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## Continuous requirement for the T cell receptor for regulatory T cell function

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### Abstract

Foxp3<sup>+</sup> regulatory T cells (T<sub>reg</sub> cells) maintain immunological tolerance and their deficiency results in fatal multi-organ autoimmunity. Although heightened T cell receptor (TCR) signaling is critical for the differentiation of T<sub>reg</sub> cells, the role of TCR signaling in T<sub>reg</sub> cell function remains largely unknown. Here we demonstrate inducible ablation of the TCR results in T<sub>reg</sub> cell dysfunction which cannot be attributed to impaired Foxp3 expression, decreased expression of T<sub>reg</sub> cell signature genes or altered ability to sense and consume interleukin 2. Rather, TCR signaling was required for maintaining the expression of a limited subset of genes comprising 25% of the activated T<sub>reg</sub> cell transcriptional signature. Our results reveal a critical role for the TCR in T<sub>reg</sub> cell suppressor capacity.

Regulatory CD4<sup>+</sup> T cells expressing the transcription factor Foxp3 play an essential role in maintaining immune tolerance<sup>1</sup>. In the thymus, increased affinity T cell receptor (TCR) engagement in immature CD4 single positive thymocytes is required for initiation of the T<sub>reg</sub> cell differentiation program and induction of Foxp3 expression<sup>2</sup>. As a consequence, T<sub>reg</sub> cells exported to the periphery exhibit a TCR repertoire skewed towards self-recognition<sup>3,4</sup>. However, the requirement for TCR signaling in mediating T<sub>reg</sub> cell suppressive function in the periphery remains largely unclear.

In comparison to conventional CD4<sup>+</sup> T cells, T<sub>reg</sub> cells exhibit impaired calcium flux, Akt activation and Erk phosphorylation upon TCR stimulation, and Foxp3 is known to potently

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### Author contributions

A.G.L. and A.Y.R. designed the experiments, and A.G.L. conducted experiments and wrote the manuscript. A.Y.R. supervised the research and edited the manuscript. W.J. prepared samples for microarray analysis and A.A. and A.G.L. conducted the analysis.

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repress at least some TCR-induced genes, as well as some genes involved in the TCR signaling pathway<sup>5–8</sup>. At the same time, Foxp3<sup>+</sup> T<sub>reg</sub> cells have high basal expression of several cell surface molecules which are known to contribute to T<sub>reg</sub> cell function (such as CD25, CD39 and CTLA-4) and whose expression in conventional CD4<sup>+</sup> T cells is dependent upon TCR stimulation<sup>9–14</sup>. Whether or not high affinity TCR interactions with self contribute to constitutive expression of these genes and consequently to T<sub>reg</sub> cell function is not known.

T<sub>reg</sub> cells—despite their intrinsically dampened response to TCR stimulation—acquire an activated phenotype and expand in response to their cognate antigens in settings of immune activation, such as infection and autoimmunity<sup>15, 16</sup>. These observations imply that self-antigen recognition helps maintain T<sub>reg</sub> cells of particular specificities and may potentiate their suppressive capacity during immune challenge<sup>17</sup>. Nevertheless, strict reliance on TCR expression for T<sub>reg</sub> cell activation—as opposed to preferential activation of antigen-specific T<sub>reg</sub> cells—has not been demonstrated, nor has TCR engagement *in vivo* been shown to be required for T<sub>reg</sub> cell function in any context.

We used inducible genetic ablation of cell surface TCR complexes to directly address the requirement of TCR expression for T<sub>reg</sub> cell immunosuppressive capacity. Notably, the TCR was largely dispensable for Foxp3 expression, lineage stability and for high expression of many T<sub>reg</sub> cell signature genes. Nevertheless, these features were not sufficient to preserve T<sub>reg</sub> cell function and to prevent immune activation. Loss of suppressor capacity in the absence of TCR was not due to impaired T<sub>reg</sub> cell ability to gain access to interleukin 2 (IL-2), and accordingly administration of exogenous IL-2 failed to rescue systemic autoimmunity. Instead, TCR expression was essential for T<sub>reg</sub> cell activation and maintenance of a limited set of genes which were found to be expressed almost exclusively in activated T<sub>reg</sub> cells. Among these genes, expression of the transcription factor IRF4 contributed to optimal T<sub>reg</sub> cell function and homeostasis. These results demonstrate an essential role for the TCR in eliciting the suppressor function of differentiated T<sub>reg</sub> cells.

## Results

### Maintenance of T<sub>reg</sub> cell identity in the absence of the TCR

In order to investigate the consequences of TCR signaling in T<sub>reg</sub> cell function, we crossed *Foxp3<sup>eGFP-CreERT2</sup>* mice to *Ca<sup>FL</sup>* mice to allow inducible deletion of *Ca* encoding the TCR $\alpha$  constant chain specifically in T<sub>reg</sub> cells<sup>18, 19</sup>. In this model, Cre-induced loss of the conditional *Ca* allele upon tamoxifen administration eliminates TCR $\alpha$  expression, preventing cell surface TCR $\alpha\beta$  heterodimer formation. Tamoxifen was administered via oral gavage on days 0 and 1 and mice were analyzed on day 9. Allelic exclusion at the *Ca* locus in heterozygous *Foxp3<sup>CreERT2</sup>Ca<sup>FL/WT</sup>* mice yielded a minor population of TCR-deficient (TCR<sup>-</sup>) T<sub>reg</sub> cells (~25%), while in homozygous *Foxp3<sup>CreERT2</sup>Ca<sup>FL/FL</sup>* mice the majority of T<sub>reg</sub> cells (~60–70%) lacked cell surface TCR $\beta$  (Fig. 1a). Although we cannot definitively exclude the possibility that few residual TCR complexes were present in minute amounts (below the detection limit of flow cytometric analyses) on the TCR<sup>-</sup> T<sub>reg</sub> cells, functional *in vitro* analyses confirmed loss of TCR crosslinking-dependent activation of TCR<sup>-</sup> T<sub>reg</sub> cells (Supplementary Fig. 1a–d).

Because binding sites for the transcription factors NFAT and c-Rel have been identified within the *Foxp3* locus, and TCR engagement-driven NF- $\kappa$ B signaling is critical for induction of *Foxp3* expression, we speculated that the TCR might be essential to maintain *Foxp3* expression<sup>20–22</sup>. However, *Foxp3* expression was only marginally reduced in TCR<sup>-</sup> T<sub>reg</sub> cells in *Foxp3<sup>CreERT2</sup>Ca<sup>FL/WT</sup>* mice, and was not reduced at all in TCR<sup>-</sup> T<sub>reg</sub> cells in *Foxp3<sup>CreERT2</sup>Ca<sup>FL/FL</sup>* mice, compared to TCR<sup>+</sup> T<sub>reg</sub> cells present in the same mice and in *Foxp3<sup>CreERT2</sup>Ca<sup>WT/WT</sup>* animals (Fig. 1b). Similarly, the expression of several T<sub>reg</sub> cell signature molecules, including CD25, GITR, CD39 and CD73, was largely unaffected in TCR<sup>-</sup> T<sub>reg</sub> cells from both *Foxp3<sup>CreERT2</sup>Ca<sup>FL/WT</sup>* and *Foxp3<sup>CreERT2</sup>Ca<sup>FL/FL</sup>* mice (Supplementary Fig. 1e). These results indicate that, at steady state, continuous TCR-mediated recognition of self does not contribute significantly to *Foxp3*-dependent maintenance of expression for these genes<sup>11, 23</sup>. In contrast, CTLA-4 expression was notably diminished in TCR<sup>-</sup> T<sub>reg</sub> cells in *Foxp3<sup>CreERT2</sup>Ca<sup>FL/WT</sup>* mice, although not in TCR<sup>-</sup> T<sub>reg</sub> cells in *Foxp3<sup>CreERT2</sup>Ca<sup>FL/FL</sup>* mice, compared to TCR<sup>+</sup> T<sub>reg</sub> cells in these mice and in *Foxp3<sup>CreERT2</sup>Ca<sup>WT/WT</sup>* animals (Supplementary Fig. 1e).

Compared to *Foxp3<sup>CreERT2</sup>Ca<sup>WT/WT</sup>* mice, percentages and absolute numbers of *Foxp3*<sup>+</sup> cells in the spleens and lymph nodes of *Foxp3<sup>CreERT2</sup>Ca<sup>FL/WT</sup>* and *Foxp3<sup>CreERT2</sup>Ca<sup>FL/FL</sup>* mice were unaltered (Supplementary Figure 1f). However, to address the possibility that a portion of T<sub>reg</sub> cells completely lost *Foxp3* expression upon ablation of the TCR and that these “former” T<sub>reg</sub> cells were not accounted for in this experimental setup, we crossed *Foxp3<sup>CreERT2</sup>Ca<sup>FL/WT</sup>* mice with mice expressing the recombination reporter *Rosa26<sup>YFP</sup>* allele. TCR $\beta$  and *Foxp3* expression was assessed in CD4<sup>+</sup>YFP<sup>+</sup> cells sorted from the spleens and lymph nodes of *Foxp3<sup>CreERT2</sup>Ca<sup>FL/WT</sup>Rosa<sup>YFP</sup>* mice on day 9 or 50 following tamoxifen administration on two consecutive days. YFP-expressing CD4<sup>+</sup>TCR $\beta$ <sup>-</sup> and CD4<sup>+</sup>TCR $\beta$ <sup>+</sup> cell subsets contained similarly minor frequencies of *Foxp3*<sup>-</sup> cells at both time points (data not shown). Furthermore, following *in vivo* IL-2 neutralization – a condition known to promote loss of *Foxp3* expression – in *Foxp3<sup>CreERT2</sup>Ca<sup>FL/WT</sup>Rosa<sup>YFP</sup>* mice, CD4<sup>+</sup>YFP<sup>+</sup>TCR $\beta$ <sup>-</sup> cells retained a higher percentage of *Foxp3*<sup>+</sup> cells than did CD4<sup>+</sup>YFP<sup>+</sup>TCR $\beta$ <sup>+</sup> cells. Together, these data indicate that TCR signaling is dispensable for the maintenance of the T<sub>reg</sub> cell phenotype and lineage stability, and moreover, that TCR signaling can drive the loss of *Foxp3* when IL-2 amounts are limiting (Fig. 1c)<sup>19</sup>.

### Requirement for the TCR in T<sub>reg</sub> cell effector differentiation

Although the T<sub>reg</sub> cell phenotype was largely preserved upon ablation of the TCR, we observed a relative enrichment of naïve-like CD44<sup>lo</sup>CD62L<sup>hi</sup> cells among TCR<sup>-</sup> T<sub>reg</sub> cells in lymph nodes and spleens of *Foxp3<sup>CreERT2</sup>Ca<sup>FL/WT</sup>* and *Foxp3<sup>CreERT2</sup>Ca<sup>FL/FL</sup>* mice (Fig. 2a and Supplementary Fig. 1g). T<sub>reg</sub> cell proliferation is almost exclusively restricted to the CD44<sup>hi</sup> subset, and in part, the enrichment we observed appeared to be a consequence of the severely impaired proliferative capacity of CD44<sup>hi</sup> T<sub>reg</sub> cell in the absence of the TCR (Fig. 2b)<sup>24</sup>. The minor population of TCR<sup>-</sup> T<sub>reg</sub> cells in *Foxp3<sup>CreERT2</sup>Ca<sup>FL/WT</sup>* mice was predominantly non-dividing: these cells showed minimal expression of Ki67, failed to incorporate BrdU over a 24 hour labeling period and contained the largest percentage of CD44<sup>lo</sup>CD62L<sup>hi</sup> cells among all TCR<sup>+</sup> and TCR<sup>-</sup> T<sub>reg</sub> cell populations in *Foxp3<sup>CreERT2</sup>Ca<sup>WT/WT</sup>*, *Foxp3<sup>CreERT2</sup>Ca<sup>FL/WT</sup>*, and *Foxp3<sup>CreERT2</sup>Ca<sup>FL/FL</sup>* mice (Fig 2a,b

and Supplementary Fig. 1h). In *Foxp3<sup>CreERT2</sup>C $\alpha$ <sup>FL/FL</sup>* mice, however, TCR<sup>-</sup> T<sub>reg</sub> cells exhibited considerable proliferative activity, albeit reduced in comparison to TCR<sup>+</sup> T<sub>reg</sub> cells present in the same mouse (Fig 2a). Increased Ki67 staining in CD44<sup>hi</sup> Treg cells inversely correlated with a decreased percentage of CD44<sup>lo</sup>CD62L<sup>hi</sup> cells within all TCR<sup>+</sup> and TCR<sup>-</sup> T<sub>reg</sub> cell populations present in *Foxp3<sup>CreERT2</sup>C $\alpha$ <sup>WT/WT</sup>*, *Foxp3<sup>CreERT2</sup>C $\alpha$ <sup>FL/WT</sup>*, and *Foxp3<sup>CreERT2</sup>C $\alpha$ <sup>FL/FL</sup>* animals (Fig 2a, b).

