Regulatory T Cell Function in Established Systemic Autoimmune Inflammation: Reversal and Containment of a Fatal Disease

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Regulatory T Cell Function in Established Systemic Autoimmune Inflammation: Reversal and Containment of a Fatal Disease

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The immunosuppressive function of regulatory T (Treg) cells is dependent on continuous expression of the transcription factor Foxp3. Foxp3 loss-of-function or induced ablation of Treg cells results in a fatal autoimmune disease featuring all known types of inflammatory responses with every manifestation stemming from Treg cell paucity, highlighting a vital function of Treg cells in preventing fatal autoimmune inflammation. However, a major question remains whether Treg cells can persist and effectively exert their function in a disease state, where broad spectrum of inflammatory mediators can either inactivate Treg cells or render innate and adaptive pro-inflammatory effector cells insensitive to suppression. By reinstating Foxp3 protein expression and suppressor function in cells expressing a reversible Foxp3 null allele in severely diseased mice, we found that the resulting single pool of “redeemed” Treg cells normalized immune activation, quelled severe tissue inflammation, reversed fatal autoimmune disease and provided long-term protection against them. Thus, Treg cells are functional in settings of established broad spectrum systemic inflammation and are capable of affording sustained reset of the immune homeostasis.
Regulatory T (Treg) cells expressing the X chromosome-encoded transcription factor Foxp3 have been implicated in the control of inflammation in diverse physiological and pathological settings\textsuperscript{1-9}. Mice and humans lacking functional Foxp3 gene develop fatal autoimmune and inflammatory disease affecting skin, lung, endo- and exocrine glands, and gastro-intestinal tract, autoimmune anemia, lymphadenopathy and splenomegaly, eosinophilia, hyper IgE syndrome, and markedly increased systemic levels of a wide range of pro-inflammatory cytokines, including IL-6, TNF\textsubscript{α}, IL-4, IL-5, IL-13, IFN\textsubscript{γ}, IL-17, IL-23, and GM-CSF\textsuperscript{1-3}. Foxp3 protein expression is required for Treg cell differentiation\textsuperscript{4-7}. Previous analyses of mice harboring a functional Foxp3\textsuperscript{GFP} reporter and a Foxp3\textsuperscript{GFPKO} reporter null allele revealed a critical requirement for Foxp3 protein for Treg cell suppressor function. Foxp3-deficient GFP\textsuperscript{+} Treg “wannabes”, while exhibiting considerable similarity to their Foxp3-sufficient GFP\textsuperscript{+} counterparts, are devoid of suppressor capacity\textsuperscript{8,9}. Accordingly, retroviral expression of a Foxp3 transgene in Treg “wannabes” confers characteristic Treg cell gene expression and suppressor function\textsuperscript{10}. Foxp3-deficient mice present with marked immune activation, increased pro-inflammatory cytokines, IgE and IgG1 levels, blepharitis, dermatitis, and lymphadenopathy by day 10 of life with the majority of mice succumbing to the disease by 3-4 wks of age\textsuperscript{5,11,12}. Likewise, Treg cell ablation induced upon diphtheria toxin (DT) treatment of healthy adult Foxp3\textsuperscript{DTR-GFP} mice, which express a simian diphtheria toxin receptor (DTR)-GFP fusion protein encoded by the endogenous Foxp3 locus, leads to similar widespread autoimmune inflammation to which they succumb within 2 wks\textsuperscript{13}. Adoptive transfer of wild-type Treg cells into Foxp3\textsuperscript{DTR-GFP} mice at the time of DT administration or into 1-day-old Foxp3 mutant mice prevents the disease\textsuperscript{5,13}. These studies have unequivocally demonstrated a critical role of Treg cells in preventing autoimmunity and inflammation and associated pathologies.

However, a major outstanding issue is whether Treg cells can effectively function in settings of established systemic inflammation and are capable of reversing and containing severe autoimmune diseases. In fact, a large body of work in experimental animal models of infection, inflammation, and cancer suggests that major immune effector cytokines, including IL-6, IL-1, type I and II IFNs, IL-23, and IL-4, can cause 1) loss of Foxp3 expression in, functional inactivation of, and acquisition of pro-inflammatory features by Treg cells, or 2) make immune effector cells refractory to Treg-mediated suppression\textsuperscript{14-20}. Accordingly, numerous analyses of Treg cells present at the inflammatory sites of autoimmune patients suggested that their function is impaired\textsuperscript{21-25}. Additional uncertainty stems from a possibility that the three archetypal inflammatory immune responses (types 1, 2, and 3), recognized based on their distinct spectra
of secreted and cellular effectors, may differ in their ability to compromise Treg functionality or in their sensitivity to, and requirements for, Treg mediated suppression. In this regard, variation in Treg cell frequencies was reported to result in a disproportionate dysregulation of type 2 vs. type 1 autoimmunity. Thus, to address the major outstanding question above, we sought to employ an experimental animal model which would: 1) exhibit systemic mixed type 1, 2, and 3 autoimmunity and tissue inflammation; 2) enable temporal control and efficient switching of Treg suppressor function in an established disease; 3) eschew adoptive transfer of Treg cells, which does not guarantee their migration to and accumulation at the inflammatory sites; and 4) allow for an extended longitudinal observation.

We reason that the cardinal features of the aforementioned archetypal inflammatory responses are shared by a broad spectrum of autoimmune and inflammatory diseases. The breadth and severity of the autoimmune inflammatory disease resulting from Foxp3 deficiency, characterized by the activation and expansion of all major immune cell types (Th1, Th2, Th17, CD8, B cells, monocytes, neutrophils, eosinophils, etc.) and rampant cytokine storm (IFNγ, IL-4, IL-5, IL-17, IL-6, TNFα, GM-CSF, IL-1β), make it an ideal experimental setting for testing the ability of Treg cells to operate in established inflammation. By engineering mice where an inducible Cre recombinase allowed for the installation of Treg cells in severely diseased animals, we found that Treg cells were functional in settings of established systemic mixed type 1, 2, and 3 inflammation and capable of resetting immune homeostasis and restoring health. Upon sensing the inflammatory environment, Treg cells became activated, rapidly expanded, and exhibited heightened suppressive capacity. These Treg cells stably persisted for an extended period of time with no signs of dysfunction or sexhaustion and were enriched for a sub-polulation featuring enhanced self-renewing properties.

Results
Restoration of Foxp3 expression in Treg “wannabe” cells
To investigate the ability of Treg cells to function in settings of established severe autoimmune inflammation, we generated mice harboring a reversible Foxp3^{loxP-Thy1.1-STOP-loxP-GFP} reporter null allele (Foxp3^{LSL}). In these mice, a loxP site-flanked Thy1.1 reporter coding sequence followed by a STOP cassette was inserted into the Foxp3 locus in front of the Foxp3 coding sequence preceded by a GFP reporter (Extended Data Fig. 1a, b). Since Foxp3 transcription was halted by the STOP cassette, effectively creating a null allele, expression of Thy1.1 marked a population of Treg “wannabe” cells in these mice (Extended Data Fig. 1c, d). These cells were
similar to the aforementioned cells expressing the Foxp3\textsuperscript{GFPKO} allele\textsuperscript{8}, with phenotypic features distinct from either naïve or activated conventional CD4 T cells, or Foxp3-sufficient Treg cells (Extended Data Fig. 1e). Combining the Foxp3\textsuperscript{LSL} allele with a Cd4 allele expressing a tamoxifen-inducible Cre-ERT2 fusion protein (Cd4\textsuperscript{creERT2}) enabled Cre-mediated excision of the STOP cassette upon 4-hydroxytamoxifen (4-OHT) treatment and conversion of Foxp3-deficient Thy1.1\textsuperscript{+} cells into Foxp3-sufficient GFP\textsuperscript{+} Treg cells (Fig. 1a and 1b). The latter, when co-transferred with effector Foxp3\textsuperscript{-} CD4 T cells into lymphopenic recipients, were able to suppress the wasting disease similar to Treg cells isolated from control Foxp3\textsuperscript{GFP} mice (Extended Data Fig. 1f). Thus, re-expression of Foxp3 in Treg “wannabes” confers suppressor function and fitness in accordance with previous findings.

**Reversal of established autoimmune inflammation in young mice by Treg cells**

Hemizygous male Foxp3\textsuperscript{LSL} mice developed early onset, multi-organ autoimmune inflammation indistinguishable from that of Foxp3-deficient mice and succumbed to the disease within 3-4 wks with few mice surviving for up to 6 wks after birth. To test whether Treg cells can suppress ongoing inflammation, male Foxp3\textsuperscript{LSL}Cd4\textsuperscript{creERT2} or Foxp3\textsuperscript{LSL}Cd4\textsuperscript{wt} mice were administered with a single dose of 4-OHT at 2 wks of age, when they exhibited pronounced autoimmune syndrome with frank clinical manifestations including blepharitis and macroscopic skin lesions. By measuring 4-OHT induced Cre recombinase activity in Treg cells in mice simultaneously treated with the S1P receptor antagonist FTY720, we assessed the recombination efficiency in the absence of the confounding effects of ongoing thymic output and T cell recirculation, which were abolished upon S1P receptor blockage. We found that the recombination efficiency was comparable in the secondary lymphoid organs, lung, and liver on day 3 following 4-OHT administration (Extended Data Fig. 2a). Within 4 wks after 4-OHT administration, the disease in Foxp3\textsuperscript{LSL}Cd4\textsuperscript{creERT2} mice was greatly reduced with the extent of activation of innate and adaptive immune cells approaching the level of healthy Foxp3\textsuperscript{DTR-GFP}Cd4\textsuperscript{creERT2} controls. In contrast, the disease in 4-OHT treated control Foxp3\textsuperscript{LSL}Cd4\textsuperscript{wt} mice rapidly progressed unabatedly with prominent splenomegaly, lymphadenopathy and thymic atrophy; none of the mice survived beyond 6 wks of age (Fig. 1c-e; Extended Data Fig. 2b-f).

4-OHT treatment led to the recombination of the Foxp3\textsuperscript{LSL} allele in CD4 expressing cells in the thymus and the periphery. Therefore, the observed reversal of inflammation could be due to either transient restoration of thymic Treg cell output following Foxp3 restoration in double positive and CD4 single positive thymocytes or due to conversion of peripheral Treg “wannabe”
cells into Treg cells. To assess whether rescue of the peripheral pool of Treg cells in disease settings is sufficient to restrain immune activation and inflammation, we induced Foxp3 expression in Treg “wannabe” cells upon 4-OHT treatment in the presence of continuous administration of FTY720 (Extended Data Fig. 3a). We observed diminished immune activation and inflammation in Foxp3LSL Cd4creERT2 mice, compared to Foxp3LSL Cd4wt controls, on day 14 following combined 4-OHT and FTY720 treatment, suggesting that peripheral “redeemed” Treg cells were effective at controlling inflammation and could rescue disease in the absence of thymic output (Extended Data Fig. 3b-c).

Detailed kinetic analysis revealed that aggressive lymphoproliferation and activation of conventional CD4 (Foxp3 Thy1.1) and CD8 T cells were suppressed within a wk after 4-OHT-induced restoration of Treg cell function, as evidenced by reduced expression of Ki67, CD44, CD25, IFNγ, and IL-4 (Fig. 1d; Extended Data Fig. 2d-e). Likewise, myelo-proliferation marked by expansion of neutrophils, monocytes and eosinophils also retreated (Fig. 1e; Extended Data Fig. 2f). During the course of the disease, control Foxp3LSL Cd4wt mice displayed increasingly severe acute phase response reflected in high levels of serum amyloid P (SAP) and haptoglobin, both of which were brought to basal levels in treated Foxp3LSL Cd4creERT2 mice (Fig. 1f and data not shown). In addition, levels of IgE, IgG1 and IgM, sharply increased in control Foxp3LSL Cd4wt mice, were brought down by newly minted Treg cells in Foxp3LSL Cd4creERT2 mice (Fig. 1g, Extended Data Fig. 2g). Histopathological analysis of the skin, lung and liver showed manifest immune infiltration and lymphocytic and myeloid inflammation in both control Foxp3LSL Cd4wt and untreated Foxp3LSL Cd4creERT2 mice; however, the tissue pathology disappeared in Foxp3LSL Cd4creERT2 mice within 4 wks after 4-OHT administration (Fig. 1h and 1i). A closer examination of the skin pathology in both Foxp3LSL Cd4creERT2 and Foxp3LSL Cd4wt mice before 4-OHT treatment revealed lichenoid interface dermatitis with edema in the dermis and focal pyknotic keratinocytes, epidermal hyperplasia and hyperkeratosis, with sizeable areas of serocellular crust. These autoimmune manifestations were ameliorated in Foxp3LSL Cd4creERT2 mice, but continued to worsen in Foxp3LSL Cd4wt mice 4 wks following 4-OHT treatment (Extended Data Fig. 4a-b). Additionally, TUNEL staining showed a pronounced increase in apoptotic cells in the liver of Foxp3LSL Cd4creERT2 mice before the treatment, which was completely reverted to control levels 4 wks post restoration of Treg function following 4-OHT treatment (Extended Data Fig. 4c-d). Consistent with the observed liver damage, serum albumin concentration in Foxp3LSL Cd4creERT2 mice at 2 wks of age was significantly diminished but restored to normal within 4 wks after 4-OHT treatment. (Extended Data Fig. 4e).
The reversed immune activation and tissue inflammation was unlikely to be a mere consequence of subtracting the Treg “wannabe” cells from the CD4 T cell pool, as our previous studies demonstrated that the disease severity in Foxp3-deficient mice harboring Treg “wannabe” cells was identical to that of neonates subjected to chronic Treg cell ablation, suggesting that Treg “wannabe” cells do not provide a notable non-redundant contribution to the severity and time course of the disease in Foxp3-deficient mice. These results show that Treg cells are capable of effectively exerting their function in settings of established inflammatory disease and suppressing multiple arms of the inflammatory immune response, causing their reversal. It can be argued, however, that the observed Treg cell-mediated restoration of the “normalcy” might be limited to an early age when the immune system is not fully developed.

