nature immunology

Continuous requirement for the TCR in regulatory T cell function

Andrew G Levine[1,2], Aaron Arvey[1,2,4], Wei Jin[1,2,4] & Alexander Y Rudensky[1–3]

Foxp3$^+$ regulatory T cells (T_{reg} cells) maintain immunological tolerance, and their deficiency results in fatal multiorgan autoimmunity. Although heightened signaling via the T cell antigen receptor (TCR) is critical for the differentiation of T_{reg} cells, the role of TCR signaling in T_{reg} cell function remains largely unknown. Here we demonstrated that inducible ablation of the TCR resulted in T_{reg} cell dysfunction that could not be attributed to impaired expression of the transcription factor Foxp3, decreased expression of T_{reg} cell signature genes or altered ability to sense and consume interleukin 2 (IL-2). Instead, TCR signaling was required for maintaining the expression of a limited subset of genes comprising 25% of the activated T_{reg} cell transcriptional signature. Our results reveal a critical role for the TCR in the suppressor capacity of T_{reg} cells.

Regulatory CD4$^+$ T cells that express the transcription factor Foxp3 have an essential role in maintaining immune tolerance[1]. In the thymus, increased affinity for engagement of the T cell antigen receptor (TCR) by immature CD4$^+$ single-positive thymocytes is required for the initiation of a program for the differentiation of regulatory T cells (T_{reg} cells) and induction of Foxp3 expression[2]. As a consequence, T_{reg} cells exported to the periphery exhibit a TCR repertoire skewed toward self-recognition[3,4]. However, it remains unclear whether TCR signaling is needed to mediate the suppressive function of T_{reg} cells in the periphery.

T_{reg} cells exhibit impaired calcium flux, activation of the kinase Akt and phosphorylation of the kinase Erk upon TCR stimulation relative to that of conventional CD4$^+$ T cells, and Foxp3 is known to potently repress at least some TCR-induced genes, as well as some genes encoding molecules involved in the TCR signaling pathway[5–8]. At the same time, Foxp3$^+$ T_{reg} cells have high basal expression of several cell surface molecules that are known to contribute to T_{reg} cell function (such as CD25, CD39 and CTLA-4), and the expression of these molecules in conventional CD4$^+$ T cells is dependent upon TCR stimulation[9–14]. It is not known whether high-affinity interactions of the TCR with complexes of self peptide and major histocompatibility complex (MHC) class II contribute to constitutive expression of these genes and, consequently, to T_{reg} cell function.

T_{reg} cells, despite their intrinsically dampened response to TCR stimulation, acquire an activated phenotype and expand their populations in response to their cognate antigens in settings of activation of the immune system, such as infection and autoimmunity[15,16]. These observations indicate that recognition of self antigen helps maintain T_{reg} cells of particular specificities and may potentiate their suppressive ability during immunological challenge[17]. Nevertheless, strict reliance on TCR expression for T_{reg} cell activation, rather than 'preferential' activation of antigen-specific T_{reg} cells, has not been demonstrated, nor has engagement of the TCR *in vivo* been shown to be required for T_{reg} cell function in any context.

We used inducible genetic ablation of cell-surface TCR complexes to directly address the requirement for TCR expression in the immunosuppressive ability of T_{reg} cells. Notably, the TCR was largely dispensable for Foxp3 expression, for lineage stability and for high expression of many signature genes in T_{reg} cells. Nevertheless, these features were not sufficient to preserve T_{reg} cell function or to prevent activation of the immune system. Loss of suppressor capacity in the absence of the TCR was not due to an impaired ability of T_{reg} cells 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 the activation of T_{reg} cells and for T_{reg} cells to maintain expression of a limited set of genes found to be expressed almost exclusively in activated T_{reg} cells. Among those genes, expression of the transcription factor IRF4 contributed to the optimal function and homeostasis of T_{reg} cells. Our results demonstrate an essential role for the TCR in eliciting the suppressor function of differentiated T_{reg} cells.

RESULTS

Maintenance of T_{reg} cell identity in the absence of the TCR

To investigate the role of TCR signaling in T_{reg} cell function, we crossed *Trac*^{FL} mice (which have a *lox*P-flanked allele encoding the TCR α-chain constant region (C_α or TCRα)) with *Foxp3*$^{eGFP\text{-}Cre\text{-}ERT2}$ mice (with expression of enhanced green fluorescent protein (eGFP) fused to a Cre recombinase–estrogen-receptor-ligand-binding-domain protein from the 3′ untranslated region of *Foxp3*; called '*Foxp3*$^{Cre\text{-}ERT2}$' here) to achieve tamoxifen-inducible deletion of *Trac* specifically in T_{reg} cells[18,19]. In this model, Cre-induced loss of the conditional

[1]Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, New York, USA. [2]Immunology Program, Memorial Sloan-Kettering Cancer Center, New York, New York, USA. [3]Ludwig Center, Memorial Sloan-Kettering Cancer Center, New York, New York, USA. [4]Present addresses: Gilead Sciences, Foster City, California, USA (A.A.) and Tsinghua University School of Medicine, Beijing, China (W.J.). Correspondence should be addressed to A.Y.R. (rudenska@mskcc.org).

Received 4 August; accepted 5 September; published online 28 September 2014; doi:10.1038/ni.3004

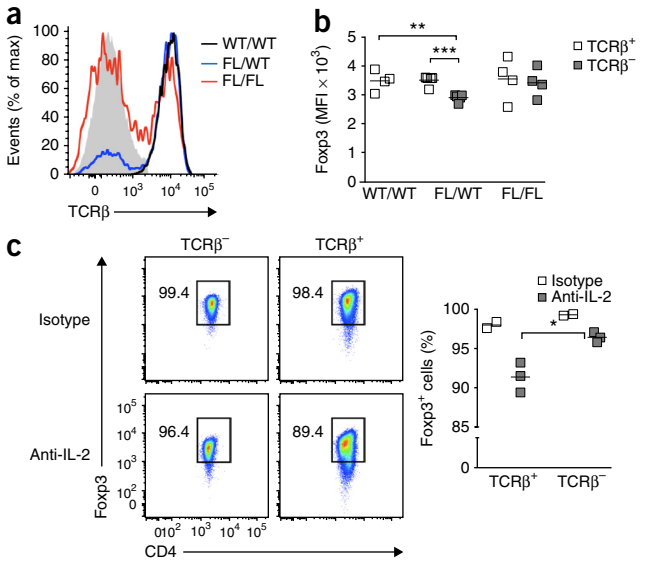

Figure 1 Maintenance of T_{reg} cell identity in the absence of the TCR. (**a**,**b**) Expression of TCRβ (**a**) and median fluorescence intensity (MFI) of Foxp3 (**b**) in CD4$^+$Foxp3$^+$ lymph node cells from 8- to 10-week-old *Trac*$^{WT/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ mice (WT/WT), *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ mice (FL/WT) and *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice (FL/FL) treated with tamoxifen by gavage on days 0 and 1 and analyzed on day 9. Gray shading (**a**), TCRβ staining on CD4$^-$TCRβ$^-$ cells. (**c**) Flow cytometry of Foxp3$^+$ T cells among CD4$^+$YFP$^+$ cells sorted to >99% purity from the spleens and lymph nodes of *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$*Rosa26*^{YFP} mice on day 13 following treatment with tamoxifen on days 0 and 1 and intraperitoneal injection of IL-2-neutralizing antibody (Anti-IL-2) or isotype-matched control antibody (Isotype) on days 4 and 8. Numbers adjacent to outlined areas (left) indicate percent Foxp3$^+$ cells among TCRβ$^+$ cells (right) or TCRβ$^-$ cells (left). Each symbol (**b**,**c** (right)) represents an individual mouse; small horizontal lines indicate the mean. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (two-tailed unpaired *t*-test). Data are representative of two independent experiments with four or more (**a**,**b**) or two or more (**c**) mice per group in each.

Trac allele upon tamoxifen administration eliminates TCRα expression, which prevents formation of heterodimers of TCRα and TCRβ (TCRαβ) at the cell surface. We administered tamoxifen via oral gavage on days 0 and 1 and analyzed mice on day 9. Allelic exclusion at the *Trac* locus in heterozygous *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ mice yielded a small population (25%) of TCR-deficient (as assessed by flow cytometry (TCRβ$^-$; called 'TCR$^-$' here)) T_{reg} cells (~25%), whereas in homozygous *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice, the majority of T_{reg} cells (~60–70%) lacked cell surface TCRβ (**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), functional *in vitro* analyses confirmed loss of TCR crosslinking–dependent activation of TCR$^-$ T_{reg} cells (**Supplementary Fig. 1a–d**).

Because binding sites for the transcription factors NFAT and c-Rel have been identified in the *Foxp3* locus, and because TCR engagement–driven signaling via the transcription factor NF-κB is critical for induction of Foxp3 expression, we speculated that the TCR might be essential for maintaining Foxp3 expression[20–22]. However, Foxp3 expression was reduced only marginally in TCR$^-$ T_{reg} cells in *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ mice and was not reduced at all in TCR$^-$ T_{reg} cells in *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice, relative to its expression in TCR-expressing (as assessed by flow cytometry (TCRβ$^+$; called 'TCR$^+$' here)) T_{reg} cells in the same mice and in *Trac*$^{WT/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ mice (**Fig. 1b**). Similarly, the expression of genes encoding several T_{reg} cell signature molecules, including CD25, GITR, CD39 and CD73, was largely unaffected in TCR$^-$ T_{reg} cells from both *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ and *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice (**Supplementary Fig. 1e**). These results indicated that in the steady state, continuous TCR-mediated recognition of self did not contribute substantially to Foxp3-dependent maintenance of expression of these genes[11,23]. In contrast, CTLA-4 expression was notably diminished in TCR$^-$ T_{reg} cells in *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ mice, but not in TCR$^-$ T_{reg} cells in *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice, relative to its expression in TCR$^+$ T_{reg} cells in *Trac*$^{WT/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ mice (**Supplementary Fig. 1e**).