To address the possibility that continuous peripheral differentiation of naïve-like T<sub>reg</sub> cells into CD44<sup>hi</sup> cells was impeded in the absence of TCR expression and that such a differentiation block contributed to the predominantly CD44<sup>lo</sup>CD62L<sup>hi</sup> phenotype of TCR<sup>-</sup> T<sub>reg</sub> cells, we bred *C $\alpha$ <sup>FL</sup>* mice to *Foxp3<sup>YFP-Cre</sup>* mice to induce ablation of the TCR in newly generated, “naïve” T<sub>reg</sub> cells<sup>25, 26</sup>. We reasoned that if the TCR was critical for T<sub>reg</sub> cell effector differentiation in the periphery, TCR<sup>-</sup> T<sub>reg</sub> cells in these mice would retain a CD44<sup>lo</sup>CD62L<sup>hi</sup> naïve-like phenotype.

Immature HSA<sup>hi</sup>CD4<sup>+</sup>Foxp3<sup>+</sup> cells in the thymi of *Foxp3<sup>Cre</sup>C $\alpha$ <sup>FL/FL</sup>* mice showed similar cell surface expression of TCR complexes as their wild type counterparts, while more mature HSA<sup>lo</sup>CD4<sup>+</sup>Foxp3<sup>+</sup> thymocytes showed only slightly reduced TCR expression (Supplementary Fig. 2a.). Among Foxp3<sup>+</sup> cells present in the spleens and lymph nodes of *Foxp3<sup>Cre</sup>C $\alpha$ <sup>FL/WT</sup>* mice, ~5–15% were TCR $\beta$ <sup>-</sup> whereas ~80% of Foxp3<sup>+</sup> cells in *Foxp3<sup>Cre</sup>C $\alpha$ <sup>FL/FL</sup>* mice lacked surface TCR $\beta$  expression (Fig 2e and Supplementary Fig. 2b). We again observed a slight decrease in the amount of Foxp3 protein in the TCR<sup>-</sup> T<sub>reg</sub> cells in *Foxp3<sup>Cre</sup>C $\alpha$ <sup>FL/WT</sup>* mice, but not in *Foxp3<sup>Cre</sup>C $\alpha$ <sup>FL/FL</sup>* mice, while expression of T<sub>reg</sub> cell signature genes was variably affected in the TCR<sup>-</sup> T<sub>reg</sub> cell populations in both mice as compared to TCR<sup>+</sup> Treg cells in *Foxp3<sup>Cre</sup>C $\alpha$ <sup>WT/WT</sup>*, *Foxp3<sup>Cre</sup>C $\alpha$ <sup>FL/WT</sup>*, and *Foxp3<sup>Cre</sup>C $\alpha$ <sup>FL/FL</sup>* mice (Supplementary Fig. 2c,d).

Despite their generally intact T<sub>reg</sub> cell surface phenotype, nearly all TCR<sup>-</sup> T<sub>reg</sub> cells in healthy *Foxp3<sup>Cre</sup>C $\alpha$ <sup>FL/WT</sup>* mice had a naïve-like CD62L<sup>hi</sup>CD44<sup>lo</sup> phenotype and lacked expression of all T<sub>reg</sub> cell differentiation markers tested, including KLRG1, CD103 and CXCR3 (Fig. 2c). Notably, this pattern was also observed under severe inflammatory conditions in *Foxp3<sup>Cre</sup>C $\alpha$ <sup>FL/FL</sup>* mice, which were moribund by three weeks of age (Fig. 2c and Supplementary Fig. 3e,f). Lack of CD44<sup>hi</sup> cells among TCR<sup>-</sup> populations in *Foxp3<sup>Cre</sup>C $\alpha$ <sup>FL/WT</sup>* and *Foxp3<sup>Cre</sup>C $\alpha$ <sup>FL/FL</sup>* mice correlated with decreased proliferation and markedly diminished percentages and numbers of TCR<sup>-</sup> T<sub>reg</sub> cells in lymph nodes and, particularly, in the spleens and tissues such as liver and lung as compared to TCR<sup>+</sup> T<sub>reg</sub> cells in the same mice and in *Foxp3<sup>Cre</sup>C $\alpha$ <sup>WT/WT</sup>* animals (Fig. 2d,e and Supplementary Fig. 2g,h). Together, these data are consistent with an absolute requirement for TCR expression—loss of which cannot be compensated for even in conditions of extreme immune activation—for peripheral effector differentiation of naïve-like T<sub>reg</sub> cells and acquisition of an activated CD44<sup>hi</sup> phenotype.

### TCR-dependent effector function of mature Treg cells

Foxp3 expression and T<sub>reg</sub> cell expansion are facilitated by IL-2R signaling<sup>27–29</sup>. The increase in Foxp3 protein expression and proliferative activity of TCR<sup>-</sup> T<sub>reg</sub> cells in *Foxp3<sup>CreERT2</sup>C $\alpha$ <sup>FL/FL</sup>* mice vs. *Foxp3<sup>CreERT2</sup>C $\alpha$ <sup>FL/WT</sup>* mice led us to suspect that ablation

of TCR expression, even on mature T<sub>reg</sub> cells, might precipitate immune activation and elevate production of IL-2 and other cytokines by activated CD4<sup>+</sup> T cells. Indeed, analysis of *Foxp3<sup>CreERT2</sup>Cα<sup>FL/FL</sup>* mice treated twice with tamoxifen and analyzed on day 9 post-treatment revealed increased percentages of CD44<sup>hi</sup> T cells and increased numbers of IL-2-producing CD4<sup>+</sup> T cells compared to tamoxifen-treated *Foxp3<sup>CreERT2</sup>Cα<sup>WT/WT</sup>* and *Foxp3<sup>CreERT2</sup>Cα<sup>FL/WT</sup>* mice (data not shown).

To more rigorously assess the role for TCR expression in mature T<sub>reg</sub> cell function, we administered four doses of tamoxifen to mice (on days 0, 3, 7 and 10) in order to maximize Cre-ERT2-mediated recombination. On day 13, we noted deletion of TCR expression in ~75–80% of T<sub>reg</sub> cells in *Foxp3<sup>CreERT2</sup>Cα<sup>FL/FL</sup>* and ~25–30% in T<sub>reg</sub> cells in *Foxp3<sup>CreERT2</sup>Cα<sup>FL/WT</sup>* mice (Fig. 3a,b). Despite normal or even increased percentages of total Foxp3<sup>+</sup> cells in the lymph nodes and spleens of *Foxp3<sup>CreERT2</sup>Cα<sup>FL/FL</sup>* mice, elevated numbers of CD4<sup>+</sup>Foxp3<sup>-</sup> and CD8<sup>+</sup> T cell were found in the lymph nodes, and increased percentages of CD44<sup>hi</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T cells were found in the lymph nodes and spleens of these mice (Fig. 3c–d). CD8<sup>+</sup> T cells and CD4<sup>+</sup> T cells from *Foxp3<sup>CreERT2</sup>Cα<sup>FL/FL</sup>* mice produced increased amounts of interferon-γ (IFN-γ) and IFN-γ, IL-2, IL-4, IL-13, IL-5 and IL-17, respectively, when compared to T cells from *Foxp3<sup>CreERT2</sup>Cα<sup>WT/WT</sup>* and *Foxp3<sup>CreERT2</sup>Cα<sup>FL/WT</sup>* mice (Fig. 3e and data not shown).

Immune activation in *Foxp3<sup>CreERT2</sup>Cα<sup>FL/FL</sup>* mice was milder than that resulting from complete T<sub>reg</sub> cell depletion in *Foxp3<sup>DTR</sup>* mice, which express the human diphtheria toxin receptor (DTR) concomitantly with *Foxp3<sup>30</sup>*. Thus, it was possible that the large numbers of TCR<sup>-</sup> T<sub>reg</sub> cells in *Foxp3<sup>CreERT2</sup>Cα<sup>FL/FL</sup>* mice retained measurable TCR-independent suppressor capacity and were still capable of immune regulation. Alternatively, the minor population of remaining TCR-sufficient T<sub>reg</sub> cells in these mice might have limited the activation of effector T cells and the associated autoimmunity to some degree. To address these possibilities, we attempted to reduce the T<sub>reg</sub> cell percentages among CD4<sup>+</sup> cells in *Foxp3<sup>DTR</sup>* mice to approximate the percentages of the residual TCR-sufficient T<sub>reg</sub> cells in *Foxp3<sup>CreERT2</sup>Cα<sup>FL/FL</sup>* mice following four doses of tamoxifen<sup>31</sup>. We reasoned that if TCR<sup>-</sup> T<sub>reg</sub> cells were capable of significant suppression, autoimmunity in *Foxp3<sup>DTR</sup>* mice subjected to only partial depletion of T<sub>reg</sub> cells would be more severe than in *Foxp3<sup>CreERT2</sup>Cα<sup>FL/FL</sup>* mice. As diphtheria toxin (DT) injection depletes T<sub>reg</sub> cells within 24 hours, while tamoxifen-induced Cre-ERT2 mediated recombination progressively increases over a four day period, we treated *Foxp3<sup>DTR</sup>*, *Foxp3<sup>CreERT2</sup>Cα<sup>WT/WT</sup>* and *Foxp3<sup>CreERT2</sup>Cα<sup>FL/FL</sup>* mice each with DT four days after their first dose of tamoxifen (which like DT was administered to mice of all genotypes) in order to account for the time needed for complete Cre-ERT2-mediated deletion of Cα (Supplementary Fig. 3a,b and data not shown). Partial depletion of the T<sub>reg</sub> cell compartment in *Foxp3<sup>DTR</sup>* mice resulted in CD4<sup>+</sup>Foxp3<sup>-</sup> T cell activation and cytokine production grossly comparable to that observed in *Foxp3<sup>CreERT2</sup>Cα<sup>FL/FL</sup>* mice harboring populations of TCR-sufficient T<sub>reg</sub> cells of a similar or even larger size (Supplementary Fig. 3b–d). Together, these results demonstrate that T<sub>reg</sub> cells require continuous TCR expression for the effective elaboration of their suppressor function, and suggest that TCR<sup>-</sup> T<sub>reg</sub> cells, which are abundant in *Foxp3<sup>CreERT2</sup>Cα<sup>FL/FL</sup>* mice, are grossly devoid of detectable suppressor capacity.

## TCR<sup>-</sup> T<sub>reg</sub> cell dysfunction is not secondary to impaired IL-2R signaling

We considered that the apparent loss of T<sub>reg</sub> cell suppressive capacity in the absence of the TCR might be an indirect consequence of an impaired ability to localize in a TCR- and antigen-dependent manner to sites of CD4<sup>+</sup> T cell activation and to, thereby, acquire IL-2, a cytokine known to be critical for T<sub>reg</sub> cell function and homeostasis. This scenario would explain the decreased expression of Foxp3 and minimal proliferation of TCR<sup>-</sup> T<sub>reg</sub> cells in healthy *Foxp3<sup>CreERT2</sup>Cα<sup>FL/WT</sup>* mice, in which IL-2 amounts were not elevated and would not be able to partially remedy these defects<sup>32</sup>.

However, direct *ex vivo* analysis of phosphorylation of Stat5—which occurs downstream of IL-2 signaling in T<sub>reg</sub> cells—in spleen and lymph nodes showed at least equivalent proportions of p-Stat5 in TCR<sup>-</sup> and TCR<sup>+</sup> T<sub>reg</sub> cells in both *Foxp3<sup>CreERT2</sup>Cα<sup>FL/WT</sup>* and *Foxp3<sup>CreERT2</sup>Cα<sup>FL/FL</sup>* mice (Fig. 4a). In *Foxp3<sup>CreERT2</sup>Cα<sup>FL/FL</sup>* mice, TCR<sup>-</sup> T<sub>reg</sub> cells had increased p-Stat5 compared to TCR<sup>+</sup> T<sub>reg</sub> cells, mirroring expression of CD25 and CD62L, which remained high on TCR<sup>-</sup> cells, but was decreased on the residual, activated TCR<sup>+</sup> T<sub>reg</sub> cells present in these mice (Fig. 4a and Supplementary Figure 1e,f). These results are consistent with the observation that p-Stat5<sup>+</sup> T<sub>reg</sub> cells are largely found within the CD62L<sup>hi</sup>CD44<sup>lo</sup> (and CD25<sup>hi</sup>) T<sub>reg</sub> cell subset which, in contrast to the activated CD44<sup>hi</sup>CD62L<sup>lo</sup> (and CD25<sup>int</sup>) T<sub>reg</sub> cell subset, rely on IL-2R signaling rather than co-stimulatory receptor engagement for their maintenance<sup>24</sup>.

