Thymic regulatory T cells arise via two distinct developmental programs

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The developmental programs that generate a broad repertoire of regulatory T cells (Treg cells) able to respond to both self antigens and non-self antigens remain unclear. Here we found that mature Treg cells were generated through two distinct developmental programs involving CD25⁺ Treg cell progenitors (CD25⁺ TregP cells) and Foxp3⁺ Treg cell progenitors (Foxp3⁺ TregP cells). CD25⁺ TregP cells showed higher rates of apoptosis and interacted with thymic self antigens with higher affinity than did Foxp3⁺ TregP cells, and had a T cell antigen receptor repertoire and transcriptome distinct from that of Foxp3⁺ TregP cells. The development of both CD25⁺ TregP cells and Foxp3⁺ TregP cells was controlled by distinct signaling pathways and enhancers. Transcriptomics and histocytometric data suggested that CD25⁺ TregP cells and Foxp3⁺ TregP cells arose by coopting negative-selection programs and positive-selection programs, respectively. Treg cells derived from CD25⁺ TregP cells, but not those derived from Foxp3⁺ TregP cells, prevented experimental autoimmune encephalomyelitis. Our findings indicate that Treg cells arise through two distinct developmental programs that are both required for a comprehensive Treg cell repertoire capable of establishing immunotolerance.

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egulatory T cells (Treg cells) play key roles in protecting against autoimmune responses to tissues, preventing inappropriate responses to commensal organisms and dampening effector T cell responses following clearance of pathogens. However, the mechanisms leading to the development of a population of Treg cells that can mediate such diverse functions remain unclear. Treg cells were shown to develop through a two-step process in the thymus. The first step is driven by strong signals sent through the T cell antigen receptor (TCR), which leads to upregulation of CD25, the key component of the high-affinity receptor for the cytokine IL-2, as well as the tumor necrosis factor (TNF) receptor superfamily members GITR, OX40 and TNFR2, but not to upregulation of the transcription factor Foxp3. A second, TCR-independent step involves the conversion of CD25⁺ TregP cells into mature CD25⁺Foxp3⁺ Treg cells in a manner dependent on IL-2 and the transcription factor STAT5. A distinct Treg cell progenitor population, characterized by low expression of Foxp3 and lacking detectable expression of CD25, was also described in the thymus. This Foxp3⁻ TregP cell shows high expression of GITR and OX40 and can differentiate into mature CD25⁺Foxp3⁺ Treg cells following stimulation with IL-2. The relative contributions of these TregP cell populations to the mature Treg cell pool remain controversial.

Here we demonstrate that CD25⁺Foxp3⁻ TregP cells (called ‘CD25⁺ TregP cells’ here) and CD25⁺Foxp3⁺ TregP cell progenitors (called ‘Foxp3⁺ TregP cells’ here) generated mature Treg cells with relatively comparable efficiency both in vitro and in vivo. The two developmental pathways for Treg cell generation differed in many aspects, including distinct transcriptomes and TCR repertoires. CD25⁺ TregP cells exhibited increased apoptosis, developed into mature Treg cells with faster kinetics and exhibited greater reactivity with self antigens in the thymus than Foxp3⁺ TregP cells. Development of the two Treg cell progenitor subsets was controlled in the thymus by different cytokines, signaling pathways, gene enhancers and stromal cells. Finally, Treg cells derived from CD25⁺ TregP cells, but not those derived from Foxp3⁺ TregP cells, protected against experimental autoimmune encephalomyelitis (EAE). Our data suggest a model in which two distinct Treg cell progenitor subsets both contribute to generate a broad Treg cell repertoire able to protect against immune responses to self antigens, limit immune responses to commensal organisms and resolve immune responses to foreign pathogens.

Results
CD25⁺ and Foxp3⁺ TregP cells differentiate into Treg cells. To determine whether CD25⁺ TregP cells and Foxp3⁺ TregP cells are...
both bona fide thymic Treg cell progenitors, we compared their ability to convert into mature Treg cells in response to low doses of IL-2 for 3 days in vitro. Sorted CD25+ Treg cells and Foxp3+ Treg cells responded to very low amounts of IL-2 (0.2–1.0 U ml−1) by converting to mature CD25+Foxp3+ Treg cells (Supplementary Fig. 1a), indicating that although they lack CD25 expression, Foxp3+ Treg cells, which express the low-affinity IL-2R consisting of the chains IL-2Rβ and IL-2Rγ, were responsive to IL-2. Mature CD25+Foxp3+ Treg cells exhibited even greater sensitivity to IL-2, as they maintained their phenotype and viability at concentrations of IL-2 (0.04 U ml−1) to which CD25+ Treg cells and Foxp3+ Treg cells did not respond (Supplementary Fig. 1a). To confirm these findings in vivo, we used ultrasound-guided intrathymic injection to co-transfer sorted, congenically distinct (CD90.2+CD45.2- or CD90.1+CD45.2+) CD25+ or Foxp3+ Treg cells into the thymus of CD45.1+ mice (Fig. 1b), indicating that although they lack CD25 expression, Foxp3+ Treg cells and Foxp3+ Treg cells converted into mature CD25+Foxp3+ Treg cells at approximately the same frequency, although CD25+ Treg cells did so slightly more efficiently (~45% ± 16.9% and 37% ± 23.5%, respectively; Fig. 1b). Thus, both CD25+ Treg cells and Foxp3+ Treg cells contributed to the generation of mature Treg cells with high efficiency both in vitro and in vivo.

CD25+ Treg cells and Foxp3+ Treg cells have distinct TCR repertoires. To address whether CD25+ Treg cells and Foxp3+ Treg cells represent distinct subsets of cells with different TCR repertoires, or whether the Treg cell developmental pathway chosen reflects only the stochastic expression of CD25 or Foxp3, we used mice expressing a Foxp3RFP Foxp3– red fluorescent protein reporter7–9. To address whether CD25+ Treg cells and Foxp3+ Treg cells have distinct TCR repertoires, we compared their ability to convert into mature Treg cells in response to low doses of IL-2 for 3 days in vitro. Sorted CD25+ Treg cells and Foxp3+ Treg cells responded to very low amounts of IL-2 (0.2–1.0 U ml−1) by converting to mature CD25+Foxp3+ Treg cells (Supplementary Fig. 1a), indicating that although they lack CD25 expression, Foxp3+ Treg cells, which express the low-affinity IL-2R consisting of the chains IL-2Rβ and IL-2Rγ, were responsive to IL-2. Mature CD25+Foxp3+ Treg cells exhibited even greater sensitivity to IL-2, as they maintained their phenotype and viability at concentrations of IL-2 (0.04 U ml−1) to which CD25+ Treg cells and Foxp3+ Treg cells did not respond (Supplementary Fig. 1a). To confirm these findings in vivo, we used ultrasound-guided intrathymic injection to co-transfer sorted, congenically distinct (CD90.2+CD45.2- or CD90.1+CD45.2+) CD25+ or Foxp3+ Treg cells into the thymus of CD45.1+ mice (Fig. 1b), indicating that although they lack CD25 expression, Foxp3+ Treg cells and Foxp3+ Treg cells converted into mature CD25+Foxp3+ Treg cells at approximately the same frequency, although CD25+ Treg cells did so slightly more efficiently (~45% ± 16.9% and 37% ± 23.5%, respectively; Fig. 1b). Thus, both CD25+ Treg cells and Foxp3+ Treg cells contributed to the generation of mature Treg cells with high efficiency both in vitro and in vivo.