The frequency and absolute number of Foxp3$^+$ cells in the spleens and lymph nodes of *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ and *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice were unaltered compared with that in *Trac*$^{WT/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ mice (**Supplementary Fig. 1f**). However, to address the possibility that a portion of T_{reg} cells completely lost Foxp3 expression upon ablation of the TCR and that these 'former' T_{reg} cells were not accounted for in this experimental setup, we crossed *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ mice with mice that express the recombination reporter *Rosa26*^{YFP} (with sequence encoding yellow fluorescent protein (YFP) expressed form the ubiquitous *Rosa26* locus). We assessed the expression of TCRβ and Foxp3 in CD4$^+$YFP$^+$ cells sorted from the spleens and lymph nodes of *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$*Rosa26*^{YFP} mice on day 9 or 50 following tamoxifen administration on two consecutive days. YFP-expressing CD4$^+$TCRβ$^-$ and CD4$^+$TCRβ$^+$ cell subsets contained similarly low frequencies of Foxp3$^-$ cells at both time points (data not shown). Furthermore, following *in vivo* neutralization of IL-2, a condition known to promote loss of Foxp3 expression[19], in *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$*Rosa26*^{YFP} mice, populations of CD4$^+$YFP$^+$ TCRβ$^-$ cells retained a higher percentage of Foxp3$^+$ cells than did populations of CD4$^+$YFP$^+$TCRβ$^+$ cells (**Fig. 1c**). Together these data indicated that TCR signaling was dispensable for the maintenance of the T_{reg} cell phenotype and lineage stability and, moreover, that TCR signaling drove the loss of Foxp3 when IL-2 amounts were limiting.

Requirement for the TCR in effector differentiation of T_{reg} cells

Although the T_{reg} cell phenotype was largely preserved upon ablation of the TCR, we observed relative enrichment for naive-like CD44^{lo}CD62L^{hi} cells among TCR$^-$ T_{reg} cells in lymph nodes and spleens of *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ and *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice (**Fig. 2a** and **Supplementary Fig. 1g**). T_{reg} cell proliferation is restricted almost exclusively to the CD44^{hi} subset, and in part the enrichment we observed appeared to be a consequence of severely impaired proliferative capacity of CD44^{hi} T_{reg} cells in the absence of the TCR[24] (**Fig. 2b**). The small population of TCR$^-$ T_{reg} cells in *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ mice was predominantly nondividing: these cells showed minimal expression of the proliferation marker Ki67, failed to incorporate the thymidine analog BrdU over a 24-hour labeling period and contained the greatest frequency of CD44^{lo}CD62L^{hi} cells among all TCR$^+$ or TCR$^-$ T_{reg} cell populations in *Trac*$^{WT/WT}$*Foxp3*$^{Cre\text{-}ERT2}$, *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ and *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice (**Fig. 2a**,**b** and **Supplementary Fig. 1h**). In *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice, however, TCR$^-$ T_{reg} cells exhibited considerable proliferative activity, albeit reduced relative to that of TCR$^+$ T_{reg} cells present in the same mouse (**Fig. 2b** and **Supplementary Fig. 1h**). Increased Ki67 staining in CD44^{hi} T_{reg} cells was inversely correlated with a lower frequency of CD44^{lo}CD62L^{hi} cells among all TCR$^+$ or TCR$^-$ T_{reg} cell populations in mice of all three genotypes (**Fig. 2a**,**b**).

To address the possibility that continuous peripheral differentiation of naive-like T_{reg} cells into CD44^{hi} cells was impeded in the absence of TCR expression and that such a differentiation block contributed to the

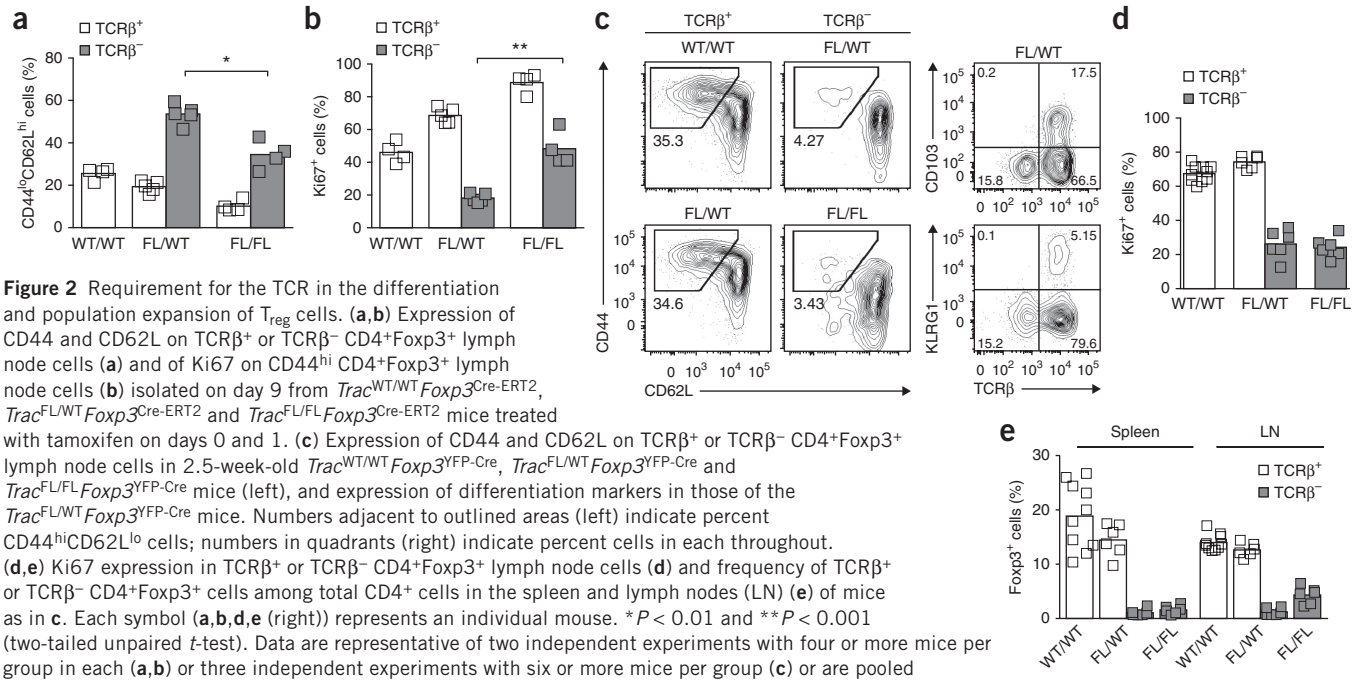

Figure 2 Requirement for the TCR in the differentiation and population expansion of T_{reg} cells. (**a**,**b**) Expression of CD44 and CD62L on TCRβ$^+$ or TCRβ$^-$ CD4$^+$Foxp3$^+$ lymph node cells (**a**) and of Ki67 on CD44^{hi} CD4$^+$Foxp3$^+$ lymph node cells (**b**) isolated on day 9 from *Trac*$^{WT/WT}$*Foxp3*$^{Cre\text{-}ERT2}$, *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ and *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice treated with tamoxifen on days 0 and 1. (**c**) Expression of CD44 and CD62L on TCRβ$^+$ or TCRβ$^-$ CD4$^+$Foxp3$^+$ lymph node cells in 2.5-week-old *Trac*$^{WT/WT}$*Foxp3*$^{YFP\text{-}Cre}$, *Trac*$^{FL/WT}$*Foxp3*$^{YFP\text{-}Cre}$ and *Trac*$^{FL/FL}$*Foxp3*$^{YFP\text{-}Cre}$ mice (left), and expression of differentiation markers in those of the *Trac*$^{FL/WT}$*Foxp3*$^{YFP\text{-}Cre}$ mice. Numbers adjacent to outlined areas (left) indicate percent CD44^{hi}CD62L^{lo} cells; numbers in quadrants (right) indicate percent cells in each throughout. (**d**,**e**) Ki67 expression in TCRβ$^+$ or TCRβ$^-$ CD4$^+$Foxp3$^+$ lymph node cells (**d**) and frequency of TCRβ$^+$ or TCRβ$^-$ CD4$^+$Foxp3$^+$ cells among total CD4$^+$ cells in the spleen and lymph nodes (LN) (**e**) of mice as in **c**. Each symbol (**a**,**b**,**d**,**e** (right)) represents an individual mouse. $^*P < 0.01$ and $^{**}P < 0.001$ (two-tailed unpaired *t*-test). Data are representative of two independent experiments with four or more mice per group in each (**a**,**b**) or three independent experiments with six or more mice per group (**c**) or are pooled from three independent experiments with six or more mice per group (**d**,**e**).

predominantly CD44^{lo}CD62L^{hi} phenotype of TCR$^-$ T_{reg} cells, we bred *Trac*^{FL} mice with *Foxp3*$^{YFP\text{-}Cre}$ mice (which express a fusion of Cre and YFP from the 3′ untranslated region of *Foxp3*) to induce ablation of the TCR in newly generated, 'naive' T_{reg} cells[25,26]. We reasoned that if the TCR were critical for the effector differentiation of T_{reg} cells in the periphery, TCR$^-$ T_{reg} cells in these mice would retain a CD44^{lo}CD62L^{hi} naive-like phenotype.

Immature HSA^{hi}CD4$^+$Foxp3$^+$ cells in the thymi of *Trac*$^{FL/FL}$ *Foxp3*$^{YFP\text{-}Cre}$ mice had cell surface expression of TCR complexes similar to that in their wild-type counterparts; more mature HSA^{lo}CD4$^+$Foxp3$^+$ thymocytes showed only slightly reduced TCR expression (**Supplementary Fig. 2a**). Among Foxp3$^+$ cells present in the spleens and lymph nodes of *Trac*$^{FL/WT}$*Foxp3*$^{YFP\text{-}Cre}$ mice, ~5–15% were TCRβ$^-$, whereas ~80% of Foxp3$^+$ cells in *Trac*$^{FL/FL}$*Foxp3*$^{YFP\text{-}Cre}$ mice lacked surface TCRβ expression (**Fig. 2e** and **Supplementary Fig. 2b**). We again observed a slight decrease in the amount of Foxp3 protein in the TCR$^-$ T_{reg} cells in *Trac*$^{FL/WT}$*Foxp3*$^{YFP\text{-}Cre}$ mice, but not in *Trac*$^{FL/FL}$*Foxp3*$^{YFP\text{-}Cre}$ mice, while expression of T_{reg} cell signature genes was variably affected in the TCR$^-$ T_{reg} cell populations in both mouse strains relative to expression in TCR$^+$ T_{reg} cells in *Trac*$^{WT/WT}$ *Foxp3*$^{YFP\text{-}Cre}$, *Trac*$^{FL/WT}$*Foxp3*$^{YFP\text{-}Cre}$ and *Trac*$^{FL/FL}$*Foxp3*$^{YFP\text{-}Cre}$ mice (**Supplementary Fig. 2c**,**d**).

