Rbpj expression in regulatory T cells is critical for restraining TH2 responses

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The transcriptional regulator Rbpj is involved in T-helper (TH) subset polarization, but its function in Treg cells remains unclear. Here we show that Treg-specific Rbpj deletion leads to splenomegaly and lymphadenopathy despite increased numbers of Treg cells with a polyclonal TCR repertoire. A specific defect of Rbpj-deficient Treg cells in controlling TH2 polarization and B cell responses is observed, leading to the spontaneous formation of germinal centers and a TH2-associated immunoglobulin class switch. The observed phenotype is environment-dependent and can be induced by infection with parasitic nematodes. Rbpj-deficient Treg cells adopt open chromatin landscapes and gene expression profiles reminiscent of tissue-derived TH2-polarized Treg cells, with a prevailing signature of the transcription factor Gata-3. Taken together, our study suggests that Treg cells require Rbpj to specifically restrain TH2 responses, including their own excessive TH2-like differentiation potential.

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Regulatory T cells (T\textsubscript{reg}) are important mediators of peripheral tolerance, and their absence leads to catastrophic autoimmunity in men (IPEX\textsuperscript{1}) and mice (Scurfy\textsuperscript{2}). T\textsubscript{reg} cells are characterized by both expression of the hallmark transcription regulator Foxp3\textsuperscript{3,4} and a unique epitogenic profile\textsuperscript{5,6}. T\textsubscript{reg} cells specialize to fulfill their diverse regulatory functions\textsuperscript{7,8}. They can engage defined molecular pathways to specifically suppress either TH1-polarized, TH2-polarized, or TH17-polarized immune effector cells\textsuperscript{9,10}. For instance, under TH1 conditions, T\textsubscript{reg} cells up-regulate expression of the T\textsubscript{H}1-specific transcription factor T-box 21 (T-bet) and accumulate at inflammatory sites\textsuperscript{11}. Correspondingly, under TH2 conditions, T\textsubscript{reg} cells express Gata-binding protein 3 (Gata-3) and interferon regulatory factor 4 (Irf4), and T\textsubscript{reg}-specific Iرف4-deletion leads to IL-4 cytokine production of effector T cells and lymphoproliferative disease\textsuperscript{12,13}. Up-regulation of signal transducer of activated T cells 3 (Stat3) is critical for the capacity of T\textsubscript{reg} cells to control TH17-mediated inflammation, while its T\textsubscript{reg}-specific deletion results in enhanced IL-17 production by effector cells and intestinal inflammation\textsuperscript{14,15}. Therefore, T\textsubscript{reg} cells integrate unique parts of T\textsubscript{H}1 subtype-specific transcriptional programs to specifically control the respective T\textsubscript{H}1-polarized immune response.

Recombination signal-binding protein for immunoglobulin kappa J region (Rbpj) is a transcription factor commonly known for its function as a co-factor during Notch signaling, translating extracellular signals into gene expression changes\textsuperscript{16}. In the context of T cell differentiation and function, Rbpj has been associated with T\textsubscript{H}1/T\textsubscript{H}2 cell fate decisions\textsuperscript{17,18}. Indeed, in CD4\textsuperscript{+}Foxp3\textsuperscript{−} conventional T (T\textsubscript{con}) cells, Rbpj is expressed in a complex with the Notch intracellular domain (NICD) was shown to be critical for regulation of Gata-3, an important molecular switch for optimal TH2 responses\textsuperscript{19}. In contrast to this, forced expression of the NICD in T\textsubscript{reg} cells rendered them incapable of suppressing T effector cells and caused autoimmunity\textsuperscript{20}. This indicates that, based on the cellular context, Rbpj and Notch have a different impact on cellular responses. While the importance of Rbpj is well documented in T\textsubscript{H}2 subset polarization, its function in T\textsubscript{reg} cells remains unclear.

Here we unveil a previously unappreciated role of Rbpj in regulating the capacity of T\textsubscript{reg} cells to restrain T\textsubscript{H}2 responses. Loss of Rbpj renders T\textsubscript{reg} cells more sensitive to T\textsubscript{H}2-inducing conditions and fosters the extensive generation of Gata-3-positive tissue-type T\textsubscript{reg} cells.

Results
Deletion of Rbpj causes defined organ pathology. We specifically deleted Rbpj in T\textsubscript{reg} cells by crossing Foxp3\textsubscript{Cre,YFP} mice with mice harboring floxed Rbpj alleles (called Δ/Δ). We compared these to littermate control Foxp3\textsubscript{Cre,YFP} mice with wildtype Rbpj alleles (termed WT). We closely monitored our mice for 20 weeks, and about 40% of mice spontaneously developed splenomegalia and lymphadenopathy within this time interval, while about 60% of animals remained healthy (Fig. 1a, b). We confirmed the T\textsubscript{reg}-specific deletion of Rbpj on DNA, RNA, and protein level (Supplementary Fig. 1a–d). First, we analyzed Δ/Δ and WT mice for the presence of CD4\textsuperscript{+}CD25\textsuperscript{−}Foxp3\textsuperscript{+} T\textsubscript{reg} cells in spleen and other tissues (Fig. 1c). We observed a strong increase in the fraction of T\textsubscript{reg} cells among CD4\textsuperscript{+} T cells from about 12% in WT spleens to 28% in spleens from affected Δ/Δ animals. In absolute numbers, lymph nodes and spleen from Δ/Δ animals harbored about 10–20 times more T\textsubscript{reg} cells than their WT counterparts (Fig. 1c, right panel). This increase was not seen in the thymus, indicating normal thymic T\textsubscript{reg} cell output, or in mesenteric lymph nodes. Analysis of CD44 and L-selectin (CD62L) indicated activation of the T\textsubscript{reg} compartment in affected Δ/Δ mice (Fig. 1d). Furthermore, affected Δ/Δ animals showed a higher density of Foxp3\textsuperscript{+} cells by immunohistology in spleen (Fig. 1e) and lymph nodes (Supplementary Fig. 1e). Affected animals developed noticeable skin pathology at snout, abdominal and tail regions, which served as useful biomarker to identify sick animals. Affected skin areas showed thickening of the epidermis and mononuclear cell infiltrates in the corium (Fig. 1f). Global defects in T\textsubscript{reg} cell function normally lead to a severe autoimmune manifestation, with destructive immune cell infiltration in a diverse set of organs\textsuperscript{2}. Detailed histological examination of different organs including small intestine, large intestine, stomach, kidneys, salivary gland, eye, liver, and lung showed no signs of obvious immune cell infiltration and tissue destruction (Supplementary Figure 2a, b), indicating that Rbpj deficiency in T\textsubscript{reg} cells did not lead to a global loss of T\textsubscript{reg}-mediated immune control. This was supported by data from a standard in vitro suppression assay with TCR-stimulated T\textsubscript{con} responder cells, were we did not detect significant changes in the in vitro suppressive potential (Supplementary Fig. 3). In summary, these data indicate that RBPJ deficiency affected a more specific segment of T\textsubscript{reg} function.

Germlen cell formation and B-cell polarization. Given that the T\textsubscript{reg}-specific deletion of Rbpj affected secondary lymphoid organs and skin, we performed gene expression analysis of total LN RNA from WT and affected Δ/Δ animals (Fig. 2a). The most strongly up-regulated genes among the 4388 differentially expressed probes were involved in immune globulin (Ig) chain rearrangement and antibody production (Fig. 2a, highlighted in blue). Furthermore, B cell-specific markers such as Cd22 and Cd19 were over-expressed in LN from Δ/Δ mice. In addition, Il4 was increased and pointed towards T\textsubscript{H}2 subtype polarization (Fig. 2a and Supplementary Fig. 4a). B cells also down-modulated Il4r in affected animals (Fig. 2b). To determine whether this increase in absolute B cell numbers and their maturation lead to changes in antibody Ig subtype distribution, we analyzed blood serum Ig levels via ELISA (Fig. 2c). Interestingly, we detected a significant up-regulation of IgG1 and IgE, while IgG3 was repressed in the serum (Fig. 2c), indicative of a classical IL-4 (=TH2)-induced B cell antibody class switch\textsuperscript{21}. As another hallmark of ongoing B cell differentiation and antibody production, we detected the formation of numerous germinal centers in LNs from affected Δ/Δ animals, but not in healthy WT animals (Fig. 2d and Supplementary Fig. 4b). Since IgE levels showed an almost 100-fold increase in affected Δ/Δ animals (Fig. 2c), and skin-resident mast cells express a high-affinity IgE receptor, we performed Giemsa staining of affected skin tissue. Indeed, we found an accumulation of mast cells rich in dark-stained granulae, presumably contributing to skin pathology in Δ/Δ animals (Fig. 2e and Supplementary Fig. 4c).

