<sup>lo</sup>CD62L<sup>+</sup> central Tregs (cTregs) and CD44<sup>hi</sup>CD62L<sup>lo/-</sup> effector Tregs  
13 (eTregs).<sup>16</sup> cTregs are quiescent, primarily reside in secondary lymphoid tissues,  
14 express high levels of CD25 and are IL-2 dependent. In contrast, eTregs, the  
15 dominant Treg population in nonlymphoid tissues, are CD25<sup>low</sup>, highly proliferative,  
16 but prone to apoptosis. It's been suggested that eTreg maintenance is driven by  
17 TCR and co-stimulatory signals, but not IL-2.<sup>16</sup>

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

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

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

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25

## 1 **Results**

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

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

### 13 **Triple<sup>high</sup> and Triple<sup>low</sup> Tregs originate in the thymus**

14 Although Triple<sup>high</sup> and Triple<sup>low</sup> 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.<sup>28,29</sup> To resolve this, we examined thymic Tregs in mice  
17 expressing FoxP3-RFP and Rag-GFP reporters (Fig 2b). RFP<sup>+</sup>GFP<sup>+</sup> CD4 SP  
18 thymocytes are de novo generated thymic Tregs since they are still Rag-GFP<sup>+</sup>,  
19 while RFP<sup>+</sup>GFP<sup>-</sup> CD4 SP cells in the thymus are recirculating Tregs from the  
20 periphery.<sup>29</sup> The frequency of de novo generated (RFP<sup>+</sup>GFP<sup>+</sup>) Triple<sup>high</sup> and  
21 Triple<sup>low</sup> Tregs in the thymus is similar to what's observed among LN Tregs. The  
22 fact that both Triple<sup>high</sup> and Triple<sup>low</sup> Tregs develop in the thymus argues against  
23 the idea that either population are induced Tregs (iTregs). To address the  
24 possibility that Triple<sup>high</sup> and Triple<sup>low</sup> Tregs might be induced by foreign antigens  
25 or inflammation, we examined Tregs in germ-free (GF) and antigen-free (AF)

1 mice. AF mice are offspring of GF mice that were weaned onto and raised on the  
2 elemental diet of glucose and amino acids.<sup>30</sup> As these animals lack a microbiome  
3 and are not exposed to dietary antigens, they contain exclusively self-antigens.  
4 GF and AF mice contain similar frequencies of LN Tregs compared to standard  
5 SPF animals (Fig 2c, top row). Importantly, SPF, GF and AF mice contain similar  
6 frequencies of Triple<sup>high</sup> and Triple<sup>low</sup> Tregs (Fig 2c, bottom row), which also  
7 express similar levels of NRP1 and Helios (Fig S3). These data rule out the idea  
8 that the Triple<sup>high</sup> and Triple<sup>low</sup> phenotypes are a response to inflammation.  
9 Furthermore, these results strongly suggest that Triple<sup>high</sup> and Triple<sup>low</sup> Tregs are  
10 generated through recognition of self-antigens.

### 11 **Triple<sup>high</sup> and Triple<sup>low</sup> Tregs express distinct TCR repertoires**

12 To directly compare the TCR repertoires of Triple<sup>low</sup> and Triple<sup>high</sup> Tregs to CD4  
13 Tconvs, all three populations expressing the Vα2 family (Fig 3a), were sorted from  
14 a Rag<sup>+</sup>, single TCR β chain strain (Yae62, Vβ8.2, TCRα<sup>+/-KO</sup>, FoxP3<sup>GFP-KI</sup>; Fig S4)  
15 and subjected to deep sequencing. The 500 most frequent clonotypes in each  
16 group were analyzed for their similarity (Fig 3 b-d) and diversity (Fig 3e,f).  
17 Morisita-Horn analysis shows that the CD4 Tconv sequences from three  
18 independent groups of mice (see Methods) are similar to each other but  
19 significantly different from Triple<sup>low</sup> and Triple<sup>high</sup> Treg sequences obtained from  
20 the same mice (Fig 3b). Triple<sup>low</sup> sequences isolated from different groups of mice  
21 are similar to each other as well, but different from CD4 Tconv and Triple<sup>high</sup> TCRs  
22 (Fig 3c). Interestingly, Triple<sup>high</sup> TCR sequences are not only different from CD4  
23 Tconv and Triple<sup>low</sup> sequences, but they are also variable between different  
24 groups of mice (Fig 3d). Despite their significant sequence differences, the TCR  
25 repertoires of CD4 Tconvs and Triple<sup>low</sup>s are similarly diverse (Fig 3 e,f). The

1 repertoire of Triple<sup>high</sup> Treg TCRs may be less diverse than the CD4 Tconv and  
2 Triple<sup>low</sup> repertoires, at least according to Shannon Entropy analysis. Taken  
3 together, deep sequencing analyses showed that Triple<sup>high</sup> Tregs, Triple<sup>low</sup> Tregs  
4 and CD4 Tconvs have clearly distinct TCR repertoires, implying that TCR  
5 specificity is important in selecting these Treg subtypes.

### 6 **Triple<sup>high</sup> Tregs are more self-reactive than Triple<sup>low</sup> Tregs**

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

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



1 affinity negative selector) induced only Triple<sup>high</sup> CD4SP thymocytes. FoxP3<sup>+</sup>  
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<sup>KO</sup>  
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<sup>+</sup> 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<sup>low</sup>,  
9 Triple<sup>intermediate</sup> and Triple<sup>high</sup> 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<sup>high (and intermediate)</sup> but not Triple<sup>low</sup> Tregs in the  
15 thymus. Taken together, these data imply that Triple<sup>high</sup> and Triple<sup>low</sup> 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<sup>high</sup> Tregs suppress lymphoproliferation**

20 To compare the regulatory properties of these two populations, FoxP3<sup>DTR</sup> mice  
21 received sorted Triple<sup>high</sup> or Triple<sup>low</sup> Tregs from B6 mice (Fig S6a), which are  
22 unaffected by diphtheria toxin (DTx). Three days later, endogenous Tregs from the  
23 FoxP3<sup>DTR</sup> 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<sup>high</sup> Tregs control the extensive proliferation of Tcells and

1 B cells in peripheral LNs of mice depleted of their endogenous Tregs (Fig 5b),  
2 while Triple<sup>low</sup> Tregs function poorly in this respect. As expected, Triple<sup>high</sup> Tregs  
3 limit the activation of CD4 Tconvs (Fig 5c).