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CD25+ Treg cells and Foxp3+ Treg cells have distinct affinity for self antigen. Next we examined the types of antigens with which CD25+ Treg cells and Foxp3+ Treg cells interacted. The relative abundance of either Treg cell subset germ-free mice was similar to that in specific-pathogen-free mice (Supplementary Fig. 2a,b) or in C57Bl/6 mice co-housed with pet-store mice, which have a normalized microbial experience1, compared with that of specific-pathogen-free mice (Supplementary Fig. 2c), suggesting that interactions with self antigens are the major driver of Treg cell selection in the thymus. We used expression of the transcription factor Nur77, whose abundance is directly proportional to TCR signal strength, to assess the strength of the interaction of CD25+ Treg cells and Foxp3+ Treg cells with self antigens in the thymus. In Nur77-GFP mice, in which green fluorescent protein (GFP) expression correlates with the strength of TCR stimulation12, mature CD25+Foxp3+ Treg cells showed higher expression of Nur77-GFP than that of conventional CD4+Foxp3+ T cells13, corresponding with the higher degree of self-reactivity attributed to Treg cells1. In this system, expression of Nur77-GFP in CD25+ Treg cells was significantly higher than that recorded in mature Treg cells (Fig. 2c,d), while its expression in Foxp3+ Treg cells was significantly lower than that of CD25+ Treg cells (Fig. 2c,d). These findings indicate that CD25+ Treg cells and Foxp3+ Treg cells have a distinct affinity for self antigens in the thymus.

We next assessed transcriptomic differences between Treg cell subsets using single-cell RNA sequencing (RNA-seq). CD25+ Treg cells, Foxp3+ Treg cells and mature CD25+Foxp3+ Treg cells, sorted from the thymus of Foxp3-GFP mice, in which an IRES–GFP construct was knocked into the 3' untranslated region of the Foxp3 gene, were used to create individual single-cell RNA-seq libraries that were subjected to high-throughput sequencing. The transcriptomic data from these individual libraries were then combined for joint analysis. Individual cells were color-coded based on sort origin, and a combined dimensional reduction with a graph-based clustering approach followed by a shared nearest-neighbor modularity optimization-based clustering algorithm (Seurat R package) was then applied to this combined dataset to identify cell groups with...
Fig. 2 | CD25+ TregP cells and Foxp3lo TregP cells are distinct thymic Treg cell lineages. a. Morisita–Horn indices comparing the similarity of Vj2 CD3 repertoires generated by TCR sequencing of CD4+CD25 Foxp3+ cells, CD4+CD25 Foxp3+ TregP cells, and CD4+CD25 Foxp3+ mature TregP cells isolated from TciiIf Tcrj−/− mice. b. Plot of Morisita–Horn similarity indices comparing TCRs between CD25+ and Foxp3lo TregP cell populations, and TCRs in CD25+ TregP cells and mature TregP cells or Foxp3lo TregP cells and mature TregP cells. CD25+ TregP cell comparisons are shown in green and Foxp3lo TregP cell comparisons in orange. Data represent two independent experiments, n=4 mice. Data were analyzed by a two-sided paired t-test. c.d Flow cytometry analysis of Nur77-GFP MFI in CD25+Foxp3+ TregP cells, CD4+Foxp3+ TregP cells and CD25+Foxp3+ mature TregP cells obtained from the thymus of Nur77-GFP reporter mice. Dots represent individual mice. Data are displayed as mean ± s.d. and represent seven independent experiments, n=19 mice. Data were analyzed using a two-sided paired Friedman test with Dunn’s multiple comparisons test. e. Left: Three-dimensional (3D) nonlinear dimensional reduction (tSNE) plots from 10x Genomics single-cell RNA-seq dataset for sorted CD25+ TregP cells, Foxp3+ TregP cells and CD25+Foxp3lo TregP cells, displaying relationships between individual cells with color coding based on flow cytometry sort origin. Right: 3D tSNE plots of data identical to those at left but color-coded based on gene expression profiles. f. Heatmap of the top ten differentially regulated genes from each cluster derived from e. Each column represents gene expression for an individual cell; yellow is up and purple is down. Data from f. are representative of three independent experiments, n=3 mice. *P<0.05, **P<0.0005, ***P<0.0001. DP, double-positive thymocyte; MFI, mean fluorescence intensity.

CD25+ TregP cells and Foxp3lo TregP cells are at distinct developmental stages. The cell surface markers CD24 and Qa2, or CD69 and MHCI, can be used as surrogates to analyze thymocyte age15. We found that CD25+ TregP cells were largely CD24hiQa2lo (87%) and CD69hi MHC1, and thus representative of immature CD4+CD8+ thymocytes. 

Distinct gene expression. This analysis generated clusters of cells that closely confirmed the original sorted populations (Fig. 2e) based on expression of Il2ra and Foxp3 (Supplementary Fig. 3a). The analysis also identified a small subset of contaminating thymocytes that expressed Rag1, Cdb1 and Dntt and therefore probably represented CD4+CD8+ double-positive thymocytes (Fig. 2f). Heatmaps based on the top ten differentially expressed genes for each cell subset discriminated all four cell clusters (Fig. 2f). Analysis of differentially expressed genes indicated that there were ~180 reproducibly differentially expressed genes between CD25+ and Foxp3lo TregP cell subsets (Fig. 2f and Supplementary Table 2). Similar results were obtained in an independent single-cell RNA-seq study with individually sorted CD25+ TregP cell, Foxp3lo TregP cell and mature TregP cell libraries (Supplementary Fig. 3b), or when CD25+ TregP cell, Foxp3lo TregP cell and mature TregP cell subsets were combined into one library (data not shown). CD25+ TregP cells were enriched in pro-apoptotic genes and genes involved in negative selection (Nria1 and Bcl2l111) (Supplementary Fig. 3c and Supplementary Table 2), consistent with stronger TCR signaling in this subset. Foxp3lo TregP cells showed increased expression of Ms4a4b and Ms4a6b (Fig. 2f) encoding MS4A4B and MS4A6B, which bind to GITR and enhance signaling via TCR and GITR14 and may facilitate differentiation of lower-affinity CD4+ thymocytes into Foxp3lo TregP cells and enhance the sensitivity of Foxp3lo TregP cells to IL-2. Thus, CD25+ TregP cells and Foxp3lo TregP cells had distinct interactions with self antigens present in the thymus and had unique transcriptomes indicative of distinct modes of selection and differentiation.
controlled by Rag2 gene-regulatory elements, to determine the kinetics of differentiation for CD25+ TregP cells and Foxp3+ TregP cells. The Rag2-GFP transgene turns off after positive selection in CD4+CD8+ thymocytes, after which the GFP protein decays with a relatively slow half-life, allowing GFP+ recent thymic emigrants to be distinguished from older GFP+ T cells in peripheral blood and lymphoid organs. We reasoned that the gradient of RAG2-GFP signal among CD4+ single-positive (CD4SP) thymocytes could be used to distinguish thymocytes at different stages of development. For these studies, we took bins from RAG2-GFP+ (youngest CD4SP) to RAG2-GFPdim (oldest CD4SP) and examined expression of CD25 and Foxp3. RAG2-GFPintminus cells (bin 1) were all CD25 Foxp3+ CD4SP thymocytes (Fig. 3a), CD25+ TregP cells, but not Foxp3+ TregP cells or mature CD25+Foxp3+ TregP cells, were detected in bin 2, while Foxp3+ TregP cells and mature CD25+Foxp3+ TregP cells appeared in bin 3. CD25+ TregP cells, Foxp3+ TregP cells and mature CD25+Foxp3+ TregP cells were all detected in bins 4–6, while CD25+ TregP cells were no longer detected in bin 7 because these cells differentiate, die or leave the thymus (Fig. 3a and Supplementary Video 1). Bin 8 contained GFP– cells (Fig. 3a), consistent with agonist-driven Treg selection occurring in the thymic medulla. In contrast, while Foxp3+ TregP cells were found in the thymic medulla, a substantial proportion (20%) were located in the thymic cortex (Fig. 4a,b), suggesting that at least some Foxp3+ TregP cells were selected on cortical antigens. Thus, CD25+ and Foxp3+ TregP cell subsets differentiate with distinct kinetics and exhibit different rates of apoptosis; moreover, a fraction of the Foxp3+ TregP cell subset shares features with conventional (that is, non–Treg) T cells undergoing positive selection, which also occurs in the thymic cortex.