Because of the spontaneous germlinal center formation, we analyzed whether the produced antibodies could bind self-antigens. To this end, protein was extracted from different organs isolated from Rag2-deficient animals, separated by SDS–PAGE, and incubated with serum from WT or affected Δ/Δ animals. We observed antibody binding to self-proteins extracted from organs such as lung, stomach, small intestine, pancreas, and eye with blood serum from Δ/Δ animals, although antibody-binding patterns were different between individual animals (Supplementary Fig. 5). In conclusion, our B-cell analysis revealed spontaneous germlinal center formation with TH2-specific Ig class-switch.

T\textsubscript{H}2 polarization of T\textsubscript{con} cells. Il4 expression was increased in LN from Δ/Δ mice (Fig. 2a and Supplementary Fig. 4a), therefore
we studied T<sub>conv</sub> polarization. In spleen and LN, T<sub>conv</sub> cells became activated and differentiated into effector/memory T cells by down-regulation of CD62L and up-regulation of CD44. Furthermore, the absolute number increased by about 5–10-fold (Fig. 3a). Treatment of T cells from WT and affected Δ/Δ animals with phorbol-12-myristate-13-acetate (PMA) and Ionomycin to measure intracellular cytokine expression revealed higher frequencies of T<sub>conv</sub> cells from affected Δ/Δ animals producing IL-2, IL-4, and IL-13, while T<sub>reg</sub> cells remained unchanged (Fig. 3b). This indicated a TH2 polarization. Since Gata-3 is the master transcription factor of TH2 cells, we analyzed Gata-3 expression in the T<sub>conv</sub> compartment and detected increased frequencies of Gata-3-positive T<sub>conv</sub> cells from about 3% in WT to 13% in Δ/Δ animals by flow cytometry (Fig. 3c, d), which was confirmed on RNA level (Fig. 3d, right panel). Co-staining of stimulated T<sub>conv</sub> cells with IL-4, IL-13, and Gata-3 revealed that IL-4 and IL-13 was mainly produced by Gata-3-positive TH2 cells (Fig. 3c). To examine closely the link between the observed TH2 bias and the
Fig. 1 Treg-specific deletion of Rbpj causes lymphoproliferative disease. a Kaplan-Meier survival curve illustrating disease development in Foxp3CreRbpjfl/fl, Foxp3CreRbpjYFPfl/Δ, and Foxp3CreRbpjYFPΔ/Δ animals within 20 weeks after birth. Disease defined by inflammatory skin lesions, lymphadenopathy, and splenomegaly. We observed 79 animals with Treg lineage-specific bi-allelic Rbpj deletion (Foxp3CreRbpjfl/Δ), 67 animals with mono-allelic Rbpj deletion (Foxp3CreRbpjYFPfl/Δ), and 79 wildtype animals (Foxp3CreRbpjYFPΔ/Δ). Statistical testing log-rank Mantel-Cox test ($p < 0.0001$). Treg-specificity of Rbpj deletion in Supplementary Fig. 1. b Splenomegaly and lymphadenopathy in affected Δ/Δ animals, representative picture (Ax: axial; Br: brachial; Iug: inguinal; Cer: cervical). Right panel, spleen weight in milligram ($n = 6-19$, Mann-Whitney test) in a dot plot where error bars indicate standard deviation and center line mean value. c Quantification of Treg (CD3+CD4+CD69+CD25+Foxp3+) cell number and frequency in WT vs. affected Δ/Δ animals. Left panel, representative pseudocolor plots of splenic Treg cells of CD4+ T cells, frequencies shown as number. Middle graph, Treg frequencies in spleen and LN (% of CD4+; n = 6–26, Mann-Whitney test). Black dots Treg cells from WT, open circle dots Treg cells from Δ/Δ animals, dots represent individual mice, line mean value. Right graph, total Treg cell numbers in various tissues ($n = 6-10$, Mann-Whitney test, Mes: mesenteric). d Left panel, CD44 and CD62L expression in Treg cells from spleens of WT and affected Δ/Δ animals, quantification for several tissues to the right ($n = 7–10$, Mann-Whitney test). e Immunohistochemistry (IHC) of spleen from WT and affected Δ/Δ animals. Foxp3 staining in brown with hematoxylin staining. f Hematoxylin and eosin (H&E) staining of non-inflamed and inflamed skin tissue of representative WT and affected Δ/Δ mouse. Additional stainings in Supplementary Fig. 2. Data representative of two or more independent experiments with individual mice. Asterisks indicate statistical significance with *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$. In IHC and H&E stainings, original magnification scale bars have been magnified for better visibility. Source data are provided as a Source Data file

Environmental conditions influence disease development. Approximately 40% of Foxp3CreYFPRbpjfl/Δ mice developed a T$_{H}$2-polarized disease, while 60% of animals did not get sick during a 20-week observation period (Fig. 1a). Since most mice that developed a pathology were older than 10 weeks, environment-related causes might influence the phenotype in Δ/Δ animals. Therefore, we transferred embryos into a new breeding facility with individually ventilated cages and a defined altered Schaedler flora (details in Methods Section). In this new environment, the incidence rate dropped and about 90% of animals remained healthy until 20 weeks of age (Fig. 4a). These findings indicate that environmental factors influence T$_{H}$2-disease onset in Δ/Δ animals. To further investigate this, we infected healthy young Δ/Δ and control animals from the new breeding facility with the parasitic nematode Strongyloides ratti (S. ratti). Infective larvae penetrate the skin and migrate within 3 days to the small intestine. Normally, immune competent mice mount a canonical T$_{H}$2 response and resolve the infection within 2–4 weeks$^{22}$. Final expulsion of parasites from the intestine is predominantly mediated by IL-9 and activated mucosal mast cells$^{23}$. Especially, Foxp3+ Treg cells have been shown to expand following S. ratti infection$^{24}$, Therefore, we infected two cohorts of Δ/Δ and WT animals with S. ratti infectious larvae into the footpad and examined animals after 6 (cohort 1) and 14 days (cohort 2, Fig. 4b). Already 6 days post infection (p.i.), Treg frequency of CD4+ T cells in the spleen increased in Δ/Δ animals to about 28%, while WT animals had normal Treg frequencies at that time point (about 9%) (Fig. 4c). The three-fold increase in Treg frequency was persistent and also measured at 14 days p.i. (Fig. 4c). At the 14-day time point, Tconv cells expressed more Gata-3 in Δ/Δ animals (Fig. 4d), as well as more IL-4, IL-13, and IL-9, but not IFN-γ (Fig. 4e–g). The fraction of Gata-3-high expressing Treg cells increased in the Δ/Δ animals from day 6 to day 14 p.i. to 56%, while at the same 14-day time point, the WT animals had significant lower numbers (27%, Fig. 4d). Furthermore, we detected increased levels of IgE, but not IgM, in blood serum of Δ/Δ animals (Fig. 4h), mirroring the phenotype observed during our initial breeding (Fig. 2c). Interestingly, on day 6, Δ/Δ animals with stronger T$_{H}$2-polarization and increased IL-9 production displayed reciprocally reduced output of S. ratti DNA (Fig. 4i) and a strong trend towards reduced numbers of parasitic females in the intestine (Fig. 4j). In summary, our data indicate that disease development in Δ/Δ animals was influenced by environmental factors, such as the breeding environment, and that the T$_{H}$2-inducing parasite S. ratti could trigger the onset of this phenotype in Δ/Δ animals.

