An essential role for the IL-2 receptor in Treg cell function

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Regulatory T cells (Treg cells), which have abundant expression of the interleukin 2 receptor (IL-2R), are reliant on IL-2 produced by activated T cells. This feature indicates a key role for a simple network based on the consumption of IL-2 by Treg cells in their suppressor function. However, congenital deficiency in IL-2R results in reduced expression of the Treg cell lineage–specification factor Foxp3, which has confounded experimental efforts to understand the role of IL-2R expression and signaling in the suppressor function of Treg cells. Using genetic gain- and loss-of-function approaches, we found that capture of IL-2 was dispensable for the control of CD4+ T cells but was important for limiting the activation of CD8+ T cells, and that IL-2R-dependent activation of the transcription factor STAT5 had an essential role in the suppressor function of Treg cells separable from signaling via the T cell antigen receptor.

Regulatory T cells (Treg cells) that express the transcription factor Foxp3 restrain immune responses to self and foreign antigens1–3. Treg cells have abundant expression of the interleukin 2 receptor α-chain (IL-2Rα; CD25) but are unable to produce IL-2. IL-2 binds with low affinity to IL-2Rα or to heterodimers of the common γ-chain (γC; CD132) and IL-2Rβ (CD122), but receptor affinity increases ~1,000-fold when these three subunits collectively interact with IL-2 (ref. 4). IL-2 and the transcription factor STAT5, a key target downstream of JAK kinases associated with IL-2R, are indispensable for inducing the expression of Foxp3 and differentiation of Treg cells in the thymus5–11. IL-2Rβ and γC are shared with the IL-15 receptor, whose signaling can also contribute to the induction of Foxp3 expression12. IL-2, in cooperation with the cytokine TGF-β, is also required for extrathymic Treg cell differentiation13.

While the role of IL-2R signaling in the induction of Foxp3 expression and Treg cell differentiation in the thymus is well established, the importance of IL-2R expression in mature Treg cells is not well understood. Although deficiency in STAT5 abolishes Foxp3 expression, it can be restored by increased amounts of the anti-apoptotic molecule Bcl2. That finding raised the possibility that a chief role of IL-2 might be in the survival of differentiating Treg cells or their precursors14. It has also been reported that ablation of the pro-apoptotic protein Bim can rescue Treg cells or their precursors from apoptosis associated with deficiency in IL-2 or IL-2R and restore the number of Treg cells, but it does not prevent fatal autoimmunity15. However, a profound effect of congenital deficiency in IL-2, Bcl2 and Bim on the differentiation and selection of Treg cells and self-reactive effector T cells (Teff cells) has confounded interpretation of that observation. Antibody-mediated neutralization of IL-2 in adult mice that have undergone removal of the thymus reduces the number of Treg cells and Foxp3 expression in Treg cells16,17. Thus, IL-2 supports stability of the Treg cell lineage after differentiation18,19. However, expression of a transgene encoding IL-2Rβ exclusively in thymocytes has been reported to rescue Il2rb−/− mice from lethal autoimmune disease, which suggests that IL-2R expression is dispensable in thymocytes but critical in peripheral Treg cells5,11. Thus, a role for IL-2R expression and signaling in peripheral Treg cells has remained uncertain. Hypothetically, a role for IL-2R in peripheral Treg cells could be threefold: guidance for Treg cells to sense their targets, which are activated self-reactive T cells that serve as a source of IL-2; Treg cell–mediated deprivation of IL-2 as a mechanism of suppression; and cell-intrinsic IL-2 signaling in differentiated Treg cells to support their maintenance, proliferation or function due to triggering of JAK–STAT5, PI3K–Akt or Ras–ERK signaling pathways. Previous studies have focused mainly on the induction or maintenance of Foxp3, while other aspects of IL-2R function have not been firmly established due to the aforementioned limitations.

Despite their considerable reliance on IL-2 for the maintenance of Foxp3 expression, Treg cells are unable to produce IL-2. The reason for the inhibition of autologous activation of STAT5 in Treg cells and the potential biological importance of this IL-2–based Treg cell–Teff cell

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regulatory loop also remain unknown. It has been suggested that repression of IL-2 is needed to maintain the 'unbound' state of high-affinity IL-2R on Treg cells, and unbound IL-2R serves a key role in Treg cell-mediated suppression by depriving Treg cells of IL-2 (refs. 20–24); however, whether this mechanism has a non-redundant role in suppression in vivo is unknown. To address the role of IL-2R and downstream signaling pathways in differentiated Treg cells, we ablated IL-2Rα, IL-2Rβ and STAT5 in Foxp3-expressing cells. By simultaneously inducing expression of an active form of STAT5, we assessed the differential requirements for IL-2R expression and IL-2 signaling in Treg cell homeostasis versus Treg cell suppressor activity.

RESULTS

IL-2R is indispensable for Treg cell function

To definitively establish a role for IL-2R in Treg cell function in vivo, we generated mice with Treg cell–specific conditional knockout of IL-2Rβ by using Cre recombinase driven by the endogenous Foxp3 locus (Foxp3 Cre) to deleteloxP-flanked Ii2rb alleles (Ii2rb fl/fl) in Treg cells after Foxp3 was expressed. Ii2rb fl/flFoxp3 Cre mice developed systemic fatal autoimmune inflammatory lesions and lymphoproliferation, albeit somewhat milder than that observed in mice with germline Foxp3 deficiency (Fig. 1a–c). The expression of IL-2Rβ and IL-2Rα was lower in Ii2rb fl/flFoxp3 Cre peripheral Treg cells than in their Ili2rb β/βFoxp3 Cre counterparts (Fig. 1d), and tyrosine-phosphorylation of STAT5 in response to IL-2 was lacking in Ii2rb fl/flFoxp3 Cre peripheral Treg cells (Fig. 1e). The frequency of Foxp3+ cells among CD4+ T cells and the expression of Foxp3 on a per-cell basis were both lower in Ii2rb fl/flFoxp3 Cre mice than in Ii2rb β/βFoxp3 Cre mice (Fig. 1f). In healthy Ii2rb β/βFoxp3 Cre/wt female mice, in which IL-2Rβ–sufficient Treg cells and IL-2Rβ–deficient Treg cells co-exist due to random inactivation of the X chromosome, IL-2Rβ–deficient Treg cells were under-represented (Fig. 1g,h). It has been suggested that IL-2 is selectively required for the maintenance of CD62L β/CD44lo Treg cells but is dispensable for CD62L β/CD44 hi Treg cells25. However, we found that the abundance of both CD62L β/CD44lo Treg cells and CD62L β/CD44 hi Treg cells was significantly lower in the absence of IL-2Rβ than in its presence in healthy Ii2rb β/βFoxp3 Cre/wt female mice (Supplementary Fig. 1a). In these mice, IL-2Rβ–deficient (Ii2rb fl/flFoxp3 Cre/wt) Treg cells had lower expression of Foxp3 and the Treg cell ‘signature’ molecules IL-2Rα, CTLA-4, GITR and CD103 than that of IL-2Rβ–sufficient (Ii2rb β/βFoxp3 Cre/wt) Treg cells regardless of their expression of CD62L and CD44 (Fig. 1i,j) and Supplementary Fig. 1a). Although in diseased Ii2rb β/βFoxp3 Cre mice, the majority of Treg cells were CD62L β/CD44 hi, this was probably a consequence of severe inflammation, because the frequency of Treg cells was also much lower in Ii2rb β/βFoxp3 Cre mice than in Ii2rb fl/flFoxp3 Cre mice at sites at which CD62L β/CD44 hi cells were prevalent—i.e., the small and large intestines (Supplementary Fig. 1b). Accordingly, the expression of many characteristic Treg cell markers, except for CD25 and Foxp3, was upregulated as the result of Treg cell activation in Ii2rb β/βFoxp3 Cre mice (Supplementary Fig. 1c). These observations suggested that both the CD62L β/CD44 hi Treg cell subset and the CD62L β/CD44 hi Treg cell subset, including those residing in non-lymphoid tissues, were dependent on IL-2, although under inflammatory conditions the latter was sustained to some extent by IL-2R-independent signals. Despite their upregulation of the expression of CTLA-4, GITR, the costimulatory receptor ICOS and CD103, the ‘activated’ IL-2Rβ–deficient Treg cells from Ii2rb fl/flFoxp3 Cre mice were still unable to control inflammation in the diseased mice and were not depressive when transferred together with T eff cells into lymphopenic recipients (data not shown).