*In vitro* analysis confirmed that lack of TCR expression did not significantly influence Stat5 phosphorylation in response to IL-2, nor impair T<sub>reg</sub> cell ability to capture and deplete IL-2 from culture media, suggesting that in the absence of TCR expression T<sub>reg</sub> cell-mediated IL-2 deprivation may not be a significant mechanism of immune suppression (Supplementary Fig. 4a,b). Furthermore, treatment of *Foxp3<sup>CreERT2</sup>Cα<sup>WT/WT</sup>*, *Foxp3<sup>CreERT2</sup>Cα<sup>FL/WT</sup>* and *Foxp3<sup>CreERT2</sup>Cα<sup>FL/FL</sup>* mice with neutralizing anti-IL-2 (or isotype control) antibody reduced Foxp3 expression and decreased cell percentages among total CD4<sup>+</sup> cells comparably between TCR<sup>+</sup> and TCR<sup>-</sup> T<sub>reg</sub> cells (Fig. 4b). Together, these data suggest that TCR<sup>-</sup> T<sub>reg</sub> cells efficiently capture IL-2 during immune activation and at steady state. Although it remains to be determined what signal(s) drive the proliferation of TCR<sup>-</sup> T<sub>reg</sub> cells selectively in diseased *Foxp3<sup>CreERT2</sup>Cα<sup>FL/FL</sup>* mice, we observed increased CD80 and CD86 expression on lymph node dendritic cells (DCs) in *Foxp3<sup>CreERT2</sup>Cα<sup>FL/FL</sup>* mice, and activated DCs were able to induce limited proliferation of TCR<sup>-</sup> T<sub>reg</sub> cells *in vitro* (Supplementary Fig. 4c,d).

Lastly, administration of IL-2-anti-IL-2 complexes to *Foxp3<sup>CreERT2</sup>Cα<sup>FL/FL</sup>* mice did not diminish to any measurable extent the activation and lymphoproliferation of effector T cells caused by loss of TCR expression in T<sub>reg</sub> cells (Supplementary Fig. 5). Conversely, IL-2 depletion did not further exacerbate autoimmunity (data not shown). Notably, this was the case in spite of a 1.5-fold expansion of TCR<sup>-</sup>, but not TCR<sup>+</sup>, T<sub>reg</sub> cells following IL-2 administration (a likely consequence of higher CD25 expression and heightened IL-2 responsiveness by TCR<sup>-</sup> T<sub>reg</sub> cells) and a greater than two-fold reduction in TCR<sup>-</sup> T<sub>reg</sub> cells following IL-2 depletion (Fig. 4b and Supplementary Fig. 5b). These observations further confirm that TCR<sup>-</sup> T<sub>reg</sub> cells—even when present in elevated numbers—possess minimal

suppressive capacity. Together, these results indicate that neither TCR-dependent interactions with antigen presenting cells nor continuous TCR-mediated localization within lymphoid organs are required for T<sub>reg</sub> cell acquisition of IL-2, and that T<sub>reg</sub> cell dysfunction in the absence of TCR cannot be attributed to altered IL-2R signaling.

### TCR expression promotes T<sub>reg</sub> cell adhesive properties *in vitro*

The *in vitro* suppressive capacity of T<sub>reg</sub> cells requires TCR engagement, possibly involving pathways independent of Zap70 catalytic activity—which is essential for conventional T cell effector function—but dependent on membrane proximal inside-out activation of integrins and subsequent enhancement of T<sub>reg</sub> cell interactions with antigen presenting cells<sup>8, 33, 34</sup>. To test this possibility, we cultured TCR<sup>+</sup> or TCR<sup>-</sup> T<sub>reg</sub> cells isolated from *Foxp3<sup>CreERT2</sup>Cα<sup>WT/WT</sup>*, *Foxp3<sup>CreERT2</sup>Cα<sup>FL/WT</sup>* or *Foxp3<sup>CreERT2</sup>Cα<sup>FL/FL</sup>* mice with DCs and assessed DC-T<sub>reg</sub> cell conjugate formation. We did not detect any differences in conjugate formation between DCs and TCR<sup>+</sup> or TCR<sup>-</sup> T<sub>reg</sub> cells following 30 min of incubation (data not shown). However, TCR<sup>-</sup> T<sub>reg</sub> cells isolated from *Foxp3<sup>CreERT2</sup>Cα<sup>FL/WT</sup>* or *Foxp3<sup>CreERT2</sup>Cα<sup>FL/FL</sup>* mice were less efficient than TCR<sup>+</sup> T<sub>reg</sub> cells at forming conjugates with DCs (3.7% vs. 7.5% of Treg cells) following overnight culture (Supplementary Fig. 6a). Conjugate formation was unaffected by the presence or absence of MHC class II molecules on DCs, which may imply that the increased adhesion of TCR<sup>+</sup> vs. TCR<sup>-</sup> T<sub>reg</sub> cells in this assay was not a result of MHC class II-TCR interactions and may instead have been a consequence of the overall heightened activation status of TCR<sup>+</sup> vs. TCR<sup>-</sup> T<sub>reg</sub> cells (Supplementary Fig. 6a). As LFA-1 expression is higher on CD44<sup>hi</sup> T<sub>reg</sub> cells in comparison to CD44<sup>lo</sup>CD62L<sup>hi</sup> T<sub>reg</sub> cells, it is possible that superior conjugate formation by TCR<sup>+</sup> T<sub>reg</sub> cells, which are enriched for CD44<sup>hi</sup> cells compared to TCR<sup>-</sup> T<sub>reg</sub> cells, may be due, at least in part, to increased expression of this integrin (Supplementary Figure 6b). Although further work is necessary to determine precisely how TCR engagement *in vivo* affects signaling pathways to modulate T<sub>reg</sub> cell adhesive properties, our results indicate that TCR expression contributes to optimal T<sub>reg</sub> cell contact-dependent interactions with APCs, which may contribute to TCR-dependent immunosuppressive function.

### TCR signaling modulates the effector T<sub>reg</sub> cell transcriptional signature

In order to explore whether TCR signals, apart from influencing T<sub>reg</sub> cell adhesion, might drive transcriptional events to license suppressor function *in vivo*, we conducted gene expression analysis of TCR<sup>+</sup> and TCR<sup>-</sup> T<sub>reg</sub> cells. Flow cytometric analysis showed that loss of TCR expression had a stronger effect on effector-like CD44<sup>hi</sup>CD62L<sup>lo</sup> T<sub>reg</sub> cells compared to naïve-like CD44<sup>lo</sup>CD62L<sup>hi</sup> T<sub>reg</sub> cells (data not shown) prompting us to investigate the gene expression profiles of these two populations separately within the TCR<sup>+</sup> and TCR<sup>-</sup> T<sub>reg</sub> cell populations isolated from healthy *Foxp3<sup>CreERT2</sup>Cα<sup>FL/WT</sup>* mice (to avoid confounding effects of immune activation). We found 155 genes were significantly down-regulated, and only five genes were significantly up-regulated, by at least 2-fold in effector-like CD44<sup>hi</sup>CD62L<sup>lo</sup> TCR<sup>-</sup> T<sub>reg</sub> cells compared to CD44<sup>hi</sup>CD62L<sup>lo</sup> TCR<sup>+</sup> T<sub>reg</sub> cells (Fig. 5a). Sixteen genes were significantly down-regulated in the naïve-like CD44<sup>lo</sup>CD62L<sup>hi</sup> TCR<sup>-</sup> T<sub>reg</sub> cells compared to CD44<sup>lo</sup>CD62L<sup>hi</sup> TCR<sup>+</sup> T<sub>reg</sub> cells (all of them also down-regulated in CD44<sup>hi</sup>CD62L<sup>lo</sup> TCR<sup>-</sup> vs. TCR<sup>+</sup> T<sub>reg</sub> cells), whereas one gene was up-regulated (Fig 5a). 535 genes showed higher expression (2-fold or greater) in effector-like

CD44<sup>hi</sup>CD62L<sup>lo</sup> TCR<sup>+</sup> T<sub>reg</sub> cells compared to naïve-like CD44<sup>lo</sup>CD62L<sup>hi</sup> TCR<sup>+</sup> T<sub>reg</sub> cells, and expression of 136 of them (25%) was TCR-dependent (Fig. 5b). Importantly, 127 of the 155 genes (82%) down-regulated in the absence of TCR in CD44<sup>hi</sup>CD62L<sup>lo</sup> T<sub>reg</sub> cells showed at least 2-fold higher expression in effector-like vs. naïve-like T<sub>reg</sub> cells.

Foxp3 has been proposed to solidify and amplify a transcriptional program initiated in T<sub>reg</sub> cell precursors by TCR engagement<sup>9, 10, 35, 36</sup>. We compared expression of the 155 genes identified above as being maintained by TCR in T<sub>reg</sub> cells (which we call TCR-dependent genes) to Foxp3-dependent genes, identified as upregulated in wild-type T<sub>reg</sub> cells compared to *Foxp3*<sup>null</sup>-expressing T cells from *Foxp3*<sup>GFPKO</sup> mice (which express a Foxp3 reporter null allele).<sup>36</sup> We found that a significantly enriched percentage of the TCR-dependent genes, compared to all genes, were also Foxp3-dependent (Fig. 5c). These observations suggest that the TCR-driven transcriptional program in T<sub>reg</sub> cells is enhanced by Foxp3 expression, but that Foxp3 alone is not sufficient to maintain the full effector T<sub>reg</sub> cell transcriptional signature.

Examination of the TCR-dependent genes identified several transcription factors that were upregulated in TCR<sup>+</sup> compared to TCR<sup>-</sup> CD44<sup>hi</sup>CD62L<sup>lo</sup> T<sub>reg</sub> cells as well as compared to TCR<sup>+</sup> and TCR<sup>-</sup> CD44<sup>lo</sup>CD62L<sup>hi</sup> T<sub>reg</sub> cells, including NFATc1, c-Rel, Bcl6 and IRF4 the latter two of which were previously shown to be important for T<sub>reg</sub> cell effector differentiation and function (Fig 5d).<sup>37–39</sup> Of the 155 TCR-dependent genes, we identified only one gene encoding an adhesion molecule, *Vcam1*, which was similarly upregulated in CD44<sup>hi</sup>CD62L<sup>lo</sup> T<sub>reg</sub> cells compared to CD44<sup>lo</sup>CD62L<sup>hi</sup> T<sub>reg</sub> cells in a manner that depended upon TCR expression (Fig. 5d). Several genes encoding potential effector molecules were also found to be upregulated in CD44<sup>hi</sup>CD62L<sup>lo</sup> vs. CD44<sup>lo</sup>CD62L<sup>hi</sup> T<sub>reg</sub> cells in a TCR-dependent manner, including IL-1R2, a decoy receptor for IL-1, and the immune inhibitory molecules CD83, CD200, and LAG-3, as well as IL-10 and EBI3, a subunit of the cytokines IL-27 and IL-35, all of which have been implicated in T<sub>reg</sub> cell function<sup>26, 40–45</sup>. In addition, the chemokine-encoding genes *Cxcl10* and *Ccl11*, as well as *Ccr8* encoding the receptor for CCL1, were significantly down-regulated in TCR<sup>-</sup> compared to TCR<sup>+</sup> T<sub>reg</sub> cells, suggesting that T<sub>reg</sub> cells may signal each other through the expression of chemokines and their corresponding receptors, or may recruit into close proximity the targets of their suppressive activity<sup>46</sup>.

All together, these data indicate that, under physiologic conditions, a substantial portion of the effector—but not the naïve-like—T<sub>reg</sub> cell transcriptional program characterized by elevated expression of several potential T<sub>reg</sub> cell effector molecules, is maintained by continuous TCR signaling.

### IRF4 expression promotes T<sub>reg</sub> cell function and homeostasis

To begin to assess the importance of the TCR-dependent transcriptional program for continuous T<sub>reg</sub> cell function *in vivo*, we focused on IRF4 as a downstream target of the TCR signaling pathway in T<sub>reg</sub> cells. We confirmed that elevated IRF4 expression in T<sub>reg</sub> cells was restricted to CD44<sup>hi</sup> cells and was reduced to basal levels upon TCR ablation in both *Foxp3*<sup>CreERT2</sup>*Cα*<sup>FL/WT</sup> and *Foxp3*<sup>CreERT2</sup>*Cα*<sup>FL/FL</sup> mice (Supplementary Fig. 7a). *Foxp3*<sup>YFP-Cre</sup>*Ir4*<sup>FL/-</sup> mice, in which IRF4 is constitutively deleted in T<sub>reg</sub> cells, have been



shown to develop a severe T<sub>H</sub>2 cytokine-dominated autoimmunity by 8 weeks of age<sup>37</sup>. T<sub>reg</sub> cells in *Irf4*<sup>-/-</sup> mice were previously demonstrated to have an almost exclusively naïve-like phenotype, and we similarly found that T<sub>reg</sub> cells in *Foxp3*<sup>YFP-Cre</sup>*Irf4*<sup>FL/FL</sup> mice were largely CD44<sup>lo</sup>CD62L<sup>hi</sup> even in the context of severe inflammation (data not shown), suggesting impaired differentiation, survival and/or expansion of effector T<sub>reg</sub> cells<sup>38</sup>. In order to determine whether fully differentiated T<sub>reg</sub> cells require IRF4 expression downstream of TCR engagement for their *in vivo* suppressive function, we administered tamoxifen on days 0, 3, 7 and 10 to *Foxp3*<sup>CreERT2</sup>*Irf4*<sup>FL/FL</sup> and *Foxp3*<sup>CreERT2</sup>*Irf4*<sup>WT/WT</sup> littermates<sup>47</sup>. On day 13, we observed a slight but reproducible decrease in IRF4 protein expression in lymph node CD44<sup>hi</sup> T<sub>reg</sub> cells in *Foxp3*<sup>CreERT2</sup>*Irf4*<sup>FL/FL</sup> mice compared to *Foxp3*<sup>CreERT2</sup>*Irf4*<sup>WT/WT</sup> mice, and an increase in GFP expression in the T<sub>reg</sub> cells as a sum of fluorescence of the *Irf4*-deletion GFP reporter (whose expression is switched on in the *Irf4* locus upon Cre-mediated deletion of the *Irf4*<sup>FL</sup> allele) and the GFP-CreERT2 fusion protein expressed concomitantly with Foxp3 (Fig. 6a and Supplementary Fig. 7b). qPCR analysis indicated a ~50% reduction in *Irf4* mRNA transcript amount in T<sub>reg</sub> cells sorted from pooled spleens and lymph nodes of tamoxifen-treated *Foxp3*<sup>CreERT2</sup>*Irf4*<sup>FL/FL</sup> compared to *Foxp3*<sup>CreERT2</sup>*Irf4*<sup>WT/WT</sup> mice (Fig. 6a).