**NF-kB is critical for the development of Foxp3+ TregP cells.** We next assessed the effect of co-stimulation and downstream signaling pathways on the development of CD25+ TregP cells and Foxp3+ TregP cells. Gld−− thymus exhibited a decrease in abundance of 1.7-fold in CD25+ TregP cells but a larger decrease of 6.7-fold in Foxp3+ TregP cells, compared to thymi from WT mice (Fig. 5a). Similar results were found in CD28-AAYAA mice, which express a mutant CD28 lacking the PYAP motif that links CD28 to activation of the transcription factor NF-kB (data not shown), but not in CD28-Y170F mice, which express a mutant CD28 lacking Y170 that links CD28 to signaling through the kinase PI3K (data not shown). Conversely, IKK-CA mice, which express a constitutively active form of the kinase IKK that leads to constitutive NF-kB activation, showed a selective increase in Foxp3+ TregP cells (data not shown). A caveat of the analysis in CD28-AAYAA, CD28-Y170F and IKK-CA mice is the lack of CD73 staining to exclude recirculating mature effector T cells and Treg cells from the CD25+Foxp3+ TregP cell and CD25+Foxp3+ TregP cell gates, respectively. Finally, we examined the effect of the downstream transcription factor NF-kB1 on Treg cell development. Nfkb1−− mouse thymus showed a 3.6-fold reduction in the abundance of CD73 Foxp3+ TregP cells compared to that from WT mice, while CD73+ CD25+ TregP cell abundance was unaffected (Fig. 5b). These results suggest that co-stimulation, and especially activation of NF-kB1, are selectively required for the formation of Foxp3+ TregP cells.

**CD25+ TregP cell and Foxp3+ TregP cell development is regulated by distinct enhancers.** To examine whether Foxp3 is required for the development of Treg cells from both CD25+ TregP cells and Foxp3+ TregP cells we used Foxp3-GFPknock-in mice, in which a GFP reporter construct is knocked into the Foxp3 locus and generates a GFP-Foxp3 fusion protein. These mice express normal amounts of Foxp3-Foxp3 protein but have been described as functional Foxp3 hypomorphs. Following gating on CD4+CD73− thymocytes, a significant reduction in the frequency of Foxp3+ TregP cells was found in the thymus of Foxp3-GFPknock-in mice compared with that of

**Table 1 | List of representative TCR V,2 CDR3 sequences and the relative read distribution in each sorted population (conventional defined as CD4+CD8+ Foxp3+)**

| Amino acid sequence | Reads, conventional | Reads, CD25+ TregP cells | Reads, Foxp3+ TregP cells | Reads, TregP cells |
|---------------------|---------------------|--------------------------|---------------------------|-------------------|
| CAAGSAGNKLTF        | 0                   | 0                        | 2,737                      | 7,714             |
| CAAKSGSFNLTF        | 0                   | 1,888                     | 0                          | 3,587             |
| CAAKSHGLTFRF        | 0                   | 0                        | 1,176                      | 3,093             |
| CAAPSSQWQLIF        | 0                   | 2,028                     | 659                        | 1,697             |
| CAASYYQKLIF         | 0                   | 349                      | 0                          | 1,801             |
| CAASKGYNQKLW        | 0                   | 569                      | 0                          | 1,729             |
| CAASAPYNQKLIF       | 0                   | 0                        | 4,221                      | 1,428             |
| CAASSGNSQNLFL       | 0                   | 0                        | 1,392                      | 1,399             |
| CAALDLSNRLTL        | 0                   | 1,015                     | 863                        | 1,097             |
| CAARASSQWLIF        | 0                   | 224                      | 1,044                      | 2,080             |
| CAASQSNQKLIF        | 0                   | 432                      | 268                        | 1,177             |
| CAARNVQKLIF         | 1092                | 5,278                     | 2,532                      | 7,041             |
| CAASGTGGYKVF        | 199                 | 317                      | 2,870                      | 1,128             |

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WT mice, while the abundance of CD25+ TregP cells was unaffected (Fig. 5c). To examine this in more detail, we analyzed Foxp3-GFP^{loxP} mice lacking the Foxp3 regulatory element Cns3 (called ‘Cns3-/- mice’ here). Cns3-/- mice, which are known to have a ~40% reduction in the frequency of Treg cells in the thymus\(^{44}\), have selective defects in immune tolerance and a Treg cell bias towards higher self-reactivity\(^{45}\). Cns3-/- mice lacked Foxp3^{lo} TregP cells (Fig. 5c). Consistent with previous reports\(^{46}\), mature CD25^{hi}Foxp3^{lo} TregP cell numbers were also substantially reduced in Cns3-/- mice compared to WT mice, and the defect was about twice as large (~85% reduction) when gating on CD73^{hi} cells to eliminate mature recirculating TregP cells (Fig. 5c). Importantly, mature CD25^{hi}Foxp3^{lo} CD73^{hi} TregP cells in Cns3-/- and WT mice expressed comparable amounts of Foxp3 (Supplementary Fig. 6a), indicating that Cns3 is not required for Foxp3 expression. CD25^{hi} TregP cells isolated from Foxp3-GFP^{loxP} and Cns3-/- mice cultured in vitro upregulated Foxp3 and differentiated into mature CD25^{hi}Foxp3^{hi} TregP cells with comparable efficiency when stimulated with IL-2 (Fig. 5d), suggesting that Cns3 is not required for upregulation of Foxp3 in CD25^{hi} TregP cells. These results indicated that the development of TregP cells from Foxp3^{hi} TregP cells was blocked in the absence of Cns3 and that mature TregP cells developed only from CD25^{hi} TregP cells in Cns3-/- mice.