Characterization of Rbpj-deficient Treg cells. Mice with Rbpj-deficiency in Treg cells spontaneously developed a T$_{H}$2-type disease. Since the Rbpj-deficiency was specific to Treg cells, we aimed at dissecting the molecular properties of those. Therefore, we measured the expression of classical Treg associated proteins, such as Foxp3, cytotoxic T-lymphocyte-associated protein 4 (Cd4-α), or Ikaraos family zinc finger 2 (Helios), and did not detect obvious differences between Treg cells from WT or affected Δ/Δ animals (Fig. 5a). Analogously, the methylation status of the Treg-specific demethylated region (TSDR), a well-described methylation-sensitive cis-regulatory region required for durable expression of the Foxp3 gene$^{25}$, was unchanged (Fig. 5b). The investigation of the T cell receptor (TCR) repertoire revealed no abnormal TCR beta J-chain usage (Fig. 5c), nor any dominant clones (Fig. 5d), and only a small increase in overall clonality and decreased entropy (Fig. 5e). As Treg cells were abundantly present in lymphatic organs of affected Δ/Δ animals, we analyzed peripheral tissues. Treg frequency and total numbers were either equal or elevated (40-fold in affected skin tissue and about three-fold in the lung, Fig. 5f–g and Supplementary Fig. 6) in affected Δ/Δ animals. Since Treg cells co-opt parts of T$_{H}$1 subset programs to specifically suppress those T$_{H}$1 subset responses, we analyzed Gata-3 protein expression. Indeed, we could detect a strong increase of Gata-3 high-expressing Treg cells isolated from affected Δ/Δ compared to WT animals (about 45% vs. 12%, respectively), which could be confirmed on RNA level (Fig. 5h). Furthermore, we investigated T follicular regulatory cells based on the expression of Cxcr5$^{+}$ and PD1$^{+}$ (Tfr; CD4+CD25+Foxp3+CXCR5+PD1+ in WT and Δ/Δ animals. The total number of PD1$^{+}$ Tfr cells was increased in LNs of affected Δ/Δ animals (Fig. 5i), excluding the possibility that a loss of Tfr cells was responsible for the observed phenotype. In addition, we co-stained PD-1 and Gata-3 and noticed a strong increase in Gata-3+PD-1$^{+}$ Treg cells in Δ/Δ mice (Fig. 5j).

Altered gene expression in Rbpj-deficient Treg cells. To dissect molecular characteristics, we performed array-based gene expression analysis of Treg cells isolated from affected Δ/Δ and WT animals (Fig. 6a). Six hundred and ninety probes were found to be differentially expressed. For example, we observed a strong up-regulation of IL-7 receptor (Il7r) and Killer cell lectin-like receptor subfamily G member 1 (KlrG1) genes, while Bcl-2-like
protein 11 (Bcl2l11) and Deltex-1 (Dtx1) were under-represented in Δ/Δ Treg cells (Fig. 6a, b). Bcl2l11 serves as translocator of apoptosis-inducing factors and regulator of mitochondrial depolarization26,27. Indeed, Rbpj-deficient Treg cells showed less caspase-3 activity when compared to WT Treg cells (Fig. 6c and Supplementary Fig. 7). Besides differences in apoptosis, Rbpj-deficient Treg cells also up-regulated the Il7r gene encoding for the IL-7 receptor (IL-7R, CD127). In thymus and spleen, about 75% of Tconv cells expressed the IL-7R, and this was not different in WT and Δ/Δ animals. In contrast to this, Treg cells from Δ/Δ mice showed a strong increase in IL-7R expression in the spleen (Fig. 6d). Since the IL-7R is involved in survival and...
proliferation, we correlated IL-7R expression with the overall frequency of Treg cells in unaffected, as well as phenotypically affected Δ/Δ animals of varying age. A good correlation between both parameters could be observed (r = 0.81), suggesting that IL-7R expression supports Treg accumulation (Fig. 6e). To further validate this, we co-stained IL-7R expression with Ki-67, a widely accepted cell proliferation marker (Fig. 6f). The majority of Treg cells in Δ/Δ animals expressed both IL-7R and Ki-67, which was not seen in WT animals (Fig. 6f), indicating that IL-7R high-expressing Treg cells were the proliferating fraction in Δ/Δ animals. Next, we measured the phosphorylation of Stat-5, a downstream component of the IL-7R signaling cascade. With escalating doses of IL-7, Rbpj-deficient Treg cells phosphorylated significantly more Stat-5, indicating elevated cytokine sensitivity (Fig. 6g). Since Klr1 was up-regulated in the expression profile (Fig. 6a), we co-stained Klr1 and IL-7R expression (Fig. 6h). Indeed, while a Klr1^{+/+}ILR^{+/+} double-positive population was almost absent in T constitutional cells or WT Treg cells, the spleens of affected Δ/Δ animals harbored about 60% Klr1^{−/−}ILR^{+} Treg cells (Fig. 6h). Therefore, both IL-7R and Klr1 were valuable parameters to identify the intensively proliferating subpopulation of Treg cells in affected Δ/Δ mice.

**IL-7R^{+}Klr1^{+} Treg cells are reminiscent of tisTregST2.** In a recent publication, we described a tissue-resident Treg population characterized by the expression of, amongst others, IL-7R, Klr1, IL-33 receptor alpha (ST2), and Gata-3. This Treg subset, mainly present within tissues and T12polarized, was called tisTregST2. Since we detected a strong enrichment of Gata-3, Klr1, and IL-7R-expressing Treg cells specifically in affected Δ/Δ animals, we performed a co-staining for ST2 and Klr1 (Fig. 7a). About 20% of Treg cells from spleens of WT animals co-express ST2 and Klr1 compared to 50–60% of spleen Treg cells from affected Δ/Δ animals (Fig. 7a). A co-staining with Ki-67 and Gata-3 revealed that Klr1^{+}ST2^{+} Treg cells in both WT and Δ/Δ animals were T12 polarized and proliferating (Fig. 7a, middle panel). Klr1^{+}ST2^{+} were also significantly increased in lymph nodes (WT: 2%; Δ/Δ: 27%) and skin tissue (WT: 48%; Δ/Δ: 65%, Fig. 7b). To compare the tissue-like gene expression program of Klr1^{+} tisTregST2-like cells in Δ/Δ animals on a broader scale, we sorted Δ/Δ Klr1^{+/−}, Δ/Δ Klr1^{−/−}, WT Klr1^{+} and WT Klr1^{−} Treg cells from spleen and performed RNA sequencing (RNA-seq) analysis. We extracted RNA-seq data from fat, skin and LN-derived bulk Treg cells from a previous study and normalized all datasets. We then plotted 106 reported tisTregST2 genes in a heatmap (Fig. 7c). Interestingly, there was a strong gene overlap between fat and skin Treg-differentially regulated genes with spleen Δ/Δ-derived and WT-derived Klr1^{+} Treg cells, indicating that the majority of Δ/Δ-Treg cells from affected mice indeed displayed a tisTregST2-like signature. Still, when listing key genes identifying tissue Treg cells from fat (Pparg) or skin (Gpr55), as well as tissue Treg effector molecules such as Il10 and amphiregulin (Areg), the Δ/Δ Klr1^{+} tisTregST2-like population in the spleen of Δ/Δ animals did not express comparable levels of these markers (Fig. 7d). This indicated that they were generated in the lymphoid tissue rather than extravasated from non-lymphoid tissues. To analyze differences in more detail, we prepared MA plots for comparisons between all four groups (Fig. 7e). The comparison between WT Klr1^{+} vs. Δ/Δ Klr1^{+} Treg cells revealed 2036 differential expressed genes, which include the molecular changes associated with loss of Rbpj (Fig. 7e; left panel). This group contains well-known Rbpj target, such as Dtx1,32, as well as suppression-related proteins such as Id-3.33 The comparison between WT Klr1^{−} vs. Δ/Δ Klr1^{+} Treg cells revealed 3330 differential expressed genes (Fig. 7e; right panel), which include tissue-Treg-related genes, such as Klr1 and Il1r1, as well as suppression-related proteins such as Bach2.34 In summary, we showed that affected Δ/Δ animals harbor a strongly increased tisTregST2-like population in their lymphoid tissues.