Reversal of established autoimmune inflammation by Treg cells in adulthood

To assess whether Treg cells are capable of suppressing ongoing inflammatory responses in adulthood, we took advantage of the fact that Foxp3, as an X chromosome-encoded gene, is subject to random X-inactivation. Thus, we generated healthy mosaic heterozygous female Foxp3<sup>DTR-GFP</sup>/L<sup>SL</sup>-Cd4<sup>creERT2</sup> mice, which harbor both a functional Treg population and a non-functional Treg “wannabe” population expressing the Foxp3<sup>DTR-GFP</sup> and Foxp3<sup>L<sup>SL</sup></sup> allele, respectively. Acute depletion of Treg cells in homozygous Foxp3<sup>DTR-GFP</sup> females or hemizygous Foxp3<sup>DTR-GFP</sup> males upon recurrent diphtheria toxin (DT) administration causes an even more aggressive widespread autoimmune inflammatory disease to which mice succumb within 10-14 days. Indeed, DT-mediated Treg cell depletion in adult (8-10-wks-old) Foxp3<sup>DTR-GFP/L<sup>SL</sup></sup> heterozygous females caused similarly massive immune activation by day 7, at which point the diseased Foxp3<sup>DTR-GFP/L<sup>SL</sup>-Cd4<sup>creERT2</sup></sup> and control Foxp3<sup>DTR-GFP/L<sup>SL</sup>-Cd4<sup>wt</sup></sup> mice were administered with tamoxifen to induce conversion of Treg “wannabes” to functional Treg cells (Fig. 2a). Within 2 wks post tamoxifen treatment, the rehabilitated Treg cells suppressed T cell activation, whereas in tamoxifen-treated control Foxp3<sup>DTR-GFP/L<sup>SL</sup>-Cd4<sup>wt</sup></sup> mice the disease progressed (Fig. 2b, Extended Data Fig. 5). Although the numbers of CD4 and CD8 T cells no longer decreased beyond wk 2 post Treg cell restoration (data not shown), the percentages of activated CD4 and CD8 T cells continued to decline during the 5 wk observation period post tamoxifen treatment (Fig. 2b). Furthermore, the rehabilitated Treg cells ameliorated the inflammatory lesions in the skin, lung, and liver induced upon Treg cell depletion in Foxp3<sup>DTR-GFP/L<sup>SL</sup>-Cd4<sup>creERT2</sup></sup> mice by wk 5 post tamoxifen administration (Fig. 2c-e). Therefore, the revived Treg cells were able to protect the adult mice from autoimmune inflammation until at least 5 wks post tamoxifen administration,
the longest period of time we could confidently ensure that the mice would not develop DT-specific antibodies, which would interfere with DT-mediated ablation of Foxp3<sup>DTR-GFP</sup> Treg cells. Thus, Treg cells can function under inflammatory settings and suppress established lethal inflammation in both neonates and adults.

**Foxp3 expression imparts Treg cell functionality in inflammatory environment**

The remarkable effect of restored Foxp3 expression in a cohort of Treg “wannabe” cells led us to explore the intrinsic and extrinsic phenotypic shifts it afforded to these cells in inflammatory vs. non-inflammatory settings. Compared to Foxp3<sup>DTR-GFP Cd4<sup>creERT2</sup> healthy littermate control mice, we observed a transient increase in Treg cell proportion in male Foxp3<sup>LSL Cd4<sup>creERT2</sup> mice, which peaked on day 7 post 4-OHT treatment and decreased to the level of control healthy mice after 4 wks, when the autoimmune disease was largely eradicated (Fig. 3a). On day 7, the Thy1.1<sup>+</sup>GFP<sup>+</sup> Treg cell population in rescued Foxp3<sup>LSL Cd4<sup>creERT2</sup> mice was more proliferative than Treg cells in healthy Foxp3<sup>DTR-GFP Cd4<sup>creERT2</sup> controls, as indicated by increased proportion of Ki67<sup>+</sup> cells. Furthermore, the former cells expressed higher amounts of GITR and CTLA4, both of which contribute to their functionality (Fig. 3b, c). To gain a transcriptome-wide view of the effects of Foxp3 expression in diseased Foxp3<sup>LSL Cd4<sup>creERT2</sup> mice, we performed RNA-seq analysis of Thy1.1<sup>+</sup>GFP<sup>+</sup> Treg cells and Thy1.1<sup>+</sup> GFP<sup>+</sup> Treg “wannabes” isolated from Foxp3<sup>LSL</sup> males on day 7 following 4-OHT treatment, and of control GFP<sup>+</sup> Treg cells isolated from similarly treated Foxp3<sup>DTR-GFP Cd4<sup>creERT2</sup> mice. Principal component analysis (PCA) of the RNA-seq data showed that Thy1.1<sup>+</sup>GFP<sup>+</sup> Treg cells from diseased mice were considerably different from control Treg cells from Foxp3<sup>DTR-GFP Cd4<sup>creERT2</sup> mice, with the differentially regulated genes in the former enriched for previously reported Treg cell and STAT5<sup>27</sup> activation gene signatures (Fig. 3d). Notably, the transcriptomes of both Treg cell groups were markedly different – equidistant in PCA – from Thy1.1<sup>+</sup>GFP<sup>+</sup> Treg “wannabes” cells (Fig. 3d). These results suggested that Treg cells in Foxp3<sup>LSL Cd4<sup>creERT2</sup> mice were more activated, potentially due to heightened STAT5 signaling. These cells expressed lower levels of Tcf7, Sell, and Ccr7 transcripts consistent with their pronounced activation. Moreover, they expressed higher levels of cell cycle and anabolism-related genes as well as genes whose products have been implicated in Treg cell mediated suppressor function (Gzma, Gzmb, Lgals1, Lgals3, Il10, Fgl2, Ctlia4, Entpd1, Icos, and Tigit)<sup>29,30</sup>, suggesting that these cells were more metabolically active and potentially more suppressive than Treg cells present in healthy control mice (Fig. 3e). Indeed, we found that Thy1.1<sup>+</sup>GFP<sup>+</sup> Treg cells from diseased Foxp3<sup>LSL Cd4<sup>creERT2</sup> mice isolated on day 7 post 4-OHT administration were significantly more potent on a per cell
basis at suppressing T cell proliferation in vitro than their counterparts from control healthy Foxp3^{DTR-GFP}Cd4^{creERT2} mice (Fig. 3g).

Notably, activated Treg “wannabe” cells from DT treated Foxp3^{LSL/DTR-GFP} sick female mice, compared to their resting counterparts from similarly treated healthy Foxp3^{LSL/WT} mice, shared some gene expression changes observed in activated Foxp3-sufficient Treg cells in rescued Foxp3^{LSL/y} mice vs. resting Treg cells in control Foxp3^{DTR-GFP} male mice described above (Fig. 3d, Extended Data Fig. 6). However, this acquisition of activated phenotype by the Treg “wannabe” cells did not endow them with any appreciable suppressive capacity, as Foxp3^{LSL/DTR}Cd4^{WT} mice, in which tamoxifen treatment did not restore Foxp3-expressing Treg cells, died of systemic multi-organ inflammation similar to that seen in the male Foxp3^{LSL/y} mice (data not shown). These results suggested that restoration of Foxp3 expression allows rehabilitated Treg cells to mobilize their functionality upon exposure to an inflammatory environment, resulting in their heightened suppressor capacity as compared to their basal state in non-inflammatory settings. Alternatively, the observed functional superiority of the rehabilitated Treg cells in Foxp3^{LSL}Cd4^{creERT2} mice could have been a cell-intrinsic effect of a delayed Foxp3 expression regardless of the inflammatory environment. To distinguish between these two possibilities, we analyzed the corresponding Thy1.1^GFP^+ Treg and Thy1.1^GFP^-Treg “wannabe” cell subsets under basal non-inflammatory conditions in healthy heterozygous female Foxp3^{LSL/WT}Cd4^{creERT2} mice and control GFP^+ Treg cells in heterozygous female Foxp3^{DTR-GFP}WT Cd4^{creERT2} mice on day 7 post 4-OHT treatment. In contrast to Treg cells exposed to the pro-inflammatory environment in male mice, Thy1.1^GFP^+ Treg cells in 4-OHT treated mosaic Foxp3^{LSL/WT}Cd4^{creERT2} females failed to exhibit increased proliferative activity, or enhanced CTLA4 and GITR expression, compared to GFP^+ Treg cells expressing the Foxp3^{WT} allele in the same mice (Fig. 3f). Expression of these markers was also similar to that of control GFP^+ Treg cells in heterozygous female Foxp3^{DTR-GFP}WT Cd4^{creERT2} mice (Fig. 3f). Likewise, comparable suppressor activity was observed for Thy1.1^GFP^+ Treg cells and control GFP^+ counterparts on day 7 from 4-OHT treated healthy Foxp3^{LSL/WT}Cd4^{creERT2} and Foxp3^{DTR-GFP}WT Cd4^{creERT2} female mice, respectively (Fig. 3g). To the contrary, sick Foxp3^{DTR-GFP}LSL^Cd4^{creERT2} mosaic female mice, subjected to DT-induced ablation of Foxp3^{DTR-GFP} Treg cells and tamoxifen-induced Foxp3 restoration in Foxp3^{LSL} cells, showed a transient increase in Treg cell percentages at 1 and 2 wks post tamoxifen-mediated rescue, which disappeared by wk 5 (Fig. 3h). This trend was similar to that observed in sick Foxp3^{LSL/y}Cd4^{creERT2} male mice. Likewise, Treg cells rehabilitated under inflammatory conditions exhibited enhanced proliferation...
as well as heightened activation evidenced by elevated expression of Ki67, CTLA4, GITR and ICOS on day 7 post tamoxifen treatment (Fig. 3i, j). Thus, the enhanced proliferative potential and superior suppressive capacity of Treg cells in diseased Foxp3<sup>LSL-Cd4<sup>creERT2</sup> male and DT-treated Foxp3<sup>DTR-GFPLSL-Cd4<sup>creERT2</sup> female mice was likely due to sensing inflammation by these cells assisted by restored Foxp3 expression.

**A single cohort of Treg cells provides long-term protection against autoimmunity**

Our observation that Treg cells introduced into diseased Foxp3<sup>LSL-Cd4<sup>creERT2</sup> males were highly functional in disease settings raised the question whether the containment of autoimmunity afforded by a sole cohort of Treg cells was durable. To address this question, we monitored 4-OHT treated male Foxp3<sup>LSL-Cd4<sup>creERT2</sup> mice for an extended period of time (Fig. 4a). To “time stamp” the Treg cell populations in these studies, we introduced into Foxp3<sup>LSL-Cd4<sup>creERT2</sup> and control Foxp3<sup>DTR-GFP-Cd4<sup>creERT2</sup> mice a Rosa26<sup>loxP-STOP-loxP-tdTomato</sup> (<sup>R26</sup><sub>Tom</sub>) recombination reporter allele, which allows for irreversible labeling of all CD4 T cells undergoing Cre-mediated recombination induced by 4-OHT with the red fluorescent tdTomato protein. Within a month after 4-OHT treatment, the Foxp3<sup>LSL-Cd4<sup>creERT2-R26<sub>Tom</sub> mice recovered completely from otherwise lethal disease and continued to gain weight (Extended Data Fig. 7a). When analyzed at the 4-month time point, Foxp3<sup>+</sup> Treg cells detected in the thymus of these mice were exclusively CD73 positive and were devoid of the CD73-negative subset representing newly generated thymic Treg cells (Fig. 4b)31. This observation implied a lack of new thymic output of Treg cells with all thymic Foxp3<sup>+</sup> cells being recirculating cells, in agreement with our observation that CD4-creERT2-mediated recombination, reported by the <sup>R26</sup><sub>Tom</sub> allele, ceased within 48 to 72 hours after 4-OHT administration (Extended Data Fig. 7b). The peripheral “4-month-old” Treg cell pool in these mice was well maintained both in the lymphoid and non-lymphoid tissues (Fig. 4c), and remained functionally competent as documented using *in vitro* suppression assay (Extended Data Fig. 7c). Nearly all Thy1.1<sup>+</sup>GFP<sup>-</sup> Foxp3<sup>+</sup> Treg cells at all sites examined in Foxp3<sup>LSL-Cd4<sup>creERT2-R26<sub>Tom</sub> mice expressed the tdTomato reporter whereas the Tshy1.1<sup>-</sup>GFP<sup>-</sup> Treg “wannabe” cells and conventional Thy1.1<sup>+</sup>GFP<sup>-</sup> Foxp3<sup>-</sup>CD4 T cells in the same mice, as well as Treg and conventional CD4 T cell subsets in control Foxp3<sup>DTR-GFP-Cd4<sup>creERT2-R26<sub>Tom</sub> mice contained only a small fraction of tdTomato “fate-mapped” cells (Fig. 4d). Thus, Treg cells generated as a single cohort in diseased Foxp3<sup>LSL-Cd4<sup>creERT2</sup> mice continued to persist while the other analyzed T cell subsets turned over most likely due to continuous thymic output. Impressively, even after 4 months, the reversal of autoimmune disease seemed complete with no pathology observed in the skin, lung, liver and small intestine (Fig. 4e). Likewise, T cell...
activation and effector cytokine production, as well as myelo-proliferation, at best minimally increased (Fig. 4f-h, Extended Data Fig. 7d-f). Thus, a single cohort of Treg cells is capable of reversing established inflammation and affording long-term protection against autoimmunity. Remarkably, the rescued mice continued to survive for at least 7 months post 4-OHT treatment (Fig. 5a). Even at this time point, the rescued mice remained largely healthy with only moderately increased T cell activation and effector cytokine production with Treg cell population well maintained in both the lymphoid and non-lymphoid tissues (Extended Data Fig. 8a-c). Despite moderately increased serum IgG1 and IgE levels in comparison to the 4-wk time point, the other Ig isotype levels remained comparable to those in control Foxp3^{DTR-GFP/y}Cd4^{creERT2} mice (Extended Data Fig. 8d and data not shown). Neither did we observe prominent immune infiltrates in the skin, liver, and small intestine (Extended Data Fig. 8e).