A non-coding single-nucleotide polymorphism that contributes to an increased risk for autoimmunity in humans was previously described in the Il2ra locus\(^{28-34}\), specifically in enhancer CalE4, which is required for rapid induction of Il2ra following TCR activation\(^{35}\). Next we examined the role of this autoimmunity-associated Il2ra enhancer in TregP cell development. Deletion of the CalE4 Il2ra enhancer in a non-obese diabetic (NOD) mouse background led to a significant reduction in the percentages of thymic

Fig. 3 | CD25^{hi} and Foxp3^{lo} TregP cells are in discrete selection stages. a. Representative histograms of RAG2-GFP expression in CD4SP thymocytes obtained from the thymus of RAG2-GFP mice. Bins are displayed according to high (bin 1) to low (bin 8) RAG-GFP expression, and cells in each bin are plotted for CD25 versus Foxp3 expression. Data shown are concatenated results from three mice and are representative of seven independent experiments, n = 9 mice. b. Representative flow cytometry plots of CD73 staining in CD4SP thymocytes and RAG2-GFP expression in CD73^{hi} and CD73^{lo} compartments. Data are representative of five experiments, n = 5 mice. c. Representative flow cytometry plots (top) and quantification (below) of the percentage of CD4^{hi}CD73^{lo} thymocytes differentiating into each TregP cell population in WT vs Il2ra-/- thymus. Data represent three independent experiments, n = 16 WT and Il2ra-/- mice, n = 12 Il2ra-/- mice. Data were analyzed by two-sided Mann-Whitney test. d. Lef: representative example of annexin V staining on CD4^{hi}CD73^{lo}CD25^{hi}Foxp3^{lo} TregP cells (top green histogram) and CD4^{hi}CD73^{lo}CD25^{hi}Foxp3^{lo} TregP cells (bottom orange histogram) from Foxp3-GFP mice. Right: quantification of annexin V staining for CD25^{hi} TregP cells and Foxp3^{lo} TregP cells. Data represent three independent experiments, n = 10 mice. Data were analyzed by two-sided Wilcoxon matched-pairs signed-rank test. All data are displayed as mean ± s.d., *P < 0.05, **P < 0.005, NS not significant.
CD25\(^+\) T\(_{reg}\) cells and mature T\(_{reg}\) cells compared with that of WT NOD mice (Fig. 5e). In contrast, the percentage of Foxp3\(^+\) T\(_{reg}\) cells was slightly increased, perhaps as a compensatory mechanism. This decrease in CD25\(^+\) T\(_{reg}\) cells was not due to a lack of CD25 expression in general, as the expression of CD25 on mature T\(_{reg}\) cells, or on the remaining CD25\(^+\) T\(_{reg}\) cells, was not reduced compared with that of WT controls (Supplementary Fig. 6b; data not shown). Collectively these data suggest that the development of T\(_{reg}\) cells from CD25\(^+\) T\(_{reg}\) cells and Foxp3\(^+\) T\(_{reg}\) cells is controlled by distinct regulatory circuits.

**Inhibition of negative selection pathways expand CD25\(^+\) T\(_{reg}\) cell generation**. To further probe whether CD25\(^+\) T\(_{reg}\) cells undergo negative selection, we examined the development of T\(_{reg}\) cells in Itk\(^--\) mice, because mice lacking the tyrosine kinase ITK have defects in negative selection\(^a\). The thymus of Itk\(^--\) mice showed higher frequencies of mature thymic CD25\(^+\)Foxp3\(^+\) T\(_{reg}\) cells\(^a\), as well as CD25\(^+\) T\(_{reg}\) cells and Foxp3\(^+\) T\(_{reg}\) cells (Fig. 6a). The adaptor ADAP, which is downstream of ITK, is also required for efficient negative selection\(^b\). Thymi from Adap\(^--\) mice showed an increase in the abundance CD25\(^+\) T\(_{reg}\) cells, but no change in Foxp3\(^+\) T\(_{reg}\) cells, compared with that of WT mice (Fig. 6b). The frequency of mature CD25\(^+\)Foxp3\(^+\) T\(_{reg}\) cells in the thymus was also significantly increased in Adap\(^--\) mice compared with that of WT mice (Fig. 6b). Thus, Adap\(^--\) mice have a selective increase in CD25\(^+\) T\(_{reg}\) cells compared with that of WT mice. A potential explanation for the discrepancy between Itk\(^--\) mice and Adap\(^--\) mice is that ITK deficiency is known to induce increased production of IL-4 in invariant nature killer T cells (iNKT cells) in the thymus\(^3,4\), while ADAP deficiency does not. To determine whether the different phenotypes, in terms of T\(_{reg}\) cell development, in Adap\(^--\) mice and Itk\(^--\) mice are linked to IL-4 production, we examined T\(_{reg}\) cell development in Itk\(^--\) and Itk\(^--\) \times Il4ra\(^--\) mice. Compared to Itk\(^--\) mouse thymus, no increase in abundance of Foxp3\(^+\) T\(_{reg}\) cells was seen in Itk\(^--\) \times Il4ra\(^--\) mice (Fig. 6a,c), suggesting that the increase in Foxp3\(^+\) T\(_{reg}\) cells in the former was due to increased amounts of IL-4 present in the thymus. Thus, CD25\(^+\) T\(_{reg}\) cells are selectively pruned by the ITK–ADAP pathway required for negative selection.

**CD25\(^+\) T\(_{reg}\) cells and Foxp3\(^+\) T\(_{reg}\) cells have distinct cytokine responsiveness**. IL-2 and, to a lesser degree, IL-15 are the predominant cytokines driving the STAT5-dependent differentiation of CD25\(^+\) T\(_{reg}\) cells into mature T\(_{reg}\) cells\(^1,2,4,13,16\). Because the ability of Foxp3\(^+\) T\(_{reg}\) cells to differentiate into mature T\(_{reg}\) cells in response to IL-4 has not been evaluated, we queried whether IL-4 could affect the differentiation of CD25\(^+\) T\(_{reg}\) cells or Foxp3\(^+\) T\(_{reg}\) cells subsets into mature T\(_{reg}\) cells. As reported previously\(^1,6,16\), IL-4 did not result in the robust conversion of thymically derived CD25\(^+\) T\(_{reg}\) cells into mature T\(_{reg}\) cells but supported substantial conversion of thymus-derived Foxp3\(^+\) T\(_{reg}\) cells into mature CD25\(^+\)Foxp3\(^+\) T\(_{reg}\) cells in an in vitro assay (Fig. 7a). Foxp3\(^+\) T\(_{reg}\) cells showed slightly higher expression of the IL-4 receptor (IL-4Ra) than that of CD25\(^+\) T\(_{reg}\) cells (Fig. 7b), which we consider unlikely to account for the difference in the differentiation of CD25\(^+\) T\(_{reg}\) cells and Foxp3\(^+\) T\(_{reg}\) cells in response to IL-4. In addition, although IL-4 converted Foxp3\(^+\) T\(_{reg}\) cells into mature T\(_{reg}\) cells, these cells expressed less CD25 and Foxp3 than those stimulated with IL-2 alone (Supplementary Fig. 7a–d).