Our findings raised the question whether ablation of IL-2Rα (which, in addition to facilitating IL-2 signaling, enables its sequestration from T eff cells) would result in Treg cell deficiency and disease similar to that of Ii2rb fl/flFoxp3 Cre mice. Thus, we generated mice with a loxp-flanked Il2ra allele (J.D.F., data not shown) and induced its conditional ablation in Treg cells by means similar to those described above. We found that Treg cell–specific deficiency in IL-2Rα resulted in a disease with an early onset and severity similar to that observed after ablation of IL-2Rβ (Supplementary Fig. 1d–f). Of note, germine deficiency in either Il2ra or Il2rb in mice on the same C57BL6/j strain as that of our mice with conditional knockout resulted in a considerably less aggressive disease with a delayed onset (data not shown), probably due to the role of IL-2R signaling in T eff cells. Our findings also indicated that IL-15 was unable to effectively compensate for the loss of IL-2 signaling in differentiated Treg cells, because in Ii2rb β/βFoxp3 Cre mice, Treg cells lacked only signaling via IL-2, whereas in Ii2rb fl/flFoxp3 Cre mice, they lacked signaling via both IL-2 and IL-15, yet they were affected similarly. This was in contrast to Treg cell differentiation in the thymus, in which IL-15 can contribute in part to Foxp3 induction12.

Since IL-2R activates PI3K–Akt, MAPK, and JAK–STAT5 signaling pathways, we next sought to assess the role of STAT5 activation downstream of IL-2R signaling in Treg cells. We found that ablation of STAT5 impaired Treg cell function similarly to ablation of IL-2Rβ and that Stat5 fl/flStat5 fl/flFoxp3 Cre mice were affected by fatal autoimmunity in a way similar to that of mice harboring IL-2R-deficient Treg cells (Supplementary Fig. 1g–k). Thus, in agreement with IL-2–neutralization studies, these results indicated that IL-2R signaling was required for Treg cell fitness in a cell-intrinsic manner.

Restoring STAT5 signaling in IL-2R-deficient Treg cells

The findings reported above indicated that activation of STAT5 downstream of IL-2R was continuously required for Treg cell function. However, the marked decrease in IL-2R expression observed in STAT5-deficient Treg cells (Supplementary Fig. 1g) made it impossible to separate loss of STAT5 from impairment in all IL-2R functions (i.e., detection of IL-2, transduction of STAT5-dependent and STAT5–independent signals, and consumption and deprivation of IL-2) as a key contributor to the observed severe dysfunction of Treg cells.

To address that major caveat and to understand the role of STAT5 versus that of IL-2R, we sought to determine whether expression of a gain-of-function form of STAT5b was able to restore Treg cell function in the absence of IL-2R. A published study using a transgene encoding a constitutively active form of STAT5b (STAT5b–CA) driven by the proximal promoter of the gene encoding the kinase Lck in the absence of IL-2R showed restoration of Treg cell differentiation in the thymus but not rescue from lymphoproliferative syndrome9. However, the expression of this transgene early during thymopoiesis leads to leukemic lymphoproliferation, which complicated the interpretation of those findings. In addition, both the activity of the proximal Lck promoter and the expression of the transgene became lower over time in peripheral T cells in these mice9. Therefore, we generated a gene-targeted mouse strain using the Rosa26 ‘gene-trap’ locus26 in which a transgene encoding STAT5b–CA driven by a CAG promoter (chicken β-actin promoter with cytomegalovirus enhancers)27 is preceded by a loxp-flanked STOP cassette (Supplementary Fig. 2a). In the resulting Rosa26 fl(Stat5 bCA) mice, STAT5b–CA is expressed only when the loxp sites undergo Cre–mediated recombination. Introduction of the Rosa26 fl(Stat5 bCA) allele into Ii2rb fl/flFoxp3 Cre mice and the consequent expression of STAT5b–CA in IL-2R-deficient Treg cells rescued the mice from the systemic inflammation and early fatal disease (Supplementary Fig. 2b). In these mice, the frequency and
Figure 1  IL-2Rβ is indispensable for Treg cell function. (a) Histopathology of various tissues (above images) from Il2rb+/fl Foxp3Cre and Il2rbfl/fl Foxp3Cre mice (left margin). Scale bars, 100 μm. (b) LN cellularity of Il2rb+/fl Foxp3Cre and Il2rbfl/fl Foxp3Cre mice (key). (c) Frequency of IFN-γ+ cells (left), IL-4+ cells (middle) and IL-17+ cells (right) among splenic CD4+Foxp3− cells stimulated for 5 h with antibody to the invariant signaling protein CD3ε and antibody to the co-receptor CD28. (d) Expression of the IL-2R subunits IL-2Rα (CD122), IL-2Rγc (CD132) and IL-2Rα (CD25) by CD4+Foxp3+ cells from Il2rb+/fl Foxp3Cre and Il2rbfl/fl Foxp3Cre mice (key); gray shaded curve, isotype-matched control antibody. (e) Intracellular tyrosine-phosphorylated STAT5  (pY-STAT5) in Il2rb+/fl Foxp3Cre and Il2rbfl/fl Foxp3Cre Treg cells left unstimulated (US) or stimulated in vitro for 20 min with recombinant mouse IL-2 (1,000 U/ml). (f) Frequency of Foxp3+ (Treg) cells among LN CD3+CD4+ cells (left) and Foxp3 expression by the CD3+CD4+ Foxp3+ cells, presented as mean fluorescence intensity (MFI) (right). (g) Flow cytometry of gated CD3+CD4+ cells from the thymus, spleen and LNs (left margin) of healthy heterozygous female Il2rb+/fl Foxp3Cre/fl or Il2rbfl/fl Foxp3Cre mice (above plots). Numbers adjacent to outlined areas indicate percent cells with intracellular Foxp3 staining Treg cells) with (top right) or without (top left) expression of Cre (assessed as yellow fluorescent protein (YFP), which is fused to Cre in Foxp3Cre mice). (h) Frequency of Foxp3+ cells among CD3+CD4+ cells (left) and of YFP+ (Cre-expressing) cells among Foxp3+ cells (right) from organs of mice as in g. (i) Foxp3 expression in YFP+Foxp3+ cells (left) and YFP+Foxp3+ cells (right) from organs of mice as in g. (j) Expression of the markers Il2Rα (CD25), CTLA-4, GITR and CD103 by YFP+Foxp3+ cells from organs of mice as in g, analyzed by flow cytometry. Each symbol (b,c,f,h–j) represents an individual mouse (among 3- to 5-week-old sex- and age-matched mice); small horizontal lines indicate the mean (± s.e.m.). NS, not significant (*P > 0.05); **P < 0.05, ***P < 0.01 and +++P < 0.001 (two-tailed unpaired Student’s t-test). Data are representative of two experiments with more than five mice per group (a) or three experiments with more than ten mice per group (d,e,g) or are from one experiment representative of three independent experiments with similar results, with three or more mice per group in each (b,c,f,h–j).