#### 4 **Triple<sup>low</sup> Tregs suppress induction of colitis**

5 To examine whether any of these Treg subsets control colitis, CD3<sup>KO</sup> mice were  
6 injected with sorted naïve CD4 Tconvs (Fig 6a), a treatment, which results in  
7 colitis (Fig 6d, upper left panel) and weight loss (Fig 6b, solid blue line) as  
8 previously described<sup>7</sup>. Co-transfer of Triple<sup>low</sup> (Fig 6b, solid brown line; Fig 6d,  
9 upper middle panel) but not Triple<sup>high</sup> (Fig 6b, upper panel, solid red line; Fig 6d,  
10 upper right panel) Tregs prevented weight loss and limited lymphocyte infiltration  
11 of the colonic mucosa. Analysis of LN cells from these mice indicated that co-  
12 transferred Triple<sup>low</sup> Tregs facilitated the conversion of some CD4 Tconv into  
13 induced Tregs (iTregs) (Fig 6e,f). Mice receiving Triple<sup>low</sup> Tregs had the highest  
14 percentage of iTregs (Fig 6e,f), very limited infiltration of the colonic mucosa (Fig  
15 6d, upper middle panel) and maintained their weight (Fig 6b).

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

1 facilitate conversion of some CD4 Tconv into FoxP3<sup>+</sup> iTregs, which in aggregate  
2 limit development of colitis.

3 Taken together, the data (Fig 5,6) argue for two populations of Tregs: Triple<sup>high</sup>s,  
4 which control lymphoproliferation in peripheral LNs and Triple<sup>low</sup>s, which limit the  
5 development of colitis (at least in a lymphopenic setting). It should be noted that  
6 the phenotypes of Triple<sup>high</sup>s are stable over the 11d time course of the experiment  
7 in Fig 5 while Triple<sup>low</sup>s are stable over the 6 week time course of the experiment  
8 in Fig 6. (see also Fig S6b). As Triple<sup>low</sup>s don't suppress lympho-proliferation and  
9 Triple<sup>high</sup>s 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<sup>high</sup> and Triple<sup>low</sup> CD4 T cells induce different pathologies**

13 While FoxP3<sup>KO</sup> (scurfy) mice cannot develop Tregs due to the lack of functional  
14 FoxP3, they do carry out negative selection.<sup>21</sup> For this reason, we wondered  
15 whether FoxP3<sup>KO</sup> (scurfy) mice contain Triple<sup>high</sup>- like and Triple<sup>low</sup>- like CD4 T  
16 cells despite their lack of a functional FoxP3 molecule. Flow cytometric analysis  
17 shows that these mice contain GITR<sup>high</sup>, PD1<sup>high</sup>, CD25<sup>high</sup> (scurfy Triple<sup>high</sup>) and  
18 GITR<sup>low</sup>, PD1<sup>low</sup>, CD25<sup>low</sup> (scurfy Triple<sup>low</sup>) CD4 T cells. Scurfy Triple<sup>high</sup> T cells  
19 resembled B6 Triple<sup>high</sup> 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<sup>high</sup>s may be similar to previously reported Treg  
22 "wannabes".<sup>21,33,34</sup> Scurfy Triple<sup>low</sup> 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<sup>low</sup> and scurfy Triple<sup>high</sup>  
25 CD4 T cells were sorted (Fig S7a) and separately transferred into T cell deficient,

1 CD3<sup>KO</sup> hosts (Fig 7b). Transferred scurfy Triple<sup>low</sup> 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  $\alpha 4\beta 7$ , an integrin that enables homing to the  
4 gut<sup>35</sup> (Fig S7b). In contrast, transferred scurfy Triple<sup>high<sup>s</sup></sup> do not cause weight loss  
5 (Fig 7c) and preferentially accumulate in peripheral but not mesenteric LNs (Fig  
6 7d,e). Moreover, scurfy Triple<sup>high</sup> 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<sup>high<sup>s</sup></sup> 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<sup>high</sup> and  
15 Triple<sup>low</sup> Tregs are generated as an offshoot of negative selection. The high affinity  
16 self-reactive TCRs expressed by Triple<sup>high</sup> Tregs likely drives their selection in the  
17 thymus as well as their proliferation and suppressive activity in peripheral LNs.<sup>36</sup>  
18 On the other hand, thymic precursors expressing lower affinity self-reactive TCRs  
19 plausibly differentiate into Triple<sup>low</sup> Tregs, which facilitate conversion of CD4  
20 Tconvs into iTregs. We also demonstrate that FoxP3<sup>KO</sup> (scurfy) mice contain  
21 Triple<sup>high</sup>- and Triple<sup>low</sup>- like CD4 T cells, which are presumably derived from  
22 negative selection<sup>21</sup> and have distinctive pathological activities: scurfy Triple<sup>high</sup> T  
23 cells preferentially migrate to peripheral LNs and elicit skin pathology (scurfy skin)  
24 while scurfy Triple<sup>low</sup> cells are found in the mesenteric LNs and cause colitis.

25 Triple<sup>high</sup> and Triple<sup>low</sup> Tregs are distinct from central and effector Treg subsets.

1 With respect to CD44, CD62L and ICOS expression, Triple<sup>high</sup> Tregs resemble  
2 eTregs and Triple<sup>low</sup> Tregs resemble cTregs; however, expression of CD25, CCR7,  
3 CD103, Helios and NRP1 indicates that Triple<sup>highs</sup> and Triple<sup>lows</sup> are distinct from  
4 these previously described subsets.<sup>16</sup>

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

18 Several reports show that Tregs and CD4 Tconvs cells are differently selected  
19 and have dissimilar TCR repertoires.<sup>24,25</sup> A comparison of the TCR repertoires  
20 expressed in thymic and peripheral (induced) Tregs is difficult due to the absence  
21 of specific markers for cell sorting.<sup>8,9,14,37,38</sup> However, analysis of peripheral  
22 (assumed to be thymus-derived) and colonic (assumed to be peripherally induced)  
23 Tregs revealed different TCR repertoires expressed in these two populations.<sup>26</sup>  
24 Deep sequencing of Triple<sup>high</sup> and Triple<sup>low</sup> Tregs as well as CD4 Tconvs indicates

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

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

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

1 lymphoproliferation in peripheral LNs by either modifying DCs towards a  
2 tolerogenic phenotype<sup>41</sup> or by directly interacting with T cells.<sup>42</sup>

3 Experimental colitis induced by transfer of CD4 Tconvs into T cell deficient  
4 hosts is a slow, progressive disease mediated by microbiota-specific CD4<sup>+</sup> T cells  
5 and characterized by a massive cell infiltration into the colon and weight loss  
6 within four to six weeks.<sup>7,43</sup> A number of reports show that co-transfer of Tregs, in  
7 particular microbiota-specific Tregs prevents the onset or even cures mice from  
8 colitis.<sup>43, 44</sup> iTregs are essential for maintaining immune homeostasis, especially at  
9 mucosal interfaces; additionally iTregs contribute to fetal tolerance.<sup>5,12,13</sup> In the gut,  
10 naïve CD4<sup>+</sup> T cells are converted into iTregs following TCR stimulation in the  
11 presence of TGFβ and IL-2; other compounds such as retinoic acid (RA) or short-  
12 chain-fatty-acids from microbiota mediate conversion as well.<sup>7</sup> In addition, IL-10 is  
13 a key player in maintaining lymphocyte homeostasis in the gut as IL-10 deficient  
14 mice suffer from spontaneous colitis.<sup>7</sup>