It is unclear which cells drive the production of IL-4 in the thymus. In humans, Hassall’s corpuscles, a distinct anatomical feature of the thymus containing cells that resemble tuft cells, are important for T\(_{reg}\) cell development\(^7\). Tuft cells were also reported in the murine thymus\(^9,10\) and are major producers of IL-25, which induces IL-4 production in other cell types\(^10\). To test whether tuft cells influence T\(_{reg}\) cell differentiation, we examined T\(_{reg}\) cell development in Pou2f3\(^--\) mice, which lack the transcription factor POU2F3 required for the development of tuft cells. We observed a decrease in frequency of ~30% for the development of Foxp3\(^+\) T\(_{reg}\) cells, but
Fig. 5 | Foxp3 hyp. TregP cells are dependent on Nfkb1 activation and the Foxp3 regulatory element Cns3. **a**, Left: representative flow cytometry plots for CD4+CD8 CD73+ gated thymocytes from WT and Cd28−/− mice stained with antibodies to CD25 and Foxp3 (one experiment, n = 4 WT and 5 Cd28−/− mice). Right: cumulative data for all mice, depicting the relative percentages of CD25+ TregP cells and Foxp3 hyp. TregP cells in CD4+CD8 CD73+ thymocytes from WT and Cd28−/− mice. **b**, Representative flow cytometry plots for CD4+CD8 CD73+ gated thymocytes from WT and Nfkb1−/− mice stained with antibodies to CD25 and Foxp3 (three independent experiments, n = 19 WT and 11 Nfkb1−/− mice). Right: cumulative data for all mice depicting the relative percentages of CD25+ TregP cells and Foxp3 hyp. TregP cells among CD4+CD8 CD73+ thymocytes from WT and Nfkb1−/− mice. **c**, Top: representative flow cytometry plots for CD4+CD8 CD73+ gated thymocytes from WT, Foxp3-GFPKI and Cns3−/− mice stained with antibodies to CD25 and Foxp3 (four independent experiments, n = 8 WT, 14 Foxp3-GFPKI and 14 Cns3−/− mice). Right panels: quantification of cumulative data for all mice, depicting relative percentages of CD25+ TregP cells, Foxp3 hyp. TregP cells and mature CD25+ Foxp3 hyp. TregP cells in CD4+CD8 CD73+ thymocytes from WT (black circles), Foxp3-GFPKI (black squares) and Cns3−/− (black triangles) mice. **d**, Percentages of CD25+Foxp3 hyp. mature TregP cells generated after stimulating sorted CD4+CD8 CD73+CD25+ TregP cells from the thymus of Foxp3-GFPKI and Cns3−/− mice for 3 days with 0 U ml−1 IL-2 (black circles, n = 5 Foxp3-GFPKI and 5 Cns3−/− replicates), 0.2 U ml−1 IL-2 (black squares, n = 4 Foxp3-GFPKI and 5 Cns3−/− replicates), 1 U ml−1 IL-2 (black triangles, n = 5 Foxp3-GFPKI and 6 Cns3−/− replicates) and 100 U ml−1 IL-2 (open circles, n = 5 Foxp3-GFPKI and 5 Cns3−/− replicates), derived from two independent experiments. **e**, Quantification of cumulative data from two independent experiments showing the relative percentages of CD25+ TregP cells, Foxp3 hyp. TregP cells and mature CD25+Foxp3 hyp. TregP cells from CD4+CD8 CD73+ gated thymocytes from WT (black circles) or EDEL mice, which lack the CaRE4 enhancer (black squares). Data represent two independent experiments, n = 10 wild-type and 10 EDEL mice. In **a, b**, data were analyzed by two-sided unpaired t-test; in **c**, CD25+ TregP cells (%) and Foxp3 hyp. TregP cells (%) were analyzed by one-way analysis of variance (ANOVA) with Tukey’s multiple comparison test, and TregP cells (%) were analyzed by Kruskal–Wallis test with Dunn’s multiple comparisons test; in **d**, data were analyzed by Kruskal–Wallis test with Dunn’s multiple comparisons test; and in **e**, data were analyzed by two-way ANOVA with Sidak multiple comparisons test. All data are displayed as mean ± s.d. **P < 0.005, ***P < 0.0001, NS not significant.

not for that of CD25+ TregP cells, in Pou2f3 hyp. thymus compared to WT thymus (Fig. 7c). To determine whether iNKT cells, the canonical producers of IL-4 in the thymus, selectively affect the development of Foxp3 hyp. TregP cells, we examined the frequency of TregP cells in Cd1d−/− mice, which lack NKT cells. There was a reduction of ~20% in the abundance ofFoxp3 hyp. TregP cells in the Cd1d−/− thymus compared with that of WT BALB/c control thymus (Fig. 7d). This effect was observed only in BALB/c mice, which produce abundant
NKT cell–derived IL-4, not in C57Bl/6 mice, in which NKT cells produce very little IL-4 (data not shown). Thus, the development of both CD25+ TregP cells and Foxp3lo TregP cells shows a degree of dependence on thymic tuft cells, and this is probably partially mediated via tuft cell induction of IL-4 production in NKT cells.

**CD25+ and Foxp3lo TregP cells exhibit distinct functions.** Next we investigated whether the mature TregP cells derived from CD25+ TregP cells or Foxp3lo TregP cells revealed differences in their ability to prevent distinct types of autoimmune disease driven by different autoantigens. To test whether mature TregP cells generated from CD25+ TregP cells differed in their ability to suppress autoimmunity in the central nervous system in comparison to Foxp3lo TregP cell–generated TregP cells, we isolated thymic CD25+ TregP cells and Foxp3lo TregP cells, transferred them separately into individual C57Bl/6 host mice 1 day before immunization with the peptide MOG35–55 and monitored the development of EAE signs. Transfer of mature TregP cells is known to prevent or ameliorate signs of disease in this EAE model. Mice receiving Foxp3lo TregP cells showed disease scores throughout the study similar to those of mice not receiving TregP cell transfer (Fig. 8a). In contrast, disease progression and severity in mice that had received CD25+ TregP cells was significantly ameliorated from days 14–19 after disease induction compared with that of mice receiving no TregP cells or Foxp3lo TregP cells (Fig. 8a,b). The number of congenerically marked donor Foxp3+ TregP cells observed following transfer of CD25+ or Foxp3lo TregP cells was similar in the spleen at the endpoint of the experiment, 20 days after TregP cell transfer (Fig. 8c), suggesting that conversion of CD25+ TregP cells and Foxp3lo TregP cells into mature TregP cells was similar. However, transferred Foxp3lo TregP cells prevented weight loss in a T cell–transfer model of colitis (Supplementary Fig. 8), demonstrating that suppressor activity in Foxp3lo TregP cell–derived TregP cells was normal. Finally, we used MOG1–Aα tetramers in combination with magnetic bead enrichment approaches to identify MOG1–Aα–specific TregP cells and

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*Fig. 6 | Itk−/− mice show increased TregP cell production from both TregP cell pathways via distinct molecular mechanisms. a, Left: representative flow cytometry plots of CD4+CD8−CD73+ gated thymocytes from WT vs. Itk−/− mice stained with antibodies to CD25 and Foxp3. Right: cumulative data for all mice depicting the relative percentages of CD25+ TregP cells and Foxp3lo TregP cells in CD4+CD8−CD73+ thymocytes from WT, Itk−/− and Itk−/− × Il4ra−/− mice. b, Left: representative flow cytometry plots of CD4+CD8−CD73+ gated thymocytes from WT vs. Adap−/− mice stained with antibodies to CD25 and Foxp3. Right: cumulative data for all mice, depicting the relative percentages of CD25+ TregP cells and Foxp3lo TregP cells in CD4+CD8−CD73+ thymocytes from WT and Adap−/− mice (three independent experiments, n = 12 WT and 12 Adap−/− mice). c, Representative flow cytometry plots of CD4+CD8−CD73+ gated thymocytes from WT, Itk−/− and Itk−/− × Il4ra−/− mice stained with antibodies to CD25 and Foxp3. In a, CD25+ TregP cells (%) were analyzed by one-way ANOVA with Tukey’s multiple comparisons test; Foxp3lo TregP cells (%) were analyzed by Kruskal-Wallis test with Dunn’s multiple comparisons test and in b, data were analyzed by two-sided Mann-Whitney test. All data are displayed as mean ± s.d. *P < 0.05, ***P < 0.0001, NS not significant.*
TregP cells in the thymus of WT mice. We found MOG-I-A\(^{\alpha}\)-specific T cells in the CD25\(^{+}\) TregP cell and mature TregP cell subsets but observed only one MOG-I-A\(^{\alpha}\)-specific T cell among Foxp3\(^{lo}\) TregP cells in the 15 mice examined (Fig. 8d,e), suggesting that MOG-I-A\(^{\alpha}\)-specific TregP cells are almost always generated from CD25\(^{+}\) TregP cells and are specifically required for protection against EAE.