The observed restoration of the suppressor function of IL-2Rβ-deficient Treg cells and rescue from the early fatal disease via expression of STAT5b-CA raised the possibility that the reintroduced high levels of IL-2Rα were responsible for these effects. However, expression of STAT5b-CA similarly rescued Il2rbfl/fl Foxp3Cre mice from the early fatal disease (Supplementary Fig. 2c–h). Notably, although the impaired ability of Treg cells to capture and consume IL-2 in both Il2rbfl/fl Foxp3Cre mice and Il2rbfl/fl Foxp3Cre mice was not ‘rescued’ via expression of STAT5b-CA (Fig. 2c), the reactivity of CD4+ T cells was

number of Treg cells were comparable to or even surpassed those in IL-2Rα-deficient Il2rbfl/fl Foxp3Cre mice (Fig. 2a). Notably, the expression of IL-2Rα was higher in Treg cells from Rosa26Stat5bCAIl2rbfl/fl Foxp3Cre mice than in those from Il2rbfl/fl Foxp3Cre or Il2rbfl/fl Foxp3Cre mice, despite the absence of the IL-2Rβ chain (Fig. 2a), which suggested that the expression of IL-2Rα on Treg cells was controlled mainly by STAT5-dependent signaling but not by STAT5-independent signaling. Notably, these IL-2Rβ-deficient Treg cells with heightened IL-2Rα expression remained unresponsive to IL-2 (Fig. 2b).
fully controlled in these mice (Fig. 2d and Supplementary Fig. 2d–h). These results suggest that the ability to capture and compete for IL-2 was dispensable for Treg cell-mediated suppression of CD4+ T cell responses. In contrast, however, the population expansion of CD8+ T cells, in particular that of activated CD62LhiCD44hi CD8+ T cells, was restrained only marginally in these mice (Fig. 2d and Supplementary Fig. 2f,h). Although expansion of the CD8+CD62LhiCD44hi subset was relatively well controlled, albeit not perfectly controlled, in neonatal mice (Fig. 2d and Supplementary Fig. 2f), this subset also gradually started to expand in these mice as early as 3 weeks after birth (Supplementary Fig. 2i). Although both Rosa26Stat5bCAIl2rbfl/fl Foxp3Cre mice and Rosa26Stat5bCAIl2rbfl/flFoxp3Cre mice were rescued from premature death and showed substantially improved clinical status comparable to that of healthy controls, they gradually failed to...
thrive and started to succumb to disease accompanied by massively expanded activated CD62L<sup>hi</sup>CD4<sup>+</sup> and CD62L<sup>lo</sup>CD4<sup>+</sup> CD8<sup>+</sup> T cell subsets in lymph nodes (LNs) and tissues by approximately 12 weeks of age (Supplementary Fig. 2i,j). These findings raised the possibility that IL-2 consumption by T<sub>reg</sub> cells, while dispensable for the control of CD4<sup>+</sup> T cells, was important for the restraint of CD8<sup>+</sup> T cells.

**T<sub>reg</sub> cells suppress CD8<sup>+</sup> T cell responses via IL-2 depletion**

To determine if the impairment in consumption of IL-2 by T<sub>reg</sub> cells accounted for the proliferation of CD8<sup>+</sup> T cells in Rosa26Stat5bCAIl2rb<sup>fl/fl</sup> Foxp3<sup>Cre</sup> mice, we administered IL-2-neutralizing antibodies to those mice starting from 5–7 d of age. As IL-2 supports the differentiation of T<sub>reg</sub> cells in all groups of mice and induced immunoinactivation in control Il2rb<sup>h/h</sup>Foxp3<sup>Cre</sup> mice (Fig. 2e and Supplementary Fig. 3a). In Il2rb<sup>h/h</sup>Foxp3<sup>Cre</sup> mice, which spontaneously developed disease, production of the T<sub>1</sub>2 (T helper type 2) cytokines IL-4 and IL-13 by CD4<sup>+</sup> T cells was significantly reduced by neutralization of IL-2; however, the activation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells was at best reduced only marginally or unaffected. In contrast, the activation and proliferation of CD8<sup>+</sup> T cells observed in Rosa26Stat5bCAIl2rb<sup>h/h</sup>Foxp3<sup>Cre</sup> mice was almost completely suppressed by this treatment.

The relative reduction in the CD8<sup>+</sup>CD62L<sup>lo</sup>CD44<sup>hi</sup> T cell subset and more pronounced proliferation of CD8<sup>+</sup>CD62L<sup>hi</sup>CD44<sup>hi</sup> T cell subset in Rosa26Stat5bCAIl2rb<sup>h/h</sup>Foxp3<sup>Cre</sup> and Rosa26Stat5bCAIl2rb<sup>h/h</sup>Foxp3<sup>Cre</sup> mice raised the possibility that loss of IL-2-consumption by T<sub>reg</sub> cells might selectively impair their suppression of the population expansion of memory CD8<sup>+</sup> T cells but not the recruitment of naive CD8<sup>+</sup> T cells into the effector-cell pool. We tested this idea by adoptive transfer of CD4<sup>+</sup> and CD8<sup>+</sup> cell subsets into lymphopenic recipients. Consistent with observations of Foxp3<sup>Cre</sup> mice, the impaired suppression of the population expansion and activation of CD4<sup>+</sup> T cells by IL-2-deficient T<sub>reg</sub> cells was completely ‘rescued’ by STAT5b-CA; in contrast, their ability to suppress memory CD8<sup>+</sup> T cells was not restored, whereas suppression of the population expansion and activation of naive CD8<sup>+</sup> T cells was recovered only partially (Fig. 2f). Thus, IL-2 consumption by T<sub>reg</sub> cells seemed to have a non-redundant role in suppressing the population expansion and activation of both the naive CD8<sup>+</sup> T cell subset and memory CD8<sup>+</sup> T cell subset, although this mechanism seemed to be particularly prominent in control of the latter subset.

Although the majority of activated CD8<sup>+</sup> T cells in Il2rb<sup>h/h</sup>Foxp3<sup>Cre</sup> and Rosa26Stat5bCAIl2rb<sup>h/h</sup>Foxp3<sup>Cre</sup> mice did not have detectable expression of IL-2Rα (Supplementary Fig. 3a), these cells were able to activate STAT5 in response to IL-2, albeit to a lesser extent than that observed in cells expressing IL-2Rα (Supplementary Fig. 3b). A small proportion of activated CD4<sup>+</sup> T cells with undetectable IL-2Rα expression also responded to IL-2, but the majority of them did not (Supplementary Fig. 3b). naive CD8<sup>+</sup> T cells also responded to IL-2, while naive CD4<sup>+</sup> T cells did not (Supplementary Fig. 3b). Thus, both naive CD8<sup>+</sup> T cells and activated CD8<sup>+</sup> T cells seemed to be more sensitive to IL-2 than were CD4<sup>+</sup> T cells, and IL-2 consumption by T<sub>reg</sub> cells might have markedly affected their activation. A corollary to that idea was that activation of STAT5 in CD8<sup>+</sup> T cells but not in CD4<sup>+</sup> T cells might render the former resistant to T<sub>reg</sub> cell–mediated suppression. Thus, we tested the effect of STAT5 activation on the proliferation of CD4<sup>+</sup> or CD8<sup>+</sup> T cells in the presence of T<sub>reg</sub> cells. For this purpose, we sorted CD4<sup>+</sup>Foxp3<sup>+</sup> and CD8<sup>+</sup>Foxp3<sup>+</sup> T cells from Rosa26Stat5bCAFoxp3<sup>Cre</sup> mice and induced expression of STAT5b-CA in these cells by treating them with recombinant Cre protein containing a membrane-permeable TAT peptide (trans-activating transcriptional activator from human immunodeficiency virus) (TAT-Cre). We adoptively transferred the treated cells into lymphopenic recipients with or without T<sub>reg</sub> cells. Although treatment with TAT-Cre initially induced STAT5b-CA expression in approximately 30% of the treated CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells, more than 95% of CD8<sup>+</sup> T cells expressed STAT5b-CA 3 weeks after the cell transfer, whereas STAT5b-CA expressing CD4<sup>+</sup> T cells increased their frequency to only 40–50% (Fig. 2g). Notably, STAT5b-CA–STAT5<sup>+</sup> T cell populations robustly expanded in the presence of either control (Il2rb<sup>h/h</sup>Foxp3<sup>Cre</sup>) T<sub>reg</sub> cells or STAT5b-CA<sup>+</sup> T<sub>reg</sub> cells (Fig. 2g,h). Although some degree of suppression of STAT5b-CA–STAT5<sup>+</sup> T cells by T<sub>reg</sub> cells was still observed, it was more mild compared with the suppression of STAT5b-CA–CD8<sup>+</sup> T cells (Fig. 2h). In contrast, the proliferation and cytokine production of activated CD4<sup>+</sup> T cells, regardless of the expression of STAT5b-CA, were well controlled by T<sub>reg</sub> cells (Fig. 2h).