**Genome-wide chromatin accessibility of WT and Δ/Δ Treg cells.** In affected Δ/Δ animals, Klr1^{+} tissue-like Treg cells constitute the majority of all spleen Treg cells, while in WT animals, Klr1^{−} non-tissue Treg cells dominate the Treg pool at large. To obtain insights into their gene-regulatory landscapes, we isolated both populations and performed the Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq)35. In total, across cell types and replicate experiments, we detected 68,214 ATAC-seq peaks throughout the genome (Fig. 8a). Reference genome annotation revealed that 17.5% of the peaks located to promoters, 41.5% to introns, 2.0% to exons, and 39% to intergenic genomic regions (Fig. 8b). In Δ/Δ Treg cells, about 3400 regions were more accessible compared to WT Treg cells, while in WT Treg cells, 10,816 regions were more accessible in comparison to Δ/Δ Treg cells (Fig. 8b, c). We next asked which transcription factor motifs were enriched in differential accessible chromatin regions to identify potential drivers of WT-specific and Δ/Δ Treg-specific gene-regulatory programs. De novo motif analysis revealed a strong Gata transcription factor signature in Δ/Δ Treg-specific regions (24.38%) vs. background sequences (13.22%) with a high p-value (10^{-68}) and score (0.95) (Fig. 8d, Supplementary Fig. 8a). Of the relevant Gata family members, only Gata-3 was significantly induced in Δ/Δ Treg cells (Fig. 8d, lower panel). In addition, we identified enrichment of Ets, Klf, and AP-1-binding sites in Δ/Δ Treg-specific regions (Supplementary Fig. 8a). WT Treg-specific regions were dominated by Ets, Tcf, Stat, as well as Nur77 motifs, and we also identified significant enrichment of a motif highly similar to the recently described Rbpj consensus-
**Fig. 3** T\(_{\text{conv}}\) cells derived from affected Δ/Δ animals are T\(_{\text{fr}}\)2 polarized. **a** T\(_{\text{conv}}\) cells (CD3\(^+\)CD4\(^+\)CD25\(^-\)Foxp3\(^-\)) from spleens of WT and affected Δ/Δ animals stained for CD44 and CD62L expression, quantification for spleen and LN (n = 7–10, Mann-Whitney test) adjacent to pseudocolor plots. Right panel, T\(_{\text{conv}}\) cell numbers per tissue (n = 6–10, Mann-Whitney test). Black dots T\(_{\text{conv}}\) cells in WT animals, open circles Δ/Δ-derived T\(_{\text{conv}}\) cells, individual mice are shown. **b** Restimulation of splenic T cells followed by intracellular cytokine staining. Upper panel intracellular cytokines in T\(_{\text{conv}}\) cells, lower panel in T\(_{\text{reg}}\) cells (CD3\(^+\)CD4\(^+\)CD25\(^+\)Foxp3\(^+\)). Statistical testing with Mann-Whitney test (IL-2: n = 12–14; IL-4: n = 8–14; IL-13: n = 7–13; IL-17: n = 3–9; IFN-γ: n = 12–14). **c** Co-staining of stimulated T\(_{\text{conv}}\) cells with cytokine and Gata-3 antibody. Upper panel representative dot plots of PMA/Ionomycin-stimulated WT (left) or Δ/Δ (right) T\(_{\text{conv}}\) cells, with IL-4 staining on X-axis and Gata-3 staining on Y-axis. Lower panel, IL-13 staining. **d** Left, dot plots of Gata-3 protein staining in spleen-derived T\(_{\text{conv}}\) cells; middle panel, quantification of Gata-3-positive cells (% of T\(_{\text{conv}}\), n = 8–11, unpaired t-test); right panel, Gata3 mRNA in T\(_{\text{conv}}\) cells (% Hprt, n = 5, unpaired t-test). **e** Identification of T follicular helper cells in lymph nodes from WT vs. affected Δ/Δ animals. Tfh cells identified as CD3\(^+\)CD4\(^+\)CD8\(^-\)CD25\(^-\)Foxp3\(^-\)Cxcr-5\(^-\)PD-1\(^+\) T cells and quantified (n = 7, unpaired t-test). Total numbers of Tfh cells per LN to the right (n = 4, unpaired t-test). **f** CD3\(^+\)CD4\(^+\)CD8\(^-\)CD25\(^-\)Foxp3\(^-\) T cells in lymph nodes of WT or affected Δ/Δ animals co-stained with PD-1 and Gata-3. Quantification to the right (n = 8, unpaired t-test). Data are representative of two or more independent experiments with individual mice (a, b, d, e, f) or a single experiment with individual mice (c). Source data are provided as a Source Data file.
binding site (Supplementary Fig. 8b). Examples of ATAC-seq signals along with occurrences of Rbpj motifs (both de novo identified and previously identified consensus motifs) at key genes are shown in Fig. 8e–h and Supplementary Fig. 8c. Earlier, we showed that Foxp3 expression was unchanged between Δ/Δ and WT Treg cells (Fig. 5a) and Foxp3 TSDR demethylation was unaffected by loss of Rbpj (Fig. 5b). Accordingly, the ATAC-seq profile at the Foxp3 locus remained unchanged between both groups (Fig. 8e). Peak calling identified the TSDR region as highly accessible region in both WT and Δ/Δ Treg cells, but no Rbpj-binding motif has been detected in this or any other part of the Foxp3 gene. The Rbpj locus was accessible in both WT and Δ/Δ Treg cells, indicating that Rbpj was not required to open its own locus (Supplementary Fig. 8c). In contrast to this, tissue-Treg

\[ \text{S. ratti footpad inj.} \]

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\[ \text{Cohort 1} \]

\[ \text{Cohort 2} \]
related genes, such as Klf1 or Il1rl1 (ST2) showed significant ATAC-seq signals around the promoter and potential enhancer sites in Δ/Δ Treg cells, and Rbpj-binding motifs were also found in these regions (Fig. 8f). These changes in ATAC-seq signals translated into enhanced expression of Il1rl1 and Klf1 (Fig. 8i). In addition to tissue Treg-related genes, Treg suppressive function-associated genes, such as Il2ra, Dtx1, and Bach2 also displayed a distinct ATAC-seq profile: in intragenic and/or enhancer sites, WT Treg cells had enriched signals compared to Rbpj-deficient Treg cells (Fig. 8g). Again, Rbpj-binding motifs were detected in differential peaks and ATAC-seq profiles correlated well with changes in gene expression: Il2ra gene expression was significantly down-modulated in Δ/Δ Treg cells, and expression of Dtx1 and Bach2 was almost completely lost (Fig. 8i). Interestingly, Bach2 has recently been described as a key transcription factor involved in regulating T12-polarization by inhibiting Gata-3 expression. Other T12-polarized-related regions were also differentially accessible in Δ/Δ Treg cells, e.g., the T12 locus control region (Rad50), Il10 and Arg2, but the corresponding gene sets were not expressed (Fig. 8b, Fig. 7d and Supplementary Fig. 8c). Taken together, our data suggest that both Rbpj and Gata3 influence the expression of key tiSTregST2-related genes, and that the genomic deletion of Rbpj in concert with a Gata-3-inducing T12-type inflammatory environment lead to the massive differentiation and expansion of tiSTregST2-like cells in Δ/Δ animals.