Transcriptional features of long-lived protective Treg cells

The extraordinarily long-term protection against autoimmunity by a single cohort of Treg cells, as well as their impressive self-renewing potential, suggested that these cells, despite their prolonged persistence and continuous self-antigen stimulation, do not exhibit dysfunction typical of chronically stimulated CD4 and CD8 T cells. However, population level analyses of these Treg cells may obscure their potential heterogeneity in relation to their self-renewing potential or exhaustion. Thus, we sought to analyze this long-lived Treg cell population at a single-cell resolution using single cell RNA sequencing (scRNA-seq). We compared GFP^{+} Treg cells from Foxp3^{LSL/y}Cd4^{creERT2}R26^{Tom} mice and Foxp3^{DTR-GFP/y}Cd4^{creERT2}R26^{Tom} control mice 7 months after 4-OHT treatment (Fig. 5b). Treg cells in experimental and control mice were efficiently and comparably labeled by tdTomato protein shortly after 4-OHT induction (Fig. 5c). The percentage of tdTomato^{+} cells among Thy1.1^{+}GFP^{+} Treg cells in Foxp3^{LSL/y}Cd4^{creERT2}R26^{Tom} mice remained high (>80%) over time, demonstrating that this long-lived cell population persisted in an impressively stable manner. The percentage of tdTomato^{+} cells among control Foxp3^{DTR-GFP} Treg cells, however, gradually declined with only about 15% tdTomato^{+} cells found within GFP^{+} Treg cells at the time of analysis, most likely as a result of continuous thymic output replenishing the peripheral Treg cell pool with non-tdTomato-labeled Treg cells (Fig. 5c).

We pooled the scRNA-seq data from Foxp3^{LSL/y}Cd4^{creERT2}R26^{Tom} and Foxp3^{DTR-GFP/y}Cd4^{creERT2}R26^{Tom} mice and performed uniform manifold approximation and projection (UMAP) for visualization. To distinguish “old” and “young” cells among the control Treg cells from Foxp3^{DTR-GFP/y}Cd4^{creERT2}R26^{Tom} mice, we computationally separated them into tdTomato...
positive ("old") and negative ("young") cells based on the expression of the tdTomato transcript by individual cells. Thy1.1^GFP^ Treg cells in Foxp3^{LSL/yCd4^{creERT2}R26_{Tom}} mice, as well as the tdTomato^+ and tdTomato^- control GFP^ Treg cells in Foxp3^{DTR-GFP,Cd4^{creERT2}R26_{Tom}} mice, exhibited distinct distribution patterns in the UMAP plot (Fig. 5d). To better understand the most significant sources of variation among the three Treg cell populations, we performed diffusion map analysis. The first diffusion component (DC1) had a distribution of gene expression highly similar to that of activated Treg cell gene signature^{27} (Fig. 5e). The long-lived Thy1.1^GFP^ Treg cells had a DC1 density similar to that of the tdTomato^+ cells in control mice, which was higher than that of the tdTomato^- cells (Fig. 5f). This result indicates that long-lived Treg cells in Foxp3^{LSL/yCd4^{creERT2}R26_{Tom}} mice and control mice tagged with tdTomato 7 month earlier and their offsprings exhibited a similarly activated phenotype. Upon examining the genes whose expression patterns highly correlated with this diffusion component, we found transcripts associated with Treg cell activation (Cd44, Sell, Tnfrsf18, Icos, Ctl4). Additionally, we also found genes whose products promote cell migration to non-lymphoid tissues (Ccr4, Cxcr3, Ccr6, Itgae), as well as tissue adaptation and suppressor function^{32,33} (Maf, Ahr) (Fig. 5g, Extended Data Fig.9a). Flow cytometric analysis of expression of some of these genes confirmed the scRNA-seq results across multiple tissues. The CD62L^hi (encoded by Sell) cells were greatly reduced while cells expressing CD103, encoded by Itgae, were markedly increased within the Thy1.1^GFP^ Treg population in Foxp3^{LSL/yCd4^{creERT2}R26_{Tom}} mice (Extended Data Fig. 9b). Furthermore, flow cytometric analysis of the tdTomato^+ fraction of control Treg cells showed enrichment for Ly6C^-CD103^+ and CD44^high^CD62L^lo cells, consistent with their heightened activation state (Extended Data Fig. 9c). Despite the burden of being the sole Treg cells responsible for immunosuppression, the Thy1.1^GFP^ Treg cells did not appear less fit than their "age-matched" counterparts in control mice with continuous Treg cell turnover. Compared to tdTomato^+ GFP^ Treg cells in Foxp3^{DTR-GFP,Cd4^{creERT2}R26_{Tom}} mice, Thy1.1^GFP^ Treg cells in Foxp3^{LSL/yCd4^{creERT2}R26_{Tom}} mice exhibited comparable expression of genes associated with T cell exhaustion^{34} and apoptosis, and similar levels of Mcl1, an anti-apoptotic gene critical for the Treg cell survival^{35} (Fig. 5f). Genes involved in cell cycle progression and various metabolic pathways were also comparably expressed by tdTomato^+ GFP^ Treg cells in Foxp3^{DTR-GFP,Cd4^{creERT2}R26_{Tom}} mice and Thy1.1^GFP^ Treg cells in Foxp3^{LSL/yCd4^{creERT2}R26_{Tom}} mice (Extended Data Fig. 9d). These shared transcriptional features highlight the similarities in metabolic status and proliferative potential between the long-lived Treg cells in Foxp3^{LSL/yCd4^{creERT2}R26_{Tom}} mice and age matched tdTomato^+ control Foxp3^{DTR-GFP} Treg cells.
The observed heightened activation and self-renewal of functional Thy1.1\(^{\text{GFP}}\) Treg cells in Foxp3\(^{\text{LSL/Y}}\)Cd4\(^{\text{CreERT2}}\)R26\(^{\text{Tom}}\) mice might represent a cell state unique to these mice or characteristic features of long-lived cells present within the peripheral Treg cell pool under physiologic conditions. To address this question, we performed trajectory analysis on combined experimental and control Treg cell scRNA-seq datasets using the Palantir algorithm, which generates a high-resolution pseudotime ordering of cells at different developmental stages\(^{36}\). The pseudotime gradient started from resting (“naïve”) Treg cells, proceeded towards activated Treg cells, and ended with activated cells with transcriptional features of “tissue” Treg cells found in non-lymphoid organs in agreement with a previous report\(^{37}\) (Fig. 5h). The analysis successfully recapitulated known gene expression dynamics during Treg cell activation including downregulation of Tcf7, Sell, and Klf2, and upregulation of Foxp3, Ctla4, and Ikzf2 transcripts (Fig. 5i). Next, we compared the pseudotemporal ordering of Thy1.1\(^{\text{GFP}}\) Treg cells from Foxp3\(^{\text{LSL/Y}}\)Cd4\(^{\text{CreERT2}}\)R26\(^{\text{Tom}}\) mice, originating from cells whose Foxp3 expression was restored 7 months ago, with that of similarly “old” tdTomato\(^{\text{GFP}}\) and of “young” tdTomato\(^{\text{GFP}}\) Treg cells from control Foxp3\(^{\text{DTR-GFP/Y}}\)Cd4\(^{\text{CreERT2}}\)R26\(^{\text{Tom}}\) mice. Both “old” Treg cell populations were positioned late in the inferred differentiation trajectory in comparison to tdTomato\(^{\text{GFP}}\) Treg cells —s consistent with their activated phenotype — and were characterized by similarly high pseudotime values (Fig. 5j). Thus, Thy1.1\(^{\text{GFP}}\) Treg cells in Foxp3\(^{\text{LSL/Y}}\)Cd4\(^{\text{CreERT2}}\)R26\(^{\text{Tom}}\) mice closely resemble a set of long-lived cells present in the normal Treg cell pool under physiologic conditions.

To gain further insights into the long-term persistence of Thy1.1\(^{\text{GFP}}\) Treg cells in Foxp3\(^{\text{LSL}}\)Cd4\(^{\text{CreERT2}}\)R26\(^{\text{Tom}}\) mice, we performed a refined clustering analysis of the Thy1.1\(^{\text{GFP}}\) and Foxp3\(^{\text{DTR-GFP}}\) Treg cell scRNA-seq datasets. Among the 13 clusters identified, clusters 0 and 3 were primarily composed of tdTomato\(^{\text{GFP}}\) control Treg cells, whereas clusters 1, 6, 7 and 8 were enriched for Thy1.1\(^{\text{GFP}}\) Treg cells (Fig. 6a). Importantly, cells from cluster 1 were enriched for IL-2-Stat5 and Wnt-β-catenin pathway gene signatures (Fig. 6b), which have been implicated in Treg cell maintenance and self-renewal\(^{38-40}\). Moreover, these cells were lower in pseudotime values compared to the majority of the Thy1.1\(^{\text{GFP}}\) Treg cells, suggesting that they were less differentiated and had the potential to differentiate into the rest of the cells to sustain the Treg cell pool (Fig. 6c). Gene expression analysis revealed transcripts enriched (Ifrngr1, Epcam, Lrc32, Il4ra, etc.) and depleted (Sell, etc.) in cluster 1 cells (Fig. 6d, Extended Data Table 1). Using flow cytometry, we corroborated these results by demonstrating a marked enrichment of IL4Rα\(^{\text{hi}}\)IFNγR1\(^{\text{hi}}\)Epcam\(^{\text{hi}}\)GARP\(^{\text{hi}}\)CD25\(^{\text{hi}}\) (γREG\(^{\text{hi}}\)) cell subset within rescued
activated CD62L<sup>lo</sup> Treg population in Foxp3<sup>LSL</sup>Cd4<sup>creERT2</sup>R26<sup>Tom</sup> mice (Fig. 6e, Extended Data Fig. 10a-c). In unperturbed mice, these cells were also present, albeit at a lower frequency, within Treg cell population in the secondary lymphoid organs and at even lower frequency in non-lymphoid tissues such as liver and lung, but were virtually undetectable among newly differentiated CD73<sup>-</sup> thymic Treg cells (Fig. 6f). γREG<sup>+</sup> Treg cells were enriched in the parenchyma of highly vascularized organs such as the spleen and the lung, as evidenced by their higher frequency among cells not labeled by intravenously administered CD45 antibody, suggesting that these cells were largely non-circulatory and likely contributed to local maintenance of the Treg cell pools (Extended Data Fig. 10d). To compare the capacity of perinatal and adult Treg cell populations to give rise to γREG<sup>+</sup> Treg cells, we labeled Treg cells in healthy 2-wk-old and 8-wk-old Foxp3<sup>DTR-GFP</sup>Cd4<sup>creERT2</sup>R26<sup>Tom</sup> mice upon 4-OHT treatment and assessed the frequencies of γREG<sup>+</sup> Treg cells within tdTomato<sup>+</sup> and tdTomato<sup>-</sup> Treg cell subsets 4 months later. In the former experimental group, tdTomato<sup>+</sup> Treg subset harbored higher frequencies of γREG<sup>+</sup> Treg cells than their tdTomato<sup>-</sup> counterparts (Fig. 6g). In contrast, tdTomato<sup>+</sup> Treg cell population “time-stamped” in adult Foxp3<sup>DTR-GFP</sup>Cd4<sup>creERT2</sup>R26<sup>Tom</sup> mice failed to show an enrichment of γREG<sup>+</sup> Treg cells over their tdTomato<sup>-</sup> counterpart; rather, both populations contained similar percentages of γREG<sup>+</sup> Treg cells as the tdTomato<sup>-</sup> Treg cells in perinatally labeled mice, which is reduced in comparison to the tdTomato<sup>+</sup> Treg cells in these mice (Fig. 6g). These observations were independently confirmed by similar “time-stamping” of Treg cells in young vs. adult Foxp3<sup>creERT2</sup>R26<sup>Tom</sup> mice (Fig. 6g). These results were consistent with the enrichment in γREG<sup>+</sup> Treg cells observed within the peripheral Treg populations in the secondary lymphoid organs and non-lymphoid tissues in unmanipulated 2-wk-old vs. 8-wk-old mice (Extended Data Fig. 10e). Our results suggest γREG<sup>+</sup> Treg cells, capable of persisting in peripheral lymphoid and non-lymphoid organs, are selectively prominent within Treg cell population generated early in life.