These observations suggested that STAT5 activation in CD8<sup>+</sup> T cells prompted robust population expansion of cells and conferred pronounced resistance to T<sub>reg</sub> cell–mediated suppression, but STAT5 activation in CD4<sup>+</sup> T cells did not. Consistent with those findings, gain-of-function experiments in which IL-2 was provided in the form of immunocomplexes of IL-2 and antibody to IL-2 showed population expansion of CD8<sup>+</sup> T cells and CD4<sup>+</sup> T<sub>reg</sub> cells but not of CD4<sup>+</sup> T<sub>eff</sub> cells<sup>28</sup>. Thus, while the ability to capture and compete for IL-2 was dispensable for T<sub>reg</sub> cell–mediated suppression of CD4<sup>+</sup> T cell responses, this mode of suppression appeared to be essential for the control of CD8<sup>+</sup> T cells, which responded to excessive IL-2 more robustly than did CD4<sup>+</sup> T cells.

**STAT5 activation in T<sub>reg</sub> cells boosts immunosuppression**

The lack of detectable STAT5 activation in response to IL-2 and of STAT5b-CA-driven population expansion of IL-2R-sufficient T<sub>reg</sub> cells that escaped Cre-mediated recombination (counter-selection) in both Rosa26Stat5bCAIl2rb<sup>h/h</sup>Foxp3<sup>Cre</sup> mice and Rosa26Stat5bCAIl2rb<sup>h/h</sup>Foxp3<sup>Cre</sup> mice indicated that the expression of an active form of STAT5 relieved T<sub>reg</sub> cells from their dependence on IL-2 signaling. This finding offered a unique opportunity to explore the biological importance of the aforementioned IL-2-dependent T<sub>reg</sub> cell–T<sub>eff</sub> cell regulatory network by uncoupling T<sub>reg</sub> cell function from IL-2 production by T<sub>eff</sub> cells. To address this issue, we generated Rosa26Stat5bCAFoxp3<sup>Cre</sup>–ERT2 mice, with tamoxifen-inducible expression of STAT5b-CA in differentiated T<sub>reg</sub> cells (via the tamoxifen-sensitive estrogen receptor variant ERT2<sup>27</sup>). Induction of STAT5b-CA expression in ~20–30% of T<sub>reg</sub> cells by a single administration of tamoxifen was followed by their rapid increase in number at the expense of T<sub>reg</sub> cells with a non-recombined Rosa26Stat5bCA allele (Supplementary Fig. 4a,b). It was notable that these cells exhibited a highly diverse use of the T cell antigen receptor (TCR) β-chain variable region similar to that in Rosa26<sup>h/h</sup>Foxp3<sup>Cre</sup>–ERT2 (control) mice (Supplementary Fig. 4c). The experimental Rosa26Stat5bCAFoxp3<sup>Cre</sup>–ERT2 mice remained healthy (Supplementary Fig. 4d,e). In these mice, the proliferated STAT5b-CA–T<sub>reg</sub> cell population had larger amounts of Foxp3, CD25, CLA-4 and GITR than those in STAT5b-CA<sup>+</sup> T<sub>reg</sub> cells in tamoxifen-treated Rosa26<sup>h/h</sup>Foxp3<sup>Cre</sup>–ERT2 mice and had a higher proportion of CD62L<sup>hi</sup>CD44<sup>hi</sup> cells than CD62L<sup>lo</sup>CD44<sup>lo</sup> cells (Fig. 3a–d and Supplementary Fig. 4f), indicative of a STAT5b-CA–imposed biasing of the T<sub>reg</sub> cell population toward an activated state or a memory state. Consistent with those possibilities, surface expression of IL-7R, the activation marker KLRG1 and CD103 was higher in STAT5b-CA<sup>+</sup> T<sub>reg</sub> cells in tamoxifen-treated Rosa26<sup>h/h</sup>Foxp3<sup>Cre</sup>–ERT2 mice than in STAT5b-CA<sup>+</sup> T<sub>reg</sub> cells in tamoxifen-treated Rosa26<sup>h/h</sup>Foxp3<sup>Cre</sup>–ERT2 mice (Fig. 3d). Notably, in the LNs and Peyer’s patches, the number
of Treg cells did not increase after the administration of tamoxifen, despite the predominance of STAT5b-CA+ Treg cells in the former mice (Supplementary Fig. 4b,g); this suggested that Treg cells with activated STAT5 are preferentially distributed in non-lymphoid tissues. The abundance of CD8+Foxp3+ cells also increased after the induction of STAT5b-CA (Supplementary Fig. 4b). The ‘autonomous’ Treg cells expressing active STAT5 showed heightened in vivo suppressor activity (Supplementary Fig. 4i) and effectively suppressed the basal state of activation and proliferative activity of CD4+ and CD8+ T cells in vivo as well, as indicated by the decreased number of Ki67+ cells and CD62LloCD44hi Treg cells and much larger CD62LloCD44lo naive T cell pool (Fig. 3c and Supplementary Fig. 5a,b). Accordingly, the production of pro-inflammatory cytokines, most prominently IL-4, by CD4+ T cells and expression of the costimulatory molecules CD80 and CD86 by B cells and dendritic cells (DCs) were reduced despite the predominance of STAT5b-CA+ Treg cells in the latter mice (Fig. 3d and Supplementary Fig. 5c). Serum IgA and IgE also showed a tendency toward a decrease, but this was not statistically significant (Supplementary Fig. 5d). These results were in agreement with the increase in TH17 responses and in both TH2-type immunoglobulin class switching and TH1-type immunoglobulin class switching observed upon acute ablation of Treg cells31.

Since altered intestinal immune responses have been linked to the promotion of colonic carcinogenesis, we explored an effect of a gain in Treg cell suppressor function afforded by activated STAT5 in the ApcMin model of colorectal cancer. Mice harboring the ApcMin mutation develop multiple adenomatous polyps in the small intestine22. ApcMin Rosa26Stat5bCAFoxp3CreERT2 mice developed a number of polyps similar to or fewer than that of ApcMin Foxp3CreERT2 mice, but the average polyp size was greater in ApcMin Rosa26Stat5bCAFoxp3CreERT2 mice than in ApcMin Foxp3CreERT2 mice (Supplementary Fig. 5e). These results were consistent with the idea that suppression of inflammation by Treg cells in tumor microenvironments promotes the growth of tumors once tumors or pre-cancerous lesions are already formed. However, the early stages of colonic carcinogenesis seemed to be not promoted but potentially suppressed by Treg cells with augmented suppressor activity.

In addition to restraining the basal immunological reactivity in physiological settings and modulating colon-carcinoma development, ‘autonomous’ Treg cells afforded superior protection against autoantigen-induced autoimmunity. We found that at 2–3 months after a single tamoxifen treatment, Rosa26Stat5bCAFoxp3CreERT2 mice were very resistant to experimental autoimmune encephalomyelitis...
induced by immunization with myelin oligodendrocyte glycoprotein peptide in complete Freund’s adjuvant (Fig. 4a-c). The frequency of CD4+Foxp3+ cells was significantly greater in the brain and spinal cord of these mice (Fig. 4b), and the infiltration of inflammatory cells, including neutrophils and IL-17-producing CD4+ Foxp3− cells, was also augmented in the presence of DCs (middle and right) or four (e) mice per group in each (a-c,e) or are pooled from four independent experiments with n = 11 (UI), n = 15 (Inf, Foxp3CreERT2) or n = 20 (Inf, Rosa26Stat5bCA Foxp3CreERT2) mice per group (d).