Despite their generally intact T_{reg} cell surface phenotype, nearly all TCR$^-$ T_{reg} cells in healthy *Trac*$^{FL/WT}$*Foxp3*$^{YFP\text{-}Cre}$ mice had a naive-like CD62L^{hi}CD44^{lo} phenotype and lacked expression of all T_{reg} cell differentiation markers tested, including KLRG1, CD103 and CXCR3 (**Fig. 2c** and data not shown). Notably, we also observed this pattern under severe inflammatory conditions in *Trac*$^{FL/FL}$*Foxp3*$^{YFP\text{-}Cre}$ mice, which were moribund by 3 weeks of age (**Fig. 2c** and **Supplementary Fig. 2e**,**f**). The lack of CD44^{hi} cells among TCR$^-$ populations in *Trac*$^{FL/WT}$*Foxp3*$^{YFP\text{-}Cre}$ and *Trac*$^{FL/FL}$*Foxp3*$^{YFP\text{-}Cre}$ mice correlated with the decreased proliferation and markedly diminished frequency and number of TCR$^-$ T_{reg} cells relative to that of TCR$^+$ T_{reg} cells in the same mice and in *Trac*$^{WT/WT}$*Foxp3*$^{YFP\text{-}Cre}$ mice; this pattern was evident in lymph nodes and was particularly pronounced in the spleen and other tissues such as the liver and lungs (**Fig. 2d**,**e** and **Supplementary Fig. 2g**,**h**). Together these data were consistent with an absolute requirement for TCR expression, the loss of which could not be compensated for even in conditions of extreme activation of the immune system, for the peripheral effector differentiation of naive-like T_{reg} cells and acquisition of an activated CD44^{hi} phenotype.

TCR-dependent effector function of mature T_{reg} cells

Foxp3 expression and T_{reg} cell population expansion are facilitated by signaling via the receptor for IL-2 (IL-2R)[27–29]. The increase in Foxp3 protein expression and proliferative activity of TCR$^-$ T_{reg} cells in *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice compared with that in such cells in *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ mice led us to suspect that ablation of TCR expression, even on mature T_{reg} cells, might precipitate activation of the immune system and elevate the production of IL-2 and other cytokines by activated CD4$^+$ T cells. Indeed, analysis of *Trac*$^{FL/FL}$ *Foxp3*$^{Cre\text{-}ERT2}$ mice treated twice with tamoxifen and analyzed on day 9 after treatment revealed increased percentages of CD44^{hi} T cells and increased numbers of IL-2–producing CD4$^+$ T cells compared with that of tamoxifen-treated *Trac*$^{WT/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ and *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ mice (data not shown).

To more rigorously investigate the role of TCR expression in mature T_{reg} cell function, we administered four doses of tamoxifen to mice (on days 0, 3, 7 and 10) to maximize Cre-ERT2–mediated recombination. On day 13, we noted loss of TCR expression in ~75–80% of T_{reg} cells in *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice and ~25–30% of T_{reg} cells in *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ mice (**Fig. 3a**,**b**). Despite the normal or even increased frequency of total Foxp3$^+$ cells in the lymph nodes and spleens of *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice, we found elevated numbers of CD4$^+$Foxp3$^-$ and CD8$^+$ T cells in the lymph nodes of these mice and a higher frequency of CD44^{hi}CD4$^+$ and CD8$^+$ T cells in their lymph nodes and spleens (**Fig. 3c**,**d**). CD8$^+$ T cells from *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice produced more interferon-γ (IFN-γ), and CD4$^+$ T cells from these mice produced more IFN-γ, IL-2, IL-4, IL-13, IL-5 and IL-17, than did T cells from *Trac*$^{WT/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ and *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ mice (**Fig. 3e** and data not shown).

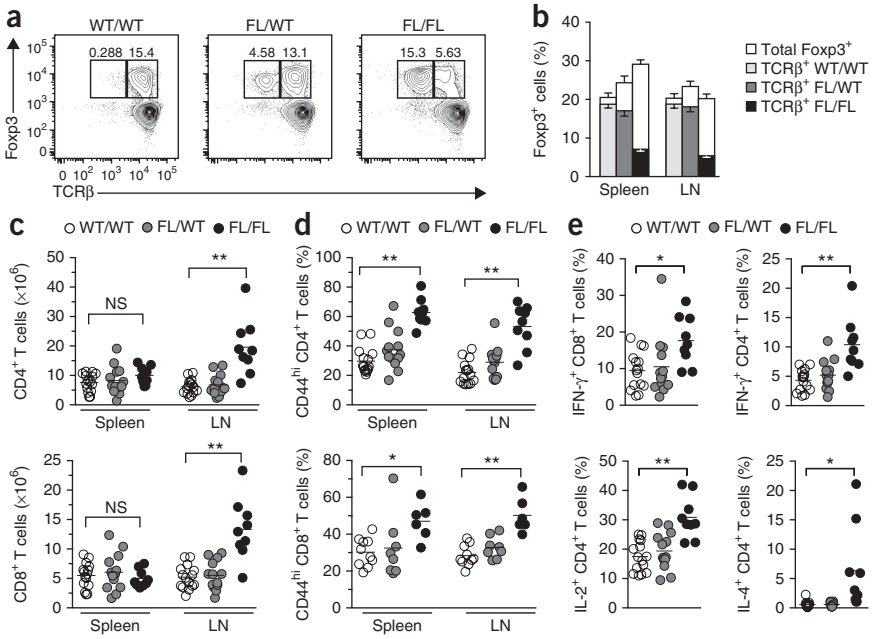

Figure 3 TCR-dependent effector function of mature T_{reg} cells in adult mice. (**a**) Expression of Foxp3 and TCRβ by CD4$^+$ cells in the lymph nodes of *Trac*$^{WT/WT}$*Foxp3*$^{Cre\text{-}ERT2}$, *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ and *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice on day 13 following tamoxifen treatment on days 0, 3, 7 and 10. Numbers adjacent to outlined areas indicate percent Foxp3$^+$TCRβ$^+$ cells (right) or Foxp3$^+$TCRβ$^-$ cells (left). (**b**) Frequency of TCRβ$^+$ Foxp3$^+$ cells among splenic and lymph node CD4$^+$ cells in *Trac*$^{WT/WT}$*Foxp3*$^{Cre\text{-}ERT2}$, *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ and *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice, and of total Foxp3$^+$ cells among CD4$^+$ cells for each genotype (Total Foxp3$^+$). (**c**–**e**) Number (**c**), CD44 expression (**d**) and cytokine production (**e**) of CD4$^+$ Foxp3$^-$ or CD8$^+$ T cells in the spleen (**c**–**e**) and lymph nodes (**c**,**d**) of mice as in **a**. Each symbol (**c**–**e**) represents an individual mouse; small horizontal lines indicate the mean. NS, not significant ($P \geq 0.05$); *$P < 0.005$ and **$P \leq 0.0001$ (two-tailed unpaired *t*-test). Data are representative of three experiments with three mice or more per group in each (**a**) or are pooled from three experiments with nine or more per group (**b**–**e**; error bars (**b**), mean ± s.e.m.).

The activation of the immune system in *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice was milder than that resulting from complete depletion of T_{reg} cells in *Foxp3*^{DTR} mice, which express the human diphtheria toxin receptor (DTR) concomitantly with *Foxp3* (ref. 30). Thus, it was possible that the large number of TCR$^-$ T_{reg} cells in *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice retained measurable TCR-independent suppressor ability and were still capable of immunoregulation. Alternatively, the small population of remaining TCR-sufficient T_{reg} cells in these mice might have limited, to some degree, the activation of effector T cells and the associated autoimmunity. To examine these possibilities, we attempted to reduce the proportion of T_{reg} cells among CD4$^+$ cells in *Foxp3*^{DTR} mice to approximate the frequency of residual TCR-sufficient T_{reg} cells in *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice following four doses of tamoxifen[31]. We reasoned that if TCR$^-$ T_{reg} cells were capable of substantial suppression, autoimmunity in *Foxp3*^{DTR} mice subjected to only partial depletion of T_{reg} cells would be more severe than that in *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice. Injection of diphtheria toxin depletes mice of T_{reg} cells within 24 h, whereas tamoxifen-induced, Cre-ERT2–mediated recombination progressively increases over a 4-day period (data not shown). Therefore, we treated *Foxp3*^{DTR}, *Trac*$^{WT/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ and *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice with diphtheria toxin 4 d after their first dose of tamoxifen to account for the time needed for complete Cre-ERT2–mediated deletion of *Trac* (we administered both taximofen and diphtheria toxin to all genotypes; **Supplementary Fig. 3a**,**b**). Partial depletion of the T_{reg} cell compartment in *Foxp3*^{DTR} mice resulted in activation and cytokine production of CD4$^+$Foxp3$^-$ T cells grossly similar to that observed in *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice with populations of TCR-sufficient T_{reg} cells of a similar or even larger size (**Supplementary Fig. 3b**–**d**). Together these results demonstrated that T_{reg} cells required continuous TCR expression for the effective elaboration of their suppressor function, and suggested that TCR$^-$ T_{reg} cells, which were abundant in *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice, were largely devoid of detectable suppressor ability.

TCR$^-$ T_{reg} cell dysfunction is not secondary to impaired IL-2R signaling

We considered that the apparent loss of suppressive ability of T_{reg} cells in the absence of the TCR might be an indirect consequence of impaired ability to localize in a TCR- and antigen-dependent manner to sites of CD4$^+$ T cell activation and to thereby acquire IL-2, a cytokine known to be critical for the function and homeostasis of T_{reg} cells. This would explain the decreased expression of Foxp3 and minimal proliferation of TCR$^-$ T_{reg} cells in healthy *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ mice, in which IL-2 amounts were not elevated and would not be able to partially remedy these defects[32].