**Discussion**

The cardinal features of the adaptive immune system, including its essentially unlimited antigen recognition specificity, amplification of innate immune responses, novel effector functions and memory, afford vertebrates with a versatile anticipatory defense against rapidly evolving infectious agents. This superior protection is confounded by a major fitness constraint due to a threat of autoimmunity and inflammation posed by the inherent self-reactivity of T cells. A singular vital role and broad purview of Treg cells in countering this threat - forestalling
autoimmunity – has been most vividly demonstrated by wide-ranging clinical manifestations of human monogenic disorders resulting from Treg cell deficiency or dysfunction due to $\text{FOXP3}$, $\text{STAT5B}$, $\text{IL2RA}$, and $\text{LRBA}$ mutations or $\text{CTLA4}$ haplo-insufficiency$^{41,42}$. In IPEX patients, these manifestations include endocrinopathies (diabetes, thyroiditis, pancreatitis, adrenal dysfunction), hepatitis, enteropathies (autoimmune gastritis, IBD, celiac disease), skin disorders (exudative dermatitis, alopecia), food allergy, hyper-IgE syndrome, autoimmune hematologic disorders (autoimmune thrombocytopenia, hemolytic anemia), myelo- and lymphoproliferation (splenomegaly, lymphadenopathy), polyneuropathy, as well as pulmonary and nephrotic syndromes$^{43}$. In mice, Treg deficiency or depletion cause equally widespread and fatal autoimmune inflammatory disease$^{13}$.

However, the potent immunosuppressive function of Treg cells indispensable for preventing autoimmune inflammation can in turn compromise protective immunity against infection. This consideration provided rationale for the concept that in the inflammatory settings of an infection Treg cells are ineffectual. Indeed, numerous studies showed that infection associated inflammatory milieu can cause downregulation of Foxp3 expression by Treg cells, impede their suppressor and proliferative capacity, or even confer pro-inflammatory properties, i.e. effector cytokine production; furthermore, pro-inflammatory cytokines, IL-6 and IL-1 in particular, can make effector cells refractory to Treg cell mediated suppression$^{14,16,18,19,44,45}$. On the other hand, some studies suggested that Treg cells are capable of modulating virus-specific immune responses and limiting associated pathologies$^{46-51}$. However, in all of these studies, Treg cells were present from the onset of infection, making it impossible to discriminate their activity prior to, or after the onset of infection-induced inflammation. Thus, the ability of Treg cells to function in infection induced inflammation remains an open question.

Since inflammation elicited by various infectious, metabolic and genetic causes, including Treg cell deficiency, shares principal cellular and soluble mediators, a corollary to the above concept is the notion that in established autoimmune inflammation, Treg cell activity is also expected to be severely attenuated or even lacking. In this regard, autoimmune and inflammatory pathologies were reported to exert varying effects on Treg cell numbers. In contrast to the aforementioned monogenic Treg deficiency-linked disorders, many studies of polygenic autoimmune diseases have found unchanged or even increased frequencies of Treg cells at inflammatory sites and in circulation$^{52}$. Meanwhile, functional and gene expression analyses of Treg cells isolated from autoimmune patients suggested that their function is impaired in the
inflammatory environment\textsuperscript{21-23,52-54}. However, numerous genetic polymorphisms have been linked to the pathophysiological manifestations of polygenic autoimmune diseases. Thus, the reported inferior Treg functionality in autoimmune patients can be one of these phenotypic manifestations. Indeed, an enrichment for such autoimmunity associated single nucleotide polymorphisms has been observed within Treg cell-specific regulatory elements identified by histone modifications and DNA demethylation analyses\textsuperscript{10,55,56}. As a potential counterargument to the above findings, adoptive transfer of Treg cells after the initiation of T cell-induced colitis was shown to cause its amelioration, highlighting their potential to control local inflammation. In this model, however, colonic inflammation was elicited upon homeostatic expansion of a relatively small number of naïve CD4 T cells upon their transfer into lymphopenic mice. Although the disease receded, it did so very slowly after transfer of a large number of CD25\textsuperscript{+}CD4\textsuperscript{+} Treg cells in a strictly IL-10 dependent manner\textsuperscript{57,58}. Thus, it remained possible that the observed reparative activity of Treg cells in this setting may be specific to the colon aided by its continuous epithelial cell turnover, or due to the numerical imbalance between the transferred Treg and effector T cells and the limited repertoire of the latter.

By genetically installing a limited Treg cell pool in Foxp3\textsuperscript{LSL} mice, we demonstrated that Treg cells are able to function in settings of systemic autoimmune inflammation, reverse fatal disease and tissue pathology, and offer its long-term containment. The reversal of inflammation was not a simple diversion of a large number of pro-inflammatory effector T cells into Treg cells as only approximately 5-6% of CD4 T cell population is represented by Treg “wannabe” cells at the time of 4-OHT treatment (data not shown). While induced Foxp3 expression could have “subtracted” no more than 6% cells from the total CD4 T cell pool, within a week of 4-OHT administration we observed ~40% and ~60% decrease in the absolute numbers of Foxp3\textsuperscript{CD44\textsuperscript{hi}} activated CD4 T cells in the spleen and the lymph nodes, respectively (data not shown). This finding was fully consistent with the indistinguishable fatal autoimmune diseases reported in Foxp3-deficient mice harboring Treg “wannabe” cells and Foxp3\textsuperscript{DTR} neonates subjected to chronic ablation of Treg cells, which demonstrated that Treg “wannabes” do not provide notable non-redundant contribution to the pathogenic self-reactive T cell pool in Foxp3 deficient mice that would affect severity and time course of the disease\textsuperscript{5,8,13}. The ability of Treg cells to suppress established inflammation was not limited to young (2-3 wk-old) mice but was also observed in adult animals. Notably, Treg cells introduced into the inflammatory environment suppressed Th2 autoimmunity including increased serum IgG1 and IgE levels, as efficiently as Th1 and Th17 responses, even
though Th2 responses are known to be most sensitive to diminished Treg cell numbers or functionality\textsuperscript{26,59}. The observed normalization of lympho- and myeloproliferation, acute phase response, and reversal of tissue inflammation highlighted the ability of Treg cells to function in settings of autoimmunity accompanied by a cytokine storm. Rather than impeding functionality, we found that exposure to this highly inflammatory milieu endowed the newly minted Treg cells with heightened suppressor activity in comparison to Treg cells isolated from control healthy mice while the latter and Treg cells generated upon a similar conversion of Treg “wannabe” cells in healthy heterozygote female mice had indistinguishable suppressor activity. It is widely accepted that Treg cells employ multiple and partially redundant modes of suppression, including secreted immunomodulatory factors, IL-2 consumption, and ATP to adenosine conversion, and may directly partake in tissue regeneration and repair via production of amphiregulin (Areg)\textsuperscript{60,61}. Consistent with these findings and the observed efficient reversal of inflammatory disease by Treg cells in Foxp3\textsuperscript{LSL} mice, these cells exhibited increased expression of numerous molecules implicated in Treg mediated immunomodulation and tissue repair including IL-2R, IL-10, Galectin-1 and -3 (Lgals1 and Lgals3), Fgl2, CTLA4, CD39 (Entpd1), ST2 (Il1rl1), and Areg. While we cannot unambiguously pinpoint inflammation sensing pathways responsible for potentiation of Treg cell function, prominent STAT5 activation gene expression signature discerned in redeemed Treg cells in 4-OHT-treated male Foxp3\textsuperscript{LSL} mice implicates cytokines signaling through common $\gamma$-chain receptors including IL-2. Accordingly, administration of therapeutic IL-2 formulations and expression of an active form of STAT5b in Treg cells have been reported to promote their expansion and superior suppressor function\textsuperscript{28,62,63}. It is important to note that the observed suppressor activity of Treg cells in inflammatory settings was not limited to recent thymic emigrants as Foxp3 expression induced in peripheral Treg “wannabe” cells present in the secondary lymphoid organs led to efficient suppression of autoimmunity in systemically inflamed mice in the absence of thymus export upon S1P1 receptor blockage. The reversal of inflammatory disease and restoration and long-term health maintenance by a single pool of redeemed Treg cells was enabled by its stability. At a population level, these cells remained functional and did not exhibit signs of dysfunction and were similar to time-stamped long-lived “old” Treg cells in a healthy mouse. Single cell transcriptomic analyses of these redeemed Treg cells revealed a subset of preferentially tissue-residing cells, marked by increased expression of IFN$\gamma$R, IL-4R$\alpha$, EpCAM, and GARP. Based on gene set enrichment and pseudo-time analyses, it is possible that these cells may contribute
to the long-term maintenance of the Treg pool, by giving rise to terminally differentiated
progenies locally.

By demonstrating that Treg cells are functional in severe established inflammation and can
suppress and reverse all known types of and key common features of inflammation and that a
single cohort of Treg cells can afford a long-lasting protection against autoimmunity and
inflammation, our findings provide general rationale and support for the emerging efforts to
develop adoptive Treg cell therapy not only for intrauterine and neonatal IPEX syndrome and
other monogenic Treg deficiencies, but for a broad spectrum of autoimmune and inflammatory
disorders.

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W.H. and A.Y.R. conceived the study and designed experiments. W.H., Z.-M.W. and A.Y.R.
interpreted the data and wrote the manuscript; W.H. and Z.-M.W. performed the experiments.
Y.F. designed the targeting construct and generated the Foxp3<sup>LSL</sup> mouse. B.E.H. performed the
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J.V. assisted with mouse generation and animal colony maintenance. Z.-M.W. and M.S.
performed analysis of the bulk RNA-seq experiment. M.S. performed analysis of scRNA-seq
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Methods

Mice

Experiments in this study were approved by the Sloan Kettering Institute (SKI) Institutional Animal Care and Use Committee under protocol 08-10-023. Mice were housed at the SKI animal facility under SPF conditions on a 12-hour light/dark cycle with free access to water and regular chow diet. All control and experimental animals were age-matched, and littermates were used as controls unless otherwise indicated.

Foxp3<sup>DTR-GFP</sup><sup>13</sup>, Foxp3<sup>GFP</sup><sup>6</sup>, Foxp3<sup>creERT2</sup><sup>28</sup>, Foxp3<sup>GFP-BirA-AVI-TEV</sup><sup>y</sup> (in preparation), Cd4<sup>creERT2</sup><sup>64</sup>, Tcrb<sup>−/−</sup>Tcrd<sup>−/−</sup> and Rosa26<sup>LSL-tdTomato</sup><sup>66</sup> mice were maintained in the the Sloan Kettering Institute animal facility. The Rosa26<sup>LSL-tdTomato</sup> allele was bred to homozygosity for fate mapping studies.

Generation of Foxp3<sup>LSL</sup> mice

The targeting construct was made by first subcloning an 8.5 kb SphI fragment harboring the Foxp3 genomic sequence from exon -1a to exon 7 obtained from the RP23-143D8 cosmid into a cloning vector carrying a Pgk-DTA-polyA cassette allowing for negative selection of random genomic integration. The FRT-PGK-neo-polyA-FRT positive selection cassette was then cloned into the DraIII site within the intron between exons -1b and 1, and the loxP-Thy1.1 coding sequence-triple-tandem SV40 polyA-eGFP coding sequence cassette was cloned into the AvrIl site within exon 1. The Thy1.1 and eGFP sequences are preceded by a start codon (ATG). The targeting construct was then electroporated into albino C57BL/6 ES cells. After neomycin selection, Southern blotting and karyotyping, correctly targeted clones were injected into WT C57BL/6 blastocysts. The resulting chimeric mice were bred to albino C57BL/6 mice. Founders identified based on the coat color and genotyping were mated to a Flp deleter strain of mice to remove the neo cassette.

4-hydroxytamoxifen, diphtheria toxin, and tamoxifen treatment

4-hydroxytamoxifen (4-OHT, Sigma-Aldrich, H7904) stock solution was made by reconstituting in ethyl alcohol 200 proof (Sigma-Aldrich, E-7023) at a concentration of 20 mg/mL, then diluting 1:1 (v/v) with Cremophor EL (Sigma-Aldrich, 238470). 20 µg/g body weight of 4-OHT diluted in PBS was administered intraperitoneally.

Diphtheria toxin (DT, List Biological Laboratories, 150) was dissolved in PBS. 1µg (first dose) or 250 ng (subsequent doses) was injected intraperitoneally for each mouse. For tamoxifen
administration, 20 mg of tamoxifen (Sigma-Aldrich, T5648) was resuspended in 1 mL corn oil (Sigma-Aldrich, C8267) by rotating and tilting at 37°C until fully dissolved. Each mouse was orally gavaged with 4 mg of tamoxifen per treatment.

4-OHT was used whenever possible because it bypasses the conversion of tamoxifen to 4-hydroxytamoxifen in the liver. Compared to tamoxifen, 4-OHT offers a much sharper pharmacokinetics and enables a highly synchronized labeling of cells. In addition, 4-OHT has a much shorter half-life than tamoxifen, particularly in adults. Therefore, tamoxifen instead of 4-OHT was used for in vivo suppression assays (Extended Data Fig. 1f) and in studies of adult female mice (Fig. 2, Fig. 3h-j, and Extended Data Fig. 7) to achieve sufficient recombination of the Foxp3<sup>LSL</sup> allele.