A distinct role for STAT5 activation in Treg cells

Next we sought to address the question of how sustained STAT5 signaling might potentiate Treg cells’ suppressive ability. In genetic loss- and gain-of-function studies, STAT5 activity in Treg cells correlated with their proliferative capacity and expression of IL-2Rα and Foxp3. However, the in vitro suppression assays reported above, as well as the diminished activation of the immune system in the LNs and Peyer’s patches of Rosa26Stat5bCA Foxp3CreERT2 mice, in which fewer Treg cells were found than in Foxp3CreERT2 mice, suggested that the enhanced immunosuppression observed in Rosa26Stat5bCA Foxp3CreERT2 mice was not simply due to a numerical increase of Treg cells but that their suppressor activity on a per-cell basis was also augmented. It was also unlikely that mild upregulation of Foxp3 expression in the presence of STAT5–CA could account for the increased suppressor activity of Treg cells, as genome-wide binding of Foxp3 does not change after Treg cells are activated, which leads to an increase in Foxp3 expression more pronounced than the one caused by STAT5–CA. The greater abundance of Foxp3 protein in STAT5–CA+ Treg cells than in STAT5–CA− Treg cells was particularly noticeable in the CD25+ Treg cell subset (average difference in mean fluorescence intensity of Foxp3 in Foxp3+ Treg cells from Rosa26Stat5bCA Foxp3CreERT2 mice versus that in those from Foxp3CreERT2 mice (n = 6): CD25+ cells, 1.06-fold; CD25+ cells, 1.36-fold; Fig. 3b), consistent with the observation that STAT5–CA+ Treg cells were relieved from their dependence on IL-2. Nevertheless, STAT5–CA+ Treg cells exhibited more potent suppressor function than that of CD25+Foxp3+ Treg cells from Rosa26Stat5bCA Foxp3CreERT2 (control) mice when transferred together with Teff cells into lymphopenic recipients, despite comparably high expression of Foxp3 (data not shown). Thus, the enhanced suppressor activity of STAT5–CA+ Treg cells was probably not due to the increase in Foxp3.

To gain insight into the potential mechanisms underlying the heightened suppressor function conferred by sustained activation of STAT5, we sorted mature Treg cells from Foxp3CreERT2 and Rosa26Stat5bCA Foxp3CreERT2 mice with comparable expression of Foxp3 and analyzed gene expression in these cells by high-throughput
sequencing technologies for cDNA (RNA-seq). While the gene-expression profiles of naive CD4+ T cells from both groups of mice were nearly identical, gene expression in Treg cells was markedly affected by the active form of STAT5 (Fig. 5a and Supplementary Fig. 6a). Among all genes expressed (~11,000) in either the Treg cell populations or naive CD4+ T cell populations analyzed, 342 genes

Figure 5 RNA-seq analysis of Treg cells expressing an active form of STAT5. (a) Principal-component analysis of RNA-seq data sets of Treg cells from Foxp3CreERT2 mice (Control Treg), STAT5b-CA-expressing Treg cells from Rosa26Stat5bCAFoxp3CreERT2 mice (Stat5bCA Treg), naive T cells from Foxp3CreERT2 mice (Control Tnaive) or naive T cells from Rosa26Stat5bCAFoxp3CreERT2 mice (Stat5bCA Tnaive), assessed using the 15% of genes with the highest variance, presented as principal component 1 (PC1) and principal component 2 (PC2). Each symbol represents a single mouse. (b) Gene expression (log2 normalized read count) in Treg cells from Foxp3CreERT2 mice (Control Treg) or STAT5b-CA-expressing Treg cells from Rosa26Stat5bCAFoxp3CreERT2 mice (Stat5bCA Treg); diagonal lines indicate change in expression of at least 1.5-fold (top line) or 0.67-fold (bottom line); colors indicate significant (adjusted P value, <0.05) upregulation (at least 1.5-fold; red) or downregulation (at least 1.5-fold; blue) of expression above a minimal threshold based on the distribution of all genes; numbers in plots indicate total genes upregulated (red) or downregulated (blue). (c) Expression of selected genes (right margin) in cells as in (a) (three replicates per cell subset (columns)), grouped by product function (left margin); P values (far right), Foxp3CreERT2 Treg cells versus Stat5bCA-CA-expressing Treg cells. (d) Empirical cumulative distribution function for the change in expression (log2 values) of all genes expressed in Stat5bCA-CA+ Treg cells (Expression change relative to that in Foxp3CreERT2 Treg cells) and for subsets of genes upregulated (Act up) or downregulated (Act down) by inflammatory activation in Treg cells33 (left) or the subsets of genes upregulated (TCR up) or downregulated (TCR down) in a TCR-dependent manner in CD44hi Treg cells34 (right). Numbers in parentheses (key) indicate total genes in each group. (e) Signaling-pathway-impact analysis of the pathways with the most significant enrichment for the expression of genes with related function from the Kyoto Encyclopedia of Genes and Genomes (left margin) showing enrichment among genes expressed differentially in Stat5bCA-CA+ Treg cells relative to their expression in Foxp3CreERT2 Treg cells, presented as net pathway perturbation (status of pathway: activated (positive values) or inhibited (negative values)) based on activating or inhibitory relationships of genes expressed differentially in the pathway; circle size is proportional to the degree of enrichment; right margin (Overlap), genes in the pathway expressed differentially in Stat5bCA-CA+ Treg cells versus control Foxp3CreERT2 Treg cells, divided by total genes in the pathway expressed either in Stat5bCA-CA+ Treg cells or Foxp3CreERT2 Treg cells; far right, false-discovery-rate (FDR)-adjusted global P value (reflecting both enrichment and perturbation). (f) Network analysis of gene-ontology term enrichment among genes significantly upregulated in Stat5bCA-CA+ Treg cells relative to their expression in Foxp3CreERT2 Treg cells; dashed outlines (added manually) indicate groups of similar gene-ontology terms (Supplementary Table 1); line thickness and color are proportional to the similarity coefficient between connected nodes; node color is proportional to the FDR-adjusted P value of the enrichment and node size is proportional to gene-set size.

Figure 6 RNA-seq analysis of Treg cells expressing an active form of STAT5. (a) Principal-component analysis of RNA-seq data sets of Treg cells from Foxp3CreERT2 mice (Control Treg), STAT5b-CA-expressing Treg cells from Rosa26Stat5bCAFoxp3CreERT2 mice (Stat5bCA Treg), naive T cells from Foxp3CreERT2 mice (Control Tnaive) or naive T cells from Rosa26Stat5bCAFoxp3CreERT2 mice (Stat5bCA Tnaive), assessed using the 15% of genes with the highest variance, presented as principal component 1 (PC1) and principal component 2 (PC2). Each symbol represents a single mouse. (b) Gene expression (log2 normalized read count) in Treg cells from Foxp3CreERT2 mice (Control Treg) or STAT5b-CA-expressing Treg cells from Rosa26Stat5bCAFoxp3CreERT2 mice (Stat5bCA Treg); diagonal lines indicate change in expression of at least 1.5-fold (top line) or 0.67-fold (bottom line); colors indicate significant (adjusted P value, <0.05) upregulation (at least 1.5-fold; red) or downregulation (at least 1.5-fold; blue) of expression above a minimal threshold based on the distribution of all genes; numbers in plots indicate total genes upregulated (red) or downregulated (blue). (c) Expression of selected genes (right margin) in cells as in (a) (three replicates per cell subset (columns)), grouped by product function (left margin); P values (far right), Foxp3CreERT2 Treg cells versus Stat5bCA-CA-expressing Treg cells. (d) Empirical cumulative distribution function for the change in expression (log2 values) of all genes expressed in Stat5bCA-CA+ Treg cells (Expression change relative to that in Foxp3CreERT2 Treg cells) and for subsets of genes upregulated (Act up) or downregulated (Act down) by inflammatory activation in Treg cells33 (left) or the subsets of genes upregulated (TCR up) or downregulated (TCR down) in a TCR-dependent manner in CD44hi Treg cells34 (right). Numbers in parentheses (key) indicate total genes in each group. (e) Signaling-pathway-impact analysis of the pathways with the most significant enrichment for the expression of genes with related function from the Kyoto Encyclopedia of Genes and Genomes (left margin) showing enrichment among genes expressed differentially in Stat5bCA-CA+ Treg cells relative to their expression in Foxp3CreERT2 Treg cells, presented as net pathway perturbation (status of pathway: activated (positive values) or inhibited (negative values)) based on activating or inhibitory relationships of genes expressed differentially in the pathway; circle size is proportional to the degree of enrichment; right margin (Overlap), genes in the pathway expressed differentially in Stat5bCA-CA+ Treg cells versus control Foxp3CreERT2 Treg cells, divided by total genes in the pathway expressed either in Stat5bCA-CA+ Treg cells or Foxp3CreERT2 Treg cells; far right, false-discovery-rate (FDR)-adjusted global P value (reflecting both enrichment and perturbation). (f) Network analysis of gene-ontology term enrichment among genes significantly upregulated in Stat5bCA-CA+ Treg cells relative to their expression in Foxp3CreERT2 Treg cells; dashed outlines (added manually) indicate groups of similar gene-ontology terms (Supplementary Table 1); line thickness and color are proportional to the similarity coefficient between connected nodes; node color is proportional to the FDR-adjusted P value of the enrichment and node size is proportional to gene-set size.
were upregulated and 314 genes were downregulated in STAT5b-CA+ Treg cells relative to their expression in STAT5b-CA− Treg cells (Fig. Sb) and Supplementary Fig. 6b). The gene set upregulated in STAT5b-CA+ Treg cells encoded various cell-surface molecules and receptors involved in cell adhesion, migration and cytoskeletal reorganization (Fig. Sc). Several genes that were upregulated or downregulated in STAT5b-CA− Treg cells relative to their expression in naive T cells showed an opposite trend in STAT5b-CA+ Treg cells (Fig. Sc), which suggested that STAT5b-CA did not simply reinforce the Treg cell signature. A published study has shown that exposure of Treg cells to inflammation induced by transient depletion of Treg cells leads to a marked change in their gene expression and a potent increase in their suppressor function30. Consistent with the heightened suppressor function of STAT5b-CA+ Treg cells, we found that the gene-expression changes in these cells conferred by the active form of STAT5 correlated with those found in highly activated Treg cells in inflammatory settings (Fig. Sd). TCR signaling is required for the ability of Treg cells to exert their suppressor function14,35. Thus, it was possible that the TCR- and STAT5-dependent signaling pathways in Treg cells were acting on a largely overlapping set of genes whose expression they jointly regulated to potentiate the suppressor activity of Treg cells. However, our analysis revealed that the gene set affected by the
active form of STAT5 was distinct from that expressed in Treg cells in a TCR-dependent manner (Fig. 5d). Thus, both the TCR signaling pathway and STAT5 signaling pathway served an indispensable role in the suppressor activity of Treg cells in vivo by controlling largely distinct sets of genes and probably distinct aspects of Treg cell suppressor activity.