We hypothesized that the suboptimal ~50% reduction in mRNA amount and the only slight reduction in IRF4 protein expression in the spleens and lymph nodes of *Foxp3*<sup>CreERT2</sup>*Irf4*<sup>FL/FL</sup> compared to *Foxp3*<sup>CreERT2</sup>*Irf4*<sup>WT/WT</sup> mice might have resulted from a competitive disadvantage of T<sub>reg</sub> cells lacking IRF4 protein, which might lead to preferential expansion of IRF4-sufficient T<sub>reg</sub> cells remaining in *Foxp3*<sup>CreERT2</sup>*Irf4*<sup>FL/FL</sup> mice. This would be consistent with the reported observation that the survival and expansion of strongly antigen-stimulated CD8<sup>+</sup> T cells was greatly impaired in the absence of IRF4<sup>48</sup>. In order to assess whether IRF4 might similarly contribute to the maintenance of T<sub>reg</sub> cells that have been strongly activated, we looked in the colonic lamina propria, in which nearly all T<sub>reg</sub> cells were CD44<sup>hi</sup> and likely had recently experienced TCR engagement based on robust IL-10 production (data not shown). Indeed, we observed decreased percentages of colonic lamina propria T<sub>reg</sub> cells in *Foxp3*<sup>CreERT2</sup>*Irf4*<sup>FL/FL</sup> compared to *Foxp3*<sup>CreERT2</sup>*Irf4*<sup>WT/WT</sup> mice, similar to the decrease in T<sub>reg</sub> cell percentages seen in the colonic lamina propria of *Foxp3*<sup>CreERT2</sup>*Cd4*<sup>FL/FL</sup> compared to *Foxp3*<sup>CreERT2</sup>*Cd4*<sup>WT/WT</sup> mice (Fig. 6b). As opposed to spleen and lymph node T<sub>reg</sub> cells, and consistent with their decreased percentages, colonic lamina propria T<sub>reg</sub> cells in *Foxp3*<sup>CreERT2</sup>*Irf4*<sup>FL/FL</sup> mice exhibited a substantial reduction in IRF4 protein expression compared to T<sub>reg</sub> cells in *Foxp3*<sup>CreERT2</sup>*Irf4*<sup>WT/WT</sup> mice (Fig. 6b). We hypothesized that the reportedly low influx of circulating T<sub>reg</sub> cells into the colonic lamina propria at steady state may have precluded IRF4-sufficient cells from becoming activated and repopulating to wild type percentages the T<sub>reg</sub> cell niche in this tissue<sup>24</sup>.

Despite the only modest decrease in IRF4 expression in T<sub>reg</sub> cells isolated from the spleens and lymph nodes of *Foxp3*<sup>CreERT2</sup>*Irf4*<sup>FL/FL</sup> mice, we were able to detect a very mild, but statistically significant increase in the percentage of CD44<sup>hi</sup> and Ki67<sup>+</sup> lymph node Foxp3<sup>+</sup>CD4<sup>+</sup> T cells as well as increased IFN- $\gamma$ , IL-4 and IL-13 production by splenic Foxp3<sup>+</sup>CD4<sup>+</sup> T cells, suggesting that IRF4 expression downstream of TCR signaling in T<sub>reg</sub>

cells contributes to T<sub>reg</sub> cells suppressive function (Fig. 6c,d). An increase in the production of T<sub>H</sub>2 cytokines was consistent with the phenotype of mice with constitutive ablation of IRF4 in T<sub>reg</sub> cells, whereas the increased IFN- $\gamma$  was likely a consequence of the substantial T<sub>H</sub>1 bias in the C56B/L6 adult mice prior to induced *Irf4* deletion<sup>37</sup>. As a control, we confirmed that in the absence of tamoxifen treatment, CD4<sup>+</sup>Foxp3<sup>-</sup> T cell expression of CD44, Ki67, IFN- $\gamma$ , IL-4 and IL-13 in *Foxp3<sup>CreERT2</sup>Irf4<sup>FL/FL</sup>* and *Foxp3<sup>CreERT2</sup>Irf4<sup>WT/WT</sup>* mice were indistinguishable, as were T<sub>reg</sub> cell percentages in the colonic lamina propria (data not shown), suggesting that the modest differences we observed between *Foxp3<sup>CreERT2</sup>Irf4<sup>FL/FL</sup>* and *Foxp3<sup>CreERT2</sup>Irf4<sup>WT/WT</sup>* mice upon tamoxifen treatment were not a consequence of the *Irf4<sup>FL</sup>* allele itself. Together, these data indicate that even partial loss of IRF4 expression downstream of TCR engagement in T<sub>reg</sub> cells interferes with optimal suppressive function of these cells.

## Discussion

Despite major progress in understanding the molecular mechanisms of TCR engagement-driven differentiation of T<sub>reg</sub> cells, the role of the TCR in T<sub>reg</sub> cell function *in vivo* has remained unclear. Here, we demonstrate that TCR signaling in differentiated T<sub>reg</sub> cells is dispensable for the maintenance of Foxp3 expression and for expression of many T<sub>reg</sub> cell characteristic markers. Although the bulk of the T<sub>reg</sub> cell-specific gene signature was also preserved in the absence of TCR, suppressor function was critically dependent on the TCR.

Given that antigen-activated CD4<sup>+</sup>Foxp3<sup>-</sup> T cells in lymphoid organs are thought to produce IL-2 in a spatially restricted manner, we considered the possibility that T<sub>reg</sub> cells might analogously require their TCRs to correctly position themselves to gain preferential access to IL-2, which might elicit their suppressive function by stimulating IL-2R. However, our *in vivo* and *in vitro* data suggests that T<sub>reg</sub> cells acquire and likely effectively deplete IL-2 in a TCR-independent manner, and thus may instead rely predominantly on CCR7 expression to ensure sufficient IL-2 exposure, as recently proposed<sup>24</sup>.

Our observation that newly generated T<sub>reg</sub> cells in *Foxp3<sup>Cre</sup>Ca<sup>FL/WT</sup>* and *Foxp3<sup>Cre</sup>Ca<sup>FL/FL</sup>* mice remained naïve-like upon loss of TCR and did not populate non-lymphoid tissues suggests that effector differentiation and expansion are TCR- and likely antigen-dependent processes. This finding may help explain the observation that distinct T<sub>reg</sub> cell TCR repertoires are displayed by T<sub>reg</sub> cells populations found in distinct lymphoid organs and tissues in adult mice<sup>49, 50</sup>. Furthermore, the fact that inducible ablation of the TCR resulted in a far more pronounced change in gene expression in the effector-like vs. the naïve-like T<sub>reg</sub> cell subset suggests that continuous TCR signaling may be selectively driving the homeostasis and suppressor function of effector-like T<sub>reg</sub> cells. As inducible deletion of the TCR in differentiated T<sub>reg</sub> cells precipitated autoimmunity, our data may suggest that all—or the bulk—of T<sub>reg</sub> cell suppressor function *in vivo* is mediated by the CD44<sup>hi</sup> effector-like T<sub>reg</sub> cell subset.

We found that partial inducible ablation of *Irf4*, expressed downstream of TCR engagement in CD44<sup>hi</sup> T<sub>reg</sub> cells, resulted in a very mild, but highly reproducible immune activation. Although this result suggests that IRF4 expression is important for TCR-dependent Treg cell

function, given the suboptimal *Irf4* deletion and modest immune activation, to what extent and how TCR-dependent IRF4 induction in T<sub>reg</sub> cells contributes to their ability to suppress spontaneous autoimmunity remains to be determined. IRF4 may predominantly function to control maintenance of highly activated T<sub>reg</sub> cells, which was particularly evident in the colonic lamina propria and which, when altered, may affect the ability of the T<sub>reg</sub> cell pool to suppress. Alternatively, IRF4 may play a more direct role in promoting T<sub>reg</sub> cell suppressive activity, perhaps by driving expression of certain T<sub>reg</sub> cell effector molecules. Although we observed decreased ICOS expression on colonic T<sub>reg</sub> cells lacking IRF4, the remaining IRF4-sufficient T<sub>reg</sub> cells present in lymphoid organs of *Foxp3<sup>CreERT2</sup>Irf4<sup>FL/FL</sup>* mice complicate a more rigorous identification of IRF4 targets (data not shown).

It is important to note, however, that the fact that naïve-like T<sub>reg</sub> cells do not express IRF4 and are overwhelmingly unaffected by TCR deletion on a transcriptional level is not necessarily an indication that these cells are non-functional or that they are not experiencing TCR engagement. Indeed, several genes including *Egr1*, *Egr2* and *Nr4a1* were found to be down-regulated in this T<sub>reg</sub> cell subset in the absence of TCR.

Future experiments will be necessary to elucidate the contributions of other individual T<sub>reg</sub> cell TCR-dependent genes to maintenance of immune tolerance in the steady state, and to the restraint of immune responses directed against commensal microorganisms, food and environmental antigens and pathogens. In this regard, it is noteworthy that while IL-10 production by T<sub>reg</sub> cells has been implicated in the control of inflammatory responses at environmental interfaces such as the gut, lungs and skin, it has also been shown to be dispensable for T<sub>reg</sub> cell control over systemic autoimmunity<sup>26</sup>. Likewise, we found that while constitutive deletion of calcineurin B1 in *Foxp3<sup>Cre</sup>Cnb1<sup>FL/FL</sup>* mice (which eliminated calcineurin-dependent NFAT activation in T<sub>reg</sub> cells) resulted in lethal early onset autoimmunity, highly efficient inducible deletion in adult lymphoreplete *Foxp3<sup>CreERT2</sup>Cnb1<sup>FL/FL</sup>* mice had no detectable adverse consequences on T<sub>reg</sub> cell function (data not shown). Together, these findings suggest that TCR engagement on T<sub>reg</sub> cells may drive a focused transcriptional program, select aspects of which are required in a context dependent manner for mediation of a broad range of T<sub>reg</sub> cell immunosuppressive functions.

## Online Methods

### Mice

Mice were bred and housed in the pathogen-free animal facility at Memorial Sloan-Kettering Cancer Center and were used in accordance with institutional guidelines. Unless otherwise noted, 8–10 week old mice were used for all experiments. *Foxp3<sup>YFP-Cre</sup>*, *Foxp3<sup>eGFP-CreERT2</sup>*, *Rosa26<sup>YFP</sup>*, *Foxp3<sup>DTR</sup>*, and *Irf4<sup>FL</sup>* mice have been previously described<sup>19, 26, 30, 47</sup>. We thank Marc Schmidt-Supprian and Klaus Rajewsky for kindly providing *Ca<sup>FL</sup>* mice. For tamoxifen administration, 40mg tamoxifen were dissolved in 100ul ethanol and subsequently in 900uL olive oil (Sigma-Aldrich) and sonicated 4 × 30 seconds in a Bioruptor Twin (Diagenode). Mice were orally gavaged with 200 µl tamoxifen emulsion per treatment. For diphtheria toxin (DT) injections, DT was dissolved in PBS and 200 µl of indicated doses were injected i.p per mouse. For *in vivo* IL-2 depletion, mice were gavaged with tamoxifen on days 0 and 1 or on days 0, 3, 7 and 10 and injected i.p. on days 4

and 8 with a 0.5mg 1:1 mix of IL-2 neutralizing JES6- 1A12 and S4B6-1 antibodies or IgG2a isotype (BioX-Cell). For administration of IL-2-anti-IL2 complexes, 1ug recombinant mouse IL-2 (R&D Systems) was incubated for 10 minutes at room temperature with 5 µg JES6-1 (BioXCell) and diluted to 200 µl in PBS immediately before i.p injection. Mice were gavaged with taxomofen on days 0, 3, 7 and 10 and received IL-2-anti-IL-2 complexes or PBS on days 5, 7, 9 and 11.