**Discussion**

Here we found that both CD25\(^{+}\) TregP cells and Foxp3\(^{lo}\) TregP cells contributed to mature TregP cell development in the thymus and that in our hands, they did so relatively equivalently. However, these two distinct TregP cell subsets differed in many important ways. They utilized different signaling pathways and enhancers for their differentiation, were affected in distinct ways by different stromal cells and cytokines and expressed distinct TCR repertoires and RNA transcriptomes. Most importantly, TregP cells derived from the CD25\(^{+}\) TregP cell subset versus those derived from the Foxp3\(^{lo}\) TregP cell subset had distinct roles in protecting against autoimmunity. Thus, there are at least two different developmental pathways in the thymus that contribute substantially to the generation of the mature TregP cell repertoire.

Several pieces of evidence support the notion that mature TregP cells derived from both CD25\(^{+}\) TregP cells and Foxp3\(^{lo}\) TregP cells are required for full maintenance of immunotolerance. Both the Foxp3-GFP\(_{\text{KIN}}\) hypomorph and Cns3\(^{-/-}\) mice showed defects in generating Foxp3\(^{lo}\) TregP cells and specific defects in immunotolerance. Foxp3-GFP\(_{\text{KIN}}\) mice have a relatively mild defect in immunotolerance that is revealed only on distinct genetic backgrounds.\(^{22,23}\)

In contrast, Cns3\(^{-/-}\) mice have unique defects in immunotolerance, such as greater lung inflammation than that of WT mice.\(^{26,28}\) Deletion of this enhancer resulted in a selective decrease in Cns3\(^{-/-}\) mice selectively lack Foxp3\(^{lo}\) TregP cells but not CD25\(^{+}\) TregP cells, this suggests a unique role for Foxp3\(^{lo}\) TregP cell-derived mature TregP cells in preventing autoimmunity.

Additional studies suggest a unique role for CD25\(^{+}\) TregP cells in promoting immune tolerance. The CaRE4 enhancer in the Il2ra locus harbors an autoimmunity risk variant that promotes susceptibility to inflammatory bowel disease but protection against diabetes.\(^{16,24}\) Deletion of this enhancer resulted in a selective decrease in CD25\(^{+}\) TregP cells, suggesting that CD25\(^{+}\) TregP cells-derived TregP cells may have an important role in protecting against inflammatory bowel disease. Likewise, Cns3\(^{-/-}\) mice exhibit increased protection against EAE.\(^{25}\) Because TregP cells in Cns3\(^{-/-}\) mice are derived almost exclusively from CD25\(^{+}\) TregP cells, this suggests that CD25\(^{+}\) TregP cell-derived TregP cells may have an important role in protecting against inflammatory bowel disease. Thus, modulating the frequency of CD25\(^{+}\) TregP cells results in differential protection against autoimmunity. This observation has important translational implications, as it indicates that it is possible to identify TregP cells with selected TCRs that have uniquely potent efficacy against specific types of autoimmune disease.

The presence of more than one developmental pathway leading to mature TregP cells raises the question of why such a system has evolved. TregP cells are required to prevent responses to self antigens and commensal antigens, as well as to dampen anti-pathogen...
immune responses once these agents have been cleared. Establishing such a diverse repertoire requires the generation of Treg cells able to recognize thymic self antigens with high affinity. Such a population could be generated by a process of agonist selection in the thymus. However, agonist selection alone is unlikely to generate the broad repertoire of Treg cells needed to prevent immune responses to commensal organisms, or to limit responses to foreign pathogens. In many ways this resembles the problem facing conventional thymocytes, which must generate a repertoire capable of recognizing a vast array of antigens that they never encounter in the thymus. Thus, establishing a broader, non-self-focused repertoire for Treg cells through the Foxp3\textsuperscript{lo} TregP cell pathway. Finally, the genetic variability that altered the relative balance of these two developmental pathways also altered the Treg cell repertoire and correlated with susceptibility to distinct forms of autoimmunity. Given the differences in signaling pathways and cytokines that control these two developmental pathways, specific targeting of each Treg cell population could help patients with various autoimmune defects.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at [https://doi.org/10.1038/s41590-018-0289-6](https://doi.org/10.1038/s41590-018-0289-6).

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Author Contributions

D.L.O. designed and conducted experiments and wrote the manuscript. S.A.M., L.E.S., J.B.A., S.A.R., D.S.B., R.S., W.H., I.P., C.N.M., C.H., C.J.C., P.A., U.B., R.S.L., C.M.H. and Y.Z. performed some experiments or analyzed data and contributed intellectually to the work. M.A., S.S., A.A., A.M., Y.Z. and C.B.W. provided key reagents and/or animals and intellectual contributions. M.A.F. designed experiments, supervised research and assisted in the preparation of this manuscript. All authors read the manuscript and helped with final revisions.

Competing interests

A.M. is a co-founder of Spotlight Therapeutics. A.M. has served as an advisor to Juno Therapeutics and is a member of the scientific advisory board at FAPT Pharma. The Minnesota laboratory has received sponsored research support from Juno Therapeutics, Epinomics and Sanofi, and a gift from Gilead. A.A. has received sponsored research support from 3 M. MAF has received sponsored research support from Merck.

Additional information

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Methods

Mice. Mice were housed in specific pathogen-free facilities at the University of Minnesota, Cornell University, Salk Institute or University of California San Francisco, and experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committees of these respective institutions. Exceptions were germ-free mice housed in germ-free facilities at the University of Chicago, and mice with a normalized microbial experience which were housed in the University of Minnesota’s mouse vivarium. Pet store mice were purchased from various pet stores in the greater Minneapolis-St. Paul metropolitan area. Information about the age of the pet store mice was not available from the vendor. Co-housing of specific-pathogen-free mice with sex-matched pet store partner was performed as described within the University of Minnesota BSL-3 facility. Conversion efficiency was confirmed by assessing the conversion of naive CD8+ T cells into CD8+ memory T cells; effective conversion correlated with 30–60% CD25+CD44+ T cells. All relevant ethical guidelines were followed. Foxp3-GFP (006772) and Foxp3-RFP mice (008374) were obtained from the Jackson Laboratory. CD45.1 (B6.SJIL) mice were obtained from the National Cancer Institute. The following mice have all been described previously: Nurr77-GFP BAC reporter, Rag2-GFP report, Cns1–3 Foxp3-GFP, CD28–, Nkhl1–, Ilk–, Ilk–×Hfra–, Adapt–, Izsna EDEL, Ilg1–, Rag2–, Cdi1– and Telp–x TCRTgR. Mice were generally 6–8 weeks old, but ranged 4–16 weeks. Mice were randomly selected for experiments in age-matched cohorts. The investigators were not ‘blinded’ to genotype during data acquisition.