FTY720 stock solution was made by reconstituting in dimethyl sulfoxide at a concentration of 20 mg/ml. 0.8 µg/g body weight of FTY720 diluted to 0.1 mg/ml in 2% β-hydroxypropanylcyclodextrin was administered intraperitoneally.

**Reagents and antibodies**

The following clones of fluorescently conjugated or biotinylated antibodies were obtained from BD Biosciences, BioLegend, ThermoFisher, or Tonbo and were used in this study for flowcytometry: anti-Siglec-F (E50-2440), anti-I-A/I-E (M5/114.15.2), anti-NK1.1 (PK136), anti-CD45 (30-F11), anti-CD11b (M1/70), anti-CD3ε (17A2), anti-Ly-6C (HK1.4), anti-CD90.2 (30-H12), anti-Foxp3 (FJK-16s), anti-CD19 (6D5), anti-Ly-6G (1A8), anti-TCRβ (H57-597), anti-F4|80 (BM8), anti-CD90.1 (HIS51), anti-CD4 (RM4-5), anti-CD8α (53-6.7), anti-GITR (DTA-1), anti-CD73 (TY/11.8), anti-CD44 (IM7), anti-CD103 (M290), anti-CD62L (MEL-14), anti-CTLA4 (UC10-4B9), anti-Helios (22F6), anti-Ki-67 (16A8), anti-CD25 (PC61.5), anti-CD45.1 (A20), anti-CD45.2 (104), anti-IL-2 (JES6-5H4), anti-IL-17A (17B7), anti-IFNγ (XMG1.2), anti-IL-4 (BVD6-24G2), anti-GARP (YGIC86), anti-CD119 (2E2), anti-EpCAM (G8.8), anti-CD124 (mIL4R-M1).

The following antibodies were used to capture antigens for ELISA in this study: purified anti-mouse IgE (R35-72, BD Pharmingen, 553413), Goat Anti-Mouse IgG1 (RRID: AB_2794408, SouthernBiotech, 1070-01), Goat Anti-Mouse IgG3 (RRID: AB_2794567, SouthernBiotech, 1100-01), Goat Anti-Mouse IgG2a (RRID: AB_2794475, SouthernBiotech, 1080-01), Goat Anti-Mouse IgG2b (RRID: AB_2794517, SouthernBiotech, 1090-01), Goat Anti-Mouse IgG2c (RRID: AB_2794644, SouthernBiotech, 1079-01), Goat Anti-Mouse IgA (RRID: AB_2314669,
SouthernBiotech, 1040-01), Goat Anti-Mouse IgM (RRID: AB_2794197, SouthernBiotech, 1020-01), Mouse Pentraxin 2/SAP Antibody (R & D Systems, MAB2558).

The following antibodies were used to detect antigens for ELISA in this study: Goat Anti-Mouse Ig-HRP (RRID: AB_2728714, SouthernBiotech, 1010-05), Biotin Rat Anti-Mouse IgE (R35-118, BD Pharmingen, 553419), Biotinylated Pentraxin 2/SAP Antibody (R & D Systems, BAF2558).

The following reagents were used to construct standard curves for ELISA in this study: Purified Mouse IgE, kappa, Isotype Control (BD Pharmingen, 557079), Purified Mouse IgA, kappa, Isotype Control (BD Pharmingen, 553476), Purified Mouse IgG3, kappa, Isotype Control (BD Pharmingen, 553486), Purified Mouse IgG1, kappa, Isotype Control (SouthernBiotech, 0100-01), Purified Mouse IgG2a, kappa, Isotype Control (Sigma, M5409), IgM Isotype Control from murine myeloma (Sigma, M5909), IgG2b Isotype Control from murine myeloma (Sigma, M5534), Mouse IgG2c (RRID: AB_2794064, SouthernBiotech, 0122-01), Recombinant Mouse Pentraxin 2 (R & D Systems, 2558-SA-050).

**Enzyme-linked immunosorbent assay (ELISA)**

ELISA experiments were conducted as previously described. Briefly, mouse peripheral blood was collected via cardiac puncture immediately after euthanasia into BD SST microcontainer tubes (02-675-185) and sera were harvested after centrifugation. F-bottom 96-well plates were coated with capturing antibodies in 50 µL 0.1 M NaHCO₃ solution at pH 9.5 O/N at 4°C. The plates were then emptied, blocked with 200 µL 1% bovine serum albumin (VWR, 97061-422) in PBS, and washed 3 times with PBS containing 0.05% Tween-20 (Sigma-Aldrich, P1379). 50 µL of sera at appropriate dilutions was added and incubated O/N at 4°C. The plate was then incubated in sequential orders with 50 µL of biotinylated detection antibodies for 2-3 hours, 50 µL of avidin-HRP (ThermoFisher, 18-4100-51) for 30 minutes, and 100 µL of TMB solution (ThermoFisher, 00-4201-56) at room temperature, with 3-4 washes with PBS-Tween in between each incubation steps. The colorimetric reaction was stopped with 100 µL of 1M H₃PO₄ (Sigma-Aldrich, P5811) after 5-10 minutes of adding TMB and absorbance at 450 nm was measured with a Synergy HTX plate reader (BioTek). Concentrations of antigens were determined using standard curves constructed with purified recombinant proteins and calculated with Gen5 3.02.2 (BioTek).

**Isolation of cells from lymphoid organs, livers, lungs, and colonic lamina propria**
For flow cytometric analyses, animals were perfused with a total of 20 mL PBS into both left and right ventricles immediately after euthanasia. Cells were retrieved from spleens, peripheral (brachial, axillary and inguinal) lymph nodes, mesenteric lymph nodes, thymuses, and livers by meshing the organs through a 100 µm strainer (Corning, 07-201-432) with a syringe plunger. Cells in the colonic lamina propria were isolated as previously described\(^6\). Briefly, colons were cleaned by flushing the luminal content out with PBS using a syringe, defatted, opened up longitudinally and diced into 1-2 cm pieces. Tissues were then incubated in 25 mL IEL solution [1x PBS w/ 2% FBS (ThermoFisher, 35010CV), 10 mM HEPES buffer (ThermoFisher, MT 25–060-CI), 1% penicillin/streptomycin (ThermoFisher, MT 30–002-CI), 1% L-glutamine (ThermoFisher, MT 25–005-CI), plus 1 mM EDTA (Sigma-Aldrich, E4884) and 1 mM DTT (Sigma-Aldrich, D9779) added immediately before use] for 15 minutes at 37°C with vigorous shaking (250 rpm) to remove the epithelial fraction. Tissues were then retrieved, washed extensively, and digested in 25 mL LPL solution [1x RPMI 1640 w/2% FBS, 10 mM HEPES buffer, 1% penicillin/streptomycin, 1% L-glutamine, 0.2 U/mL collagenase A (Sigma, 11088793001) and 1 U/mL DNase I (Sigma-Aldrich, 10104159001)] for 30 minutes at 37°C with vigorous shaking (250 rpm). ¼inch ceramic beads (MP Biomedicals, 116540034) were added during this step (3–4 per sample) to facilitate tissue dissociation. The digested samples were passed through a 100 µm strainer, pelleted at 450 g for 5 minutes and washed extensively. Lungs were digested in the same fashion as the lamina propria fraction of the colons but for 45 minutes. Cells from non-lymphoid organs were centrifugated in 40% PBS-adjusted Percoll (v/v, ThermoFisher, 45–001-747) in PBS to enrich for lymphocytes. Erythrocytes in the spleen, lung, and liver samples were lysed with ACK lysis buffer [150 mM NH\(_4\)Cl (Sigma-Aldrich, A9434), 10 mM KHCO\(_3\) (Sigma-Aldrich, P7682), 0.1 mM Na\(_2\)EDTA, pH 7.4].

For fluorescence-activated cell sorting, cell suspension was made from pooled secondary lymphoid organs (spleen; peripheral and mesenteric lymph nodes) as above and CD4 T cells were enriched with the Dynabeads Flowcomp Mouse CD4 Kit (ThermoFisher, 11461D) according to manufacturer’s instructions, stained with antibodies, washed extensively, resuspended in isolation buffer (PBS w/ 2% FBS, 10 mM HEPES buffer, 1% L-glutamine, and 2 mM EDTA) containing 0.01% SYTOX Blue dead cell stain (ThermoFisher, S34857) to facilitate dead cell exclusion, and sorted on a FACSARia (BD) instrument. Treg cells (TCR\(\beta^+\)CD4+CD8\(^{-}\)NK1.1^Foxp3^+Thy1.1^), Treg “wannabes” (TCR\(\beta^+\)CD4+CD8^−NK1.1^Thy1.1^Foxp3^+) and naive conventional CD4 T cells (TCR\(\beta^+\)CD4+CD8^−NK1.1^Foxp3^+Thy1.1^CD44^hi^CD62L^hi^) were sorted by gating on the respective populations.
Histology and TUNEL assay
Sample embedding, sectioning, haematoxylin and eosin staining, pathology grading, and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay were conducted at HistoWiz (Brooklyn, NY). Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin before sectioned into 5-micron slices. Lymphocytic and acute (neutrophils) or chronic (monocytes) myeloid inflammation was blindly scored with the following criteria: 0-normal, 1-mild increase, 2-moderate increase, 3-medium increase, 4-sever increase.

For TUNEL assay, liver sample sections were processed under standardized conditions using the DeadEnd Fluorometric Detection System (Promega, G3250), and subsequent immunohistochemistry was carried out using BOND Polymer Refine Detection Kit (Leica, DS9800) according to manufacturers' instructions. TUNEL+ cells were quantified with ImageJ v2.0.0-rc-69/1.52p.

Measurement of serum albumin
Serum albumin level was measured by Laboratory of Comparative Pathology, SKI, using the Albumin kit (Beckman Coulter, OSR6102), according to manufacturer's instruction.

Flow cytometric analysis of cytokine production
To measure cytokine production after ex vivo restimulation, single cell suspensions were incubated at 37°C for 3-4 hours with 5% CO₂ in 96-well flat-bottom plates in the presence of 50 ng/mL phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich, P8139) and 500 ng/mL ionomycin (Sigma-Aldrich, I0634) with 1 μg/mL brefeldin A (Sigma-Aldrich, B6542) and 2 μM monensin (Sigma-Aldrich, M5273) to inhibit ER and Golgi transport. Cells were then stained with Ghost Dye Violet 510 (Tonbo, 13-0870), Ghost Dye Red 780 (Tonbo, 13-0865), or Zombie NIR Flexible Viability Kit (Biolegend, 423106) in PBS for 10 minutes at 4°C to help identify dead cells and then with purified anti-Mouse CD16/CD32 (2.4G2, Tonbo, 70-0161) in staining buffer [0.5% (w/v) BSA, 2 mM EDTA, 10 mM HEPES, 0.02% NaN₃ (Sigma-Aldrich, S2002) in 1x PBS] for 10 minutes at 4°C to block the Fc receptors. Samples were then incubated with fluorescently-conjugated antibodies against cell surface antigens in staining buffer for 25 minutes at 4°C and then washed extensively. For accessing intracellular antigens, cells were fixed and permeabilized with the BD Cytofix/Cytoperm Kit for measuring cytokine production, or with the ThermoFisher Transcription Factor Fix/Perm Kit for staining cytosolic and nuclear antigens,
according to manufacturers’ instructions. Samples were recorded on an LSR II cytometer (BD) or an Aurora cytometer (Cytek) and analyzed with FlowJo v10.6.1 (BD).

In vitro suppression assay

A 2-fold titration series of FACS-sorted Treg cells starting from 40,000 cells/well was set up in U-bottom 96-well plates. 40,000 FACS-sorted, CellTrace Violet (ThermoFisher, C34571)-labeled naïve CD4 T cells and 100,000 erythrocyte-lysed splenocytes from Tcrb⁻⁻Tcdr⁻⁻ mice as antigen-presenting cells were then added to each well. α-mouse CD3 (145-2C11, BioXCell, BE0001-1) was then added to a final concentration of 1 µg/mL. Cells were incubated in a final volume of 200 µL complete RPMI w/ 10% FBS and with 5% CO₂ at 37°C for 72 hours before analysis. Cells that have had more than 4 rounds of CTV dilution were considered divided for calculating Treg-mediated suppression using the following formula:

\[
\%\text{Suppression (Sample X)} = \frac{\%\text{divided (no Treg)} - \%\text{divided (Sample X)}}{\%\text{divided (no Treg)}}
\]

Statistics

Statistical significance was determined using tests indicated in the respective figure legends. P-values for t-test were calculated with GraphPad Prism 7 and had been corrected for multiple hypothesis testing using the Holm-Sidak method. P-values for ANOVA were computed with R for Fig. 2b and GraphPad Prism 7 elsewhere and had been corrected for multiple hypothesis testing using the Tukey method (when using R and Prism), the Dunnett’s method or the Sidak method (when using Prism, according to its recommendations). P-values for Kolmogorov-Smirnov test were calculated with R. Throughout the entire study, error bars represent mean ± s.e.m., and the following notation was used to report statistical significance: ns, non-significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.