### FACS staining and cell isolation

Cells were stained with LIVE/DEAD Fixable Yellow Dead Cell Stain (Molecular Probes) and antibodies listed in Supplementary Table 1. For BrdU experiments, mice were injected i.p. with 1mg BrdU in 1mL PBS and staining was performed using the BD Pharmingen BrdU Flow Kit. Flow cytometric analysis was performed using an LSRII flow cytometer (BD Bioscience) and FlowJo software (Tree Star). For cell isolation, CD4<sup>+</sup> T cells were purified from pooled spleen and lymph node cell suspensions using magnetic Dynabeads (Invitrogen) and further sorted using an Aria II cell sorter (BD Bioscience). Intracellular staining was performed using eBioscience Fixation Permeabilization buffers. For cytokine staining lymph node and spleen cells were stimulated with soluble anti-CD3 clone 2C11 (5ug/ml) and anti-CD28 clone 37.51 (5ug/ml) in the presence of 1ug/mL brefeldin A for 4–6 hours at 37°C, 5% CO<sup>2</sup>.

### *In vitro* proliferation assay

Dendritic cells (DCs) were expanded *in vivo* by subcutaneous injection of B16 melanoma cells secreting Flt3 ligand into the left hind flank of B6 mice. Once tumors were visible, spleens from injected animals were dissociated in RPMI 1640 medium containing 1.67 units/mL liberase TL (Roche) and 50 µg/mL DNase I (Roche) for 20 min at 37 °C with shaking. EDTA was added at a final concentration of 5 mM to stop digestion and the resulting homogenate was processed for CD11c<sup>+</sup> cell isolation using the MACS mouse CD11c (N418) purification kit (Miltenyi Biotec). FACS purified CD4<sup>+</sup>eGFP<sup>+</sup> cells from tamoxifen treated *Foxp3<sup>CreERT2</sup>Ca<sup>FL/WT</sup>* mice were labeled with CellTrace Violet (Molecular Probes) according to the manufacturer's instructions and plated in triplicate in 96-well flat bottom plates (5 × 10<sup>4</sup> cells/well) in media containing 25 U/mL human recombinant IL-2 (PeproTech) with or without equal numbers of DCs, with or without 100ng/mL LPS (*E. coli* 0111:B4, Sigma-Aldrich.)

### *In vitro* IL-2 stimulation and pSTAT5 detection

*Foxp3<sup>CreERT2</sup>Ca<sup>FL/WT</sup>* mice were treated with tamoxifen on days 0 and 1, and on day 9 CD4<sup>+</sup> T cells were purified from pooled spleen and lymph node cell suspensions using magnetic Dynabeads (Invitrogen). Cells were stained with anti-CD4 and anti-TCRβ, washed, and plated in 96-well V-bottom plates (1 × 10<sup>6</sup> cells/well) in RPMI containing 10% FBS with or without increasing concentrations of IL-2 for 20 minutes at 37°C. Cells were subsequently processed using BD Phosflow Lyse/Fix Buffer and Perm Buffer III (BD Biosciences) and stained with anti-p-STAT5 Y694 antibody according to the manufacturer's instructions. T<sup>reg</sup> cells were identified by eGFP expression. For *ex vivo* p-STAT5 staining,

spleen and lymph nodes were dissociated at 4°C in PBS (0.5% BSA) containing anti-CD4 and anti-TCR $\beta$  antibodies, stained for 10 minutes on ice, and washed twice before fixation.

### ***In vitro* IL-2 depletion**

*Foxp3<sup>CreERT2</sup>Ca<sup>FL/WT</sup>* mice were treated with tamoxifen on days 0 and 1, and on day 9 pooled spleens and lymph nodes were enriched for CD4<sup>+</sup> cells and subsequently sorted to >99% purity into eGFP<sup>+</sup>TCR $\beta$ <sup>+</sup>, eGFP<sup>+</sup>TCR $\beta$ <sup>-</sup>, and eGFP<sup>-</sup>TCR $\beta$ <sup>+</sup> populations. Each population was divided among 8 wells of a V-bottom 96-well plate (250,000 cells/well) in 25 $\mu$ L RPMI(10%) with or without increasing doses of recombinant human IL-2 for 2 hours at 37°C. IL-2 depletion from the media was assessed using the BD Cytometric Bead Array and Human IL-2 Enhanced Sensitivity Flex Set (BD Biosciences) according to the manufacturer's instructions.

### ***In vitro* conjugation assay**

*Foxp3<sup>CreERT2</sup>Ca<sup>WT/WT</sup>*, *Foxp3<sup>CreERT2</sup>Ca<sup>FL/WT</sup>*, and *Foxp3<sup>CreERT2</sup>Ca<sup>FL/FL</sup>* mice were treated with tamoxifen on days 0 and 1 and on day 9, CD4<sup>+</sup> T cells were isolated from spleen and lymph nodes using the Dynabeads Untouched Mouse CD4 Cells negative selection kit (Invitrogen.) TCR<sup>+</sup> T<sup>reg</sup> cells were sorted from *Foxp3<sup>CreERT2</sup>Ca<sup>WT/WT</sup>* mice based on eGFP expression alone. TCR<sup>-</sup> T<sup>reg</sup> cells were sorted from *Foxp3<sup>CreERT2</sup>Ca<sup>FL/WT</sup>* and *Foxp3<sup>CreERT2</sup>Ca<sup>FL/FL</sup>* mice as eGFP<sup>+</sup> TCR $\beta$ <sup>-</sup>. T<sup>reg</sup> cells were subsequently labeled with CFSE and Flt3L-expanded DCs from *Ab* (MHC class II)<sup>+/+</sup> and *Ab* (MHC class II)<sup>-/-</sup> mice were labeled with CellTrace Violet. 10<sup>4</sup> T<sup>reg</sup> cells and 6  $\times$  10<sup>4</sup> DCs were cultured together in a 96-well round bottom plate in RPMI 10% supplemented with 500 U/mL IL-2. Concanavalin A was used at a final concentration of 2.5  $\mu$ g/mL. Following a 10 hr culture at 37°C, cells were gently resuspended before flow cytometric analysis.

### **Gene expression analysis**

eGFP<sup>+</sup> TCR $\beta$ <sup>+</sup> and TCR $\beta$ <sup>-</sup> CD44<sup>hi</sup>CD62L<sup>lo</sup> (two replicates) and CD44<sup>lo</sup>CD62L<sup>hi</sup> (three replicates) cell populations were sorted from tamoxifen-treated *Foxp3<sup>CreERT2</sup>Ca<sup>FL/WT</sup>* mice (five or more mice per replicate) using an Aria II flow cytometer. Complementary DNA (cDNA) libraries were amplified and hybridized to Affymetrix 430 2.0 chips. Arrays were normalized using RMA, and genes were considered differentially expressed if they had a q value <0.01 after Benjamini-Hochberg FDR correction. Differential gene expression in T<sup>reg</sup> cells from *Foxp3<sup>GFPKO</sup>* vs. *Foxp3<sup>+</sup>* mice has been previously described.

### **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

### **References**

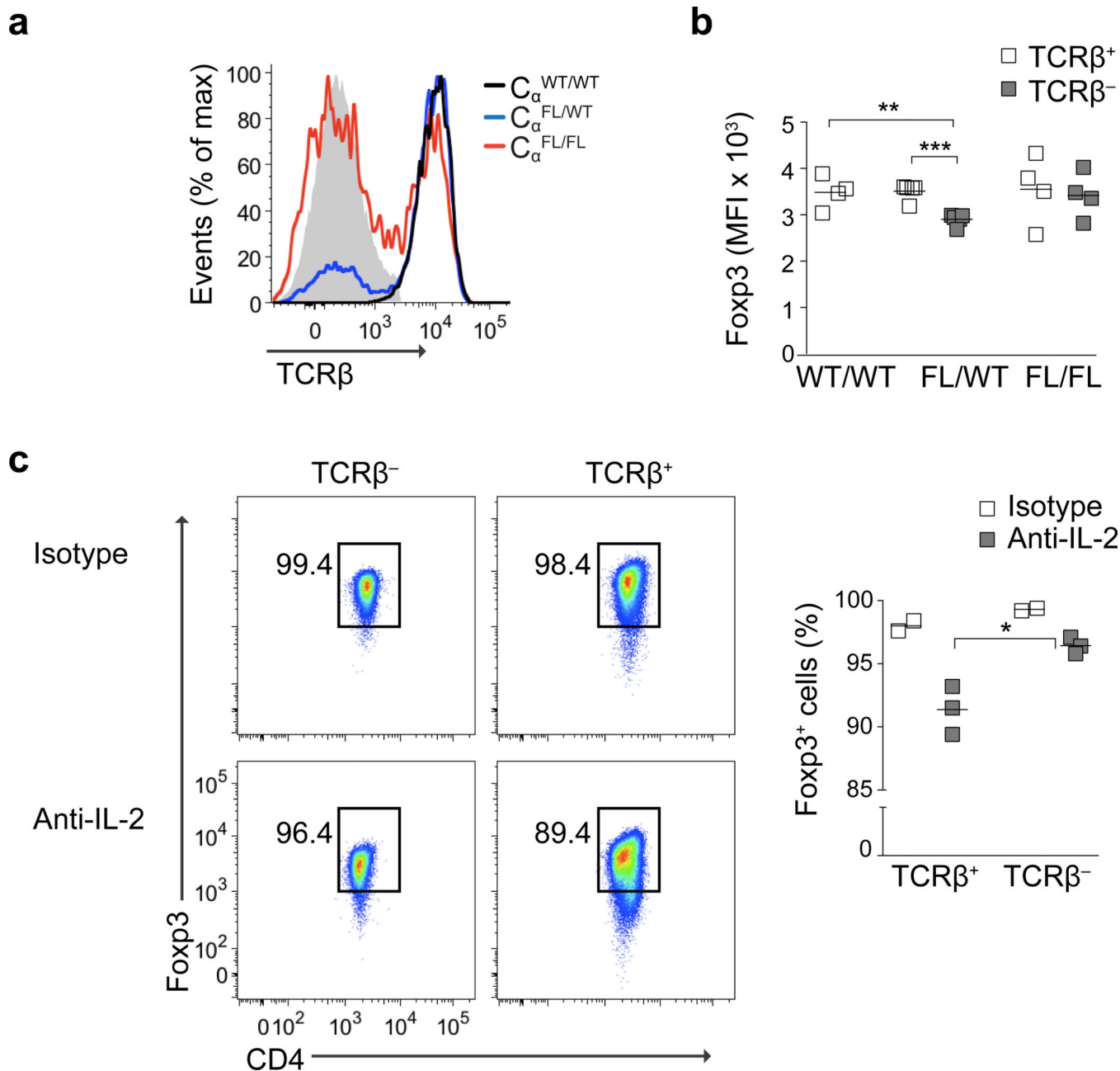
1. Josefowicz SZ, Lu LF, Rudensky AY. Regulatory T cells: mechanisms of differentiation and function. Annual review of immunology. 2012; 30:531–564.
2. Lee HM, Bautista JL, Scott-Browne J, Mohan JF, Hsieh CS. A broad range of self-reactivity drives thymic regulatory T cell selection to limit responses to self. Immunity. 2012; 37:475–486. [PubMed: 22921379]