**T12-polarized Treg fail to suppress T12 responses in vitro.** But why are these T12-polarized Treg cells not controlling the T12-response anymore? Recently, it was shown that Librb4a (encoding the protein ILT3) expressing Treg cells were unable to regulate T12-responses due to their inability to control the maturation of a specific T12-promoting DC subset. This subset of DCs is characterized by the expression of PD-L2 and IRF-4. Our ATAC-seq data identified a highly accessible region at the Librb4a promoter in Klrk1Δ/ΔST2+ Treg cells isolated from affected Δ/Δ animals, and a Rbpj-binding site was also predicted in this region (Fig. 9a). Indeed, enhanced activity at the Librb4a promoter resulted in increased Librb4a expression in Klrk1Δ/ΔST2+ Treg cells from affected Δ/Δ animals (Fig. 9b). In addition, Klrk1Δ/ΔST2+ Treg cells from WT animals expressed more ILT-3 than Klrk1Δ/ΔST2− non-tissue type Treg cells, suggesting a general mechanism of ILT3-expression during differentiation of the tiSTregST2-like gene expression program. Our de novo motif analysis indicated that Gata-3 was responsible for large parts of the tiSTregST2-like signature in Klrk1Δ/ΔST2+ Treg cells from affected Δ/Δ animals (Fig. 8d). To study the link between ILT3+Gata-3 overexpression and control of T12 responses, we performed in vitro polarization studies with Treg cells. IL-4 is the prototype cytokine to induce Gata-3 expression and T122 differentiation, and IL-33 is linked to the generation of tiSTregST2 cells. Therefore, we FACs-sorted highly pure Treg cells from Foxp3GFP animals and expanded them with anti-CD3/CD28 microbeads, IL-2, IL-4, and IL-33 or without the latter two cytokines as control, for 6 days. Both groups of expanded Treg cells stayed highly Foxp3 positive (Fig. 9c). Interestingly, we were able to co-induce ILT3 and Gata-3 expression specifically in the IL-4 and IL-33-treated Treg cells (Fig. 9c). Using this model, we studied the ability of ILT3-expressing T12-polarized Treg cells to influence DC maturation and DC-mediated T12-polarization of FACS-sorted CD4+Foxp3+CD62L+ naive T cells in vitro. Our data revealed that ILT3-expressing T12-polarized Treg cells profoundly promoted the differentiation of PD-L2+1R4F4+ DCs, a subset described to support T12-polarization in vivo. In ILT3-expressing Treg cells were unable to suppress the T12 differentiation of IL-4 and anti-CD3-stimulated naive T cells into Gata-3-polarized effector T cells in vivo. This result is consistent with the authors' demonstration that ILT3-expressing T12-polarized Treg cells are not able to suppress T12 responses in vivo. Finally, we investigated the sensitivity of Rbpj−/− Treg cells to T12-inducing conditions. To this end, we FACs-sorted and expanded Rbpj−/− Treg cells from healthy, young animals, with no pre-existing T12-polarization, and compared them to WT Treg cells. Both groups were treated with escalating doses of IL-4 in vitro (Fig. 9f). Indeed, Rbpj−/− Treg cells were more sensitive to the T12-inducing IL-4 treatment, translating into enhanced Gata-3 protein and mRNA induction in Δ/Δ Treg cells (Fig. 9f). This elevated sensitivity towards Gata-3 induction could explain the profound expansion of Gata-3+Klrk1Δ/ΔST2+ T12-polarized Treg cells in affected Δ/Δ animals, with ameliorated T12-suppressive potential.

**Discussion**

In this study, we identify a previously unrecognized role for Rbpj in Treg cell-mediated immune homeostasis. Upon Treg-specific Rbpj deletion in Foxp3 CreRbpjΔ/Δ mice, mice developed a lymphoproliferative disease with type-2 effector polarized B-cell and T-cell responses. Disease development was environment-related and could be induced by infection with the parasitic nematode S. ratti. The finding that disease development was environment-related could explain the discrepancy to a published study using mice with RbpjΔ/Δ Treg cells, where the authors did not report the lymphoproliferative characteristic.

But what happened once the proper environmental trigger has been received? Based on our data, we would argue that deleting Rbpj confined the functional capacity of Treg cells in several ways. First, augmented proliferation potential: the down-modulation of
Bcl2l11 could lead to enhanced resistance to apoptosis, while the up-regulation of the Interleukin-7 receptor promoted proliferation and supported a strong increase in total Treg numbers in lymphoid tissues. Potential Rbpj-binding sites at the Il7r promoter region have already been reported. Second, Rbpj is important to restrict the TH2 differentiation potential of Treg cells: Rbpj-deficient Treg cells were more sensitive to IL-4 polarization and concomitantly overexpressed Gata-3 and other TH2-associated proteins compared to WT Treg cells. This was also observed upon in vivo infection with parasitic nematodes. Our data showed that about two-times more Treg cells expressed high levels of Gata-3 in Δ/Δ mice as compared to infected WT mice. As a consequence of Gata-3 expression, Δ/Δ Treg cells differentiated into T1f2-polarized KIrG1^+ST2^+ TfrTregST2-like cells. This differentiation integrated a third critical restriction of Treg function and suppressive capacity: the loss of TH2-suppressive capacity via the down-modulation of Bach2, Dtx1, and Il2ra, and the induction of ILT3, Il2ra, encoding for the IL-2 receptor alpha chain (CD25), is required for Treg suppressive capacity. Bach2 has been described as an important transcription factor required...
to inhibit Gata-3 expression and T12 polarization\textsuperscript{34,41}, including the expression of ST2\textsuperscript{42}, and the Bach2–Baf1 interaction was shown to control T12-type immune responses\textsuperscript{43}. In addition, Bach2 supports the suppressive capacity of Treg cells, as a loss-of-function study demonstrated that Bach2-deficient Treg cells failed to prevent disease in a colitis model\textsuperscript{34,44,45}. D1tx1, previously reported to interact with Rbpj\textsuperscript{32}, was also shown to be important for Treg cell suppressive function in a transfer model of colitis\textsuperscript{33}. Finally, the induction of ILT3 can lead to a severe defect in controlling T12-polarized immune responses via the induction of IRF4\textsuperscript{4+}-PD-L2\textsuperscript{+} DCs\textsuperscript{36}.

This cumulative effect on Treg suppressive capacity was finally leading to a loss of T12 suppressive potential, a state where effector T12 cells produced more IL-4, leading to even more Gata-3 expression in ΔΔ Treg cells. Gata-3 expression is required to maintain high Foxp3 expression levels and it is important to prevent Treg cell differentiation into an effector phenotype\textsuperscript{44–46}. But Gata-3 does not function in a binary on-off mode. It has been reported that Gata-3 over-expression in T cell progenitors changes the identity of developing double-negative thymocytes and drives them into the mast cell lineage\textsuperscript{47–49}. These studies indicate that a well-defined Gata-3 dosage is required for proper T cell development and function. A recent report showed that the IL-4 signaling strength is important for Treg cell function. By using mice carrying an IL-4Ra chain mutation leading to enhanced IL-4 signaling, the authors demonstrated that these Treg cells, which were polarized towards a T12 cell-like phenotype with high Gata-3 expression levels, had an impaired functionality\textsuperscript{50}.

Our data indicate that Rbpj could act as a Gata-3 dosage modifier, adjusting the balance of Gata-3 expression by restricting IL-4 sensitivity as a powerful molecular switch. In addition, published findings report that the Gata3 gene itself is a direct target of Rbpj\textsuperscript{18}. Therefore, the regulation of Gata-3 expression could be both on the transcriptional, as well as the IL-4 cytokine sensitivity level. Our findings should be considered in the current discussion that T\textsubscript{12} polarized Gata-3\textsuperscript{+} T\textsubscript{reg} cells are better suppressors of the corresponding T\textsubscript{12} polarized effector T cells, a model proposed based on the complete deficiency of IRF4\textsuperscript{43}. We could show that IL-4 and IL-33-induced Gata3\textsuperscript{high} T\textsubscript{reg} cells express significantly more ILT3 upon in vitro expansion and differentiation, a surface receptor shown to interfere with efficient control of T12 effector cells\textsuperscript{30}. Gata3\textsuperscript{high} T\textsubscript{reg} cells were unable to inhibit T12 differentiation of IL-4 exposed naive CD4 cells in vitro. In contrast to this, they supported the maturation of a T12-promoting IRF4\textsuperscript{+}-PD-L2\textsuperscript{+} DC subpopulation in vitro.

Our motif analysis of the ATAC-sequencing data revealed a strong Gata signature in ΔΔ Treg\textsuperscript{specific} differentially accessible regions. Many of the affected genes were shared with tisTregST2 cells, a T12-biased Treg subset normally present within non-lymphoid tissues\textsuperscript{37}. Rbpj may regulate the Gata-3-dependent TregST2 differentiation pathway and, thereby, might limit the access to the TregST2 compartment in lymphoid organs to allow the maintenance of a diverse Treg subset pool.