15 Our results clearly show that Triple<sup>low</sup> but not Triple<sup>high</sup> Tregs suppress colitis  
16 induction. Triple<sup>low</sup> Tregs by themselves do not control colitis induction, but  
17 function by promoting the generation of iTregs from CD4 Tconvs. Mice which  
18 received Triple<sup>low</sup> Tregs, but whose recently generated iTregs were depleted with  
19 DTx develop fulminant colitis. To our knowledge, there is no study, showing that a  
20 particular Treg cell population can induce the conversion of CD4 Tconvs in iTregs  
21 *in vivo*. One recent study indicates that M2a macrophages promote a supportive  
22 environment for iTregs and directly contribute to immunological homeostasis in the  
23 gut.<sup>45</sup> Nevertheless, how Triple<sup>low</sup> Tregs facilitate the generation of iTregs is still an  
24 open question.

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

5 It's still not clear which scurfy T cell population promotes multi organ  
6 inflammation and how TCR affinity for self or foreign antigen is linked to the  
7 various pathologies seen in scurfy mice. Here, we show that scurfy Triple<sup>high</sup> T  
8 cells are similar to bona fide Triple<sup>high</sup> Tregs with respect to PD1, GITR, CD25 and  
9 Helios expression. Transferred scurfy Triple<sup>high</sup>s proliferate extensively in  
10 peripheral LNs, infiltrate the skin and cause cutaneous lesions similar to those  
11 seen in scurfy mice. Interestingly, IL-2 deficient scurfy mice do not develop skin  
12 lesions, while IL-4-, IL-6-, IL-10-, Stat6- or CD103- deficient scurfy mice do.<sup>46</sup> The  
13 authors suggested that IL-2 acts as the main mediator of skin inflammation in  
14 scurfy mice. In this context, scurfy Triple<sup>high</sup> cells cannot likely produce their own  
15 IL-2, since they express Helios, a repressor of IL-2 transcription.<sup>34</sup> For this reason,  
16 the presence of IL-2 secreting, skin resident DCs might explain the accumulation  
17 of scurfy Triple<sup>high</sup>s in the dermis.<sup>47</sup>

18 In contrast, scurfy Triple<sup>low</sup> cells do not initiate cutaneous lesions, but instead  
19 induce severe colitis within 4 weeks when transferred to T cell deficient recipients.  
20 It's unclear whether scurfy Triple<sup>low</sup> cells are the scurfy equivalent to B6 Triple<sup>low</sup>  
21 Tregs or to B6 CD4 Tconv cells. Nevertheless, at least a portion of scurfy Triple<sup>low</sup>  
22 cells are likely to be microbiota specific, since germfree scurfy mice are less prone  
23 to develop colitis compared to scurfy mice housed under SPF conditions.<sup>26,36</sup>  
24 Taken together, these results indicate that scurfy disease is pleotropic. Although  
25 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<sup>high</sup> Tregs control the homeostatic proliferation of lymphocytes, while  
6 the less self-reactive Triple<sup>low</sup> 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

## 1 **Methods**

### 2 **Mice**

3 All mice (female and male) were between 5–12 weeks old and had a C57BL/6  
4 genetic background. Male FoxP3KO mice were used at 2-3 weeks of age. CD45.1  
5 congenic C57BL/6 (B6 Ly5.1), CD45.2 congenic C57BL/6 (B6), RIP-OVA mice  
6 expressing a membrane bound form of Ova under the control of the rat insulin  
7 promoter (RIP)<sup>48,49,39</sup> OTII TCRtg mice recognizing IA<sup>b</sup>/OVA<sub>323-339</sub><sup>50</sup>, B6.Nur77-  
8 GFP<sup>31</sup> and FoxP3KO<sup>51</sup> were all obtained from The Jackson Laboratory (Bar  
9 Harbor, ME). 3BK506 TCRtg and 3BK508 TCRtg mice recognizing IA<sup>b</sup>/3K and  
10 Triple KO mice deficient for MHC class II, invariant chain and Rag<sup>KO</sup> (referred here  
11 as MHCII KO) were provided by P. Marrack and J. Kappler (Denver, USA) and are  
12 described elsewhere<sup>52</sup>. FoxP3<sup>DTR,39</sup> mice were kindly provided by A. Rudensky  
13 (New York, USA). FoxP3eGFP and CD3 $\epsilon$ <sup>-/-</sup> (CD3<sup>KO</sup>) were kindly provided by T.  
14 Rolink (Basel, Switzerland) and single TCR  $\beta$  chain (OT-I V $\beta$ 5) transgenic mice  
15 kindly provided by D. Zehn (Lausanne, Switzerland) and are described  
16 elsewhere.<sup>53,54,55</sup> Mice were housed under specific pathogen-free conditions and  
17 bred in our colony (University Hospital Basel) in accordance with Cantonal and  
18 Federal laws of Switzerland. Animal protocols were approved by the Cantonal  
19 Veterinary Office of Baselstadt, Switzerland. Mice expressing the YAe62 TCR $\beta$   
20 chain<sup>56,57</sup> and all mouse sub-lines were maintained in a pathogen-free  
21 environment in accordance with institutional guidelines in the Animal Care Facility  
22 at the University of Massachusetts Medical School. Foxp3.RFP/GFP mice on the  
23 C57BL/6 background were bred and maintained at the animal facility of the CRTD  
24 (Dresden, Germany) under specific pathogen-free conditions; animal experiments  
25 were performed in accordance with the German law on care and use of laboratory