However, direct *ex vivo* analysis of phosphorylation of the transcription factor STAT5, which occurs downstream of IL-2 signaling in T_{reg} cells, showed that in the spleen and lymph nodes of both *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ and *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice, the proportion of phosphorylated STAT5 in TCR$^-$ T_{reg} cells was at least equivalent to that in TCR$^+$ T_{reg} cells (**Fig. 4a** and data not shown). In *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice, TCR$^-$ T_{reg} cells had more phosphorylated STAT5 than did TCR$^+$ T_{reg} cells; this mirrored their expression of CD25 and CD62L, which remained high on TCR$^-$ cells but was decreased on the residual activated TCR$^+$ T_{reg} cells present in these mice (**Figs. 2a** and **4a** and **Supplementary Fig. 1e**,**g**). These results were consistent with the observation that T_{reg} cells that contain phosphorylated STAT5 are found mainly in the CD62L^{hi}CD44^{lo} (and CD25^{hi}) subset of T_{reg} cells; in contrast to the activated CD44^{hi}CD62L^{lo} (and CD25^{int}) T_{reg} cell subset, this group of cells has been reported to rely on IL-2R signaling rather than engagement of costimulatory receptors for their maintenance[24].

In vitro analysis confirmed that lack of TCR expression did not substantially influence the phosphorylation of STAT5 in response to IL-2, nor did it impair the ability of T_{reg} cells to capture and deplete IL-2 from culture media (**Supplementary Fig. 4a**,**b**), which suggested that in the absence of TCR expression, T_{reg} cell–mediated deprivation of IL-2 might not be an important mechanism of immunosuppression. Furthermore, treatment of *Trac*$^{WT/WT}$*Foxp3*$^{Cre\text{-}ERT2}$, *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ and *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice with neutralizing antibody to IL-2 (anti-IL-2) or isotype-matched control antibody had a similar effect on TCR$^+$ and TCR$^-$ T_{reg} cells, reducing Foxp3 expression and lowering the frequency of Foxp3$^+$ cells among total CD4$^+$ cells (**Fig. 4b**). Together these data suggested that TCR$^-$ T_{reg} cells efficiently captured IL-2 during activation of the immune system and at steady state. It remains to be determined what signal(s) drove

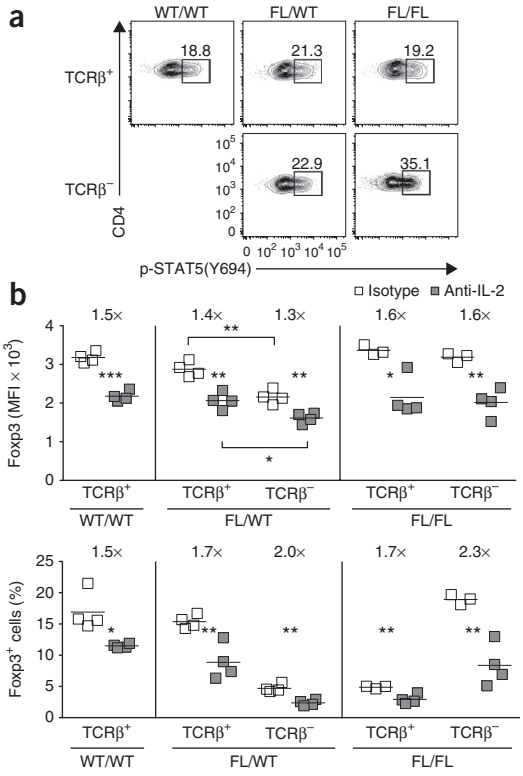

Figure 4 TCR expression by T_{reg} cells is dispensable for IL-2R signaling *in vivo*. (**a**) Phosphorylation of STAT5 at Tyr694 (p-STAT5(Y694)) in TCRβ$^+$ or TCRβ$^-$ CD4$^+$Foxp3$^+$ lymph node cells from *Trac*$^{WT/WT}$ *Foxp3*$^{Cre\text{-}ERT2}$, *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ and *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice on day 9 following tamoxifen administration on days 0 and 1. Numbers adjacent to outlined areas indicate percent CD4$^+$ Foxp3$^+$ cells with phosphorylated STAT5. (**b**) Median fluorescence intensity of Foxp3 in TCRβ$^+$ or TCRβ$^-$ Foxp3$^+$ cells (top) and frequency of TCRβ$^+$ or TCRβ$^-$ Foxp3$^+$ cells among CD4$^+$ cells (bottom) in the lymph nodes of *Trac*$^{WT/WT}$ *Foxp3*$^{Cre\text{-}ERT2}$, *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ and *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice on day 13 following tamoxifen treatment on days 0, 3, 7 and 10 and intraperitoneal injection of IL-2-neutralizing or isotype-matched contol antibody on days 4 and 8. Numbers above plots indicate comparison of results obtained for mice treated with isotype-matched control antibody relative to those for mice treated with anti-IL-2. Each symbol (**b**) represents an individual mouse; small horizontal lines indicate the mean. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ (two-tailed unpaired *t*-test). Data are representative of three experiments involving a total of three or more mice per group (**a**) or of two experiments with two or more mice per group in each (**b**).

the proliferation of TCR$^-$ T_{reg} cells selectively in diseased *Trac*$^{FL/FL}$ *Foxp3*$^{Cre\text{-}ERT2}$ mice. However, we observed increased expression of the costimulatory molecules CD80 and CD86 on lymph node dendritic cells (DCs) in *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice, and activated DCs were able to induce limited proliferation of TCR$^-$ T_{reg} cells *in vitro* (**Supplementary Fig. 4c**,**d**).

Finally, the administration of complexes of IL-2 and anti-IL-2 to *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice did not measurably diminish the activation or lymphoproliferation of effector T cells caused by loss of TCR expression in T_{reg} cells (**Supplementary Fig. 5**). Conversely, IL-2 depletion did not further exacerbate autoimmunity (data not shown). Notably, this was the case despite a 1.5-fold population expansion of TCR$^-$ T_{reg} cells, but not of TCR$^+$ T_{reg} cells, following IL-2 administration (probably a consequence of higher CD25 expression and heightened IL-2 responsiveness in TCR$^-$ T_{reg} cells) and a reduction of over twofold in TCR$^-$ T_{reg} cells following depletion of IL-2 (**Fig. 4b** and **Supplementary Fig. 5b**). These observations further confirmed that TCR$^-$ T_{reg} cells, even when present in elevated numbers, had minimal suppressive ability. Together these results indicated that neither TCR-dependent interactions with antigen-presenting cells nor continuous TCR-mediated localization within lymphoid organs were required for T_{reg} cells to acquire IL-2, and that T_{reg} cell dysfunction in the absence of the TCR could not be attributed to altered IL-2R signaling.

TCR expression promotes T_{reg} cell adhesive properties *in vitro*

The *in vitro* suppressive ability of T_{reg} cells requires engagement of the TCR[33,34]. This might involve pathways independent of the catalytic activity of the signaling kinase Zap70, which is essential for the effector function of conventional T cells, but dependent on membrane-proximal inside-out activation of integrins and subsequent enhancement of the interaction of T_{reg} cells with antigen-presenting cells[8]. To address this possibility, we cultured TCR$^+$ or TCR$^-$ T_{reg} cells isolated from *Trac*$^{WT/WT}$*Foxp3*$^{Cre\text{-}ERT2}$, *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ or *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice with DCs and assessed the formation of DC–T_{reg} cell conjugates. We did not detect any difference in conjugate formation between DCs and TCR$^+$ or TCR$^-$ T_{reg} cells following 30 min of incubation (data not shown). However, following overnight culture, TCR$^-$ T_{reg} cells isolated from *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ or *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice were less efficient than were TCR$^+$ T_{reg} cells at forming conjugates with DCs (3.7% of TCR$^-$ T_{reg} cells compared with 7.5% of TCR$^+$ T_{reg} cells; **Supplementary Fig. 6a**). Conjugate formation was unaffected by the presence or absence of MHC class II molecules on DCs (**Supplementary Fig. 6a**), which might indicate that the greater adhesion of TCR$^+$ T_{reg} cells than of TCR$^-$ T_{reg} cells in this assay was not a result of interactions between TCR and MHC class II and might have been a consequence of the overall heightened activation status of TCR$^+$ T_{reg} cells relative to that of TCR$^-$ T_{reg} cells. As expression of the integrin LFA-1 was higher on CD44^{hi} T_{reg} cells than on CD44^{lo}CD62L^{hi} T_{reg} cells (**Supplementary Fig. 6b**), it is possible that greater conjugate formation by TCR$^+$ T_{reg} cells, which show greater enrichment for CD44^{hi} cells than do TCR$^-$ T_{reg} cells, was due at least in part to increased expression of this integrin. Further work is needed to determine precisely how engagement of the TCR *in vivo* affects signaling pathways to modulate the adhesive properties of T_{reg} cells. However, our results indicated that TCR expression contributed to optimal contact-dependent interactions between T_{reg} cells and antigen-presenting cells, which might support TCR-dependent immunosuppressive function.