3. Hsieh CS, Zheng Y, Liang Y, Fontenot JD, Rudensky AY. An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires. *Nature immunology*. 2006; 7:401–410. [PubMed: 16532000]
4. Hsieh CS, et al. Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors. *Immunity*. 2004; 21:267–277. [PubMed: 15308106]
5. Gavin MA, Clarke SR, Negrou E, Gallegos A, Rudensky A. Homeostasis and anergy of CD4(+)CD25(+) suppressor T cells in vivo. *Nature immunology*. 2002; 3:33–41. [PubMed: 11740498]
6. Ouyang W, et al. Novel Foxo1-dependent transcriptional programs control T(reg) cell function. *Nature*. 2012; 491:554–559. [PubMed: 23135404]
7. Marson A, et al. Foxp3 occupancy and regulation of key target genes during T-cell stimulation. *Nature*. 2007; 445:931–935. [PubMed: 17237765]
8. Au-Yeung BB, et al. A genetically selective inhibitor demonstrates a function for the kinase Zap70 in regulatory T cells independent of its catalytic activity. *Nature immunology*. 2010; 11:1085–1092. [PubMed: 21037577]
9. Gavin MA, et al. Foxp3-dependent programme of regulatory T-cell differentiation. *Nature*. 2007; 445:771–775. [PubMed: 17220874]
10. Lin W, et al. Regulatory T cell development in the absence of functional Foxp3. *Nature immunology*. 2007; 8:359–368. [PubMed: 17273171]
11. Williams LM, Rudensky AY. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nature immunology*. 2007; 8:277–284. [PubMed: 17220892]
12. Wing K, et al. CTLA-4 control over Foxp3+ regulatory T cell function. *Science*. 2008; 322:271–275. [PubMed: 18845758]
13. Borsellino G, et al. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood*. 2007; 110:1225–1232. [PubMed: 17449799]
14. Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nature immunology*. 2007; 8:1353–1362. [PubMed: 17982458]
15. Shafiani S, et al. Pathogen-specific Treg cells expand early during mycobacterium tuberculosis infection but are later eliminated in response to Interleukin-12. *Immunity*. 2013; 38:1261–1270. [PubMed: 23791647]
16. Rowe JH, Ertelt JM, Xin L, Way SS. Pregnancy imprints regulatory memory that sustains anergy to fetal antigen. *Nature*. 2012; 490:102–106. [PubMed: 23023128]
17. Samy ET, Parker LA, Sharp CP, Tung KS. Continuous control of autoimmune disease by antigen-dependent polyclonal CD4+CD25+ regulatory T cells in the regional lymph node. *The Journal of experimental medicine*. 2005; 202:771–781. [PubMed: 16172257]
18. Polic B, Kunkel D, Scheffold A, Rajewsky K. How alpha beta T cells deal with induced TCR alpha ablation. *Proceedings of the National Academy of Sciences of the United States of America*. 2001; 98:8744–8749. [PubMed: 11447257]
19. Rubtsov YP, et al. Stability of the regulatory T cell lineage in vivo. *Science*. 2010; 329:1667–1671. [PubMed: 20929851]
20. Hsieh CS, Lee HM, Lio CW. Selection of regulatory T cells in the thymus. *Nature reviews Immunology*. 2012; 12:157–167.
21. Zheng Y, et al. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature*. 2010; 463:808–812. [PubMed: 20072126]
22. Ruan Q, et al. Development of Foxp3(+) regulatory t cells is driven by the c-Rel enhanceosome. *Immunity*. 2009; 31:932–940. [PubMed: 20064450]
23. Wan YY, Flavell RA. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. *Nature*. 2007; 445:766–770. [PubMed: 17220876]
24. Smigielski KS, et al. CCR7 provides localized access to IL-2 and defines homeostatically distinct regulatory T cell subsets. *The Journal of experimental medicine*. 2014; 211:121–136. [PubMed: 24378538]

25. Fisson S, et al. Continuous activation of autoreactive CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells in the steady state. *The Journal of experimental medicine*. 2003; 198:737–746. [PubMed: 12939344]
26. Rubtsov YP, et al. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity*. 2008; 28:546–558. [PubMed: 18387831]
27. Tang Q, et al. Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. *Immunity*. 2008; 28:687–697. [PubMed: 18468463]
28. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nature immunology*. 2005; 6:1142–1151. [PubMed: 16227984]
29. Webster KE, et al. In vivo expansion of T reg cells with IL-2-mAb complexes: induction of resistance to EAE and long-term acceptance of islet allografts without immunosuppression. *The Journal of experimental medicine*. 2009; 206:751–760. [PubMed: 19332874]
30. Kim JM, Rasmussen JP, Rudensky AY. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nature immunology*. 2007; 8:191–197. [PubMed: 17136045]
31. Tian L, et al. Foxp3(+) regulatory T cells exert asymmetric control over murine helper responses by inducing Th2 cell apoptosis. *Blood*. 2011; 118:1845–1853. [PubMed: 21715314]
32. Zou T, et al. Cutting edge: IL-2 signals determine the degree of TCR signaling necessary to support regulatory T cell proliferation in vivo. *J Immunol*. 2012; 189:28–32. [PubMed: 22623329]
33. Thornton AM, Shevach EM. CD4<sup>+</sup>CD25<sup>+</sup> immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *The Journal of experimental medicine*. 1998; 188:287–296. [PubMed: 9670041]
34. Kim JK, et al. Impact of the TCR signal on regulatory T cell homeostasis, function, and trafficking. *PLoS one*. 2009; 4:e6580. [PubMed: 19668367]
35. Ohkura N, et al. T cell receptor stimulation-induced epigenetic changes and Foxp3 expression are independent and complementary events required for Treg cell development. *Immunity*. 2012; 37:785–799. [PubMed: 23123060]
36. Samstein RM, et al. Foxp3 exploits a pre-existent enhancer landscape for regulatory T cell lineage specification. *Cell*. 2012; 151:153–166. [PubMed: 23021222]
37. Zheng Y, et al. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature*. 2009; 458:351–356. [PubMed: 19182775]
38. Cretney E, et al. The transcription factors Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells. *Nature immunology*. 2011; 12:304–311. [PubMed: 21378976]
39. Sawant DV, et al. Bcl6 controls the Th2 inflammatory activity of regulatory T cells by repressing Gata3 function. *J Immunol*. 2012; 189:4759–4769. [PubMed: 23053511]
40. Collison LW, et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature*. 2007; 450:566–569. [PubMed: 18033300]
41. Colotta F, et al. Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4. *Science*. 1993; 261:472–475. [PubMed: 8332913]
42. Huang CT, et al. Role of LAG-3 in regulatory T cells. *Immunity*. 2004; 21:503–513. [PubMed: 15485628]
43. Petermann KB, et al. CD200 is induced by ERK and is a potential therapeutic target in melanoma. *The Journal of clinical investigation*. 2007; 117:3922–3929. [PubMed: 18008004]
44. Reinwald S, et al. CD83 expression in CD4<sup>+</sup> T cells modulates inflammation and autoimmunity. *J Immunol*. 2008; 180:5890–5897. [PubMed: 18424708]
45. Hall AO, et al. The cytokines interleukin 27 and interferon-gamma promote distinct Treg cell populations required to limit infection-induced pathology. *Immunity*. 2012; 37:511–523. [PubMed: 22981537]
46. Hoelzinger DB, et al. Blockade of CCL1 inhibits T regulatory cell suppressive function enhancing tumor immunity without affecting T effector responses. *J Immunol*. 2010; 184:6833–6842. [PubMed: 20483762]
47. Klein U, et al. Transcription factor IRF4 controls plasma cell differentiation and class-switch recombination. *Nature immunology*. 2006; 7:773–782. [PubMed: 16767092]

48. Man K, et al. The transcription factor IRF4 is essential for TCR affinity-mediated metabolic programming and clonal expansion of T cells. *Nature immunology*. 2013; 14:1155–1165. [PubMed: 24056747]
49. Lathrop SK, Santacruz NA, Pham D, Luo J, Hsieh CS. Antigen-specific peripheral shaping of the natural regulatory T cell population. *The Journal of experimental medicine*. 2008; 205:3105–3117. [PubMed: 19064700]
50. Lathrop SK, et al. Peripheral education of the immune system by colonic commensal microbiota. *Nature*. 2011; 478:250–254. [PubMed: 21937990]





**Figure 1. Maintenance of T<sub>reg</sub> cell identity in the absence of the TCR**

(a, b) TCR $\beta$  expression (a) and Fc $\gamma$ 3 median fluorescence intensity (MFI) (b) in CD4 $^+$ Fc $\gamma$ 3 $^+$  lymph node cells from 8–10 week old *Foxp3<sup>CreERT2</sup>C $\alpha$ <sup>WT/WT</sup>* (black line, WT/WT), *Foxp3<sup>CreERT2</sup>C $\alpha$ <sup>FL/WT</sup>* (blue line, FL/WT) and *Foxp3<sup>CreERT2</sup>C $\alpha$ <sup>FL/FL</sup>* (red line, FL/FL) mice gavaged on days 0 and 1 with tamoxifen and analyzed on day 9. The gray histogram in (a) shows TCR $\beta$  staining on CD4 $^-$ TCR $\beta^-$  cells. (c) Percentages of TCR $\beta^+$ Fc $\gamma$ 3 $^+$  and TCR $\beta^-$ Fc $\gamma$ 3 $^+$  T cells among CD4 $^+$ YFP $^+$  cells sorted to >99% purity from the spleens and lymph nodes of *Foxp3<sup>CreERT2</sup>C $\alpha$ <sup>FL/WT</sup>Rosa<sup>YFP</sup>* mice on day 13 following treatment with tamoxifen on days 0 and 1 and i.p. injections of IL-2 neutralizing or control antibody on days 4 and 8. Data are representative of two independent experiments

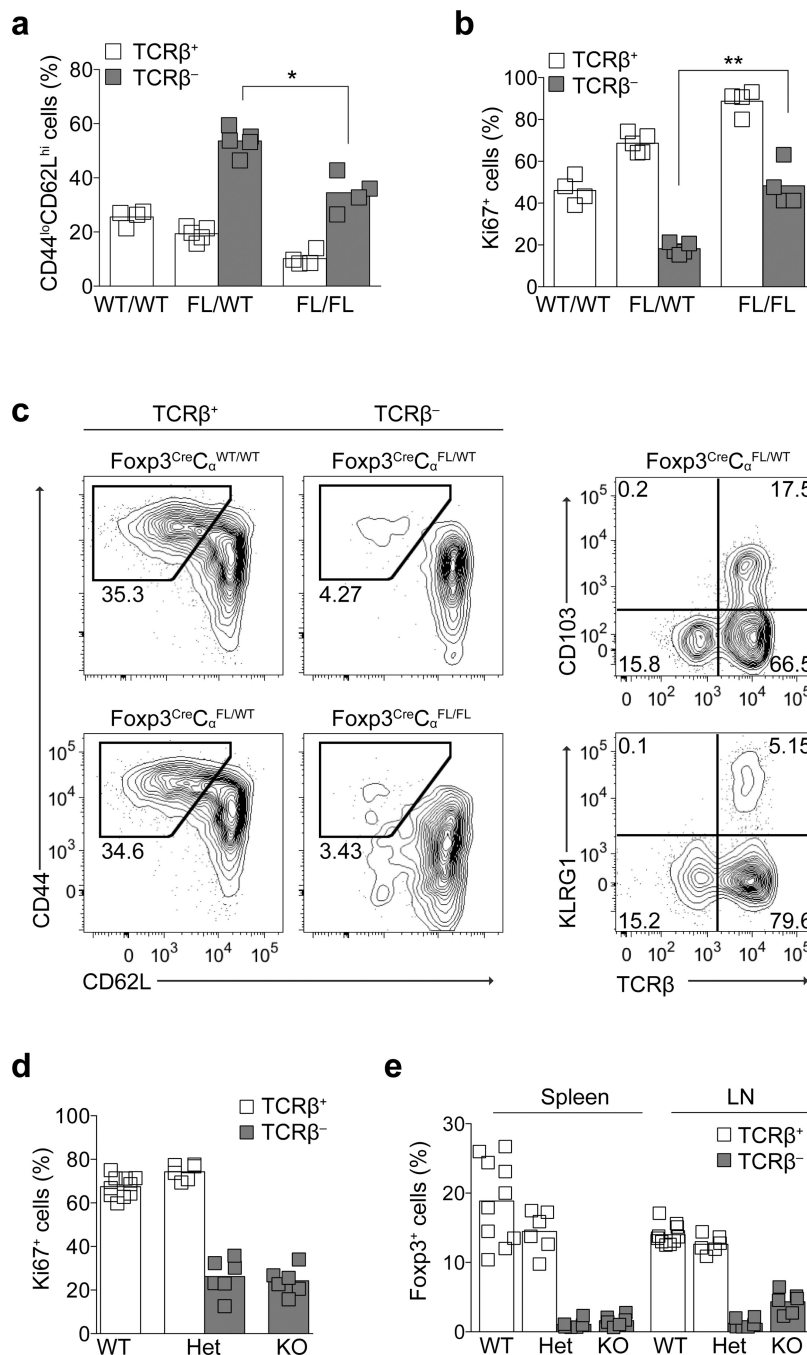
with four or more **(a,b)** or two or more **(c,d)** mice per group in each. \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ . P-values were calculated using an unpaired t-test.