1 animals and approved by the Regierungspräsidium Dresden. Antigen free and  
2 germ free C57BL/6 mice<sup>30</sup> 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<sup>29</sup> 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), TCR $\beta$  (H57-597) and  $\alpha$ 4 $\beta$ 7  
20 (DATK32). Intracellular staining for FoxP3 (FJK-16s/ 150D), Helios (22F6), pcJun  
21 (D47G9), pCD3 $\zeta$  (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 Cantoll (BD Bioscience) and FlowJo software  
3 (TreeStar) were used. For cell isolation, CD4<sup>+</sup>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<sup>KO</sup> 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  $1 \times 10^5$   
16 syngeneic (B6 or MHCII KO) BmDCs with  $3 \times 10^5$  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,  $1 \times 10^5$   
19 thymocytes from 3BK508tg mice were co-cultured with  $1 \times 10^5$  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.<sup>58</sup> 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  $\mu$ M), P2A (2  $\mu$ M) or 3K (0.2  $\mu$ M) peptides for 7  
2 days before analysis. All in vitro assays were performed at 37°C in 5% CO<sub>2</sub> 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.<sup>49</sup>  
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<sup>KO</sup> (CD45.2) were isolated and depleted of mature T  
9 cells. A mixture of 9:1 of B6 and OT-II Rag<sup>KO</sup> bone marrow cells ( $4 \times 10^6$  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 Diphtheria Toxin  
17 (DTx) (Calbiochem) every other day for 10-12 days (first and second injection  
18 50 $\mu$ g/kg; subsequent injections 25 $\mu$ g/kg). In some groups,  $2.5 \times 10^5$  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<sup>KO</sup> mice received (i.v.)  $3.2 \times 10^5$  sorted naïve CD4 T cells  
22 from pooled LNs of B6Ly5.1 (CD4<sup>+</sup>CD25<sup>-</sup>) or FoxP3<sup>DTR</sup> Ly5.1 (CD4<sup>+</sup>GFP<sup>-</sup>) mice. In  
23 some groups,  $0.8 \times 10^5$  sorted Tregs from pooled LN were co-transferred.  
24 Recipients of naïve FoxP3<sup>DTR</sup> CD4 T cells (CD4<sup>+</sup>GFP<sup>-</sup>) were injected every third  
25 day with DTx (10 $\mu$ g/kg), i.p.. For adoptive transfer of scurfy CD4 T cells, 6-10

1 week old T cell deficient CD3<sup>KO</sup> were reconstituted with 5x10<sup>5</sup> sorted CD4  
2 subpopulations from pooled LNs of 2-3 week old sick (scurfy) FoxP3<sup>KO</sup> male mice.  
3 Recipient mice were weighed weekly at the same time of day and sacrificed when  
4 initial body weight dropped 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 paraffin, sectioned and stained with hematoxylin  
8 and eosin.

### 9 **Clonotype Analysis of GITR<sup>low</sup> PD-1<sup>low</sup> versus GITR<sup>high</sup> PD-1<sup>high</sup> peripheral** 10 **Tregs**

11 Naïve CD4<sup>+</sup> (CD4<sup>+</sup> CD25<sup>-</sup> Foxp3<sup>-</sup>), Triple<sup>low</sup> T<sub>reg</sub> (CD4<sup>+</sup> CD25<sup>low</sup> Foxp3<sup>+</sup>  
12 GITR<sup>low</sup> PD-1<sup>low</sup>) or Triple<sup>high</sup> T<sub>reg</sub> (CD4<sup>+</sup> CD25<sup>high</sup> Foxp3<sup>+</sup> GITR<sup>high</sup> PD-1<sup>high</sup>) T cell  
13 populations were sorted from 3 replicate groups (2 mice per group) of single TCRβ  
14 chain transgenic (B6.YAe62βtg<sup>+</sup> TCRα<sup>+/-</sup>) 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<sup>th</sup> 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

1 using the program fastq-multx<sup>59</sup> and clonotypes for each population were  
2 tabulated using TCRklass<sup>60</sup>. A table of analyzed sequences and their frequencies  
3 is shown in Fig S8. All sequences will be made available online.

#### 4 **Similarity and Diversity of TCR clonotypes**

5 The similarity of TCRs utilized within each population was quantified using the  
6 Morisita-Horn similarity index, 0 (minimal similarity) and 1 (maximal similarity).

7 The Morisita-Horn (M-H) similarity indexes were calculated by tabulating the  
8 frequency in which the top 500 clonotypes of an individual population from one  
9 replicate sample was found in all other populations, using EstimateS Ver9.1.0<sup>61</sup>  
10 software. Statistical significance for M-H index values was assessed using a  
11 Mann-Whittney U test, GraphPad Prism version 6.04. The diversity of TCR  
12 repertoire for each population was measured using the top 500 most frequent  
13 clonotypes. The Shannon Entropy<sup>62</sup> value for each sample was calculated as  $H =$   
14  $-\sum p_i \log_2 p_i$ , where  $p_i$  is the frequency of the clonotype within the top 500  
15 clonotypes. Lower H values indicate lower diversity. Additionally, the Simpson's  
16 diversity index<sup>63</sup> using the formula  $D_s = 1 - \sum [n_i(n_i - 1)]/[N(N - 1)]$ , where  $n_i$  is the  
17 TCR clone size of the  $i$ th clonotype and  $N$  is the total number of the top 500  
18 clonotypes sampled. The index ranges from 0 to 1 with 1 indicating high diversity.

#### 19 **Statistical analysis**

20 Statistical analysis were performed using Prism 6.0 (Graphpad software). If not  
21 other indicated, Students t test (unpaired, two-tailed) was used to asses statistical  
22 significiance. P-values  $\leq 0.05$  were considered significant (\* $p \leq 0.05$ , \*\* $p \leq 0.01$ ,  
23 \*\*\* $p \leq 0.001$ , \*\*\*\* $p \leq 0.0001$ ) P values  $> 0.05$ ; non-significant (ns)

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## 13 **Author Contributions**

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

## 22 **Author Information**

23 The authors declare no competing financial interests. Correspondence and  
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1 **Figure Legends**

2

3 **Figure 1: Triple<sup>high</sup> and Triple<sup>low</sup> 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<sup>+</sup>FoxP3<sup>+</sup> cells (left panel, green

7 gate), Triple<sup>high</sup> Tregs (GITR<sup>high</sup>PD1<sup>high</sup>CD25<sup>high</sup>, second panel, red gate) and

8 Triple<sup>low</sup> Tregs (GITR<sup>low</sup>PD1<sup>low</sup>CD25<sup>low</sup>, second panel, brown gate). Bar graph (left)

9 shows frequencies of Triple<sup>high</sup> and Triple<sup>low</sup> Tregs in LNs of B6 mice (n=6 mice).