TCR modulation of the effector T_{reg} cell transcriptional signature

To explore whether TCR signals, apart from influencing T_{reg} cell adhesion, might drive transcriptional events to 'license' suppressor function *in vivo*, we analyzed the gene expression of TCR$^+$ and TCR$^-$ T_{reg} cells. Flow cytometry showed that loss of TCR expression had a stronger effect on effector-like CD44^{hi}CD62L^{lo} T_{reg} cells than on naive-like CD44^{lo}CD62L^{hi} T_{reg} cells (data not shown). This prompted us to investigate the gene-expression profiles of these two populations separately in the TCR$^+$ or TCR$^-$ T_{reg} cell populations isolated from healthy *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ mice (to avoid confounding effects of activation of the immune system). We found that 155 genes were downregulated by at least twofold and only five genes were upregulated by that amount in effector-like CD44^{hi}CD62L^{lo}TCR$^-$ T_{reg} cells relative to their expression in CD44^{hi}CD62L^{lo}TCR$^+$ T_{reg} cells (**Fig. 5a**). 16 genes were downregulated in the naive-like CD44^{lo}CD62L^{hi}TCR$^-$ T_{reg} cells relative to their expression in CD44^{lo}CD62L^{hi}TCR$^+$ T_{reg} cells

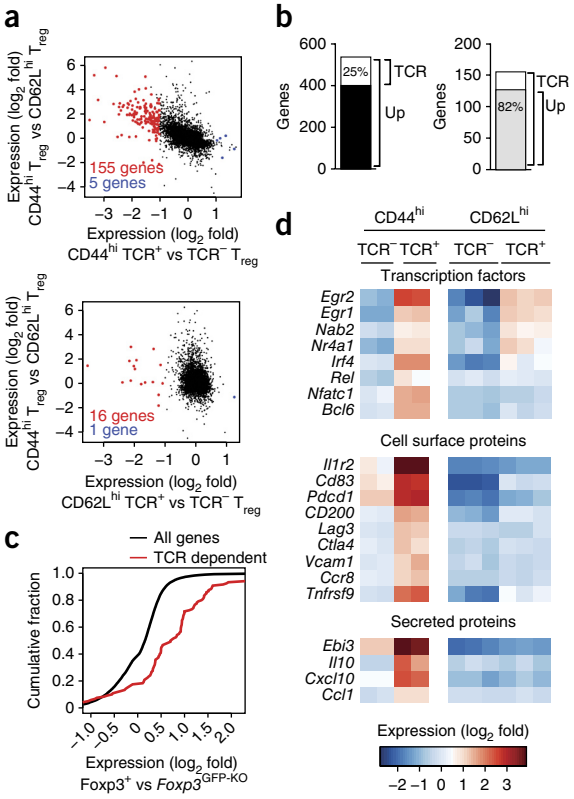

Figure 5 TCR signaling maintains the effector T_{reg} cell transcriptional signature. (**a**) Genes expressed differently in $CD44^{hi}CD62L^{lo}$ versus $CD44^{lo}CD62L^{hi}$ $TCR\beta^+$ T_{reg} cells plotted against those expressed differently in $CD44^{hi}CD62L^{lo}$ $TCR\beta^-$ versus $CD44^{hi}CD62L^{lo}$ $TCR\beta^+$ T_{reg} cells (top) or $CD44^{lo}CD62L^{hi}$ $TCR\beta^-$ versus $CD44^{lo}CD62L^{hi}$ $TCR\beta^+$ T_{reg} cells (bottom) among subpopulations sorted by flow cytometry on day 14 from $Trac^{FL/WT}Foxp3^{Cre\text{-}ERT2}$ mice treated with tamoxifen on days 0, 1 and 3; numbers in plots indicate genes upregulated (blue) or downregulated (red) by twofold or more in the absence of the TCR ($q < 0.01$). (**b**) Genes downregulated twofold or more in $CD44^{hi}CD62L^{lo}$ $TCR\beta^-$ T_{reg} cells relative to their expression in $CD44^{hi}CD62L^{lo}$ $TCR\beta^+$ T_{reg} cells (TCR) among those upregulated twofold or more in $CD44^{hi}CD62L^{lo}$ T_{reg} cells relative to their expression in $CD44^{lo}CD62L^{hi}$ T_{reg} cells (Up) (left), and genes upregulated twofold or more in $CD44^{hi}CD62L^{lo}$ T_{reg} cells relative to their expression in $CD44^{lo}CD62L^{hi}$ T_{reg} cells (Up) among those downregulated twofold or more in $CD44^{hi}CD62L^{lo}$ $TCR\beta^-$ T_{reg} cells relative to their expression in $CD44^{hi}CD62L^{lo}$ $TCR\beta^+$ T_{reg} cells (TCR) (right). (**c**) Cumulative distribution function plot of TCR-dependent genes plotted against all genes expressed differently in $Foxp3^{GFP\text{-}KO}$ T cells versus $Foxp3^+$ $CD4^+$ T cells. $P < 10^{-20}$ (two-sample Kolmogorov-Smirnov test). (**d**) Expression patterns of TCR-dependent genes encoding transcription factors, cell surface molecules and secreted molecules in T_{reg} cells. Data are representative of one experiment with two or more replicates with five or more mice per replicate.

(all of these were also downregulated in $CD44^{hi}CD62L^{lo}TCR^-$ vs. $CD44^{hi}CD62L^{lo}TCR^+$ T_{reg} cells), whereas one gene was upregulated (**Fig. 5a**). Some 535 genes showed higher expression (twofold or greater) in effector-like $CD44^{hi}CD62L^{lo}TCR^+$ T_{reg} cells than in naive-like $CD44^{lo}CD62L^{hi}TCR^+$ T_{reg} cells, and the expression of 136 of these (25%) was TCR dependent (**Fig. 5b**). Notably, 127 of the 155 genes (82%) downregulated in the absence of the TCR in $CD44^{hi}CD62L^{lo}$ T_{reg} cells showed at least twofold higher expression in effector-like T_{reg} cells than in naive-like T_{reg} cells (**Fig. 5b**).

Foxp3 has been proposed to solidify and amplify a transcriptional program initiated in T_{reg} cell precursors by engagement of the TCR[9,10,35,36]. We compared the expression of the 155 genes identified above as being maintained by the TCR in T_{reg} cells (called 'TCR-dependent genes' here) to the expression of Foxp3-dependent genes, identified as being upregulated in wild-type T_{reg} cells relative to their expression in eGFP$^+$ T cells from $Foxp3^{GFP\text{-}KO}$ mice, which express eGFP from a *Foxp3*-null allele[36]. We found that substantially more of the TCR-dependent genes than all genes were also Foxp3 dependent (**Fig. 5c**). These observations suggested that the TCR-driven transcriptional program in T_{reg} cells was enhanced by Foxp3 expression, but that Foxp3 alone was not sufficient to maintain the full transcriptional signature of effector T_{reg} cells.

Examination of the TCR-dependent genes identified several transcription factors that were upregulated in $TCR^+CD44^{hi}CD62L^{lo}$ T_{reg} cells relative to their expression in $TCR^-CD44^{hi}CD62L^{lo}$, $TCR^+CD44^{lo}CD62L^{hi}$ and $TCR^-CD44^{lo}CD62L^{hi}$ T_{reg} cells, including NFATc1, c-Rel, Bcl-6 and IRF4 (**Fig. 5d**); published data have shown that Bcl-6 and IRF4 are important for the differentiation and function of effector T_{reg} cells[37–39]. Of the 155 TCR-dependent genes, we identified only one adhesion molecule–encoding gene (*Vcam1*) whose expression was upregulated in $CD44^{hi}CD62L^{lo}$ T_{reg} cells relative to its expression in $CD44^{lo}CD62L^{hi}$ T_{reg} cells in a manner that depended on TCR expression (**Fig. 5d**). Genes encoding several other potential effector molecules were also upregulated in $CD44^{hi}CD62L^{lo}$ T_{reg} cells relative to their expression in $CD44^{lo}CD62L^{hi}$ T_{reg} cells, in a TCR-dependent manner, including those encoding IL-1R2 (a decoy receptor for IL-1); the immunoinhibitory molecules CD83, CD200 and LAG-3; IL-10; and EBI3 (a subunit of the cytokines IL-27 and IL-35)[26,40–45]. In addition, the chemokine-encoding genes *Cxcl10* and *Ccl1*, as well as *Ccr8* (which encodes the receptor for CCL1), were significantly downregulated in TCR^- T_{reg} cells relative to their expression in TCR^+ T_{reg} cells, which suggested that T_{reg} cells might signal each other through the expression of chemokines and their corresponding receptors or might recruit into close proximity the targets of their suppressive activity[46]. Together these data indicated that under physiological conditions, a substantial portion of the effector T_{reg} cell transcriptional program, but not the naive-like T_{reg} cell transcriptional program, characterized by elevated expression of several potential T_{reg} cell effector molecules, was maintained by continuous TCR signaling.

IRF4 expression promotes T_{reg} cell function and homeostasis

To begin to assess the importance of the TCR-dependent transcriptional program for continuous T_{reg} cell function *in vivo*, we focused on IRF4 as a downstream target of the TCR signaling pathway in T_{reg} cells. We confirmed that elevated IRF4 expression in T_{reg} cells was restricted to $CD44^{hi}$ cells and was reduced to basal expression upon ablation of the TCR in both $Trac^{FL/WT}Foxp3^{Cre\text{-}ERT2}$ and $Trac^{FL/FL}Foxp3^{Cre\text{-}ERT2}$ mice (**Supplementary Fig. 7a**). $Irf4^{FL/-}Foxp3^{Cre\text{-}ERT2}$ mice, in which IRF4 is constitutively deleted in T_{reg} cells, have been shown to develop severe autoimmunity dominated by type 2 helper T cell cytokines by 8 weeks of age[37]. T_{reg} cells in $Irf4^{-/-}$ mice have been shown to have an almost exclusively naive-like phenotype[38], and we similarly found that T_{reg} cells in $Irf4^{FL/FL}Foxp3^{YFP\text{-}Cre}$ mice were largely $CD44^{lo}CD62L^{hi}$, even in the context of severe inflammation (data not shown), which suggested impaired differentiation, survival and/or population expansion of effector T_{reg} cells.