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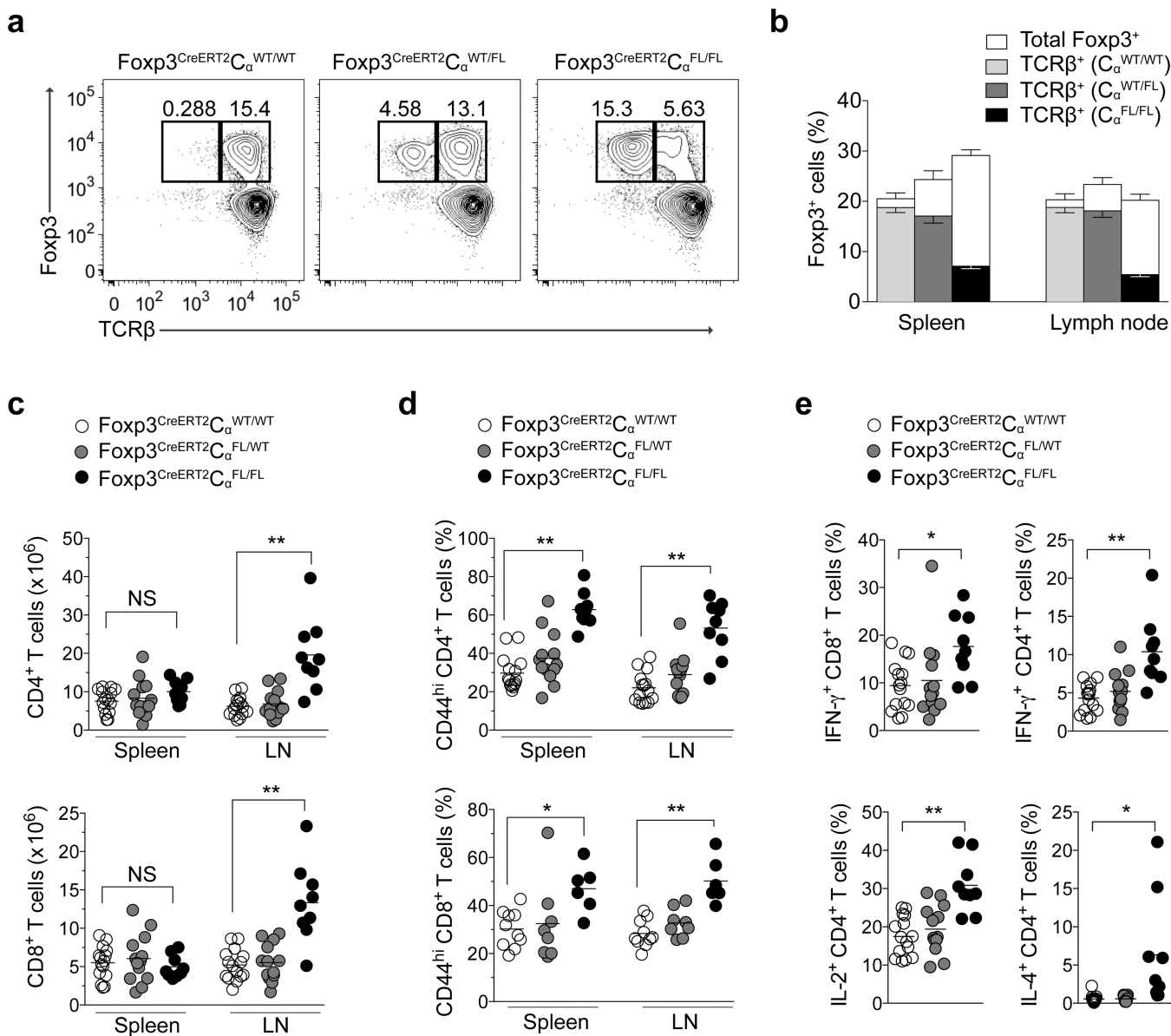
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**Figure 2. Requirement for the TCR in T<sub>reg</sub> cell differentiation and expansion**

(a, b) CD44 and CD62L expression on TCR<sup>+</sup> and TCR<sup>-</sup> CD4<sup>+</sup>Foxp3<sup>+</sup> (a) and Ki67 expression on CD44<sup>hi</sup> CD4<sup>+</sup>Foxp3<sup>+</sup> (b) lymph node cells isolated on day 9 from *Foxp3<sup>CreERT2</sup>C<sub>α</sub><sup>WT/WT</sup>*, *Foxp3<sup>CreERT2</sup>C<sub>α</sub><sup>FL/WT</sup>*, and *Foxp3<sup>CreERT2</sup>C<sub>α</sub><sup>FL/FL</sup>* mice treated with tamoxifen on days 0 and 1. Data in (a) and (b) are representative of two independent experiments with four or more mice per group in each. (c) CD44 and CD62L expression on TCRβ<sup>+</sup> and TCRβ<sup>-</sup> CD4<sup>+</sup>Foxp3<sup>+</sup> lymph node cells in 2.5 week old *Foxp3<sup>Cre</sup>C<sub>α</sub><sup>WT/WT</sup>* (WT/WT), *Foxp3<sup>Cre</sup>C<sub>α</sub><sup>FL/WT</sup>* (FL/WT) and *Foxp3<sup>Cre</sup>C<sub>α</sub><sup>FL/FL</sup>* (FL/FL) mice (left) and

differentiation marker expression on TCR $\beta^+$  and TCR $\beta^-$  CD4 $^+$ Foxp3 $^+$  lymph node cells in 2.5 week old *Foxp3<sup>Cre</sup>Ca<sup>FL/WT</sup>* mice (right). Data is representative of three independent experiments involving a total of six or more mice in each group. **(d,e)** Ki67 expression in lymph node TCR $\beta^+$  and TCR $\beta^-$  CD4 $^+$ Foxp3 $^+$  cells **(d)** and percentages of TCR $\beta^+$  and TCR $\beta^-$  CD4 $^+$ Foxp3 $^+$  cells among total CD4 $^+$  cells **(e)** in 2.5 week old *Foxp3<sup>Cre</sup>Ca<sup>WT/WT</sup>* (WT), *Foxp3<sup>Cre</sup>Ca<sup>FL/WT</sup>* (Het), and *Foxp3<sup>Cre</sup>Ca<sup>FL/FL</sup>* (KO) mice. The data in (d) and (e) represent an aggregate of three independent experiments with a total of six or more mice per group. \*\*, P < 0.001; \*, P < 0.01. P-values were calculated using an unpaired t-test.



**Figure 3. TCR-dependent effector function of mature T<sub>reg</sub> cells in adult mice**

(a,b) TCRβ and Foxp3 expression among lymph node CD4<sup>+</sup> cells (a) and percentages of Foxp3<sup>+</sup> cells among lymph node and splenic CD4<sup>+</sup> cells (b) in *Foxp3*<sup>CreERT2</sup>C<sub>α</sub><sup>WT/WT</sup>, *Foxp3*<sup>CreERT2</sup>C<sub>α</sub><sup>FL/WT</sup> and *Foxp3*<sup>CreERT2</sup>C<sub>α</sub><sup>FL/FL</sup> mice on day 13 following tamoxifen treatment on days 0, 3, 7 and 10. In (b), percentages of TCRβ<sup>+</sup>Foxp3<sup>+</sup> cells among CD4<sup>+</sup> cells in *Foxp3*<sup>CreERT2</sup>C<sub>α</sub><sup>WT/WT</sup> (light gray bars), *Foxp3*<sup>CreERT2</sup>C<sub>α</sub><sup>FL/WT</sup> (dark gray bars) and *Foxp3*<sup>CreERT2</sup>C<sub>α</sub><sup>FL/FL</sup> (black bars) are shown together with percentages of total Foxp3<sup>+</sup> cells among CD4<sup>+</sup> cells (white bars) within each genotype. Error bars in (b) indicate s.e.m. Data in (a) is representative of three experiments with three mice or more per group in each. Data in (b) represent an aggregate of three experiments with a total of nine mice or more mice per group. (c–d) Numbers of (c) and CD44 expression (d) and cytokine production (e) by CD4<sup>+</sup>Foxp3<sup>-</sup> and CD8<sup>+</sup> T cells in *Foxp3*<sup>CreERT2</sup>C<sub>α</sub><sup>WT/WT</sup> (white circles),

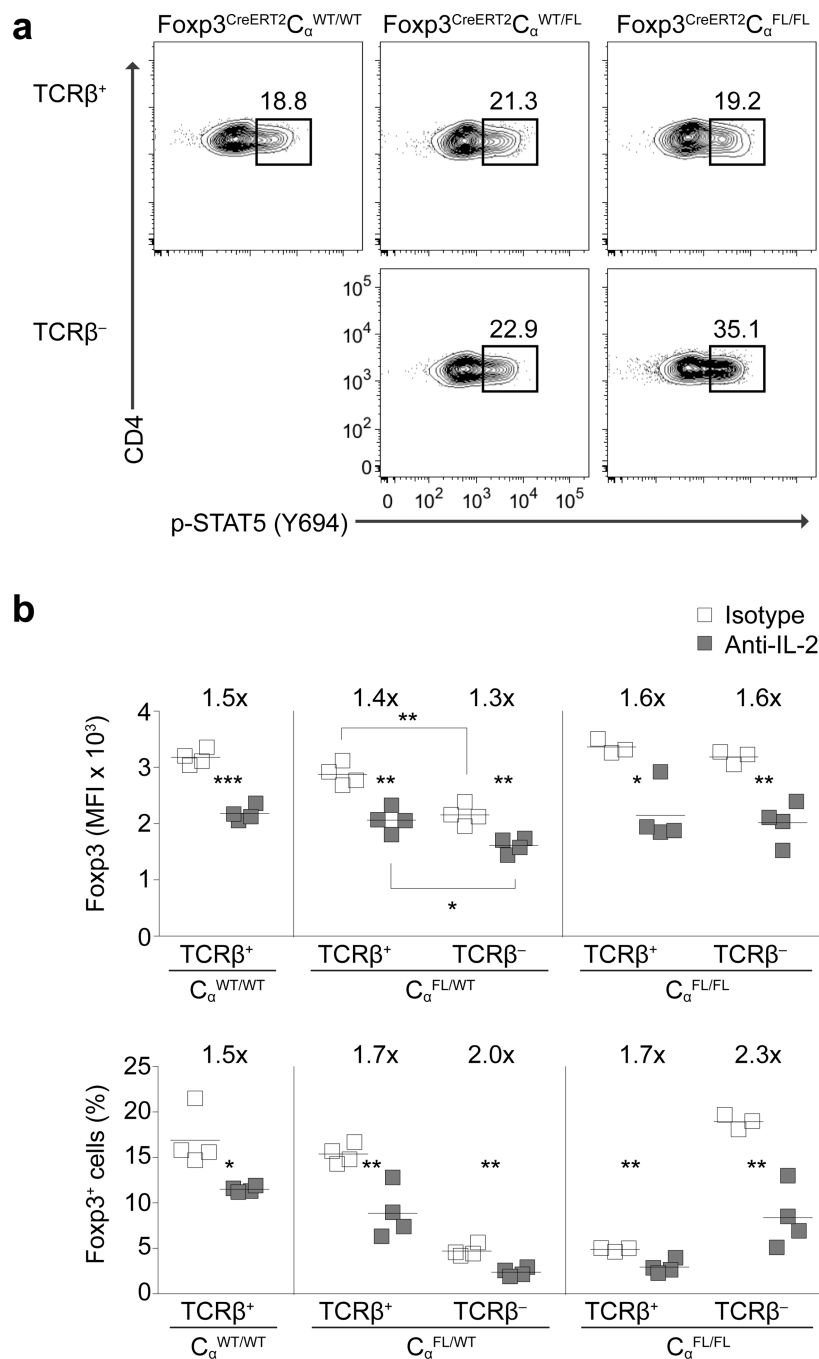
*Foxp3<sup>CreERT2</sup>Ca<sup>FL/WT</sup>* (gray circles) and *Foxp3<sup>CreERT2</sup>Ca<sup>FL/FL</sup>* (black circles) mice on day 13 following tamoxifen treatment on days 0, 3, 7 and 10 in spleens and lymph nodes for **(c,d)** and spleens for **(e)**. Data represent an aggregate of three experiments with nine or more mice per group. \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ . P-values were calculated using an unpaired t-test.

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**Figure 4. T<sub>reg</sub> cell TCR expression is dispensable for IL-2R signaling *in vivo***  
**(a)** p-Stat5 expression in lymph node TCRβ<sup>+</sup> and TCRβ<sup>-</sup> CD4<sup>+</sup>Foxp3<sup>+</sup> cells in *Foxp3<sup>CreERT2</sup>C<sub>α</sub><sup>WT/WT</sup>*, *Foxp3<sup>CreERT2</sup>C<sub>α</sub><sup>FL/WT</sup>* and *Foxp3<sup>CreERT2</sup>C<sub>α</sub><sup>FL/FL</sup>* mice on day 9 following tamoxifen administration on days 0 and 1. Data is representative of three experiments with three or more mice per group in total. **(b)** Foxp3 median fluorescence intensity (MFI) in TCRβ<sup>+</sup> and TCRβ<sup>-</sup> Foxp3<sup>+</sup> cells (upper panel) and percentages of TCRβ<sup>+</sup> and TCRβ<sup>-</sup> Foxp3<sup>+</sup> cells among CD4<sup>+</sup> cells (lower panel) in lymph nodes of *Foxp3<sup>CreERT2</sup>C<sub>α</sub><sup>WT/WT</sup>*, *Foxp3<sup>CreERT2</sup>C<sub>α</sub><sup>FL/WT</sup>* and *Foxp3<sup>CreERT2</sup>C<sub>α</sub><sup>FL/FL</sup>* mice on day 13

following tamoxifen treatment on days 0, 3, 7 and 10 and i.p. injections of IL-2 neutralizing or isotype antibody on days 4 and 8. The data in **(b)** is representative of two experiments with two or more mice per group each. \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ . P-values were calculated using an unpaired t-test.