Bulk RNA sequencing and data analysis

Doubly sorted cells were directly placed into TRIzol reagent (ThermoFisher, 15596-018) for subsequent RNA extraction. RNA was precipitated with isopropanol and linear acrylamide, washed with 75% ethanol, and resuspended in RNase-free water. After RiboGreen quantification and quality control by Agilent BioAnalyzer, 2ng of total RNA underwent amplification using the SMART-Seq v4 Ultra Low Input RNA Kit (Clonetech, 63488), with 12 cycles of amplification. 3.8 – 4 ng of amplified cDNA was then used to prepare libraries with the KAPA Hyper Prep Kit (Kapa Biosystems, KK8504) using 8 cycles of PCR. Barcoded samples
were run on a HiSeq 4000 instrument in a 50bp/50bp paired-end run, using the HiSeq 3000/4000 SBS Kit (Illumina). An average of 41 million paired reads were generated per sample with % mRNA bases per sample ranging from 67% to 77%. In experiments shown in Fig. 3, Treg cells (TCRβ+CD4+Foxp3+) from Foxp3\textsuperscript{DTR-GFP}\textsubscript{+/y} and Foxp3\textsuperscript{LSL/Cd4\textsuperscript{creERT2/+}} mice and Treg “wannabe” (TCRβ+CD4+Foxp3-Thy1.1+) cells from Foxp3\textsuperscript{LSL/Cd4\textsuperscript{+/y}} mice were analyzed (2-3 biological replicates). In experiments shown in Extended Data Fig. 6, Treg “wannabe” cells from 3 Foxp3\textsuperscript{LSL/+} mice and 2 Foxp3\textsuperscript{LSL/DTR-GFP} mice were analyzed. RNA-seq reads from fastq files were aligned to the reference mouse genome GRCm38 using the STAR aligner\textsuperscript{69}, and local realignment was performed using the Genome Analysis Toolkit\textsuperscript{70}. For each sample, raw count of reads per gene was measured using R, and the DESeq2 R package\textsuperscript{71} was used to perform differential gene expression analysis across different conditions. A cutoff of 0.05 was set on the obtained FDR-adjusted p-values to get the significant genes of each comparison. All detectable genes were rlog-normalized and then used for the principal component analysis.

**Single cell RNA sequencing and data analysis**

**Library Preparation and sequencing**

Library preparation and sequencing for the scRNA-Seq of doubly-FACS-sorted Treg cells isolated from the secondary lymphoid organs of 7 months post-4-OHT treatment Foxp3\textsuperscript{DTR-GFP} (cells pooled from 5 mice) and Foxp3\textsuperscript{LSL/Cd4\textsuperscript{creERT2/+}} mice (cells pooled from 4 mice) were performed by the Single Cell Research Initiative, SKI, using 10X genomics Chromium Single Cell 3’ Library \& Gel bead Kit V3 according to manufacturer’s protocol. Cells, with a mean viability of 75%, were encapsulated in microfluidic droplets at a dilution of ~60 cells/µL with the multiplet fraction being 3.5%. After the RT step, the barcoded-cDNA was purified with DynaBeads, followed by 12-cycles of PCR-amplification (98°C for 180 s; [98°C for 15 s, 67°C for 20 s, 72°C for 60 s] x 12-cycles; 72°C for 60 s). Next, 50 ng of PCR-amplified barcoded cDNA was fragmented with the reagents provided in the kit and purified with SPRI beads to obtain an average fragment size of 600 bp. The fragmented DNA was ligated to sequencing adapters which was then indexed with PCR (98°C for 45 s; [98°C for 20 s, 54°C for 30 s, 72°C for 20 s] x 10 cycles; 72°C for 60 s). The resulting DNA library was double-size purified (0.6-0.8X) with SPRI beads and sequenced on an Illumina NovaSeq 6000 System (R1 – 26 cycles, i7 – 8 cycles, R2 – 96 cycles) at a depth of 210 million reads per sample (average reads per single cell being 31,000 and average reads per transcript 3.96 – 4.10), with a median sequencing saturation of 74%.

**Data Pre-processing**
Fastq files were processed using Cell Ranger (10x Genomics) and reads were aligned to the mouse genome mm10 from ENSEMBL GRCm38 that was modified to include sequences corresponding to the coding region and the 3'UTR of the $R26^{Tom}$ allele. Cells containing over 5% mitochondria-derived transcripts were filtered out, resulting in 3,634 $Foxp3^{DTR-GFP}$ cells and 3,390 $Foxp3^{LSL}$ cells that passed QC metrics, with a median of 3,580 molecules/cell. Cells with total molecule counts below 1000, as determined by the lower mode of the distribution of total molecules per cell, were additionally filtered out to remove putative empty droplets. Genes that were expressed in more than 10 cells were retained for further analysis. The resulting count matrices from both samples were then combined, resulting in a final set of 7,024 cells x 12,432 genes, and normalized to median library size, where library size is defined as total molecule counts per cell. The normalized data are then log transformed as log (counts + 1) for downstream analysis.

**Principal component analysis**
For dimensionality reduction and visualization of data, we further excluded genes with very low or very high expression of transcripts (log average expression <0.02 or >3 and dispersion >0.15), and principle component analysis was then performed on the log-transformed normalized data. Using 40 principal components, where the number of principal components was determined by the knee-point in eigenvalues, yielded a good representation of the variability in the data.

**MAGIC imputation**
To account for missing values in scRNA-seq due to a high dropout rate, we employed MAGIC, a method of “de-noising” and imputing missing expression values through data diffusion between cells with similar covariate gene relationships\(^7\). We constructed the diffusion map matrix using $k = 30$, $ka = 10$, and $t = 6$ as input parameters, where $t$ specifies the number of times the affinity matrix is powered for diffusion.

**Diffusion components and pseudotime calculation**
Instead of constructing a tSNE map using 40 PCs, we followed the strategy outlined by Setty et al.\(^36\) in order to characterize potential pseudotime non-linear trajectories and to visualize single-cell gene expression in a UMAP embedding of diffusion components. Based on the eigen gap, we chose to use 15 diffusion components for downstream analysis in Palantir and for calculating diffusion distances. We scaled each included diffusion component by the factor $\lambda/(1-\omega)$. \(^7\)
\( \lambda \) where \( \lambda \) is the associated eigenvalue, to reflect ‘multi-scale’ diffusion distances. Then, we calculate each cell position in pseudotime based on modeling cell fate in a continuous probabilistic model.

**Clustering and gene ranking**

Clustering of cells was performed using PhenoGraph\textsuperscript{73} setting \( k = 15 \) nearest neighbors. A cluster was removed because of its disparity with the rest of the data (t-SNE projected this cluster as a separate component that comprised cells from both populations), and those cells also had a relatively lower number of total molecules compared with other populations. Significant differentially expressed genes in each cluster were identified using \( t \)-test (where the variance of small groups is overestimated), which was implemented in Single-Cell Analysis in Python\textsuperscript{74} with default parameters.

**tdTomato expression and gene set module score calculation**

Because of a high dropout rate of single cell sequencing, we performed MAGIC imputation of tdTomato expression (as described above) only for Foxp3\textsuperscript{DTR-GFP} cells since the overwhelming majority of the Foxp3\textsuperscript{LSL} cells were tdTomato\textsuperscript{+} which could potentially cause over-imputation. A cutoff of 1.04 was set for the imputed tdTomato expression where any cells with higher expression were categorized as tdTomato\textsuperscript{+} (~15\% of cells) and those with lower expression as tdTomato\textsuperscript{−} (~85\% of cells) in agreement with flow cytometric measurements. Gene set module scores for II2-Stat5 (GSEA HALLMARK_IL2_STAT5_SIGNALING) and Wnt/\( \beta \)-catenin (GSEA HALLMARK_WNT_BETA_CATENIN_SIGNALING) were calculated with the AddModuleScore function in Seurat v 3.1.5\textsuperscript{75} using the default parameters.
References


Figure 1 | Restoration of Foxp3 expression in Treg “wannabes” cures fulminant autoimmunity in male Foxp3SL mice. Mice were treated with 4-hydroxytamoxifen (4-OHT) on postnatal day 14. a, Schematic of the Foxp3SL allele. b, Flow cytometric analysis of splenic CD4 T cells 1 wk post 4-OHT treatment. c, Lymph nodes (top), spleens (middle), and thymi (bottom) of mice of indicated genotypes 4 wks after 4-OHT treatment. d-f, Percentages of activated, proliferating, and cytokine-producing splenic T cells (d), frequencies of splenic myeloid cell populations (e), and serum amyloid P (SAP) levels (f) at indicated time points after 4-OHT treatment. g, Levels of antibodies in the serum 4 wks post-4-OHT treatment. h, Haematoxylin and eosin staining of indicated tissues before and 4 wks after 4-OHT treatment. i, Histology scores of indicated tissues before and 4 wks after 4-OHT treatment. For d-g, data are combined from three independent experiments with 3 to 12 mice per group per time point. Data are
shown as mean ± s.e.m., two-way (d-f, i) or one-way (g) ANOVA with Tukey’s multiple comparisons test. ns, non-significant; *, p < 0.05; **, p < 0.01; ***, p<0.001; ****, p<0.0001.
Figure 2 | Restoration of Foxp3 expression in Treg “wannabe” cells in mosaic adult female Foxp3<sup>LSL/DTR-GFP</sup> mice suppresses immune activation caused by diphtheria toxin-mediated Treg cell ablation. a, Experimental scheme. 8-10-wk-old female mice were injected with diphtheria toxin (DT) intraperitoneally and given oral gavage of tamoxifen (Tam) on designated days. b, Frequencies of activated, proliferating, and cytokine producing splenic conventional CD4 and CD8 T cells at the indicated time points. Data are pooled from two independent experiments with 3 to 5 mice per group per time point. Line graphs show mean percentages ± s.e.m., two-way ANOVA with Tukey’s multiple comparison test. ns, non-significant; *, p < 0.05; **, p < 0.01; ***, p<0.001; ****, p<0.0001. c-e, Haematoxylin and eosin staining of sections of skin (c), lung (d), and liver (e) from mice of indicated genotypes at denoted time points post tamoxifen treatment (left), and their respective inflammation scores (right). Scatter plots show mean score ± s.e.m., one-way ANOVA with Tukey’s multiple comparison test.
Figure 3 | Rescued Treg cells in inflamed mice are activated and potently suppressive in inflammatory settings. a-c, Male Foxp3<sup>GFP-Cd4<sup>creERT2</sup></sup> and Foxp3<sup>LSL-Cd4<sup>creERT2</sup></sup> mice were treated with 4-OHT on postnatal day 14. Data are pooled from three independent experiments. a, Percentages of splenic Treg cells at indicated time points following 4-OHT treatment. Line graph shows mean ± s.e.m. Multiple t-tests with Holm-Sidak multiple comparison. b, c, representative histograms (b) and combined data (c) showing expression of Ki67, CTLA4 and GITR by rescued and control Treg cells on day 7 post 4-OHT treatment from the spleen analyzed by flow cytometry. Scatter plots show mean ± s.e.m. Two-tailed
unpaired t-tests. d, e, RNA-seq analysis of rescued Treg cells, control Treg cells, and Treg “wannabes” from male mice of indicated genotypes treated with 4-OHT on postnatal day 14 and analyzed 7 days afterwards. d, Principal component analysis of gene expression in the three indicated cell populations (top), and empirical cumulative distribution function plots showing gene signatures of activated Treg cells (bottom left) and Treg cells expressing a constitutively active form of STAT5b (bottom right). Two-sided Kolmogorov-Smirnov test. e, Heatmaps showing expression of curated genes in rescued and control Treg cells from mice of indicated genotypes. f, Expression of Ki67, CTLA4 and GITR by indicated Treg cell subsets from the spleen of mosaic heterozygous Foxp3^{DTR-GFP/WT}Cd4^{creERT2} and Foxp3^{LSL/WT}Cd4^{creERT2} female mice treated with 4-OHT on postnatal day 14 and analyzed after 7 days using flow cytometry. Data are shown as mean ± s.e.m., one-way ANOVA with Tukey’s multiple comparison test. g, Suppression of in vitro proliferation of conventional CD4 T cells induced by α-CD3 antibody and antigen-presenting cells by control or rescued Treg cells (GFP+), or Treg “wannabes” (Thy1.1+) from indicated strains of mice on day 7 post 4-OHT treatment. Two-way ANOVA with Tukey’s multiple comparison test. Data are shown as mean %suppression ± s.e.m. Frequencies of Treg cells at the indicated time points. Line graph shows mean percentages ± s.e.m. Two-way ANOVA with Tukey’s multiple comparison test. h-j, Adult mosaic heterozygous female Foxp3^{DTR-GFP/WT}Cd4^{creERT2} and Foxp3^{DTR-GFP/LSL}Cd4^{creERT2} mice were treated with DT and 4-OHT as in Figure 2a. h, Percentages of splenic Treg cells at indicated time points following 4-OHT treatment. Line graph shows mean ± s.e.m. Multiple t-tests with Holm-Sidak multiple comparison. i, j, Representative histograms (i) and combined data (j) showing expression of indicated markers by Treg cells at wk 1. Scatter plots show mean percentages or MFI ± s.e.m., two-tailed unpaired t-tests. ns, non-significant; *, p < 0.05; **, p < 0.01; ***, p<0.001; ****, p<0.0001.
Figure 4 | Rescued Treg cells in male Foxp3<sup>LSL</sup> mice provide long-term protection from autoimmune inflammatory disease. a, Experimental design. Mice were treated with a single dose of 4-OHT at 2 wks of age and analyzed 4 months later. b, Flow cytometric analysis of CD73 expression in Foxp3<sup>+</sup> CD4 single-positive thymocytes as a discriminating marker of recirculating vs. recently generated thymic Treg cells. Two-tailed unpaired t-test. c, Flow cytometric analysis of Treg cell percentages (upper panel) and absolute numbers (lower panel) in tissues of mice of indicated genotypes. Two-tailed multiple t-tests with Holm-Sidak multiple comparison. d, Percentages of lineage-traced (tdTomato<sup>+</sup>) Treg cells, Treg "wannabes", and conventional CD4 T cells. One-way ANOVA with Dunnett’s multiple hypothesis tests. e, Analysis of histopathology in mice of indicated genotypes. Haematoxylin and eosin staining of sections of the indicated organs. f-h, Percentages of activated, proliferating, and cytokine-producing conventional CD4 T cells (f), CD8 T cells (g) and myeloid populations (h) from indicated organs. Data are pooled from two independent experiments. Two-tailed unpaired t-tests with Holm-Sidak multiple comparison. pLN, peripheral (brachial, axillary, and inguinal) lymph nodes; mLN, mesenteric lymph nodes; LP, lamina propria. Scatter plots show mean ± s.e.m. ns, non-significant; *, p < 0.05; **, p < 0.01; ***; p<0.001; ****, p<0.0001.
Figure 5 | Single-cell transcriptomic analysis of control and long-lived rescued Treg cells. 