10 Histogram (right panel) shows CD25 expression (MFI) on Triple<sup>high</sup> and Triple<sup>low</sup>

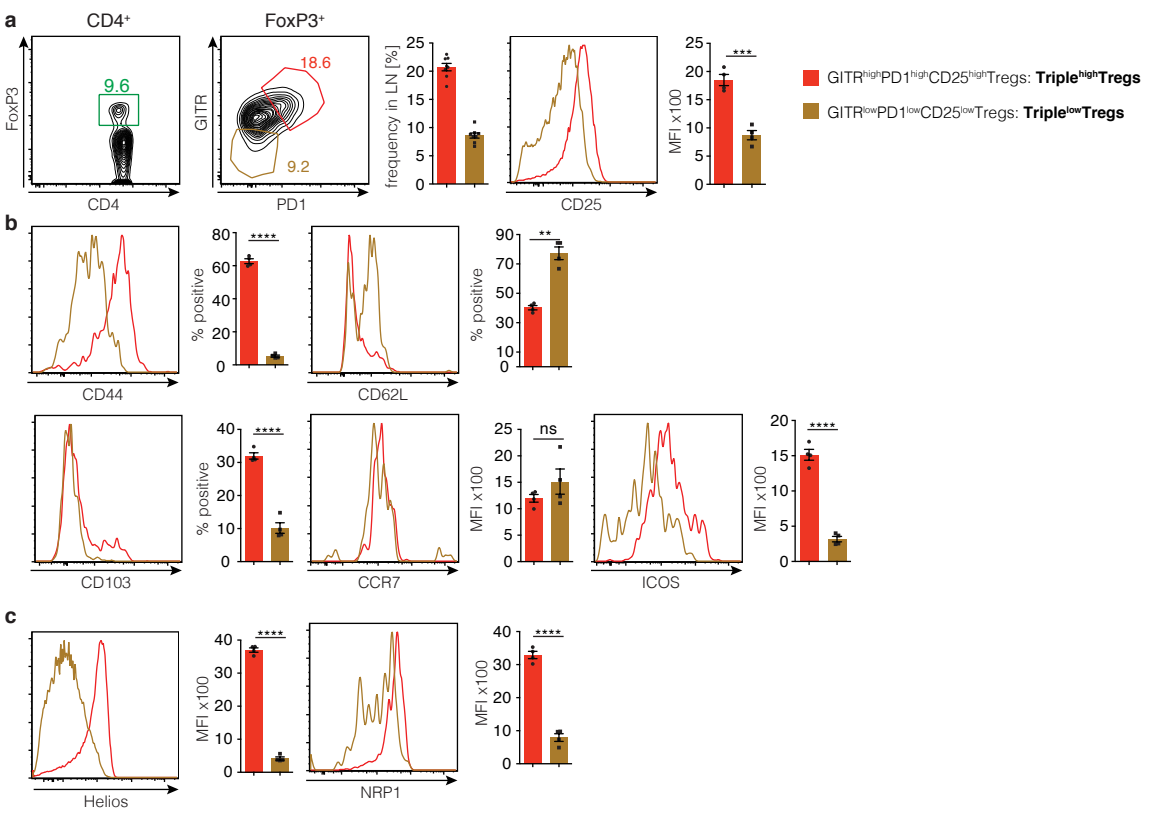
11 Tregs (n=4 mice) b and c) Expression of homing and activation markers on

12 Triple<sup>high</sup> (red) and Triple<sup>low</sup> 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

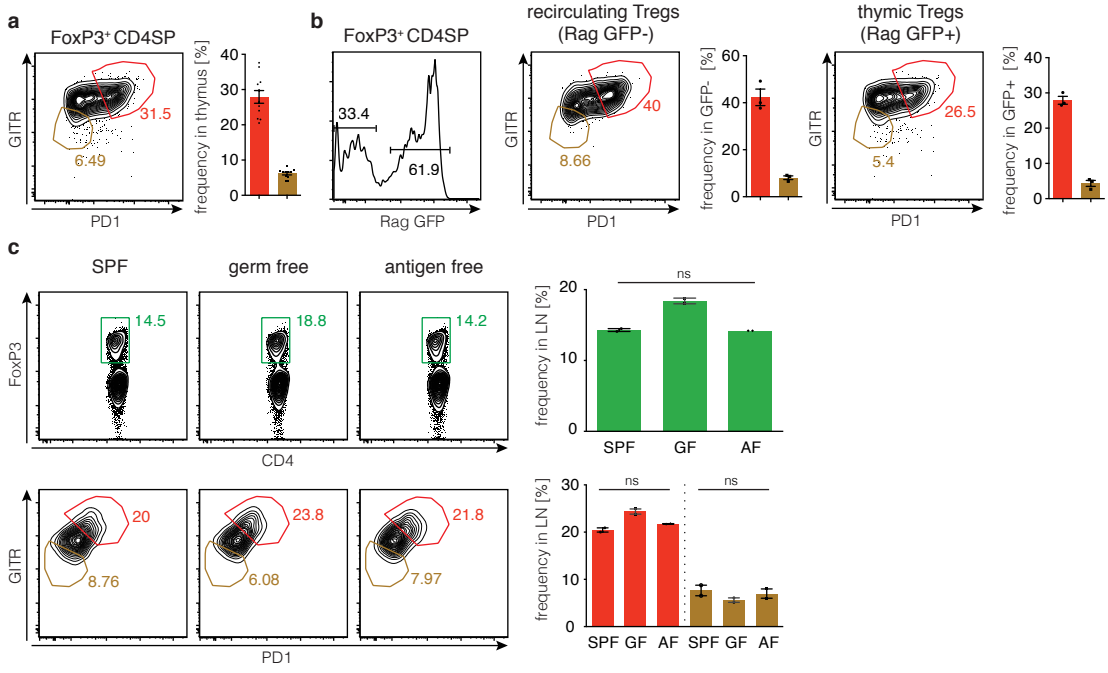
15 graphs show mean ± SEM. Data is taken from 2-3 independent experiments.

**Figure 1**

1 **Figure 2: Triple<sup>high</sup> and Triple<sup>low</sup> Tregs cells have thymic origin.**