To determine whether fully differentiated T_{reg} 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 $Irf4^{FL/FL}Foxp3^{Cre\text{-}ERT2}$ and $Irf4^{WT/WT}Foxp3^{Cre\text{-}ERT2}$ littermates[47]. On day 13, we observed slightly but reproducibly lower IRF4 protein expression in $CD44^{hi}$ T_{reg} lymph node cells from $Irf4^{FL/FL}Foxp3^{Cre\text{-}ERT2}$

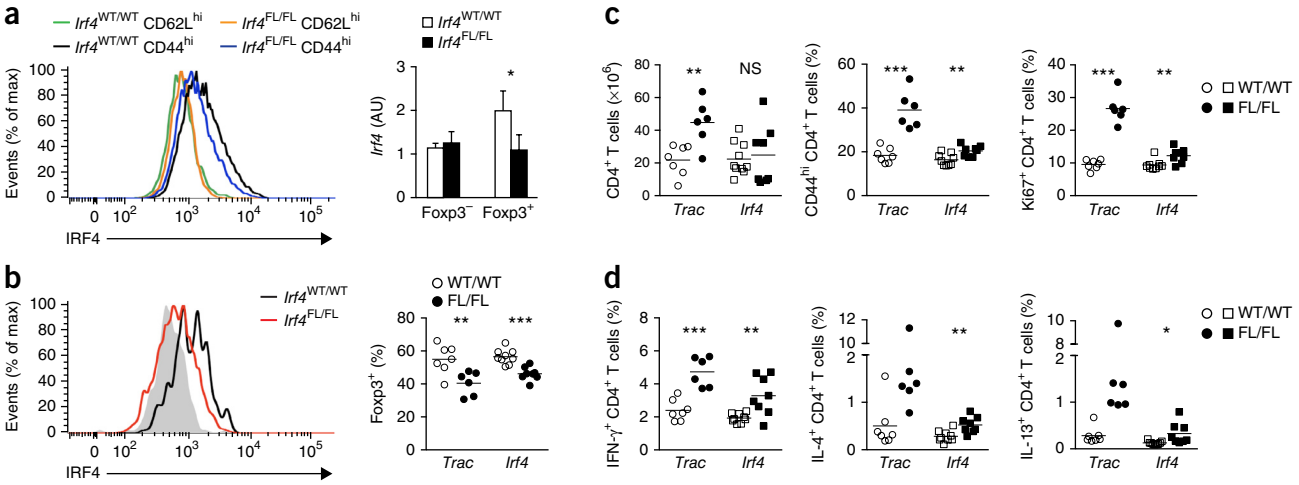

Figure 6 IRF4 expression contributes to optimal suppressive ability and homeostasis of T_{reg} cells. (**a**) IRF4 expression in $CD44^{hi}CD62L^{lo}$ ($CD44^{hi}$) and $CD44^{lo}CD62L^{hi}$ ($CD62L^{hi}$) $CD4^+Foxp3^+$ lymph node cells from $Irf4^{WT/WT}Foxp3^{Cre\text{-}ERT2}$ mice ($Irf4^{WT/WT}$) and $Irf4^{FL/FL}Foxp3^{Cre\text{-}ERT2}$ mice ($Irf4^{FL/FL}$) analyzed on day 13 following tamoxifen treatment on days 0, 3, 7 and 10 (left), quantitative PCR analysis of *Irf4* mRNA in $CD4^+eGFP^-$ and $CD4^+eGFP^+$ cells sorted from pooled spleens and lymph nodes of those mice. AU, arbitrary units. (**b**) IRF4 expression in colonic lamina propria $CD4^+Foxp3^+$ cells in $Irf4^{WT/WT}Foxp3^{Cre\text{-}ERT2}$ and $Irf4^{FL/FL}Foxp3^{Cre\text{-}ERT2}$ mice (left), and frequency of $Foxp3^+$ cells among $CD4^+$ cells in the large intestine lamina propria in $Trac^{WT/WT}Foxp3^{Cre\text{-}ERT2}$ or $Irf4^{WT/WT}Foxp3^{Cre\text{-}ERT2}$ mice (open circles) and $Trac^{FL/FL}Foxp3^{Cre\text{-}ERT2}$ or $Irf4^{FL/FL}Foxp3^{Cre\text{-}ERT2}$ mice (filled circles) analyzed on day 13 following tamoxifen treatment on days 0, 3, 7 and 10. Gray shaded curve (left), $CD4^+Foxp3^-$ cells. (**c**,**d**) Number of cells and frequency of $CD44^{hi}$ or $Ki67^+$ cells (**c**) or cytokine-producing cells (**d**) among $CD4^+Foxp3^-$ T cells in the lymph nodes (**c**) and spleens (**d**) of $Trac^{WT/WT}Foxp3^{Cre\text{-}ERT2}$ mice (open circles), $Irf4^{WT/WT}Foxp3^{Cre\text{-}ERT2}$ mice (open squares), $Trac^{FL/FL}Foxp3^{Cre\text{-}ERT2}$ mice (filled circles) and $Irf4^{FL/FL}Foxp3^{Cre\text{-}ERT2}$ mice (filled squares) analyzed on day 13 following tamoxifen treatment on days 0, 3, 7 and 10. Each symbol (**b**–**d**) represents an individual mouse; small horizontal lines indicate the mean. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (two-tailed unpaired *t*-test). Data are representative of two experiments with four or more mice per group in each (**a**,**b**, left) or are pooled from two experiments with four or more mice per group in each (**a**,**b**, right, **c**,**d** (error bars (**a**), s.d.).

mice than in cells from $Irf4^{WT/WT}Foxp3^{Cre\text{-}ERT2}$ mice (**Fig. 6a**). We also saw higher GFP expression in the T_{reg} cells, measured as a sum of fluorescence from the *Irf4*-deletion GFP reporter (whose expression is switched on in the *Irf4* locus by Cre-mediated deletion of the $Irf4^{FL}$ allele) and from the GFP-Cre-ERT2 fusion protein expressed concomitantly with Foxp3 (**Supplementary Fig. 7b**). Quantitative PCR analysis indicated ~50% lower abundance of *Irf4* mRNA in T_{reg} cells sorted from pooled spleens and lymph nodes of tamoxifen-treated $Irf4^{FL/FL}Foxp3^{Cre\text{-}ERT2}$ mice than in those of $Irf4^{WT/WT}Foxp3^{Cre\text{-}ERT2}$ mice (**Fig. 6a**).

We hypothesized that the suboptimal ~50% reduction in mRNA transcripts and the only slight reduction in IRF4 protein expression in the spleens and lymph nodes of $Irf4^{FL/FL}Foxp3^{Cre\text{-}ERT2}$ mice compared with that of $Irf4^{WT/WT}Foxp3^{Cre\text{-}ERT2}$ mice might have resulted from a competitive disadvantage of T_{reg} cells that lack IRF4 protein. Such a disadvantage might lead to 'preferential' population expansion of the IRF4-sufficient T_{reg} cells remaining in the $Irf4^{FL/FL}Foxp3^{Cre\text{-}ERT2}$ mice. This would be consistent with the published observation that the survival and expansion of strongly antigen-stimulated $CD8^+$ T cells is greatly impaired in the absence of IRF4 (ref. 48).

To determine whether IRF4 might similarly contribute to the maintenance of T_{reg} cells that have been strongly activated, we assessed the colonic lamina propria, in which nearly all T_{reg} cells were $CD44^{hi}$ and had probably recently experienced engagement of the TCR, given their robust IL-10 production (data not shown). Indeed, we observed a lower frequency of colonic lamina propria T_{reg} cells in $Irf4^{FL/FL}Foxp3^{Cre\text{-}ERT2}$ mice than in $Irf4^{WT/WT}Foxp3^{Cre\text{-}ERT2}$ mice (**Fig. 6b**), similar to lower frequency of T_{reg} cells in the colonic lamina propria of $Trac^{FL/FL}Foxp3^{Cre\text{-}ERT2}$ mice than in $Trac^{WT/WT}Foxp3^{Cre\text{-}ERT2}$ mice noted above. In contrast to spleen and lymph node T_{reg} cells, and consistent with their lower frequency, colonic lamina propria T_{reg} cells in $Irf4^{FL/FL}Foxp3^{Cre\text{-}ERT2}$ mice had much lower expression of IRF4 protein than did those in $Irf4^{WT/WT}Foxp3^{Cre\text{-}ERT2}$ mice (**Fig. 6b**).

We hypothesized that the reportedly low influx of circulating T_{reg} cells into the colonic lamina propria at steady state may have precluded IRF4-sufficient cells from becoming activated and repopulating to a wild-type frequency the T_{reg} cell niche in this tissue[24].

Despite the only slightly lower IRF4 expression in T_{reg} cells isolated from the spleens and lymph nodes of $Irf4^{FL/FL}Foxp3^{Cre\text{-}ERT2}$ mice than in their counterparts from $Irf4^{WT/WT}Foxp3^{Cre\text{-}ERT2}$ mice, we were able to detect a very mild, but statistically significant, increase in the frequency of $CD44^{hi}$ and $Ki67^+$ lymph node $Foxp3^-CD4^+$ T cells, as well as increased production of IFN-γ, IL-4 and IL-13 by splenic $Foxp3^-CD4^+$ T cells (**Fig. 6c**,**d**). This suggested that IRF4 expression downstream of TCR signaling in T_{reg} cells contributed to the suppressive function of T_{reg} cells. An increase in the production of type 2 helper T cell cytokines was consistent with the phenotype of mice with constitutive ablation of IRF4 in T_{reg} cells, whereas the increased IFN-γ was probably a consequence of the substantial type 1 helper T cell bias in C56B/L6 adult mice before induced *Irf4* deletion[37].

As a control, we confirmed that in the absence of tamoxifen treatment, the expression of CD44, Ki67, IFN-γ, IL-4 and IL-13 in $CD4^+Foxp3^-$ T cells from $Irf4^{FL/FL}Foxp3^{Cre\text{-}ERT2}$ mice was indistinguishable from that in their counterparts from $Irf4^{WT/WT}Foxp3^{Cre\text{-}ERT2}$ mice, as was the frequency of T_{reg} cells in the colonic lamina propria (data not shown). This suggested that the modest differences between $Irf4^{FL/FL}Foxp3^{Cre\text{-}ERT2}$ and $Irf4^{WT/WT}Foxp3^{Cre\text{-}ERT2}$ mice that we observed upon tamoxifen treatment were not a consequence of the $Irf4^{FL}$ allele itself. Together these data indicated that even partial loss of IRF4 expression downstream of TCR engagement in T_{reg} cells interfered with optimal suppressive function of these cells.

DISCUSSION

Despite major progress in understanding the molecular mechanisms of TCR engagement–driven differentiation of T_{reg} cells, the role of

the TCR in T_{reg} cell function *in vivo* has remained unclear. Here we demonstrated that TCR signaling in differentiated T_{reg} cells was dispensable for the maintenance of Foxp3 expression and for the expression of many characteristic markers of T_{reg} cells. Although the bulk of the T_{reg} cell–specific gene signature was also preserved in the absence of the TCR, suppressor function was critically dependent on the TCR.

Given that antigen-activated $CD4^+Foxp3^-$ T cells in lymphoid organs are thought to produce IL-2 in a spatially restricted manner, we considered the possibility that T_{reg} cells might analogously require their TCRs to correctly position themselves to gain preferential access to IL-2. This might elicit the suppressive function of T_{reg} cells by stimulating IL-2R. However, our *in vivo* and *in vitro* data suggested that T_{reg} cells acquired and probably effectively depleted IL-2 in a TCR-independent manner. Thus, these cells may instead rely predominantly on expression of the chemokine receptor CCR7 to ensure sufficient IL-2 exposure, as has been proposed[24].