a, Survival plot of 4-OHT-treated male Foxp3<sup>LSL</sup>Cd4<sup>wt</sup> and Foxp3<sup>LSL</sup>Cd4<sup>creERT2</sup> mice. Mantel-Cox test. 

b, Experimental design. Mice were treated with 4-OHT at 2 wks of age and Foxp3<sup>+</sup> Treg cells, FACS-purified based on GFP expression, were subjected to scRNA-seq analysis using 10X Genomics platform 7 months later. 

c, Percentages of fate-mapped splenic Treg cells and conventional CD4 T cells in control and rescued mice at indicated time points post 4-OHT treatment. Scatter plot shows mean ± s.e.m. Two-way ANOVA with Tukey’s multiple comparisons test. 

d, UMAP visualization of the single-cell transcriptomes of experimental Foxp3<sup>LSL</sup> and control tdTomato<sup>+</sup> or tdTomato<sup>+</sup> Foxp3<sup>DTR-GFP</sup> Treg cells. 

e, UMAP visualization colored by diffusion component 1 (left) and the expression level of activated Treg cell gene signature (right). 

f, Histograms depicting the density of Foxp3<sup>LSL</sup> and tdTomato<sup>+</sup> or tdTomato<sup>+</sup> Foxp3<sup>DTR-GFP</sup> Treg cells along diffusion component 1, Mcl1 expression, and the average expression levels of indicated gene sets. 

g, Correlation of curated genes with diffusion component 1. 

h, UMAP visualization colored by pseudotime.
generated with Palantir. Arrow indicates the direction of differentiation across the map. i, Expression of representative genes along the pseudotime trajectory. j, Violin plots showing the medians of pseudotime values of $\text{Foxp3}^{LSL}$ and tdTomato$^+$ or tdTomato$^-$ Foxp3$^{DTR-GFP}$ Treg cells. Thick vertical bars show the interquartile range.
Figure 6 | Identification and analysis of γREG+ Treg cells from the single-cell RNA-seq data. 

a, UMAP visualization colored by the clusters. Bar graph shows the numbers of cells from each sample that contributed to the individual clusters. 
b, UMAP visualization of Foxp3<sup>LSL</sup> Treg cells colored by the module scores for the indicated gene sets. 
c, Violin plots showing the medians of pseudotime values of Foxp3<sup>LSL</sup> Treg cells from the top 10 clusters. Thick vertical bars show the interquartile range. Horizontal bars show the number of Foxp3<sup>LSL</sup> Treg cells contributing to each cluster. 
d, Dot plot showing the expression of top enriched genes (p<0.05) and underrepresented genes from cluster 1 expressed by more than 20% of the cells in at least one cluster. 
e, Frequencies of γREG+ Treg cells among CD62L<sup>lo</sup> Treg cells in rescued and control mice treated with 4-OHT on postnatal day 14 and analyzed 4 months later. Two-tailed unpaired t-
tests with Holm-Sidak multiple comparison. f, Frequencies (left) and absolute numbers (right) of γREG⁺ Treg cells in various tissues of unmanipulated 8-wk-old Foxp3GFP mice. g, Percentages of γREG⁺ Treg cells among tdTomato⁺ or tdTomato⁻ Treg cells “time-stamped” in adult (8-wk-old) or perinatal (2-wk-old) mice and analyzed after 4 months. Two-way ANOVA with Tukey’s multiple comparison test.
Extended Data Figure 1 | Generation and characterization of Foxp3^{LSL} mice. a, Schematics of the targeting construct and the Foxp3^{LSL} allele before and after Flp recombinase-mediated removal of the neo cassette. Homologous regions between the targeting construct and the WT allele are demarcated with dashed gray lines. Gene map is based on RefSeq record NM_001199347.1. pA, bovine growth hormone polyadenylation signal; neo, neomycin resistance gene; PGK, mouse phosphoglycerate kinase 1 promoter; 3xSV40pA, triple-tandem SV40 early polyadenylation signals (STOP cassette); DTA, diphtheria toxin A. b, Genotyping PCR showing the WT (275 bp) and knock-in-specific (350 bp) bands using primers labeled in a. c, d, Flow cytometric analysis of T cells and TCRβ^{hi} single positive thymocytes from 3-wk-old male Foxp3^{LSL/y} (c) and 8-10-wk-old female Foxp3^{LSL/WT} (d) mice. LN, lymph nodes. e, Expression of molecules associated with T cell activation in splenic Foxp3^{+} Treg and Thy1.1^{+} Treg “wannabe” cells, and naïve (CD44^{lo}CD62L^{hi}) and activated (CD44^{hi}CD62L^{lo}) conventional CD4 T cells shown in d. f, 2x10^6 CD45.1^{+}Foxp3^{-}conventional CD4 T cells were co-transferred with 2x10^5 CD45.2^{+} Treg “wannabes” from 2-3-wk-old Foxp3^{LSL/y} mice or Treg cells from 6-8-wk-old Foxp3^{GFP} mice into Tcrb^{-/-}Tcrd^{-/-} mice. Recipients were orally gavaged with tamoxifen and injected i.p. with diphtheria toxin (DT) at the indicated time points (top). DT was administered to deplete the few contaminating DTR-expressing Treg cells in the transferred FACs-purified conventional T cells or those that were induced to express Foxp3 after transfer. Cellularity and proliferation of CD45.1^{+} responder cells from lymph nodes were analyzed using flow cytometry (bottom). Data are representative of two independent experiments. Bar graphs show mean ± s.e.m.
Extended Data Figure 2 | Restoration of Foxp3 expression reverses spontaneous lymphocyte expansion, myelo-proliferation, and cytokine production, and normalizes circulatory Ig levels in male Foxp3^{LSL} mice. a, 4-OHT mediated recombination efficiency in lymphoid and non-lymphoid organs. Experimental scheme (left) and recombination efficiency of Rosa26^Tom in Treg cells in indicated organs (right). Scatter plot shows mean ± SEM, One-way ANOVA. b-g, Male Foxp3^{LSL} mice were treated with 4-OHT on wk 2 of life and analyzed throughout the following 4 wks. b, Representative histograms showing expression of proliferation and activation markers by splenic conventional CD4 and CD8 T cells at the indicated time points. c, Representative contour plots of flow cytometric analysis of activated and cytokine-producing splenic conventional CD4 and CD8 T cell populations. d, e, frequencies (d) and numbers (e) of activated, proliferating and cytokine-producing conventional CD4 and CD8 T cells from lymph nodes of mice of indicated genotypes at designated time points after 4-OHT treatment. Data shown are mean ± s.e.m. Two-way ANOVA with Tukey’s multiple comparison test. f, Numbers of splenic myeloid cell populations at indicated time points after 4-OHT treatment. Two-way ANOVA with Tukey’s multiple comparison test. g, Serum antibody levels as in Figure 1g. Data are shown as mean Ig isotype concentrations ± s.e.m., one-way ANOVA with Tukey’s multiple comparison test. ns, non-significant; *, p < 0.05; **, p < 0.01; ***, p<0.001; ****, p<0.0001.
Extended Data Figure 3 | Restoration of Foxp3 expression in peripheral Treg “wannabes” rescues immune activation in male Foxp3<sup>Lsl</sup> mice.  

**a**, Experimental Design. Mice were treated with 4-hydroxytamoxifen (4-OHT) at 2 wks of age while being continuously treated with FTY720 to block thymic output.  

**b, c**, Numbers of activated, proliferating, and cytokine-producing conventional CD4 and CD8 T cells in the spleen (**b**) and lymph nodes (**c**).
Extended Data Figure 4 | Restoration of Foxp3 expression in Treg “wannabes” rescues tissue damage in the skin and liver of male Foxp3<sup>Lox/Lox</sup> mice. Mice were treated with 4-OHT on postnatal day 14 and examined at the indicated time points post-treatment. a, b, H&E staining of skin sections showing epidermal hyperplasia and formation of serocellular crust (arrow heads). Dashed lines demarcate the boundary between epidermis and dermis. c, TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay followed by immunohistochemistry to visualize apoptotic cells (arrow heads) in H&E counter-stained liver sections from mice of indicated genotypes. d, Quantification of TUNEL<sup>+</sup> cells. e, Measurement of serum albumin levels. d, e, Data shown are mean ± s.e.m., one-way ANOVA with Tukey’s multiple comparison test. ns, non-significant; *, p < 0.05; **, p < 0.01; ***, p<0.001.
Extended Data Figure 5 | Restoration of Foxp3 expression in Treg “wannabe” cells in mosaic adult female Foxp3<sup>LSL/DTR-GFP</sup> mice suppresses immune activation caused by diphtheria toxin-mediated Treg cell ablation. Experimental scheme shown in Figure 2a. a, Representative histograms showing expression of activation and proliferation markers in splenic conventional T cell populations. b, Representative contour plots of splenic conventional CD4 and CD8 T cells showing cytokine production.
Extended Data Figure 6 | Analysis of gene expression changes in Treg “wannabe” cells induced upon activation.  

**a**, Experimental scheme. 8-10-wk-old heterozygous female Foxp3<sup>LSL/DTR-GFP</sup> Cd4<sup>wt</sup> mice were treated with diphtheria toxin (DT) on designated days to deplete Foxp3<sup>DTR-GFP</sup>-expressing Treg cells and induce activation of Foxp3<sup>LSL</sup>-expressing Treg “wannabe” cells which were sorted and analyzed by RNA-seq.  

**b**, FC-FC plot of activation-induced gene expression changes in Treg cells and “wannabes”. Genes with mean normalized counts of >100 are shown. Differentially expressed genes (\(p < 0.05\)) are colored based on the direction of the change in either or both cell types.  

**c**, Venn diagram showing the numbers of genes with larger than a 2-fold change in activated Treg and “wannabe” cells.  

**d**, CDF plots showing expression changes in activated Treg “wannabe” cells for all genes (black) and Treg activation signature genes that are up- (red) or down- (blue) regulated. Two-sided Kolmogorov-Smirnov test.  