To better understand aspects of Treg cell function potentiated by activation of STAT5, we performed analysis of signaling pathways and molecular-function enrichment. This revealed over-representation of gene sets encoding products involved in cell-cell and extracellular matrix interactions, cell adhesion and cellular locomotion among genes expressed differentially in STAT5b-CA+ Treg cells relative to their expression in STAT5b-CA- Treg cells (Fig. 5e,f and Supplementary Fig. 6c). This result suggested that in Treg cells, activation of STAT5 might potentiate their interactions with the target cells.

Since intravital imaging of Treg cells in vivo has revealed their stable interactions with DCs in vitro, we assessed the potential effect of constitutively active STAT5 in Treg cells on their ability to form conjugates with DCs in vitro. In agreement with the gene-set-enrichment analysis, we found that STAT5b-CA expression in Treg cells promoted the formation of conjugates of Treg cells and DCs (Fig. 6a). These enhanced interactions of STAT5b-CA+ Treg cells with DCs in vitro were consistent with the decreased expression of co-stimulatory molecules by DCs observed in tamoxifen-treated Rosa26Stat5bCAFoxp3Cre-ERT2 mice (Fig. 3f).

The findings reported above raised the question of whether activation of STAT5 can potentiate the suppressor function of Treg cells in a TCR-independent manner. To investigate this, we analyzed Rosa26Stat5bCAFoxp3Cre-ERT2 mice with a Tcra null allele. Tamoxifen-inducible Cre-mediated ablation of Tcrα (and thus the TCR) in Treg cells is highly efficient in these mice and results in activation of the immune system that results from impaired suppressor function34. In heterozygous Tcrafl/wtFoxp3Cre-ERT2 mice, Cre-mediated recombination can theoretically result in ablation of the TCR in up to a half of Treg cells due to allelic exclusion at the Tcra locus. We observed a small proportion of TCR-deficient Treg cells in these mice after 2 weeks of tamoxifen administration (Fig. 6b). Although expression of the active form of STAT5 was observed in ~50% of TCR-deficient and TCR-competent Treg cells in Tcrafl/wtRosa26Stat5bCAFoxp3Cre-ERT2 mice, the proportion of only TCR-sufficient STAT5b-CA+ Treg cells, not TCR-deficient STAT5b-CA- Treg cells, was greater in these mice than in Tcrafl/wtFoxp3Cre-ERT2 mice (Fig. 6b). The marked increase in T cell activation and pro-inflammatory cytokine production was mitigated in part by expression of the active form of STAT5 in tamoxifen-treated Tcrafl/Rosa26Stat5bCAFoxp3Cre-ERT2 mice (Fig. 6c). This partial recovery of Treg cell suppressor function by the active form of STAT5 in Treg cells that had undergone ablation of the TCR was also confirmed in experiments in which flow-cytometry-sorted TCR-deficient STAT5b-CA+ Treg cells and Teff cells were adoptively transferred into lymphopenic recipients (Fig. 6d).

Since the ‘rescue’ was incomplete, these results suggested that enhanced STAT5 signaling potentiated the suppressor activity of Treg cells in the absence of contemporaneous TCR-dependent signals. Indeed, some features of Treg cells that had been observed in TCR-sufficient STAT5b-CA+ Treg cells were still present in STAT5b-CA- Treg cells that had undergone TCR ablation (Fig. 6c,d). However, we note that STAT5b-CA expression failed to restore suppressor function in Tcrafl/Rosa26Stat5bCAFoxp3Cre mice in which TCR deletion occurred immediately after the induction of Foxp3 (data not shown). It has been shown that TCR signaling is required for the acquisition of an activated, antigen-experienced phenotype and suppressor function by Treg cells34. Thus, our results suggested that activation of STAT5 potentiated TCR-independent suppressor function in mature Treg cells that had already undergone TCR-dependent maturation. That observation is reminiscent of the sequential requirements for these two signals, via the TCR and IL-2R, in the differentiation of Treg cells in the thymus, where STAT5 signaling promotes differentiation of Treg cell precursors that have experienced permissive TCR signaling57.

DISCUSSION

Published analysis of mice with germline deficiency in IL-2 and IL-2R subunits have demonstrated that IL-2 is a key cytokine required for the induction of Foxp3 expression and the differentiation of Treg cells in the thymus5–11. Furthermore, antibody-mediated neutralization of IL-2 and provision of IL-2 in the form of immunocomplexes of IL-2 and antibody to IL-2, as well as genetic delineation of regulatory elements within the Foxp3 locus, have revealed an important role for IL-2 in the maintenance of mature Treg cells and in the stabilization of Foxp3 expression during their extrathymic differentiation16,28,38. Such findings have raised the question of whether IL-2R signaling can also directly promote the suppressor ability of Treg cells and can therefore serve as a critical nexus linking the differentiation and maintenance of Treg cells with their suppressor function. A published in vitro study has proposed a role for IL-2 signaling on the basis of indirect evidence31. In addition, consumption of IL-2 by Treg cells has been suggested to serve an essential role in the suppressor function of Treg cells by causing the death of activated CD4+ T cells due to IL-2 deprivation20–24. On the other hand, other reports have suggested that IL-2R is dispensable for the ability of Treg cells to suppress the proliferation of effector T cells8,39. Furthermore, the rescue of Il2ra−/− and Il2rb−/− mice from disease observed after adoptive transfer of wild-type Treg cells suggested the existence of major mechanisms of Treg cell–mediated suppression that are independent of IL-2 deprivation67. However, the last studies left open the major question of whether IL-2 consumption by Treg cells is essential for the suppression of IL-2R-sufficient Teff cells, since IL-2 is probably a main driver of autoimmune disease in the absence of functional Treg cells.