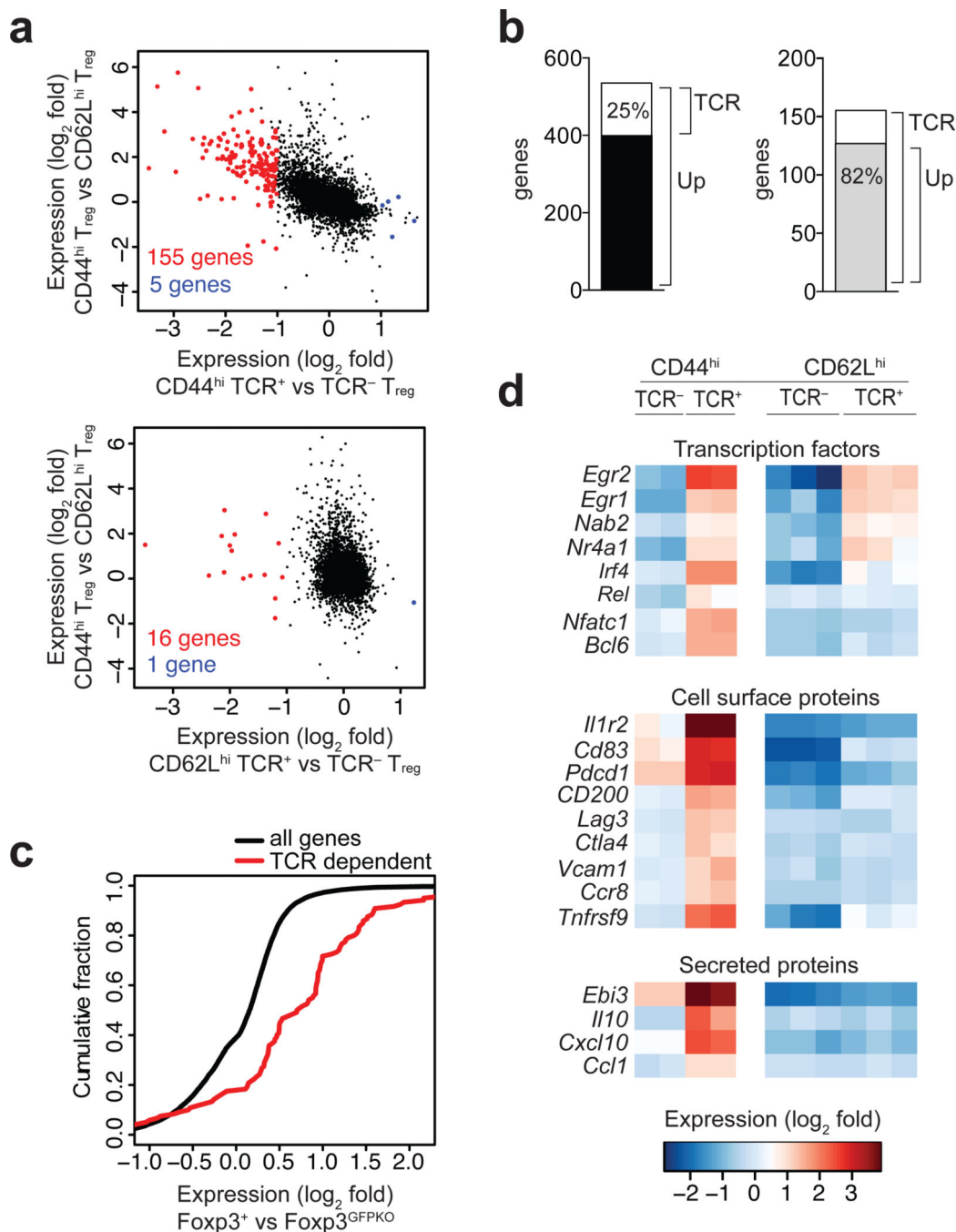
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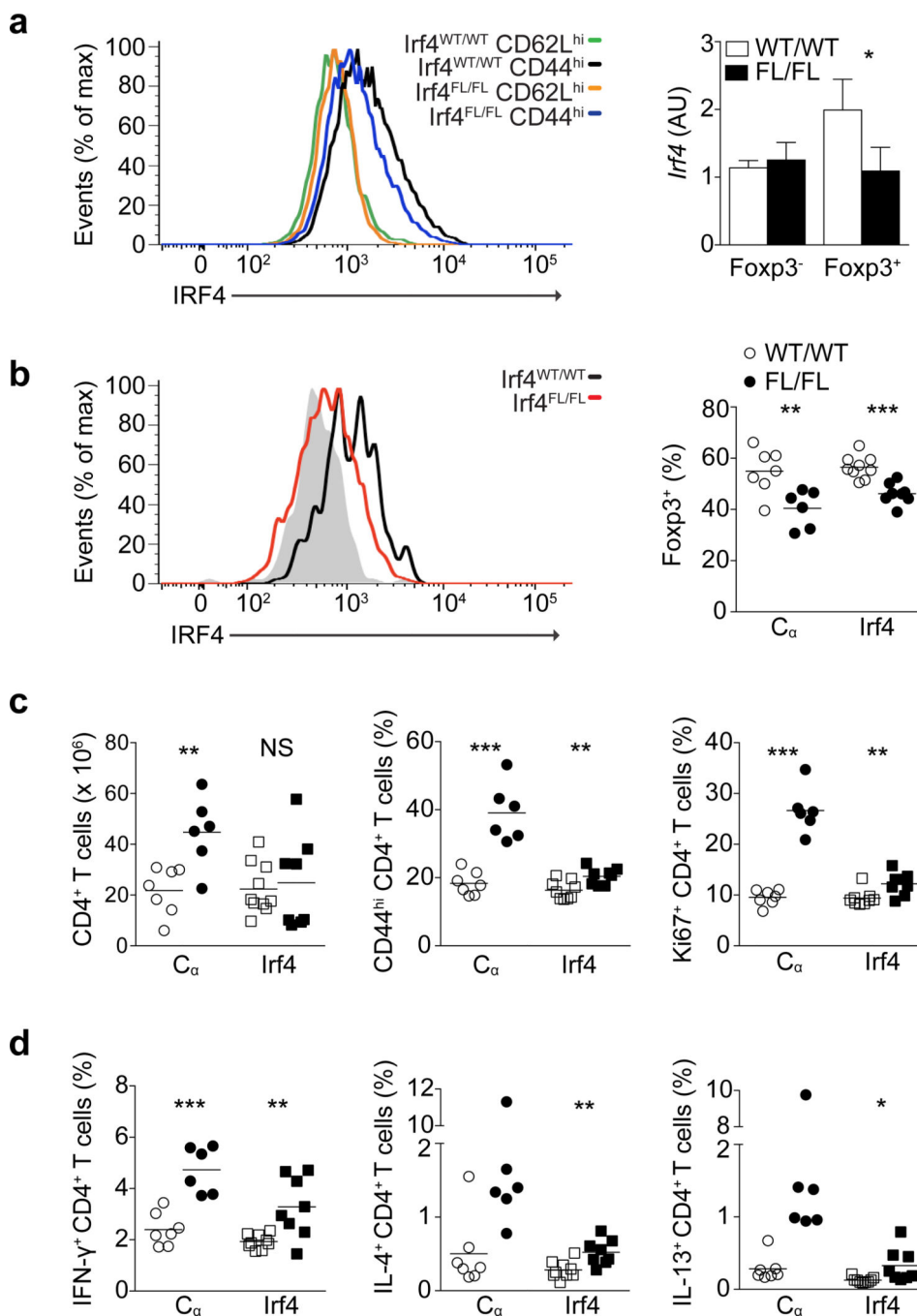




**Figure 5. TCR signaling maintains the effector T<sub>reg</sub> cell transcriptional signature**

(a) Genes differentially expressed in CD44<sup>hi</sup>CD62L<sup>lo</sup> vs. CD44<sup>lo</sup>CD62L<sup>hi</sup> TCR<sup>β</sup><sup>+</sup> T<sub>reg</sub> cells compared to genes differentially expressed in CD44<sup>hi</sup>CD62L<sup>lo</sup> TCR<sup>β</sup><sup>-</sup> vs. CD44<sup>hi</sup>CD62L<sup>lo</sup> TCR<sup>β</sup><sup>+</sup> Treg cells (two replicates, upper panel) or CD44<sup>lo</sup>CD62L<sup>hi</sup> TCR<sup>β</sup><sup>-</sup> vs. CD44<sup>lo</sup>CD62L<sup>hi</sup> TCR<sup>β</sup><sup>+</sup> Treg cells (three replicates, lower panel). The indicated cell subpopulations were sorted on day 14 from *Foxp3<sup>CreERT2</sup>Cd<sup>FL/WT</sup>* mice (five or more mice per replicate) treated with tamoxifen on days 0, 1 and 3 using an Aria II flow cytometer. Gene expression was analyzed using Affymetrix 430 2.0 mouse gene expression arrays.

Genes down- or up-regulated in the absence of TCR are shown in red or blue, respectively. The number of genes significantly differentially expressed by 2-fold or more are indicated,  $q$ -value  $< 0.01$ . **(b)** Genes down-regulated 2-fold or more in  $CD44^{hi}CD62L^{lo} TCR\beta^{-}$  vs.  $TCR\beta^{+} T_{reg}$  cells ('TCR') among genes up-regulated 2-fold or more in  $CD44^{hi}CD62L^{lo}$  vs.  $CD44^{lo}CD62L^{hi} T_{reg}$  cells ('Up') are shown as white portion of the black bar (left) and 'Up' genes among 'TCR' genes are shown as the gray portion of the white bar (right). **(c)** Cumulative distribution function plot of TCR-dependent genes vs. all genes differentially expressed in  $Foxp3^{GFPKO}$  vs.  $Foxp3^{+} CD4^{+}$  T cells.  $P < 10^{-20}$ , two-sample Kolmogorov-Smirnov test. **(d)** Treg cell TCR-dependent genes encoding transcription factors, cell surface, and secreted molecules.



**Figure 6. IRF4 expression contributes to optimal T<sub>reg</sub> cell suppressive capacity and homeostasis** (a) IRF4 expression in lymph node CD44<sup>hi</sup>CD62L<sup>lo</sup> ('CD44<sup>hi</sup>') and CD44<sup>lo</sup>CD62L<sup>hi</sup> ('CD62L<sup>hi</sup>') CD4<sup>+</sup>Foxp3<sup>+</sup> cells in *Foxp3<sup>CreERT2</sup>Irf4<sup>WT/WT</sup>* and *Foxp3<sup>CreERT2</sup>Irf4<sup>FL/FL</sup>* mice (left) and qPCR analysis of IRF4 mRNA transcript amounts in CD4<sup>+</sup>eGFP<sup>-</sup> and CD4<sup>+</sup>eGFP<sup>+</sup> cells sorted from pooled spleens and lymph nodes (right). qPCR is representative of two experiments with four or more mice per group each (b) IRF4 expression in colonic lamina propria CD4<sup>+</sup>Foxp3<sup>+</sup> cells in *Foxp3<sup>CreERT2</sup>Irf4<sup>WT/WT</sup>* and *Foxp3<sup>CreERT2</sup>Irf4<sup>FL/FL</sup>* mice (left); gray histogram represents CD4<sup>+</sup>Foxp3<sup>-</sup> cells. Percent

Foxp3<sup>+</sup> among CD4<sup>+</sup> cells in the large intestine lamina propria in *Foxp3<sup>CreERT2</sup>Ca<sup>WT/WT</sup>* and *Foxp3<sup>CreERT2</sup>Irf4<sup>WT/WT</sup>* (white circles) and *Foxp3<sup>CreERT2</sup>Ca<sup>FL/FL</sup>* and *Foxp3<sup>CreERT2</sup>Irf4<sup>FL/FL</sup>* (black circles) mice, as indicated (right). **(c,d)** Numbers, percent CD44<sup>hi</sup>, percent Ki67<sup>+</sup> **(c)** and percent cytokine-producing cells **(d)** of CD4<sup>+</sup>Foxp3<sup>-</sup> T cells in lymph nodes **(c)** and spleens **(d)** of *Foxp3<sup>CreERT2</sup>Ca<sup>WT/WT</sup>* (white circles), *Foxp3<sup>CreERT2</sup>Irf4<sup>WT/WT</sup>* (white squares), *Foxp3<sup>CreERT2</sup>Ca<sup>FL/FL</sup>* (black circles), and *Foxp3<sup>CreERT2</sup>Irf4<sup>FL/FL</sup>* (black squares) mice. All mice were analyzed on day 13 following tamoxifen treatment on days 0, 3, 7 and 10. Histograms **(a,b)** are representative of two experiments with four or more mice per group each. All other data in **(b-d)** is combined from two experiments with four or more mice per group each. \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05. P-values were calculated using an unpaired t-test.