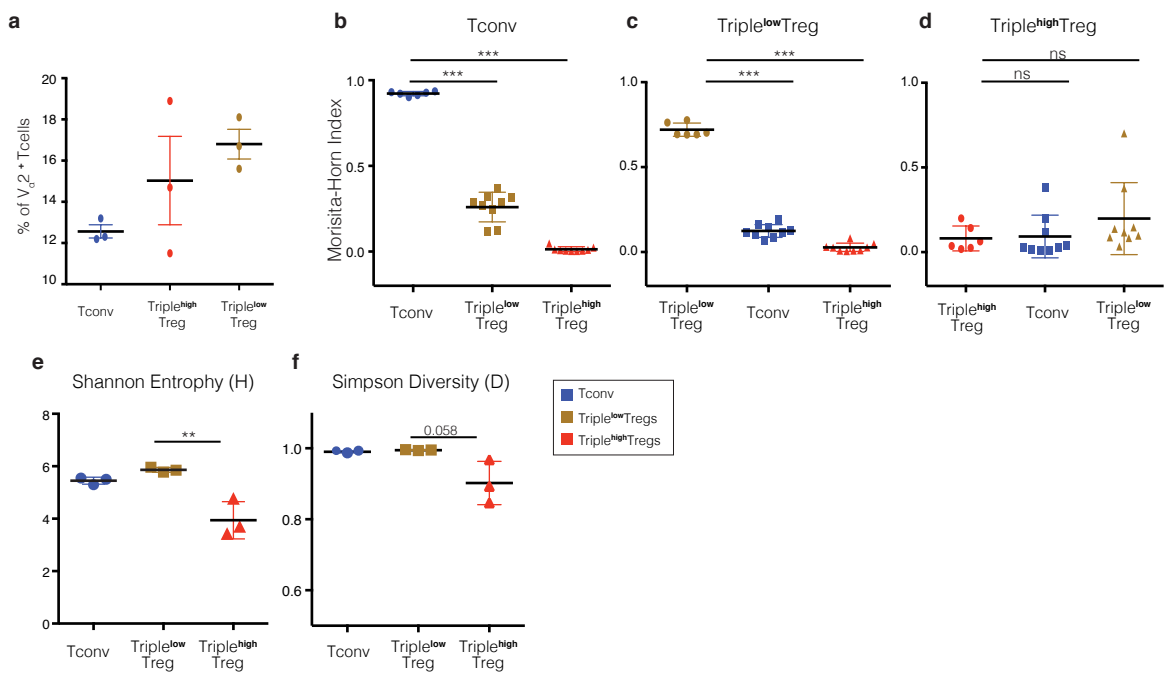
2 a) B6 thymocytes mice were analyzed for FoxP3, GITR and PD1 expression by  
3 flow cytometry. Gates show frequencies of Triple<sup>high</sup> (red) and Triple<sup>low</sup> Tregs  
4 (brown). Bar graph shows mean frequency of Triple<sup>high</sup> and Triple<sup>low</sup> Tregs in thymi  
5 (n=6 mice,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

**Figure 2**



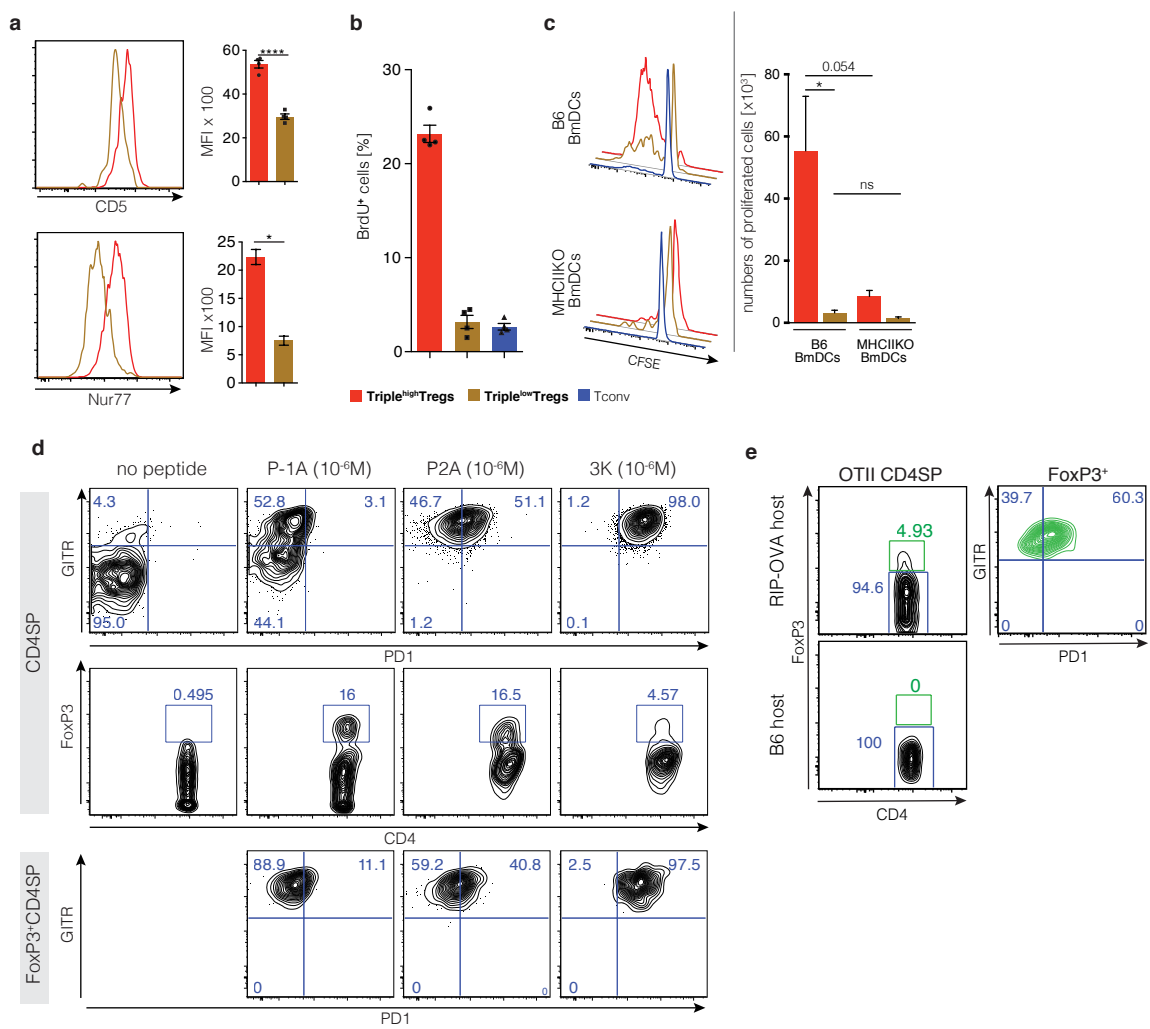


1 **Figure 3: Triple<sup>high</sup> and Triple<sup>low</sup> Tregs express distinct TCR repertoires.**  
2 Analysis TCR clonotypes in Treg subsets from Yae62 Vβ8.2, TCRα<sup>+/<sup>KO</sup> FoxP3-  
3 GFP<sup>Kl</sup> (single TCR β chain) mice. a) Vα2 expression among CD4 Tconvs (blue),  
4 Triple<sup>high</sup> Tregs (red) and Triple<sup>low</sup> Tregs (brown). b) Morisita-Horn similarity  
5 analysis of Vα2<sup>+</sup> TCR clonotypes from CD4 Tconvs (blue) compared to Triple<sup>low</sup>  
6 (brown) Treg and Triple<sup>high</sup> (red) Treg clonotypes from three independent groups  
7 of single TCRβ chain (YAe62tg, TCRα<sup>+/<sup>KO</sup>, Rag<sup>+</sup>) 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<sup>+</sup> TCR clonotypes from Triple<sup>low</sup> Tregs (brown) to CD4 Tconvs (blue) and  
11 Triple<sup>high</sup> Treg (red) clonotypes. d) Morisita-Horn similarity analysis comparing  
12 Vα2<sup>+</sup> TCR clonotypes from Triple<sup>high</sup> Tregs (red) to CD4 Tconvs (blue) and  
13 Triple<sup>low</sup> 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</sup></sup>