A chief limiting factor in efforts to experimentally assess a role for IL-2R signaling in and IL-2 consumption by Treg cells in their function in vivo has been the lack of adequate genetic tools. We addressed this issue through the conditional deletion of Il2ra and Il2rb alleles and ablation of the expression of these genes in Treg cells in combination with induced expression of an active form of STAT5. These new genetic tools enabled us to unequivocally demonstrate a cell-intrinsic, essential role for IL-2R signaling not only in the maintenance of mature Treg cells and their fitness but also in their suppressor function. Furthermore, we found that STAT5 deficiency in Treg cells resulted in a similar loss of suppressor function and that expression of an active form of STAT5 rescued mice from the lethal disease that results from IL-2R deficiency. These results suggested a key role for IL-2R–STAT5 signaling in linking the differentiation and maintenance of Treg cells and their function. STAT5 binds to the Foxp3 promoter and the intronic Foxp3 regulatory element CNS2 and is involved in the induction and maintenance of Foxp3 expression38. Complexes of the transcription factors Runx and CBFβ also bind to CNS2 and the Foxp3 promoter and affect Foxp3 expression40. Although both CNS2-deficient Treg cells and CBFβ-deficient Treg cells do exhibit reduced Foxp3 expression resembling that of STAT5- or IL-2R-deficient Treg cells, the impairment of suppressor function in the last is much more severe. Thus, the decrease in Foxp3 expression alone could not account for the severe loss of Treg cell suppressor function in the absence of STAT5 or IL-2R. Indeed, our analysis of...
gene expression and functional features conferred by expression of the active form of STAT5 pointed to a heightened ability of Treg cells to bind to DCs and suppress their activation. Furthermore, expression of an active form of STAT5 partially ‘rescues’ the nearly complete loss of Treg cell suppressor function in the absence of TCR signaling.34,35. Such results might appear to be at odds with the published finding that a transgene encoding STAT5b-CA driven by the proximal Lck promoter and Eμ enhancer fails to curtail fatal lymphoproliferative disease in Il2rb−/− mice despite restoring Foxp3 expression and Treg cell differentiation in the thymus.5 However, the interpretation of the last result is problematic due to the massive population expansion of pre-leukemic T cells and B cells and reduced expression of the STAT5b-CA-encoding transgene in peripheral Treg cells.

Our studies have demonstrated that depriving Treg cells of IL-2 by Treg cells was fully dispensable for the suppression of IL-2R-sufficient CD4+ T cells, even though IL-2R signaling was required. However, IL-2R-dependent consumption of IL-2 by Treg cells was indispen-sable for the suppression of CD8+ T cell responses. The last, seemingly unexpected finding makes sense given the observed exquisite sensitivity of both naive CD8+ T cells and activated CD8+ T cells to IL-2-induced stimulation. Furthermore, IL-2 is produced upon activation of both naive CD4+ T cells and CD8+ T cells within hours of TCR engagement, in contrast to the production of effector cytokines such as IL-4 and IFN-γ, which requires the differentiation of naive T cells into Treg cells on a much longer time scale.41. Such distinguishing features provide a likely explanation for the need for a distinct mechanism for the control of CD8+ T cell responses by Treg cells through IL-2 consumption.

It has been suggested that sensing of local IL-2 production by Treg cells enables ‘licensing’ of their suppressor function.21. However, the ‘rescue’ of the suppression of CD4+ T cell responses by IL-2R-deficient Treg cells expressing an active form of STAT5 suggested that activated Treg cells can suppress autoimmunity without identifying the cellular source of IL-2. Thus, while IL-2 is a booster for the suppressor function of Treg cells, it might not have an indispensable role as a cue for specific targeting.

Genetically modified T cells are emerging as a potent means of therapy in some forms of cancer. The observed enhanced suppressor activity of Treg cells expressing an active form of STAT5 and significantly reduced severity of organ-specific autoimmunity in their presence would suggest that such modification of Treg cells might hold promise for the optimal design of Treg cell–based therapies for a variety of autoimmune and inflammatory disorders and organ transplantation.

Our findings have highlighted a central role for IL-2R-signaling-driven activation of STAT5 in supporting and boosting the suppressor function of differentiated Treg cells. In this context, it is noteworthy that although a Foxp3 ortholog has not been identified in birds, CD4+ T cell subsets with high expression of IL-2Rα have in vitro suppressor activity in chicken and ducks.42,43. This suggests the importance of evolutionary conservation of IL-2Rα function in suppressive T cells.

**METHODS**

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

**Accession codes.** GEO: microarray data, GSE84553.

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

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**AUTHOR CONTRIBUTIONS**

T.C., J.D.F. and A.Y.R. conceived of the project, designed the experiments and wrote and edited the manuscript; T.C., A.K.K., A.G.L., X.F., Y.Z., G.G. and Y.F. conducted experiments; U.K. generated the Il2rbfl allele; and J.D.F. generated the Il2rbfl allele.

**COMPETING FINANCIAL INTERESTS**

The authors declare competing financial interests: details are available in the online version of the paper.

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**ONLINE METHODS**

**Mice.** Foxp3\(^{Cre}\) and Foxp3\(^{Cre-ERT2}\) mice were described previously\(^{17,44}\). Il2ra\(^{−/−}\) mice were generated by J.D.P. Stat5a/b\(^{−/−}\) mice were provided by L. Henningshausen. Apc\(^{Min}\) mice were purchased from the Jackson Laboratory. The targeting strategies to generate Il2rb\(^{−/−}\) (generated by UK) and Rosal2\(^{Cre}\) alleles are shown in **Supplementary Figure 7**. Tcra\(^{−/−}\) mice were described previously\(^{45}\). The experimental mice were either generated on or backcrossed onto a C57BL/6J (B6) background, bred and housed in the specific pathogen-free animal facility at Memorial Sloan Kettering Cancer Center. All animal experiments were approved by institutional animal care and use committee at Memorial Sloan Kettering Cancer Center and were performed in accordance with the institutional guidelines. For survival analysis, mice were monitored daily; unhealthy mice were euthanized once they were found lethargic and were counted as non-survivors. For tamoxifen treatment, tamoxifen (Sigma-Aldrich) was dissolved in olive oil at a concentration of 40 mg/ml. Mice were given oral gavage of 100 μl of tamoxifen emulsion per treatment. In experimental autoimmune encephalomyelitis and infection experiments, mice were challenged 2–3 months after a single gavage of tamoxifen and assessed as described previously\(^{46}\).

**Flow cytometry and cell sorting.** Cells were stained with fluorescence-tagged antibodies purchased from eBioscience, BD Biosciences, Tonbo Biosciences or BioLegend (**Supplementary Table 2**) and analyzed using a BD LSR II flow cytometer. Flow cytometry data were analyzed using FlowJo software (TreeStar). For intracellular cytokine staining, cells were stimulated for 5 h with antibodies to CD3 and CD28 (5 μg/ml each) in the presence of brefeldin A or monensin, harvested and stained with eBioscence Fixation Permeabilization kit. For intracellular phosphorylated STAT5 staining, cells were stimulated with or without rmIL-2 for 20 min, fixed and permeabilized with 4% PFA followed by 90% methanol, and stained with anti-pY-STAT5 antibody (BD Biosciences). Cell sorting of Foxp3\(^{+}\) and Foxp3\(^{−}\) cells was performed based on YFP or GFP expression using a BD FACSaria II cell sorter.

**Listeria and vaccinia infection.** Mice were given intravenously injection of *L. monocytogenes* (LM104035; 2000 cells/mouse) into the tail vein on day 0 and analyzed on day 8. For the detection of *L. monocytogenes*–specific immune responses, splenic DCs from unchallenged B6 mice sorted using CD11c microbeads (Miltenyi) were cultured in wells of a 96-well U-bottom plate (2 x 10\(^{5}\) cells/well) with heat-killed *L. monocytogenes* (2 x 10\(^{7}\) cells/well) for 6 h before the analysis. The cells were then co-cultured with splenic T cells obtained from *L. monocytogenes*–infected mice (1 x 10\(^{6}\) cells/well) for 5 h in the presence of brefeldin A, and cytokine-producing T cells were detected by flow cytometry. For vaccinia virus infection, mice were given intravenous injection of non-replicating virus (5 x 10\(^{7}\) PFU/mouse) on day 0 and vaccinia infection.