Tissue preparation and cell isolation. For analysis of thymocyte and Treg development, thymus was mechanically dissociated into 1× PBS with 2% fetal bovine serum (FBS) and 2 mM ethylenediaminetetraacetic acid, pH 7.4, using frosted-glass slides. Cell suspensions were passed through 70μm filters and washed before staining.

Flow cytometry and antibodies. All flow cytometry analysis was conducted in the University of Minnesota Flow Cytometry Core Facility using BD LSR II and Fortessa cytometers (BD Biosciences). For surface staining, cells were stained for 20 min with fluorochrome-conjugated antibodies before washing and analysis or intracellular staining. Intracellular detection of Foxp3, cleaved casp-3 and GFP was performed as previously described using the eBioscience Transcription Factor Staining Kit. When staining for GFP, Rag2-GFP thymi were fixed for 10 min at room temperature in 1.6% paraformaldehyde before intracellular staining of GFP and Foxp3 using the eBioscience Transcription Factor Staining Kit. For apoptosis assays, thymi were harvested and mechanically dissociated into 1× phosphate buffered saline (PBS) on ice. Following surface staining, cells were washed into Annexin V binding buffer (eBioscience) and stained with annexin V conjugate and PI (Invitrogen) to guide an insulin syringe (27 G) into the thymus and visualize injection of the left or right side of the sternum. The ultrasound image generated was used to guide injection. Ultrasound-guided intrathymic injection. The condition was induced by injection of 200 ng Pertussis Toxin on days 0 and 2 via intraperitoneal injection in complete RPMI supplemented with 20% of the reads in the Treg cell compartment, which influence distribution disproportionally, were excluded from analysis as described.

Treg progenitor conversion assays. Treg progenitors were isolated as previously described. Briefly, Foxp3-GFP thymi were dissociated and co-cultured, and pooled CD4SP cells were enriched by magnetic depletion with biotinylated anti-CD8 and anti-Ter119 (eBioscience) followed by secondary labeling with streptavidin-conjugated microbeads (Miltenyi Biotec). Enriched CD4SP cells were stained with fluorochrome-conjugated anti-CD4, anti-CD25, anti-CD73 and streptavidin using the eBioscience Transcription Factor Staining Kit. For apoptosis, thymi were harvested and mechanically dissociated into 1× phosphate buffered saline (PBS) on ice. Following surface staining, cells were washed into Annexin V binding buffer (eBioscience) and stained with Annexin V conjugate and PI (Invitrogen) to guide an insulin syringe (27 G) into the thymus and visualize injection of the left or right side of the sternum. The ultrasound image generated was used to guide injection. Ultrasound-guided intrathymic injection. The condition was induced by injection of 200 ng Pertussis Toxin on days 0 and 2 via intraperitoneal injection in complete RPMI supplemented with 20% of the reads in the Treg cell compartment, which influence distribution disproportionally, were excluded from analysis as described.

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Mapping and analysis of single-cell sequencing. CD4+CD73-CD25+Foxp3+, CD4+CD73-CD25+Foxp3+ and CD4+CD73+CD25+Foxp3+ cells were isolated from a single Foxp3-GFP thymi using a BD FACSAria sorter. Cells were resuspended at 10^6 ml–1 in 50% PBS in 1× PBS before being counted and captured using 10x Genomics Single Cell 3’ Solution. A custom genome was created by adding the sequence for the Foxp3-GFP construct as a new chromosome to the Ensembl GRCh38 reference and general feature format file (version 89). The gene annotation file was then filtered further to include only protein-coding genes. The 10x Genomic Cellranger pipeline (version 2.2.0; https://support.10xgenomics.com/single-cell-gene-expression/software/overview/welcome) was used to align reads and generate counts for each sorted population (or library). Sorted populations were then combined using depth normalization mode.

Heatmap analysis of different principal components and an elbow plot were created to determine whether to proceed with nine principal components for cluster analysis using the Seurat function ‘FindCluster’. To visualize the clusters, nonlinear dimensional reduction was performed using these nine principal components. Clusters were assigned to a specific population by comparing cells labeled for the original sorted population and cellular markers. R package plot3D (version 1.1.1) and threejs (version 0.3.1) were used to create 3D visualization of the nonlinear dimensional reduction data. Differential expression (‘bimod’) was calculated for each cluster against all other clusters to identify potential markers for each individual cluster. Differential expression was also calculated between individual clusters using the same method.

Experimental induction of autoimmune encephalomyelitis. The condition EAE was induced in mice as described previously. Briefly, on day –1, sorted Treg cell populations (4 × 10^5 – 5 × 10^5) were transferred intravenously into CD45.1+ congenic mice. On day 0, mice were immunized with 200 μg of MOG35-55 emulsified in complete Freund’s adjuvant (CFA) with 4 mg/ml heat-killed Mycobacterium tuberculosis (Tb). Immunization was performed with two subcutaneous injections (50 μl) of emulsion in the left or right flank of the lower back. Mice were treated with 200ng Pertussis Toxin on days 0 and 2 via intraperitoneal injection in 1xPBS. Mice were monitored for disease progression and were treated with normal saline (subcutaneously) or were given wet food on the cage floor, as described previously.

Induction of transfer colitis. Colitis was induced as previously described. Briefly, 5 × 10^5 CD4+CD45RB+CD45RB+ cells were transferred by intravenous or intraperitoneal injection into Rag2–/– recipient mice. Sorted Treg cells were transferred by intraperitoneal injection in either a single dose (5 × 10^7 on days 1 and 7) or a single dose (5 × 10^7 on day 21). Mice were weighed weekly to monitor disease progress.

Statistical analysis. Statistical tests used to analyze data are included within the figure legends. Briefly, comparisons of two groups were done by either paired t-test (paired, normal data), Wilcoxon matched-pairs test (paired, non-normal data), t-test (non-paired, normal data) or Mann–Whitney (non-paired, non-normal data); tests were always two-sided. Comparison of three or more groups was done
by one-way ANOVA (non-paired, normal data), Kruskal–Wallis (non-paired, non-normal data) or Friedman test (paired, non-normal data). $P<0.05$ was considered significant. Statistics were calculated using Prism (GraphPad Software). All data, except those specifically mentioned in Figure legends, are displayed as mean ± s.d.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Code availability statement.** The source code for bioinformatics analyses is attached as a supplementary file.

**Data availability**
The data that support the findings of this study are available from the corresponding author upon request. Single-cell RNA-seq data were deposited at Gene Expression Omnibus, with the following accession code: GSE123067.