Methods

Mice. Wildtype C57BL/6, congenic B6.SJL-Pitpcre-Pepck<sup>Cre+</sup>/BoyCrl (CD45.1<sup>+</sup>) and congenic B6.PL-Thy1<sup>+</sup>/C57 (CD90.1<sup>+</sup>) mice were obtained from Charles River Breeding Laboratories (Wilmington, MA, USA) or the Jackson Laboratory (Bar Harbor, ME, USA). B6.N129(Cg)-Foxp3<sup>tm3Ayr</sup>/mice (Foxp3<sup>ires-cre</sup>) (mice(Foxp3<sup>ires-cre</sup>) were bred to CD45.1<sup>+</sup> or CD90.1<sup>+</sup> mice in the animal facility of the German Cancer Research Center (DKFZ), B6.129(Cg)-Foxp3<sup>ges-cre</sup> (Jackson Foxp3<sup>ires-cre</sup>) were crossed to Rbpj<sup>bdf</sup> mice\textsuperscript{33} to specifically delete Rbpj in Treg cells. Age-matched littermate controls (Foxp3<sup>ires-cre</sup> mice) were used throughout the study. Details about hygiene status for the Rbpj alleles) were used throughout the study. Due to the nature of the study, the experiments involving animals. Relevant ethical regulations for animal research were complied with.

Breeding conditions and disease-free survival in barriers. Data from Fig. 1a are derived from animals housed under specific pathogen-free conditions in a specific mouse facility (called barrier 3) of the German Cancer Research Center, fulfilling the criteria given in the FELASA recommendations (animal number, health monitoring, age, agents, methods). All animals were housed in open cages allowing transmission of agents. Research personnel had access to the unit. Routine testing included testing for ectoparasites, endoparasites, bacteria and viruses. In barrier 3, murine norovirus (MNV), Pneumocystis sp. and Staphylococcus aureus were detected, along with occasional detection of additional opportunistic agents. In our breeding facility, we observed 79 animals with Treg lineage-specific Rbpj deletion (Foxp3<sup>ires-cre</sup>Rbpj<sup>+/−</sup>), of which 21 were sacrificed due to sickness and used for experimentation. Twenty-nine animals <20 weeks old were otherwise healthy but used for experimentation (Regierungspräsidium Karlsruhe, Regierung von Unterfranken Staat für Gesundheit und Verbraucherschutz Hamburg) approved all experiments involving animals. Relevant ethical regulations for animal research were complied with.

...taining unpaired T reg cells from WT and affected ΔΔ animals. Colors indicate respective TCR beta J-chain. Individual mice are shown (n = 3). d Frequency of 10 most abundant Treg TCR sequences for each individual mouse from WT and affected ΔΔ animals. All remaining sequences in blue. e Clonality (left) and entropy (right) values for all TCR sequences. Statistical testing unpaired t-test, individual mice are shown (n = 3). f Foxp3 staining in lung tissue sections from WT (left) or affected ΔΔ animals (right). Additional Foxp3 stainings and controls in Supplementary Fig. 6. g Measurement of Treg cell in peripheral tissues. Treg (CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>−</sup>CD19<sup>−</sup>CD25<sup>−</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>) percentage of CD4<sup>+</sup> (left) or total number (right) in respective tissue (n = 5, unpaired t-test). h Gata-3 protein staining of spleen-derived Treg cells (CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>−</sup>Foxp3<sup>+</sup>). Pseudocolor plots representative examples, quantification of Gata-3-positive cells (n = 9–11) and Gata3 mRNA expression of sorted Treg cells (n = 4–5) right (unpaired t-test). i Identification of T follicular regulatory cells in LN from WT vs. affected ΔΔ animals. Tfr cells as CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>−</sup>CD25<sup>−</sup>Foxp3<sup>+</sup>Cxcr5<sup>−</sup>PD-1<sup>+</sup> T cells and quantified right (n = 4, unpaired t-test). j CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>−</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells from LN of WT or affected ΔΔ animals co-stained with PD-1 and Gata-3. Percentage of Gata-3+PD-1<sup>+</sup> Treg cells quantified to the right (n = 4, unpaired t-test). Data are representative of two or more independent experiments with individual mice (a, g, h) or a single experiment with individual mice (b, c, d, f, i-j). Source data are provided as a Source Data file.
**Fig. 6** Profiling of Rbpj-deficient Treg cells. **a** Gene expression profile of Treg cells (CD3+CD4+CD8–CD25+Foxp3+) from spleens of WT vs. affected Δ/Δ animals. Selected genes labeled. Average values of three biological replicates shown. Numbers show significantly up-regulated or under-represented genes in the comparison (p < 0.05). Statistical calculations described in Methods. Genes with p-values of < 0.001 set to 0.001 and labeled with a triangle.

**b** Measurement of Rbpj (left) and Dtx1 (right) mRNA expression in FACS-isolated spleen-derived Treg and Tconv cells from WT or unaffected Δ/Δ animals via Taqman qPCR (n = 5–6, one-way ANOVA with Newman–Keuls post-test). 

**c** Splenocytes isolated from WT and affected Δ/Δ animals treated with sulfurhodamine-conjugated DEVD-FMK to label active caspase-3. Treg or Tconv cells (CD3+CD4+CD8–CD25+Foxp3+) identified. Representative pseudocolor plots illustrating active Caspase-3 expression are shown in Supplementary Fig. 7. Statistical evaluation with unpaired t-test, n = 10–14.

**d** Left panel, representative dot plots illustrating IL-7R expression on Tconv cells (upper panel) and Treg cells (lower panel) found in WT (left) or affected Δ/Δ (right) animal-derived spleens; right panel, quantification (n = 4–8, unpaired t-test).

**e** Correlation between frequency of IL-7R expression and frequency of Treg cells. Values for healthy unaffected Δ/Δ animals (blue dots) and affected Δ/Δ animals (red dots) shown (n = 17 for blue dots, and n = 8 for red dots).

**f** Values displayed. For IL-7R and Klrg-1 expression in Treg cells or Tconv cells derived from WT or affected Δ/Δ animals. Representative dot plots and quantification (n = 9–10, unpaired t-test).

**g** Intracellular expression of phosphorylated Stat5 (pStat5) after IL-7 treatment of ex-vivo isolated splenocytes from WT vs. affected Δ/Δ animals. Representative histograms on top, percentage of intracellular pStat5 expression in Treg cells below. Statistics based on two-way ANOVA with Bonferroni post-test (n = 6).

**h** IL-7R and Klrg-1 expression in Treg cells or Tconv cells from WT or affected Δ/Δ animals. Representative pseudocolor plots and quantification for Treg and Tconv cells (n = 8–10, unpaired t-test). Data are representative of two or more independent experiments with individual mice (b–f, h) or a single experiment with several individual mice (a, g). Source data are provided as a Source Data file.
Fig. 7 Enrichment of ST2+ Klrg1+ Treg cells in affected Δ/Δ animals. a Treg cells (CD4+ CD45+ CD25+ Foxp3+) identified in spleens from WT vs. affected Δ/Δ animals and sub-gated for presence of ST2+ Klrg1+ tisTreg ST2-like cells (representative pseudocolor plots to the left). Middle, histograms illustrating Ki67 and Gata3 expression in ST2+ Klrg1+ (K+) vs. ST2–Klrg1– (K–) Treg cells from WT and affected Δ/Δ animals, quantification to the right (n = 5, unpaired t-test). b Quantification of ST2+ Klrg1+ Treg cells in spleen, inguinal LN and skin of WT and affected Δ/Δ animals (% of Treg, n = 5, unpaired t-test). c RNA sequencing from spleen Δ/Δ Klrg1+ Treg cells (Δ/Δ K+ Treg, red), Δ/Δ Klrg1– Treg cells (Δ/Δ K– Treg, blue), WT Klrg1+ Treg cells (WT K+ Treg, red), and WT Klrg1– Treg cells (WT K– Treg, blue). Expression of 106 tisTregST2 signature genes derived from a previous study in a heatmap with column dendrogram clustering. The expression of these signature genes in Treg cells from fat, skin and LN Treg cells shown to the left (Fat/Skin/LN WT Treg). Heatmap and dendrogram created using R and heatmap function. d Expression of Il10, Areg, Pparg, and Gpr55 in WT Treg cells from skin, fat, or LN versus spleen (spl) Δ/Δ Klrg1+ Treg, spleen Δ/Δ Klrg1+ Treg, and spleen WT Klrg1+ Treg cells. Statistical analysis of RNA-seq data described in Methods section. e MA plots illustrating expression of genes in two comparisons. Left, spleen WT Klrg1+ Treg cells vs. spleen Δ/Δ Klrg1+ Treg cells; right, spleen WT Klrg1– Treg cells vs. spleen Δ/Δ Klrg1+ Treg cells. Individual genes are highlighted. Numbers indicate significantly expressed genes in the respective comparison. Data are representative of two or more independent experiments with individual mice (a, b) or a single experiment with several individual mice (c–e). Source data are provided as a Source Data file.
The experiment concluded. Out of the 45 animals with mono-allelic Rbpj deletion, 33 animals were between 10 and 20 weeks old when the observation was stopped and although some turned sick later and were used for experimentation. Seventeen animals older than 20 weeks and were marked healthy during the observation period, for experimentation and censored for survival analysis. Thirteen animals grew experimentation. Seventeen animals <20 weeks old were otherwise healthy but used for experimentation. Foxp3 Cre