**In vivo IL-2 neutralization.** Mice were given intraperitoneal injection of a cocktail of two different anti-IL-2 monoclonal antibodies, JES6-1 and S4B6-1 (BioXcell), or isotype-matched control antibody (rat IgG2a, 2A3; BioXcell), 200 μg each, twice a week, starting from 5–7 d after birth.

**TAT-Cre treatment of T cells.** For the induction of STAT5b-CA expression in non-Treg cells, 1 x 10\(^{6}\) CD4\(^{+}\)Foxp3\(^{−}\) or CD8\(^{+}\)Foxp3\(^{−}\) T cells sorted from the LNs and spleens of Foxp3\(^{Cre}\) and *Rosa26Stat5bCA/Foxp3\(^{Cre-ERT2}\) mice were resuspended in 2 ml of serum-free RPMI medium containing a TAT-Cre recombinase (Millipore, 50 μg/ml) and incubated at 37 °C for 45 min. The cells were washed with RPMI containing 10% FCS, resuspended in PBS, and injected into T cell–deficient (Tcrb\(^{−/−}\)Tcra\(^{−/−}\)) mice together with or without separately sorted Treg cells for in vivo suppression assay.

**In vitro IL-2 capture assay.** Pooled cells from LNs and spleens were depleted of B cells and accessory cells by panning and T cells were enriched. The cells were stained with anti-CD8 and anti-B220 (**Supplementary Table 2**), and CD4\(^{+}\) Treg cells were sorted on the basis of GFP (YFP) expression alone in CD8−negative population. The sorted cells were divided among eight wells of a 96-well V-bottomed plate (2 x 10\(^{5}\) cells/well) in 25 μl RPMI medium (10% FCS) with or without increasing doses of recombinant human IL-2 (0.016 to 12 U/ml), 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 T cell–DC conjugation assay.** Treg cells and non-Treg cells were sorted in the same manner as in the IL-2 capture assay. Splenic CD11c\(^{+}\) DCs were isolated by magnetic-activated cell sorting from B6 mice given injection of Flt3L-secreting B16 melanoma cells. Treg and non-Treg cells were stained with CFSE. DCs were stained with CellTrace Violet (Molecular Probes). 1 x 10\(^{5}\) Treg or non-Treg cells were cultured together with graded numbers of DCs (1 x 10\(^{5}\) to 1 x 10\(^{7}\)) in a 96-well round-bottomed plate for 720 min in the presence or absence of rmIL-2 (100 IU/ml). Frequencies of Treg cells conjugated with DCs (% CTYV\(^{+}\)CFSE\(^{−}\)/CFSE\(^{−}\)) were analyzed by flow cytometry.

**In vitro suppression assay.** Naive CD4\(^{+}\) T cells (responder cells) and Treg cells were purified by flow cytometry and stained with CellTrace Violet. 4 x 10\(^{4}\) naive CD4\(^{+}\) T cells were cultured with graded numbers of Treg cells in the presence of 1 x 10\(^{5}\) irradiated, T cell–depleted, CFSE-stained splenocytes and 1 μg/ml anti-CD3 (145–2C11, Bioxcell) in a 96-well round-bottom plate for 80 h. Cell proliferation of responder T cells and Treg cells (live CFSE\(^{−}\)CD4\(^{+}\)Foxp3\(^{−}\) and Foxp3\(^{+}\)) was determined by flow cytometry based on the dilution of fluorescence intensity of CellTrace Violet of the gated cells.

**Measurement of serum and fecal immunoglobulin.** Serum IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA were measured by ELISA using SBA Clonotyping System (Southern Biotech). IgE ELISA was performed using biotinylated anti-IgE (R35–118, BD Biosciences) and HRP-conjugated streptavidin. For measurement of fecal IgA, fresh fecal pellets were collected and dissolved in extraction buffer (7 μl per mg pellet) containing 50 mM Tris-HCl, 150 mM NaCl, 0.5% NP-40, 1mM EDTA, 1 mM DTT, and protease inhibitor cocktail (Complete mini; Roche). Supernatants were collected after centrifugation, titrated, and IgA was measured by ELISA.

**Statistical analysis for animal experiments.** Each mouse was tagged with a unique identification number, and researchers were blinded to the genotypes of mice, except for adjustment of sample size included in a single experiment and after data analysis was completed. Wild-type mice with suspected congenital anomalies were excluded from the study. Cell samples that showed less than 70% cell vitality after preparation or after in vitro stimulation were excluded from the study. Statistical analyses were performed using Prism software with two-tailed unequal paired Student’s t-test. Welch’s correction was applied when F-test was positive. P values of <0.05 were considered significant.

**RNA sequencing.** Male 8-week-old *Rosa26Stat5bCA/Foxp3\(^{Cre-ERT2}\) (STAT5CA) and Foxp3\(^{Cre-ERT2}\) (control) mice, nine mice for each experimental group, received a single dose (4 mg) of tamoxifen by oral gavage 4 months before isolation. Splenic CD4\(^{+}\)Foxp3\(^{−}\)/YFP/GFP) + GTRI\(^{−}\)CD125\(^{hi}\) Treg and CD4\(^{+}\)Foxp3\(^{−}\)/YFP/GFP) CD62L\(^{hi}\)CD45\(^{R}\) naive T cells were double sorted using a BD FACSaria II cell sorter, and a total of 12 samples were generated. Spleen T cell subsets isolated from three individual mice in the same experimental group were pooled into one biological replicate; three biological replicates were subjected to RNA-seq analysis for each experimental group. Total RNA was extracted and used for poly(A) selection and Illumina TruSeq paired-end library preparation following manufacturer's protocols. Samples were sequenced on the Illumina HiSeq 2500 to an average depth of 27.5 x 10\(^{6}\) bp per pair per sample. All samples were processed at a same time and sequenced on the same lane to avoid batch effects.

Read alignment and processing followed a method previously described\(^{45}\). Raw reads were trimmed using Trimmomatic v0.32 with standard settings to remove low-quality reads and adaptor contamination\(^{46}\). The trimmed reads were then aligned to the mouse genome (Ensemble assembly GRCm38) using TopHat2 v2.0.11 implementing Bowtie2 v2.2.2 with default settings. Read alignments were sorted with SAMtools v0.1.19 before being counted to genomic features using HTSeq v0.6.1p1. The overall read alignment

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rate across all samples was 74.5%. Differential gene expression was analyzed using DESeq2 1.6.3 in R (version 3.1.0)47.

**Bioinformatics analysis of RNA-seq.** The distribution of read counts across all genes was bimodal. The assumption that this corresponded to ‘expressed’ and ‘non-expressed’ genes was supported by examination of marker genes known to be expressed or not expressed in Treg cells and naïve T cells. The local minimum between the two peaks was chosen to be the threshold for expression. Using this threshold of ~60 normalized reads, 10,589 of 39,179 genes were called as present. Genes significantly upregulated (342) or downregulated genes (314) in STAT5b-CA Treg cells relative to their expression in control Treg cells were defined as expressed genes with a change in expression of at least 1.5-fold or 0.67-fold, respectively, and a FDR-adjusted P value of ≤0.05.

‘TCR-upregulated’ (i.e., TCR-dependent) genes were defined as genes downregulated (a change in expression of at least 0.5-fold) in TCR-deficient relative to their expression in TCR-sufficient CD44hiTreg cells, while ‘TCR-downregulated’ genes were upregulated (at least 1.75-fold; Padj ≤ 0.001) in TCR-deficient CD44hi Treg cells (GSE6107734). ‘Activation-upregulated’ genes were genes upregulated (twofold change; P adj ≤ 0.01) in Treg cells from Foxp3DTR mice recovering from transient depletion of Treg cells (GSE55753)33.

Signaling pathway impact analysis was performed using the R package of the same name48. Genes significantly up- and downregulated, and their changes in expression, were analyzed as one set for enrichment and perturbation of 90 Mus musculus KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways accessed on 5 October 2015. The network was visualized using the default ‘Prefuse Force-Directed Layout’ in Cytoscape with default settings and 500 iterations. Groups of similar GO terms were manually circled.

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