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Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- **n/a** Confirmed
- □ The exact sample size \((n)\) for each experimental group/condition, given as a discrete number and unit of measurement
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- □ A description of all covariates tested
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- For null hypothesis testing, the test statistic (e.g. \(F, t, r\)) with confidence intervals, effect sizes, degrees of freedom and \(P\) value noted. Give \(P\) values as exact values whenever suitable.
- □ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- □ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- □ Estimates of effect sizes (e.g. Cohen’s \(d\), Pearson’s \(r\)), indicating how they were calculated
- □ Clearly defined error bars
- State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection

- LSR II and Fortessa cytometers were used to collect flow cytometry data; 10X Genomics system was used to capture and create libraries for single cell RNA-seq; FACS Aria II sorters were used for all cell sorting

Data analysis

- Prism (8), FlowJo (10.5.3); Seurat R Package (version 1.4.0.12)

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

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Single cell RNA-seq data will be deposited in public repositories prior to publication and made available to reviewers/editors upon request.
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size          | Sample sizes were not calculated prior to performing experiments but conform to standard practices; Sample sizes were sufficient to detect differences between groups and all comparisons had a minimum of 3 data points |
|----------------------|---------------------------------------------------------------------------------------------------------------------------|
| Data exclusions      | The only data excluded from the manuscript is from cells in single-cell RNA-seq data sets which did not meet pre-established, standard quality thresholds (i.e., cells with fewer than 5 genes per cells) |
| Replication          | All replicates successfully reproduced similar results |
| Randomization        | Animals were randomly selected for experiments |
| Blinding             | Investigators were not blinded to genotypes prior to data collection as non-subjective measures were used to understand the phenotypes observed (i.e. abundance of a population); Bioinformaticists were initially blinded to sample sort origins during single-cell RNA-seq analysis; Investigators were not blinded during colitis experiments as non-subjective measures were used to track disease (weight); A blinded investigator was used for measuring EAE clinical score for 1 experiment and the majority of data points for the other experiment |

Reporting for specific materials, systems and methods

| Materials & experimental systems | Methods |
|---------------------------------|---------|
| n/a                             | n/a     |
| ☑ Involved in the study         | ☑ Involved in the study |
| ☑ Unique biological materials   | ☑ ChiP-seq |
| ☑ Antibodies                    | ☑ Flow cytometry |
| ☑ Eukaryotic cell lines         | ☑ MRI-based neuroimaging |
| ☑ Palaeontology                 |         |
| ☑ Animals and other organisms   |         |
| ☑ Human research participants   |         |

| Antibodies |
|------------|
| Antibodies used |
| anti-mouse CD4 (GK1.5) BV786, BD Biosciences, 563331, 1:100 |
| anti-mouse CD4 (RM4-5) BV510, BD Biosciences, 563106, 1:100 |
| anti-mouse CD4 (RM4-5) PE, eBioscience, 12-0042-83, 1:300 |
| anti-mouse CD4 (GK1.5) eF450, eBioscience, 48-0041-82, 1:100 |
| anti-mouse CD4 (GK1.5) APC, eBioscience, 17-0041-83, 1:300 |
| anti-mouse CD4 (RM4-5) FITC, Tonbo Biosciences, 35-0042-U500, 1:100 |
| anti-mouse CD4 (GK1.5) PE-Cy7, Biolegend, 100422, 1:300 |
| anti-mouse CD4 (RM4-5) BV605, Biolegend, 100548, 1:100 |
| anti-mouse CD8a (S3-6.7) BV786, BD Biosciences, 563332, 1:100 |
| anti-mouse CD8a (S3-6.7) APC-eF780, eBioscience, 47-0081-82, 1:100 |
| anti-mouse CD8a (S3-6.7) Biotin, Tonbo Biosciences, 30-0081-U500, 1:200 |
| anti-mouse CD8a (S3-6.7) PerCP-Cy5.5, Biolegend, 100734, 1:100 |
| anti-mouse CD3 (145-2C11) PE-Cy7, eBioscience, 25-0031-82, 1:100 |
| anti-mouse CD25 (PC61.5) PerCP-Cy5.5, Tonbo Biosciences, 65-0251-U100, 1:100 |
| anti-mouse CD25 (PC61.5) BV421, Biolegend, 102043, 1:100 |
| anti-mouse CD25 (PC61.5) PE-Cy7, eBioscience, 25-0251-82 |
| anti-mouse CD25 (PC61.5) APC, eBioscience, 17-0251-82, 1:250 (HIC) |
| anti-mouse FOXP3 (FJK-16s) PE, eBioscience, 12-5773-82, 1:100 |
| anti-mouse FOXP3 (FJK-16s) APC, eBioscience, 17-5772-82, 1:100 |
| anti-mouse FOXP3 (FJK-16s) FITC, eBioscience, 320112, 1:100 |
| anti-mouse FOXP3 (FJK-16s) AF700, eBioscience, 56-5773-82, 1:100 (IHC) |
Multiple lots of antibodies were used which did not affect results or reproducibility.

Validation data for all antibodies are available on the manufacturers website.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals
Mice were housed in specific pathogen–free facilities at the University of Minnesota, Cornell University, Salk Institute or University of California San Francisco, and experiments were in accordance with protocols approved by the Institutional Animal Care and Use Committee. The one exception are mice with a normalized microbial experience which were housed in the University of Minnesota’s mouse vivarium. Pet store mice were purchased from various pet stores in the greater Minneapolis-St. Paul metropolitan area. Information about the age of the pet store mice was not available from the vendor. Co-housing of SPF mice with sex-matched pet store partner was performed as described (Beura, et al., 2016) within the University of Minnesota BSL-3 facility. Conversion efficiency was confirmed by assessing the conversion of naïve CD8+T cells into CD8+memory T cells; effective conversion correlated with ~30-60%CD8+CD44hiT cells. All relevant ethical guidelines have been followed. Foxp3-GFPmice (006772), Foxp3-RFPmice (008374) were from the Jackson Laboratory. CD45.1+(B6.SJL) mice were from the US National Cancer Institute. Nur77-GFPBAC reporter mice, Rag2-GFP reporter mice, Cns3-/- and Foxp3-GFPKIN, CD28-/-, Nfkb1-/-, Itk-/-, Itk-/-x Il4Ra-/-, Adap-/-, Il2ra EDEL (and littermate NOD controls), Ilgal-/-, Rag2-/-, Cd1d-/- and Tc18x TCRα/- have been described previously (Boursalian et al., 2004; Fontenot et al., 2005; Hsieh et al., 2004; Huang et al., 2014a; Liao and Littman, 1995; Moran et al., 2011; Peterson et al., 2001; Sha et al., 1995; Shahinian et al., 1993; Shinkai et al., 1992; Simeonov et al., 2017; Sonoda et al., 1999; Zheng et al., 2010). Age of mice was generally between six to eight weeks but the range over experiments was four to sixteen weeks old. Mice were randomly selected for experiments, in age-matched cohorts. EAE experiments were performed with female donors and recipients however other experiments used male or female mice. The investigators were not 'blinded' to genotype during data acquisition.

Wild animals
Pet store mice were purchased from various pet stores in the greater Minneapolis-St. Paul metropolitan area. Information about the age of the pet store mice was not available from the vendor. Co-housing of SPF mice with sex-matched pet store partner was performed as described (Beura et al., 2016) within the University of Minnesota BSL-3 facility.

Field-collected samples
No field-collected samples were used in this study.
Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation Sample preparation is described in detail within the methods section.

Instrument LSR II, Fortessa, and Aria II

Software FlowJo (10.5.3)

Cell population abundance Representative post-sort data is presented in figure 1

Gating strategy Gating strategy is presented in figures 1 and 8 for thymic TRP/Treg and tetramer staining respectively. Briefly, lymphocytes were identified by SSC-A vs FSC-A then singlets by SSC-A by SSC-W; CD4 single positive cells were identified as CD8-CD4+, either bulk CD4 single positive or CD73-CD4 single positive cells were analyzed for CD25-FOX3-, CD25-FOX3lo or CD25+FOX3+ cells.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.