Rbpj genotyping. Animal tails were digested in digest buffer (50 mM KCl, 20 mM Tris–HCl pH 8.8, 0.00045% Tween20 and Igepal CA-630) with proteinase K overnight at 56 °C. Following inactivation for 10 min at 96 °C, a PCR reaction with

Tris–HCl pH 8.8, 0.00045% Tween20 and Igepal CA-630) with proteinase K overnight at 56 °C. Following inactivation for 10 min at 96 °C, a PCR reaction with

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Fig. 8 ATAC-seq of Rbpj Δ/Δ Treg cells. a ATAC-seq with three biological replicates of Δ/Δ Rbpj+ Treg cells and WT Rbpj+ Treg cells. Identification of 68,214 peaks throughout the mouse genome. Distribution of all peaks across autosomes 1–19 and allosome X in a pie chart; pie size indicates contribution in percent. b Annotation of peaks to promoter (red), intron (light blue), exon (dark blue), or intergenic regions (orange). Left, annotation for all 68,214 peaks identified in the all dataset; middle, annotation for 3392 peaks specifically upregulated in Δ/Δ Rbpj+ Treg cells; right, annotation for 10,816 peaks specifically upregulated in WT Rbpj+ Treg cells. c Heatmap showing normalized ATAC-seq read counts in window of −750 bp to +750 bp around all differential peaks (14,208) for six samples. y-axis individual peaks, x-axis distance from peak center. Color code indicates normalized ATAC-seq read count in 25 bp bins, with 0 = white and 10 = blue. d De novo motif analysis in 3392 peaks up in Δ/Δ Rbpj+ Treg cells. Top enriched de novo motif is shown, along with the corresponding p-value and the three most-similar known motifs (similarity score from 0 to 1, with 1 indicating an exact match). Further motifs and motif analysis for the 10,816 peaks up in WT shown in Supplementary Fig. 8a, b. Below, gene expression of Gata3, Gata4, and Gata6 (n = 3–7, unpaired t-test). e–h ATAC-seq genome browser tracks for eight genes, with WT Rbpj+ Treg cell data in black and Δ/Δ Rbpj+ Treg cell data in red. Gene information is shown on top, along with the genomic location. Height indicates normalized ATAC-seq signal, the scale shown in brackets. All samples are group-normalized to allow peak height comparison. Below, all peaks (black squares), differential peaks (blue squares), instances of de novo Rbpj-binding motif (purple) or literature-based Rbpj motif (orange) are shown. Displayed are: Foxp3 (e), Il17r and Irf7 (f), Il2ra, Dax1, and Bach2 (g), Arg and Illo (h). i RNA expression values (Rpkm) for genes shown in (e–g), data derived from RNA sequencing (n = 3–7, unpaired t-test). Data are representative of experiments with several individual mice. Source data are provided as a Source Data file.

Flow cytometry and fluorescence-activated cell sorting. Target organs were isolated and single-cell suspensions were established. If applicable, tissues were treated with collagenases and pre-purified according to protocol7. Red blood cells were lyzed in hypotonic buffers, and cells were either pre-enriched with magnetic bead technology (Miltenyi Biotech) or directly stained with fluorescence-labeled antibodies. Surface stainings were performed for 30 min at 4 °C with all antibodies used at 1:100 dilution if not stated otherwise. If applicable, cells were fixed and afterwards permeabilized with the Foxp3 Fix/Perm Buffer set for 60 min at RT. Upon permeabilization, cells were stained intracellularly for 60 min at RT. Flow cytometry samples were acquired on a LSR II, Fortessa II or Canto II flow cytometer (BD Biosciences) with four-way purity settings and an 85 µM nozzle. For RNA isolation, samples were sorted into 500 µL of DNA lysis buffer and DNA was purified according to manufacturer’s instructions (DNeasy Blood and Tissue Kit, Qiagen). For DNA collection, samples were sorted into 500 µL of DNA lysis buffer and DNA was purified according to manufacturer’s protocol (DNEasy Blood and Tissue Kit, Qiagen). To harvest protein, cells were sorted into FCS-containing buffer and afterwards pelleted. Cells were lysed in RIPA buffer.

Real-time PCR. For RNA isolation, antibody-labelled cells were sorted directly into RT-Plus Buffer (ThermoFisher) and hybridized according to manufacturer’s protocol (VivaFlour kit, Qiagen). RNA from whole tissues was isolated with mechanical tissue dissemination using ceramic beads followed by column-based RNA isolation (InnuPrep RNA Kit, Analytik Jena). RNA was reversely transcribed into cDNA according to manufacturer’s protocol (Reverse Transciptase II, Life technologies). cDNA was used with Taqman probes and Taqman master mix or with Sybr primer and Sybr master mix in a Vii7 real-time PCR system (all ThermoFisher).

Immunohistochemistry and slide scanning/image analysis. Foxp3 stainings of embedded tissues were prepared with a fluorescence-labeled anti-Foxp3 antibody as described in literature9. To visualize germinal centre formation, lymph node samples were removed from animals, immediately frozen in TissueTec buffer (Sakura Fineteck Europe) on cold carbon dioxide pellets and stored at −80 °C. Individual samples were cut from the tissue block and fixed with acetone for 10 min. Following blocking with 10% FCS, slides were incubated with AF488-labeled anti-mouse GL2 antibody (1:20), AF594-labeled anti-mouse IgD antibody (1:20), and AF447-labeled anti-mouse CD4 antibody (1:20) overnight at 4 °C. After washing, samples were mounted with a fluorescence mounting medium (Dako). Unstained or single-stained samples were prepared and imaged as background staining controls. Samples were imaged on a motorized Zeiss inverted Cell Observer.Z1 with a mercury arc burner HXP 120/Colibri LED module, as well as a grey scale CCD camera AxioCam and a color CCD camera AxioCam MRC. Images were sequentially scanned and assembled with ZEN 2011 lite (BLUE EDITION) software. In order to obtain full-size images of lymph nodes, we first scanned the green channel (AF488, GL-7) followed by red channel (AF594, IgD), and blue channel (AF647, CD4). Contrast and fluorescence intensity were adjusted for all samples in parallel. To reduce robotic scanning errors, images were stitched to smooth transition areas. For Foxp3 stainings, staining intensity was normalized for each image.