**Figure 3**

1 **Figure 4: Triple<sup>high</sup> and Triple<sup>low</sup> Tregs have different self-reactivity.**  
2 a) Triple<sup>high</sup> (red) and Triple<sup>low</sup> (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<sup>high</sup> (red),  
5 Triple<sup>low</sup> (brown) Tregs and Tconvs (blue). Mean percentages of proliferating  
6 (BrdU<sup>+</sup>) cells are shown (n=4 mice). c) Representative histograms show in vitro  
7 proliferation (CFSE dilution) of LN-derived Triple<sup>high</sup> Tregs (red) Triple<sup>low</sup> Tregs  
8 (brown) and CD4 Tconvs (blue) from cultures of purified CD4 LN Tcells and B6 or  
9 B6.MHCII<sup>KO</sup> 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<sup>+</sup> 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<sup>+</sup> B6 and Ly5.2<sup>+</sup> OT-II, Rag<sup>KO</sup> mice.  
20 Numbers indicate percentage of cells within gates. Right panel: Flow cytometric  
21 analysis of thymic OTII derived CD4SP FoxP3<sup>+</sup> thymocytes for GITR and PD1  
22 expression in RIP-mOVA hosts. Contour plots are representative of 4 individual  
23 chimeric mice.

**Figure 4**

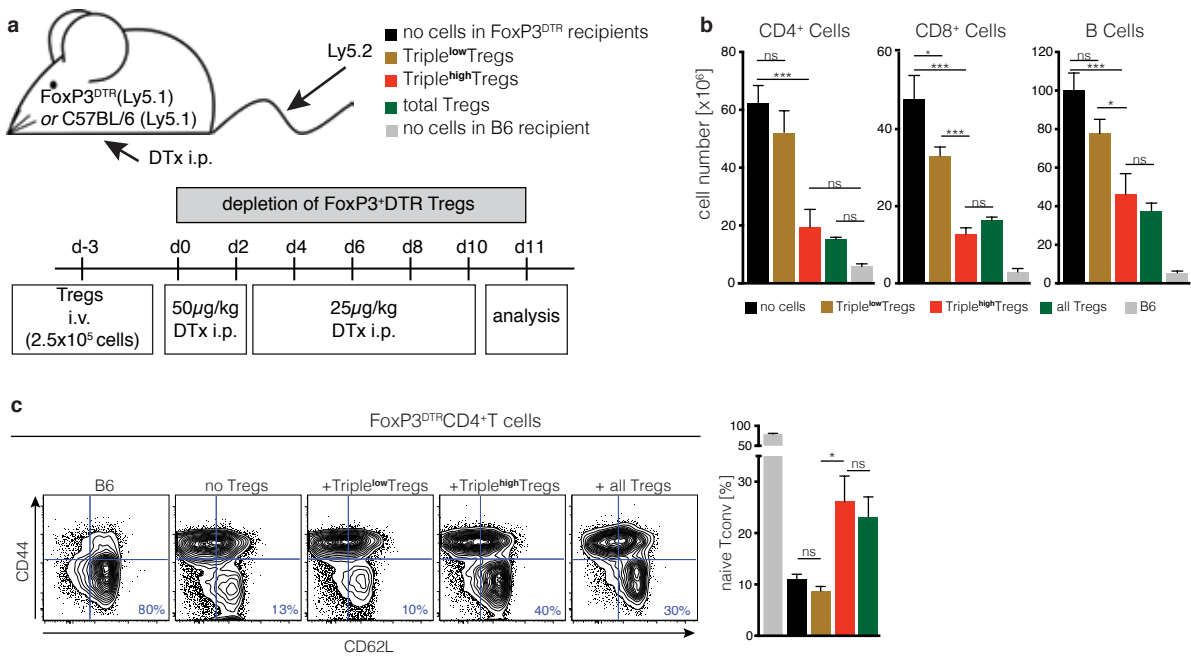


1 **Figure 5: Triple<sup>high</sup> but not Triple<sup>low</sup> Tregs suppress *in vivo***  
2 **lymphoproliferation.**

3 a)  $2.5 \times 10^5$  sorted B6 Tregs cells, (unaffected by DTx) from pooled B6 LNs were  
4 injected intravenously (i.v.) into 6-10 week old FoxP3<sup>DTR</sup> mice. Three days later,  
5 host FoxP3<sup>+</sup> 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<sup>DTR</sup> mice  
7 previously injected i.v. with either no cells (black, n=10 mice), B6 Triple<sup>low</sup> Tregs  
8 (brown, n=4 mice) B6 Triple<sup>high</sup> Tregs (red, n=6 mice), or total B6 Tregs (green,  
9 n=3 mice). FoxP3<sup>DTR</sup> 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<sup>+</sup>, CD8<sup>+</sup> 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<sup>+</sup> CD44<sup>low</sup> CD62L<sup>high</sup>) 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**