Our observation that newly generated T_{reg} cells in $Trac^{FL/WT}Foxp3^{YFP\text{-}Cre}$ and $Trac^{FL/FL}Foxp3^{YFP\text{-}Cre}$ mice remained naive-like upon loss of the TCR and did not populate nonlymphoid tissues suggested that effector differentiation and population expansion were processes dependent on the TCR and probably dependent on antigens. This finding may help explain the observation that distinct TCR repertoires are displayed by T_{reg} cell populations in different lymphoid organs and tissues in adult mice[49,50]. Furthermore, inducible ablation of the TCR resulted in a far greater change in gene expression in the effector-like T_{reg} cell subset than in the naive-like subset. This suggested that continuous TCR signaling might be selectively driving the homeostasis and suppressor function of effector-like T_{reg} cells. As inducible deletion of the TCR in differentiated T_{reg} cells precipitated autoimmunity, our data may suggest that all or most of the suppressor function of T_{reg} cells *in vivo* is mediated by the $CD44^{hi}$ effector-like T_{reg} cell subset.

We found that partial inducible ablation of *Irf4*, expressed downstream of TCR engagement in $CD44^{hi}$ T_{reg} cells, resulted in a very mild but reproducible activation of the immune system. This result suggested that IRF4 expression was important for TCR-dependent T_{reg} cell function; however, given the suboptimal deletion of *Irf4* and very modest activation of the immune system, it remains to be determined to what extent and in what way TCR-dependent induction of IRF4 in T_{reg} cells contributes to their ability to suppress spontaneous autoimmunity. IRF4 may act mainly to control the maintenance of highly activated T_{reg} cells, which was particularly evident in the colonic lamina propria and which, when altered, may affect the ability of the T_{reg} cell pool to suppress. Alternatively, IRF4 may have a more direct role in promoting the suppressive activity of T_{reg} cells, perhaps by driving the expression of certain T_{reg} cell effector molecules. Although we observed decreased expression of the inducible costimulator ICOS on colonic T_{reg} cells that lacked IRF4 (data not shown), the remaining IRF4-sufficient T_{reg} cells in lymphoid organs of $Irf4^{FL/FL}Foxp3^{Cre\text{-}ERT2}$ mice preclude more rigorous identification of IRF4 targets.

However, we note that even though naive-like T_{reg} cells did not express IRF4 and were overwhelmingly unaffected by TCR deletion on a transcriptional level, this did not necessarily indicate that these cells were nonfunctional or were not experiencing TCR engagement. Indeed, several genes, including *Egr1*, *Egr2* and *Nr4a1*, were downregulated in this T_{reg} cell subset in the absence of the TCR.

Additional experiments are needed to elucidate the contributions of other individual TCR-dependent genes in T_{reg} cells to the maintenance of immunotolerance in the steady state and to the restraint of immune responses directed against commensal microorganisms, food and environmental antigens and pathogens. In connection with this, we note that although IL-10 production by T_{reg} cells has been linked to 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_{reg} cell control over systemic autoimmunity[26]. Likewise, we found that whereas constitutive deletion of calcineurin B1 in $Cnb1^{FL/FL}Foxp3^{YFP\text{-}Cre}$ mice (which eliminated calcineurin-dependent activation of NFAT in T_{reg} cells) resulted in lethal early-onset autoimmunity, highly efficient inducible deletion in adult lymphoreplete $Cnb1^{FL/FL}Foxp3^{Cre\text{-}ERT2}$ mice had no detectable adverse effects on T_{reg} cell function (data not shown). Together these findings suggest that engagement of the TCR on T_{reg} 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_{reg} cell immunosuppressive functions.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. GEO: microarray data, GSE61077.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank M. Schmidt-Supprian (Technical University Munich) and K. Rajewsky (Max Delbrück Center) for $Trac^{FL}$ mice. Supported by the US National Institutes of Health (R37AI034206 to A.Y.R.), the Ludwig Cancer Center at Memorial Sloan-Kettering Cancer Center (A.Y.R.) and the Howard Hughes Medical Institute (A.Y.R.).

AUTHOR CONTRIBUTIONS

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

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

1. Josefowicz, S.Z., Lu, L.F. & Rudensky, A.Y. Regulatory T cells: mechanisms of differentiation and function. *Annu. Rev. Immunol.* **30**, 531–564 (2012).
2. Lee, H.M., Bautista, J.L., Scott-Browne, J., Mohan, J.F. & Hsieh, C.S. A broad range of self-reactivity drives thymic regulatory T cell selection to limit responses to self. *Immunity* **37**, 475–486 (2012).
3. Hsieh, C.S., Zheng, Y., Liang, Y., Fontenot, J.D. & Rudensky, A.Y. An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires. *Nat. Immunol.* **7**, 401–410 (2006).
4. Hsieh, C.S. *et al.* Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors. *Immunity* **21**, 267–277 (2004).
5. Gavin, M.A., Clarke, S.R., Negrou, E., Gallegos, A. & Rudensky, A. Homeostasis and anergy of $CD4^+CD25^+$ suppressor T cells *in vivo*. *Nat. Immunol.* **3**, 33–41 (2002).
6. Ouyang, W. *et al.* Novel Foxo1-dependent transcriptional programs control T(reg) cell function. *Nature* **491**, 554–559 (2012).
7. Marson, A. *et al.* Foxp3 occupancy and regulation of key target genes during T-cell stimulation. *Nature* **445**, 931–935 (2007).
8. Au-Yeung, B.B. *et al.* A genetically selective inhibitor demonstrates a function for the kinase Zap70 in regulatory T cells independent of its catalytic activity. *Nat. Immunol.* **11**, 1085–1092 (2010).
9. Gavin, M.A. *et al.* Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* **445**, 771–775 (2007).
10. Lin, W. *et al.* Regulatory T cell development in the absence of functional Foxp3. *Nat. Immunol.* **8**, 359–368 (2007).
11. Williams, L.M. & Rudensky, A.Y. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nat. Immunol.* **8**, 277–284 (2007).
12. Wing, K. *et al.* CTLA-4 control over $Foxp3^+$ regulatory T cell function. *Science* **322**, 271–275 (2008).

13. Borsellino, G. *et al.* Expression of ectonucleotidase CD39 by Foxp3$^+$ T_{reg} cells: hydrolysis of extracellular ATP and immune suppression. *Blood* **110**, 1225–1232 (2007).
14. Pandiyan, P., Zheng, L., Ishihara, S., Reed, J. & Lenardo, M.J. CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4$^+$ T cells. *Nat. Immunol.* **8**, 1353–1362 (2007).
15. Shafiani, S. *et al.* Pathogen-specific T_{reg} cells expand early during mycobacterium tuberculosis infection but are later eliminated in response to Interleukin-12. *Immunity* **38**, 1261–1270 (2013).
16. Rosenblum, M.D. *et al.* Response to self antigen imprints regulatory memory in tissues. *Nature* **480**, 538–542 (2012).
17. Samy, E.T., Parker, L.A., Sharp, C.P. & Tung, K.S. Continuous control of autoimmune disease by antigen-dependent polyclonal CD4$^+$CD25$^+$ regulatory T cells in the regional lymph node. *J. Exp. Med.* **202**, 771–781 (2005).
18. Polic, B., Kunkel, D., Scheffold, A. & Rajewsky, K. How αβT cells deal with induced TCRα ablation. *Proc. Natl. Acad. Sci. USA* **98**, 8744–8749 (2001).
19. Rubtsov, Y.P. *et al.* Stability of the regulatory T cell lineage *in vivo*. *Science* **329**, 1667–1671 (2010).
20. Hsieh, C.S., Lee, H.M. & Lio, C.W. Selection of regulatory T cells in the thymus. *Nat. Rev. Immunol.* **12**, 157–167 (2012).
21. Zheng, Y. *et al.* Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature* **463**, 808–812 (2010).
22. Ruan, Q. *et al.* Development of Foxp3$^+$ regulatory T cells is driven by the c-Rel enhanceosome. *Immunity* **31**, 932–940 (2009).
23. Wan, Y.Y. & Flavell, R.A. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. *Nature* **445**, 766–770 (2007).
24. Smigiel, K.S. *et al.* CCR7 provides localized access to IL-2 and defines homeostatically distinct regulatory T cell subsets. *J. Exp. Med.* **211**, 121–136 (2014).
25. Fisson, S. *et al.* Continuous activation of autoreactive CD4$^+$ CD25$^+$ regulatory T cells in the steady state. *J. Exp. Med.* **198**, 737–746 (2003).
26. Rubtsov, Y.P. *et al.* Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* **28**, 546–558 (2008).
27. Tang, Q. *et al.* Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. *Immunity* **28**, 687–697 (2008).
28. Fontenot, J.D., Rasmussen, J.P., Gavin, M.A. & Rudensky, A.Y. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat. Immunol.* **6**, 1142–1151 (2005).
29. Webster, K.E. *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. *J. Exp. Med.* **206**, 751–760 (2009).
30. Kim, J.M., Rasmussen, J.P. & Rudensky, A.Y. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat. Immunol.* **8**, 191–197 (2007).
31. Tian, L. *et al.* Foxp3$^+$ regulatory T cells exert asymmetric control over murine helper responses by inducing Th2 cell apoptosis. *Blood* **118**, 1845–1853 (2011).
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.* **189**, 28–32 (2012).
33. Thornton, A.M. & Shevach, E.M. CD4$^+$CD25$^+$ immunoregulatory T cells suppress polyclonal T cell activation *in vitro* by inhibiting interleukin 2 production. *J. Exp. Med.* **188**, 287–296 (1998).
34. Kim, J.K. *et al.* Impact of the TCR signal on regulatory T cell homeostasis, function, and trafficking. *PLoS ONE* **4**, e6580 (2009).
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* **37**, 785–799 (2012).
36. Samstein, R.M. *et al.* Foxp3 exploits a pre-existent enhancer landscape for regulatory T cell lineage specification. *Cell* **151**, 153–166 (2012).
37. Zheng, Y. *et al.* Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T_H2 responses. *Nature* **458**, 351–356 (2009).
38. Cretney, E. *et al.* The transcription factors Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells. *Nat. Immunol.* **12**, 304–311 (2011).
39. Sawant, D.V. *et al.* Bcl6 controls the Th2 inflammatory activity of regulatory T cells by repressing Gata3 function. *J. Immunol.* **189**, 4759–4769 (2012).
40. Collison, L.W. *et al.* The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* **450**, 566–569 (2007).
41. Colotta, F. *et al.* Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4. *Science* **261**, 472–475 (1993).
42. Huang, C.T. *et al.* Role of LAG-3 in regulatory T cells. *Immunity* **21**, 503–513 (2004).
43. Petermann, K.B. *et al.* CD200 is induced by ERK and is a potential therapeutic target in melanoma. *J. Clin. Invest.* **117**, 3922–3929 (2007).
44. Reinwald, S. *et al.* CD83 expression in CD4$^+$ T cells modulates inflammation and autoimmunity. *J. Immunol.* **180**, 5890–5897 (2008).
45. Hall, A.O. *et al.* The cytokines interleukin 27 and interferon-γ promote distinct T≠ cell populations required to limit infection-induced pathology. *Immunity* **37**, 511–523 (2012).
46. Hoelzinger, D.B. *et al.* Blockade of CCL1 inhibits T regulatory cell suppressive function enhancing tumor immunity without affecting T effector responses. *J. Immunol.* **184**, 6833–6842 (2010).
47. Klein, U. *et al.* Transcription factor IRF4 controls plasma cell differentiation and class-switch recombination. *Nat. Immunol.* **7**, 773–782 (2006).
48. Man, K. *et al.* The transcription factor IRF4 is essential for TCR affinity-mediated metabolic programming and clonal expansion of T cells. *Nat. Immunol.* **14**, 1155–1165 (2013).
49. Lathrop, S.K., Santacruz, N.A., Pham, D., Luo, J. & Hsieh, C.S. Antigen-specific peripheral shaping of the natural regulatory T cell population. *J. Exp. Med.* **205**, 3105–3117 (2008).
50. Lathrop, S.K. *et al.* Peripheral education of the immune system by colonic commensal microbiota. *Nature* **478**, 250–254 (2011).