**e**, FC-FC plot showing gene expression changes in Treg vs. “wannabe” cells isolated from sick and healthy mice. Signature genes that are up- or down-regulated in activated Treg and conventional CD4 T cells are highlighted in different colors.
Extended Data Figure 7 | Rescued Treg cells in male Foxp3^{LSL} mice exert long-term control of adaptive and innate immune cells. Mice were treated with a single dose of 4-OHT at 2 wks of age and analyzed 4 months later. a, Body weight of rescued Foxp3^{LSL} Cd4^{creERT2} and control Foxp3^{DTR-GFP} Cd4^{creERT2} mice over a 4-month time course. Two-way ANOVA with Sidak’s multiple comparison test. b, Analysis of 4-OHT “functional” pharmacokinetics in 2-wk-old male mice. Mice were injected with 4-OHT 3–72 hours before transfer of congenically marked recombination-proficient CD4 T cells from Cd4^{creERT2} R26^{Tom} mice. 4-OHT-induced recombination was assayed 7 days later by tdTomato expression among donor CD4 T cell as a readout of 4-OHT activity. Data are pooled from two independent experiments with 2 to 4 mice per time point each. Line graph shows mean ± s.e.m. Ctrl, no 4-OHT injection. c, Suppression of in vitro proliferation of conventional CD4 T cells induced by α-CD3 antibody and antigen-presenting cells by control Treg cells from Foxp3^{DTR-GFPy} or rescued Treg cells from Foxp3^{LSLy} mice. Two-way ANOVA with
Tukey's multiple comparison test. Data are shown as mean %suppression ± s.e.m. Numbers of activated, proliferating, and cytokine-producing conventional CD4 T cells (d), CD8 T cells (e) and myeloid populations (f) in indicated tissues. pLN, peripheral (brachial, axillary, and inguinal) lymph nodes; mLN, mesenteric lymph nodes; LP, lamina propria. Data are pooled from two independent experiments. Scatter plots show mean ± s.e.m. Two-tailed t-tests with multiple hypothesis testing correction using the Holm-Sidak method. ns, non-significant; *, p < 0.05; **, p < 0.01; ***, p<0.001; ****, p<0.0001.
Extended Data Figure 8 | Rescued Treg cell population persisting for 7 months in male Foxp3^{LSL} mice prevent relapse of rampant autoimmunity. Mice were treated with 4-OHT on postnatal day 14 and analyzed 7 months later. 

a-c, Frequencies of Treg cells (a) and proliferating, activated, and cytokine-producing conventional CD4 (b) and CD8 (c) T cells. Two-tailed unpaired t-tests with multiple hypothesis testing correction using the Holm-Sidak method.

d, Serum antibody levels. Scales were kept the same as in Figure 1g. Two-tailed unpaired t-test.

e, Representative images of haematoxylin and eosin-stained sections of the indicated organs. Scatter plots and bar graphs show mean ± s.e.m. ns, non-significant; *, p < 0.05; **, p < 0.01; ***, p<0.001; ****, p<0.0001.
Extended Data Figure 9 | Analysis of long-lived Treg cells in Foxp3\textsuperscript{LSL} and control Foxp3\textsuperscript{DTR-GFP} mice. Mice were treated with 4-OHT on postnatal day 14 and analyzed 7 months later. a, UMAP visualization of the single-cell transcriptomes colored by imputed expression levels of representative genes. b, Frequencies of CD62L\textsuperscript{hi} and CD103\textsuperscript{+} cells among Treg cells in rescued Foxp3\textsuperscript{LSL}Cd4\textsuperscript{creERT2} and control Foxp3\textsuperscript{DTR-GFP}Cd4\textsuperscript{creERT2} mice. Two-tailed multiple t-tests. c, Foxp3\textsuperscript{WT}Cd4\textsuperscript{creERT2}R26\textsuperscript{Tom} mice were treated with 4-OHT on postnatal day 14 and analyzed 4 months later. Representative contour plots show expression of activation markers by tdTomato\textsuperscript{+} and tdTomato\textsuperscript{-} Foxp3\textsuperscript{+} CD4 T cells. d, Histogram depicting the density of Foxp3\textsuperscript{LSL} and tdTomato\textsuperscript{+} or tdTomato\textsuperscript{-} Foxp3\textsuperscript{DTR-GFP} Treg cells along the average expression values for the indicated gene sets. Scatter plots show mean ± s.e.m. ns, non-significant; *, p < 0.05; **, p < 0.01; ***, p<0.001; ****, p<0.0001.
**Extended Data Figure 10 | Flow cytometric analysis of γREG+ Treg cells.** a, Gating strategy for γREG+ Treg cells in adult mice. b, c, Representative histogram (b) and quantification (c) of IL4Rα expression in γREG+ and other Treg cell populations identified in a. Two-way ANOVA with Tukey’s multiple comparison test. d, Percentages of γREG+ Treg cells in lymphoid organs and non-lymphoid tissues of CD45 i.v.-labeled 8-10-wk-old Foxp3GFP mice. One-way ANOVA with Sidak’s multiple comparison test. e, Percentages (left) and numbers (right) of γREG+ Treg cells in different organs of 2- or 8-wk-old Foxp3GFP BirA-AVI-TEV mice. Two-sided unpaired t-tests with correction for multiple hypothesis testing using the Holm-Sidak method. Bar graph and scatter plots show mean ± s.e.m. ns, non-significant; *, p < 0.05; **, p < 0.01; ***, p<0.001; ****, p<0.0001.
Figure 1

Restoration of Foxp3 expression in Treg “wannabes” cures fulminant autoimmunity in male Foxp3LSL mice. Mice were treated with 4-hydroxytamoxifen (4-OHT) on postnatal day 14. a, Schematic of the Foxp3LSL allele. b, Flow cytometric analysis of splenic CD4 T cells 1 wk post 4-OHT treatment. c, Lymph nodes (top), spleens (middle), and thymi (bottom) of mice of indicated genotypes 4 wks after 4-OHT treatment. d-f, Percentages of activated, proliferating, and cytokine-producing splenic T cells (d), frequencies of splenic myeloid cell populations (e), and serum amyloid P (SAP) levels (f) at indicated time points after 4-OHT treatment. g, Levels of antibodies in the serum 4 wks post-4-OHT treatment. h,
Haematoxylin and eosin staining of indicated tissues before and 4 wks after 4-OHT treatment. For d-g, data are combined from three independent experiments with 3 to 12 mice per group per time point. Data are shown as mean ± s.e.m., two-way (d-f, i) or one-way (g) ANOVA with Tukey’s multiple comparisons test. ns, non-significant; *, p < 0.05; **, p < 0.01; ***, p<0.001; ****, p<0.0001.

Figure 2

Restoration of Foxp3 expression in Treg “wannabe” cells in mosaic adult female Foxp3LSL/DTR-GFP mice suppresses immune activation caused by diphtheria toxin-mediated Treg cell ablation. a, Experimental scheme. 8-10-wk-old female mice were injected with diphtheria toxin (DT) intraperitoneally and given oral gavage of tamoxifen (Tam) on designated days. b, Frequencies of activated, proliferating, and cytokine producing splenic conventional CD4 and CD8 T cells at the indicated time points. Data are pooled from two independent experiments with 3 to 5 mice per group per time point. Line graphs show mean percentages ± s.e.m., two-way ANOVA with Tukey’s multiple comparison test. ns, non-significant; *, p < 0.05; **, p < 0.01; ***, p<0.001; ****, p<0.0001. c-e, Haematoxylin and eosin staining of sections of skin (c), lung (d), and liver (e) from mice of indicated genotypes at denoted time points post tamoxifen.
treatment (left), and their respective inflammation scores (right). Scatter plots show mean score ± s.e.m., one-way ANOVA with Tukey’s multiple comparison test.

Figure 3

Rescued Treg cells in inflamed mice are activated and potently suppressive in inflammatory settings. a-c, Male Foxp3DTR-GFPCd4creERT2 and Foxp3LSLCd4creERT2 mice were treated with 4-OHT on postnatal day 14. Data are pooled from three independent experiments. a, Percentages of splenic Treg cells at indicated time points following 4-OHT treatment. Line graph shows mean ± s.e.m. Multiple t-tests with
Holm-Sidak multiple comparison. b, c, representative histograms (b) and combined data (c) showing expression of Ki67, CTLA4 and GITR by rescued and control Treg cells on day 7 post 4-OHT treatment from the spleen analyzed by flow cytometry. Scatter plots show mean ± s.e.m. Two-tailed unpaired t-tests. d, e, RNA-seq analysis of rescued Treg cells, control 1094 Treg cells, and Treg “wannabes” from male mice of indicated genotypes treated with 4-OHT on postnatal day 14 and analyzed 7 days afterwards. d, Principal component analysis of gene expression in the three indicated cell populations (top), and empirical cumulative distribution function plots showing gene signatures of activated Treg cells (bottom left) and Treg cells expressing a constitutively active form of STAT5b (bottom right). Two-sided Kolmogorov-Smirnov test. e, Heatmaps showing expression of curated genes in rescued and control Treg cells from mice of indicated genotypes. f, Expression of Ki67, CTLA4 and GITR by indicated Treg cell subsets from the spleen of mosaic heterozygous Foxp3DTR-GFP/WTCd4creERT2 and Foxp3LSL/WTCd4creERT2 female mice treated with 4-OHT on postnatal day 14 and analyzed after 7 days using flow cytometry. Data are shown as mean ± s.e.m., one-way ANOVA with Tukey’s multiple comparison test. g, Suppression of in vitro proliferation of conventional CD4 T cells induced by α-CD3 antibody and antigen-presenting cells by control or rescued Treg cells (GFP+), or Treg “wannabes” (Thy1.1+) from indicated strains of mice on day 7 post 4-OHT treatment. Two-way ANOVA with Tukey’s multiple comparison test. Data are shown as mean %suppression ± s.e.m. Frequencies of Treg cells at the indicated time points. Line graph shows mean percentages ± s.e.m. Two-way ANOVA with Tukey’s multiple comparison test. h-j, Adult mosaic heterozygous female Foxp3DTR-GFP/WTCd4creERT2 and Foxp3DTR-GFP/LSLCd4creERT2 mice were treated with DT and 4-OHT as in Figure 2a. h, Percentages of splenic Treg cells at indicated time points following 4-OHT treatment. Line graph shows mean ± s.e.m. Multiple t-tests with Holm-Sidak multiple comparison. i, j, Representative histograms (i) and combined data (j) showing expression of indicated markers by Treg cells at wk 1. Scatter plots show mean percentages or MFI ± s.e.m., two-tailed unpaired t-tests. ns, nonsignificant; *, p < 0.05; **, p < 0.01; ***, p<0.001; ****, p<0.0001.
Rescued Treg cells in male Foxp3LSL mice provide long-term protection from autoimmune inflammatory disease. 

a, Experimental design. Mice were treated with a single dose of 4-OHT at 2 wks of age and analyzed 4 months later. 
b, Flow cytometric analysis of CD73 expression in Foxp3+ CD4 single-positive thymocytes as a discriminating marker of recirculating vs. recently generated thymic Treg cells. Two-tailed unpaired t-test. 
c, Flow cytometric analysis of Treg cell percentages (upper panel) and absolute
numbers (lower panel) in tissues of mice of indicated genotypes. Two-tailed multiple t tests with Holm-Sidak multiple comparison. d, Percentages of lineage-traced (tdTomato+) Treg cells, Treg “wannabes”, and conventional CD4 T cells. One-way ANOVA with Dunnett’s multiple hypothesis tests. e, Analysis of histopathology in mice of indicated genotypes. Haematoxylin and eosin staining of sections of the indicated organs. f-h, Percentages of activated, proliferating, and cytokine-producing conventional CD4 T cells (f), CD8 T cells (g) and myeloid populations (h) from indicated organs. Data are pooled from two independent experiments. Two-tailed unpaired t-tests with Holm-Sidak multiple comparison. pLN, peripheral (brachial, axillary, and inguinal) lymph nodes; mLN, mesenteric lymph nodes; LP, lamina propria. Scatter plots show mean ± s.e.m. ns, non-significant; *, p < 0.05; **, p < 0.01; ***, p<0.001; ****, p<0.0001.

Figure 5
Single-cell transcriptomic analysis of control and long-lived rescued Treg cells. 

a, Survival plot of 4-OHT-treated male Foxp3LSLcd4wt and Foxp3LSLcd4creERT2 mice. Mantel-Cox test. 
b, Experimental design. Mice were treated with 4-OHT at 2 wks of age and Foxp3+ Treg cells, FACS-purified based on GFP expression, were subjected to scRNA-seq analysis using 10X Genomics platform 7 months later. 
c, Percentages of fate-mapped splenic Treg cells and conventional CD4 T cells in control and rescued mice at indicated time points post 4-OHT treatment. Scatter plot shows mean ± s.e.m. Two-way ANOVA with Tukey's multiple comparisons test. 
d, UMAP visualization of the single-cell transcriptomes of experimental Foxp3LSL and control tdTomato- or tdTomato+ Foxp3DTR-GFP Treg cells. e, UMAP visualization colored by diffusion component 1 (left) and the expression level of activated Treg cell gene signature (right). 
f, Histograms depicting the density of Foxp3LSL and tdTomato+ or tdTomato- Foxp3DTR-GFP Treg cells along diffusion component 1, Mcl1 expression, and the average expression levels of indicated gene sets. 
g, Correlation of curated genes with diffusion component 1. h, UMAP visualization colored by pseudotime generated with Palantir. Arrow indicates the direction of differentiation across the 1146 map. 
i, Expression of representative genes along the pseudotime trajectory. j, Violin plots showing the medians of pseudotime values of Foxp3LSL and tdTomato+ or tdTomato- Foxp3DTR-GFP Treg cells. Thick vertical bars show the interquartile range.
Figure 6

Identification and analysis of gREG+ Treg cells from the single-cell RNA-seq data. a, UMAP visualization colored by the clusters. Bar graph shows the numbers of cells from each sample that contributed to the individual clusters. b, UMAP visualization of Foxp3LSL Treg cells colored by the module scores for the indicated gene sets. c, Violin plots showing the medians of pseudotime values of Foxp3LSL Treg cells from the top 10 clusters. Thick vertical bars show the interquartile range. Horizontal bars show the number of Foxp3LSL Treg cells contributing to each cluster. d, Dot plot showing the expression of top enriched genes (p<0.05) and underrepresented genes from cluster 1 expressed by more than 20% of
the cells in at least one cluster. e, Frequencies of gREG+ Treg cells among CD62Llo Treg cells in rescued and control mice treated with 4-OHT on postnatal day 14 and analyzed 4 months later. Two-tailed unpaired t tests with Holm-Sidak multiple comparison. f, Frequencies (left) and absolute 1162 numbers (right) of gREG+ Treg cells in various tissues of unmanipulated 8-wk-old Foxp3GFP mice. g, Percentages of gREG+ Treg cells among tdTomato+ or tdTomato- Treg cells “time-stamped” in adult (8-wk-old) or perinatal (2-wk-old) mice and analyzed after 4 months. Two-way ANOVA with Tukey’s multiple comparison test.

**Supplementary Files**

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- [ExtendedDataTable1.xlsx](ExtendedDataTable1.xlsx)