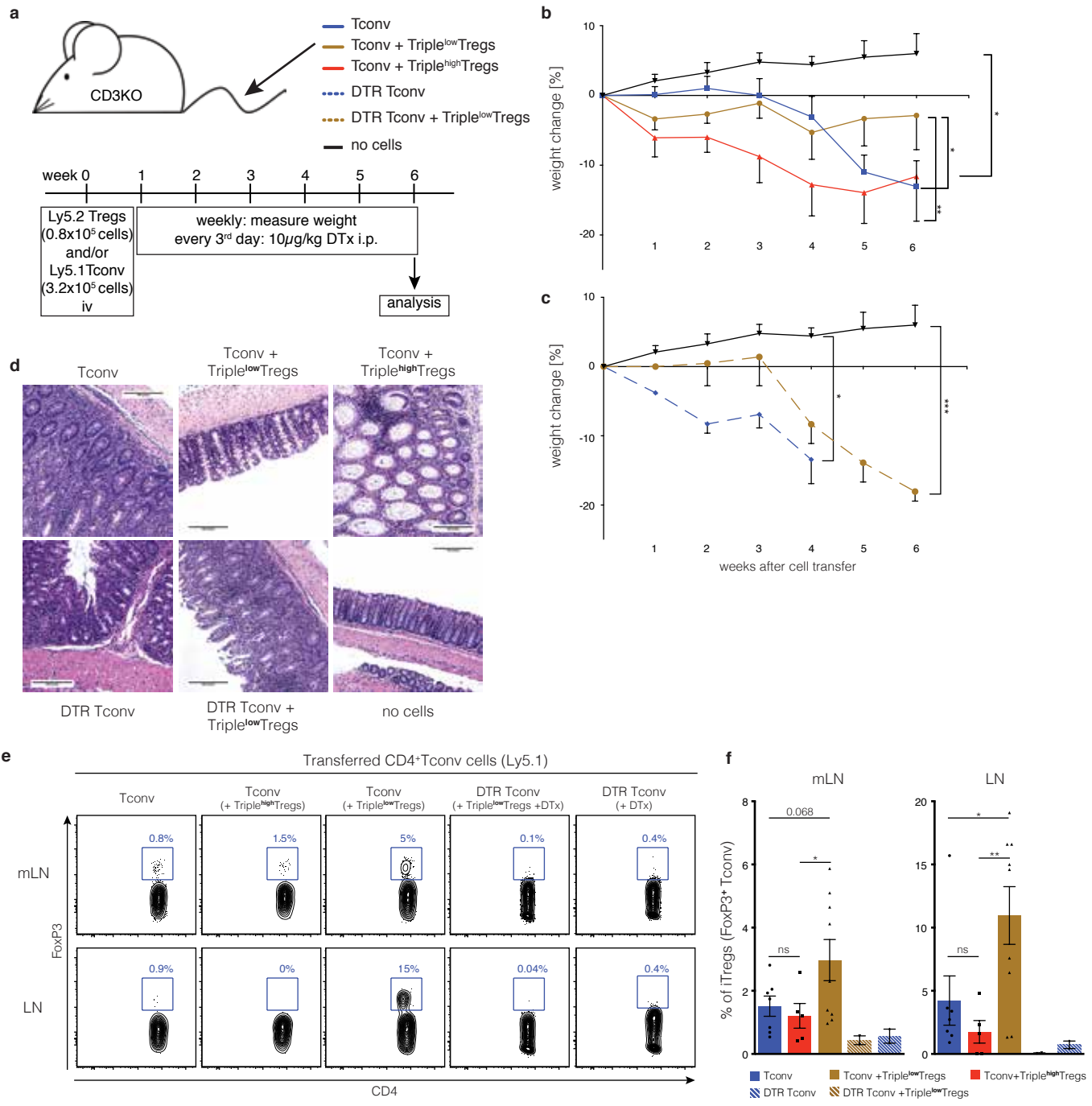
1 **Figure 6: Triple<sup>low</sup> but not Triple<sup>high</sup> Tregs suppress colitis.**

2 a) To induce colitis, 6-10 week old T cell deficient CD3<sup>KO</sup> recipients received  
3  $3.2 \times 10^5$  sorted naïve CD4 Tconvs (CD4<sup>+</sup>CD25<sup>-</sup>) isolated from B6 Ly5.1<sup>+</sup> mice (B6  
4 Tconv). In some groups,  $0.8 \times 10^5$  sorted, Ly5.2<sup>+</sup> Triple<sup>high</sup> or Triple<sup>low</sup> 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<sup>KO</sup> recipients were first injected i.v. with  $3.2 \times 10^5$  sorted, naïve CD4 Tconvs  
8 isolated from FoxP3<sup>DTR</sup>, Ly5.1<sup>+</sup> mice (FoxP3<sup>DTR</sup> Tconv). In some groups,  $0.8 \times 10^5$   
9 sorted B6 Triple<sup>low</sup> Tregs cells (unaffected by DTx) were co-transferred along with  
10 FoxP3<sup>DTR</sup> Tconvs. To deplete Tconv-derived iTregs, recipients were injected i.p.  
11 every third day with DTx (10 $\mu$ g/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<sup>KO</sup> 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<sup>low</sup>  
16 Tregs (brown, n=9 mice) or Triple<sup>high</sup> Tregs (red, n=6 mice). c) Graph shows  
17 percentage of weight change in CD3<sup>KO</sup> mice, which received either no cells (black,  
18 n=5 mice), Ly5.1 FoxP3<sup>DTR</sup> CD4 Tconvs alone (dashed blue, n=3 mice) or Ly5.1  
19 FoxP3<sup>DTR</sup> CD4 Tconvs co-transferred with B6 Triple<sup>low</sup> 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 $\leq$  0.05, \*\*p $\leq$  0.01,  
22 \*\*\*p $\leq$  0.001 (Student's t test). d) Representative H&E staining of colon sections  
23 from CD3<sup>KO</sup> 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<sup>DTR</sup> CD4 Tconvs  
25 six weeks after transfer from mice described in c). Contour plots show CD4/FoxP3

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



**Figure 6**



1 **Figure 7: Scurfy Triple<sup>high</sup> and scurfy Triple<sup>low</sup> CD4 T cells induce different**  
2 **pathologies.**

3 a) FoxP3<sup>KO</sup> (Scurfy) mice contain Treg like cells. Left panel: Flow cytometric  
4 analysis of CD4 T cells showing scurfy Triple<sup>high</sup> (PD1<sup>high</sup> GITR<sup>high</sup> CD25<sup>high</sup>; orange  
5 gate) and scurfy Triple<sup>low</sup> (PD1<sup>neg</sup> GITR<sup>neg</sup> CD25<sup>neg</sup>; purple gate) CD4<sup>+</sup> T cells.

6 Additional panels: PD1, GITR, CD25, Helios, CD5 and CD62L expression in  
7 scurfy Triple<sup>high</sup> (orange solid), scurfy Triple<sup>low</sup>, (purple solid), B6 Triple<sup>high</sup> Tregs  
8 (dotted red) and B6 CD4 Tconv (dotted blue) cells. b) Sorted scurfy Triple<sup>high</sup> or

9 scurfy Triple<sup>low</sup> T cells were transferred to T cell deficient CD3<sup>KO</sup> hosts. Host mice  
10 were weekly monitored for weight change and development of other pathologies.

11 c) Graph shows mean percentage of weight change  $\pm$  SEM of CD3<sup>KO</sup> recipients  
12 following adoptive transfer of no (black, n=3 mice), scurfy Triple<sup>high</sup> (orange, n=8  
13 mice) or scurfy Triple<sup>low</sup> (purple, n=8 mice) CD4<sup>+</sup> T cells. \*\*\*p $\leq$  0.001 (Student's t  
14 test) ns = not significant. d) Photographs of peripheral and mesenteric LNs from  
15 CD3<sup>KO</sup> mice transferred with scurfy Triple<sup>high</sup> or scurfy Triple<sup>low</sup> CD4 T cells e)

16 Absolute number of scurfy CD4 cells  $\pm$  SEM in peripheral LNs and mLNs six week  
17 after cell transfer, (orange, scurfy Triple<sup>high</sup>; violet, scurfy Triple<sup>low</sup>) (n=8 mice) ,

18 \*p $\leq$  0.05. f) Representative photographs and H&E staining of tail skin and colons  
19 sections of CD3<sup>KO</sup> recipient six weeks after adoptive cell transfer. B6 control mice  
20 are shown as well. Data is taken from 5 independent experiments.

21

22

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**Figure 7**