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- to 10-week-old male and female sex-matched mice were used for all experiments. *Foxp3*$^{YFP\text{-}Cre}$, *Foxp3*$^{eGFP\text{-}Cre\text{-}ERT2}$, *Rosa26*^{YFP}, *Foxp3*^{DTR} and *Irf4*^{FL} mice have been described[19,26,30,47]. M. Schmidt-Supprian and K. Rajewsky provided *Trac*^{FL} mice. For tamoxifen administration, 40 mg tamoxifen was dissolved in 100µl ethanol and subsequently in 900 µl olive oil (Sigma-Aldrich) and was sonicated four times for 30 s each in a Bioruptor Twin (Diagenode). Mice were given oral gavage of 200 µl tamoxifen emulsion per treatment. For injection of diphtheria toxin (DT), DT was dissolved in PBS and 200 µl of the appropriate dose was injected intraperitoneally into each mouse. For *in vivo* depletion of IL-2, mice were given tamoxifen by gavage on days 0 and 1 or on days 0, 3, 7 and 10 and were given intraperitoneal injection on days 4 and 8 of a 0.5mg 1:1 mixture of IL-2-neutralizing antibody (JES6-1A12 and S4B6-1; Bio X Cell) or IgG2a isotype-matched control antibody (Bio X Cell). For administration of IL-2–anti-IL2 complexes, 1 µg recombinant mouse IL-2 (R&D Systems) was incubated for 10 min at room temperature with 5 µg JES6-1 (Bio X Cell) and was diluted to a volume of 200 µl in PBS immediately before intraperitoneal injection. Mice were given tamoxifen by gavage on days 0, 3, 7 and 10 and received IL-2–anti-IL-2 complexes or PBS on days 5, 7, 9 and 11. No animals were excluded from analyses. No statistical method was used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments or outcome assessment.

Flow cytometry 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 given intraperitoneal injection of 1 mg BrdU (5-bromodeoxyuridine) in 1 ml PBS and cells were stained with a BD Pharmingen BrdU Flow Kit. An LSR II flow cytometer (BD Bioscience) and FlowJo software (TreeStar) were used for flow cytometry. For cell isolation, CD4$^+$ T cells were purified from pooled spleen and lymph node cell suspensions with magnetic Dynabeads (Invitrogen) and were further sorted with a FACSAria II cell sorter (BD Bioscience). Intracellular staining was performed with eBioscience Fixation Permeabilization buffers. For cytokine staining, lymph node and spleen cells were stimulated for 4–6 h at 37 °C, 5% CO_2, with soluble anti-CD3 (5 µg/ml; 2C11; Bio X Cell) and anti-CD28 (5 µg/ml; 37.51; Bio X Cell) in the presence of 1 µg/ml brefeldin A (Sigma).

***In vitro* proliferation assay.** DC populations were expanded *in vivo* by subcutaneous injection of cytokine Flt3L–secreting B16 melanoma cells into the left hind flank of B6 mice. Once tumors were visible, spleens from injected mice were dissociated for 20 min at 37 °C, with shaking, in RPMI-1640 medium containing 1.67 units/ml Liberase TL (Roche) and 50 µg/ml DNase I (Roche). EDTA was added at a final concentration of 5 mM to stop digestion, and the resulting homogenate was processed for isolation of CD11c$^+$ cells with a MACS mouse CD11c (N418) purification kit (Miltenyi Biotec). CD4$^+$eGFP$^+$ cells purified by flow cytometry from tamoxifen-treated *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ mice were labeled with CellTrace Violet according to the manufacturer's instructions (Molecular Probes) and were plated in triplicate in 96-well flat-bottomed plates (5×10^4 cells per well) in medium containing 25 U/ml human recombinant IL-2 (PeproTech) with or without equal numbers of DCs and with or without 100 ng/ml LPS (*Escherichia coli* strain 0111:B4, Sigma-Aldrich).

***In vitro* IL-2 stimulation and detection of phosphorylated STAT5.** *Trac*$^{FL/WT}$ *Foxp3*$^{Cre\text{-}ERT2}$ mice were treated with tamoxifen on days 0 and 1, and on day 9, CD4$^+$ T cells were purified from pooled spleen and lymph node cell suspensions with magnetic Dynabeads (Invitrogen). Cells were stained with anti-CD4 (RM4-5; Tonbo Biosciences) and anti-TCRβ (H57-597; Tonbo Biosciences), then were washed and plated in 96-well V-bottomed plates (1×10^6 cells per well) in RPMI medium containing 10% FBS with or without increasing concentrations of IL-2, followed by incubation for 20 min at 37 °C. Cells were subsequently processed with BD Phosflow Lyse/Fix Buffer and Perm Buffer III (BD Biosciences) and were stained with antibody to STAT5 phosphorylated at Tyr694 (47/Stat5; BD Biosciences) according to the manufacturer's instructions. T_{reg} cells were identified by eGFP expression. For *ex vivo* staining of phosphorylated STAT5, spleen and lymph nodes were dissociated at 4 °C in PBS (0.5% BSA) containing anti-CD4 and anti-TCRβ (both identified above), were stained for 10 min on ice and were washed twice before fixation.

***In vitro* depletion of IL-2.** *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ mice were treated with tamoxifen on days 0 and 1, and on day 9, pooled spleens and lymph nodes were enriched for CD4$^+$ cells and subsequently sorted to a purity of >99% into eGFP$^+$TCRβ$^+$, eGFP$^+$TCRβ$^-$ and eGFP$^-$TCRβ$^+$ populations. Each population was divided among eight wells of a 96-well V-bottomed plate (2.5×10^5 cells/well) in 25 ul RPMI medium (10%) with or without increasing doses of recombinant human IL-2, followed by incubation for 2 h at 37 °C. Depletion of IL-2 from the medium was assessed with the BD Cytometric Bead Array and Human IL-2 Enhanced Sensitivity Flex Set according to the manufacturer's instructions (BD Biosciences).

***In vitro* conjugation assay.** *Trac*$^{WT/WT}$*Foxp3*$^{Cre\text{-}ERT2}$, *Trac*$^{FL/WT}$ *Foxp3*$^{Cre\text{-}ERT2}$ and *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice were treated with tamoxifen on days 0 and 1, and on day 9, CD4$^+$ T cells were isolated from spleen and lymph nodes with the Dynabeads Untouched Mouse CD4 Cells negative selection kit (Invitrogen.) TCR$^+$ T_{reg} cells were sorted from *Trac*$^{WT/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ mice on the basis of eGFP expression alone. TCR$^-$ T_{reg} cells were sorted from *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ and *Trac*$^{FL/FL}$*Foxp3*$^{Cre\text{-}ERT2}$ mice as eGFP$^+$TCRβ$^-$ cells. T_{reg} cells were subsequently labeled with CFSE (carboxyfluorescein diacetate succinimidyl ester), and populations of DCs from mice with no deficiency or homozygous deficiency in MHC class II were expanded *in vivo* with Flt3L-secreting B16 melanoma cells and were labeled with CellTrace Violet. 1×10^4 T_{reg} cells and 6×10^4 DCs were cultured together in a 96-well round-bottomed plate in RPMI medium 10% supplemented with 500 U/ml IL-2. Concanavalin A (C2010; Sigma) was used at a final concentration of 2.5 µg/ml. Following 10 h of culture at 37 °C, cells were gently resuspended before flow cytometry.

Gene-expression analysis. eGFP$^+$ TCRβ$^+$ and TCRβ$^-$ CD44^{hi}CD62L^{lo} cell populations (two replicates) and CD44^{lo}CD62L^{hi} cell populations (three replicates) were sorted from tamoxifen-treated *Trac*$^{FL/WT}$*Foxp3*$^{Cre\text{-}ERT2}$ mice (five or more mice per replicate) with a FACSAria II flow cytometer. Complementary DNA (cDNA) libraries were amplified and hybridized to Affymetrix 430 2.0 chips. Arrays were normalized by the robust multiarray average method, and genes were considered to have a difference in expression if they had a *q* value of <0.01 after Benjamini-Hochberg false-discovery rate correction. Differences in gene expression in T_{reg} cells from *Foxp3*^{GFPKO} mice and *Foxp3*$^+$ mice has been described[36].