Gene expression analysis with bead chips and statistics. For whole lymph-node gene expression analysis, inguinal lymph nodes from WT and affected Δ/Δ animals underwent mechanical tissue dissemination using ceramic beads followed by column-based RNA isolation, as described earlier. For gene expression analysis of T cells, we FACs-isolated spleen-derived Treg (CD3+ CD4+ CD8−, Foxp3-YFP+) and Tconv cells (CD3+ CD4+ CD8+, Foxp3−) were isolated via FACS. Cells were lysed in RIPA buffer and supplemented with Laemmli buffer containing beta-mercaptoethanol. Samples were then heated to 95 °C for 10 min and afterwards separated by SDS–PAGE with pre-cast gels (Biorad). Gels were blotted onto PVDF membranes according to standard protocol. Membranes were blocked with 5% Milk-PBST for 1 hour at RT followed by incubation overnight with an anti-mouse primary monoclonal antibody (Cell signal primary monoclonal Ab (CD49d-PE, CD120a-APC, CD104A) at 1:3000 in 5% Milk-PBST). Membranes were washed and RBP antibody was labeled with an HRP-conjugated secondary antibody at 1:100,000 dilution for 1 hour at RT. Membranes were washed and developed with a chromogenic detection reagent (Thermo Fisher).

Histology and microscopy. Immediately after the animals were sacrificed, organs were carefully excised and stored overnight in freshly prepared 4% formaldehyde solution. Afterwards, samples were embedded, cut-thick (thickness between 3 and 5 µm) and stained. Haematoxylin and eosin (H&E) stains, periodic acid–Schiff reaction (PAS) stains, and Giemsa stains were prepared according to literature10. Representative sections of Aslan AA1 microscopically equipped with a AxioCam Ic3 color camera with Zen 2011 lite (BLUE EDITION) software. Intensity and contrast settings were adjusted for each organ but kept consistent between control and test sample. Foxp3-staining on embedded tissues was performed as follows: first, samples were thin-cut (3–5 µm); second, paraffin was sequentially melted (72 °C, 30 min) followed by Xylol (2 x 5 min), and alcohol treatments; third, samples were incubated at 120 °C in Tris–EDTA buffer for 5 min, followed by blocking with peroxidase-block (Dako); then, incubation with primary antibody (Foxp3–FJK–16.1, 1:50, 30 min at RT) and secondary antibody (anti-rat HRP, 30 min at RT) with intermittent washing steps for 5 min (Washing buffer from Dako). last, chromogenic detection solution was added for 10 min at RT (DAB+ substrate, Dako) followed by 1 min incubation with Hematoxylin (Merck) and washing steps.

Statistical analysis of data. Data were analyzed with Prism software. We used a log-rank Mantel–Cox test in Kaplan–Meier survival curves (Figs. 1a and 4a), Mann–Whitney testing (Figs. 1b, c, d, 2b, c, 3a, b), unpaired t-testing (Figs. 3d, e, f, 5b, c, d, g), data derived from RNA sequencing (n = 3–7, unpaired t-test). Data are representative of experiments with several individual mice. Source data are provided as a Source Data file.
ILT3<sup>+</sup>Gata3<sup>+</sup> T<sub>Treg</sub> cells failed to suppress T<sub>T</sub>2 polarization. a ATAC-seq genome browser track for the Liltb4a (ILT3) gene, with WT Klg1<sup>−/−</sup> T<sub>Treg</sub> cell data in black and Δ/Δ Klg1<sup>−/−</sup> T<sub>Treg</sub> cell data in red. Gene information on top. Height indicates normalized ATAC-seq signal, scale shown in brackets. All samples group-normalized. Below, all peaks (black squares), differential peaks (blue squares), and de novo Rbpj-binding motif (purple). b Liltb4a gene expression based on RNA-seq data derived from WT and Δ/Δ Klg1<sup>−/−</sup> and Klg1<sup>−/−</sup> T<sub>Treg</sub> populations (n = 3–7, unpaired t-test). c WT T<sub>Treg</sub> cells (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3-GFP<sup>+</sup>) expanded for 6 days in vitro with IL-4 and IL-33 (IL4<sup>+</sup>IL33 T<sub>Treg</sub> group) or without (control, Ctrl T<sub>Treg</sub> group). After 6 days, expression of Foxp3, Gata-3, and ILT3 measured by flow cytometry. Quantification right (n = 4, unpaired t-test). d 16-h DC polarization assay with different numbers of Gata3-expressing T<sub>Treg</sub> cells or control T<sub>Treg</sub> cells (Ctrl T<sub>Treg</sub>) as in c. Expression of PD-L2 and IRF-4 in DCs (CD11c<sup>+</sup>MHCII<sup>+</sup>) measured by flow cytometry. Quantification left (paired t-test, n = 5) and representative dot plots right. e 96-h T-effector cell (T<sub>T</sub>) polarization assay with 20 ng/mL IL-4 and different numbers of either Gata3-expressing T<sub>Treg</sub> cells (blue line) or control T<sub>Treg</sub> cells (black line) as in c and d, in the presence of DCs. Representative histograms showing Gata-3 expression in T<sub>T</sub> eff cells (CD4<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>) on top, quantification below (n = 5, paired t-test). f T<sub>Treg</sub> cells (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>YFP<sup>+</sup>) isolated from healthy, unaffected Δ/Δ and WT animals and stimulated with escalating doses of IL-4 followed by FACs measurement of Gata3 protein. Representative histograms on top show Gata3 expression in WT (black) or Δ/Δ (red) T<sub>Treg</sub> cells, treated with 0.0025 ng/mL IL-4 (left) or 25 ng/mL IL-4 (right). Below, Gata3 expression across different doses. Statistics based on two-way ANOVA with Bonferroni post-test (n = 6). Lower row, qPCR-based verification of flow cytometry data for Gata3 with 2.5 ng/mL IL-4 (n = 4, paired t-test). Data are representative of two or more independent experiments with individual mice (c-f) or a single experiment with several individual mice (a, b). Source data are provided as a Source Data file.
Bacterial seed Ig subtype analysis. Blood was extracted via cardiac puncture from sacrificed animals and allowed to clot for at least 15 min. Afterwards, samples were centrifuged at 13,000 × g for 15 min and serum was collected. ELISA plates (Costar #3596) were pre-coated with goat-anti-mouse IgG (Biorad #107-0068) or goat-anti-mouse IgE (Biozol #1110-01) overnight at 4 °C, followed by washing (PBS 0.2% gelatin 0.1% NaNC). Wells were incubated with serial dilutions of serum or control antibody for one hour at RT, followed by four washing steps. Peroxidase-conjugated secondary antibodies were added at 1:1000 dilution in PBS and incubated for one hour at RT. After four washing steps, Plates were developed with 1 mg/mL OPD in 0.1 M KH2PO4 (pH 6.0) solution with 1 µL/mL of 30% H2O2 solution. Once colorimetric reaction was complete, incubation was stopped with 25 µL 1 M H2SO4 and read on a ELISA photometer at 490 nm wave length. All antibodies are listed in Supplementary Table 1.

Isolation of blood plasma and blood serum. Blood was collected from sacrificed mice via cardiac puncture. Blood was mixed with Heparin-PBS to a final concentration of 20 U/mL Heparin. Samples were centrifuged at 3000 × g for 15 min at 4 °C. Blood parameters were measured by photometric analysis on the ADVIA 2400 system (Siemens Healthcare Diagnostics) in the Zentrallabor (Medical Clinic I, Analysezentrum) of the Heidelberg University Clinic. For blood serum collection, blood was extracted via cardiac puncture and allowed to clot for at least 15 min. Afterwards, samples were centrifuged at 13,000 × g for 15 min and blood serum was collected.

Autoantibody detection via Western Blot. Following organs and tissues from RAG2−/− animals were isolated: brain, eye, spleen, lymph nodes, pancreas, salivary gland, stomach, liver, kidneys, heart, lung, testis, small, and large intestine. Tissues were snap-frozen and adjusted to 100 mg, followed by addition of 500 µL RosetteSep™ T cell-Conversion (BD) antibody (concentration 1:3000). After washing, strips were re-assembled and HRP conjugate antibody solution with 1 µL/mL of 30% H2O2 solution. Once colorimetric reaction was complete, incubation was stopped with 25 µL 1 M H2SO4 and read on a ELISA photometer at 490 nm wave length. All antibodies are listed in Supplementary Table 1.

Infection of WT and Δ/Δ animals with S. ratti. Animal experimentation was conducted at the animal facility of the Bernhard Nocht Institute for Tropical Medicine in agreement with the German animal protection law under the supervision of a veterinarian. The experimental protocols have been reviewed and approved by the responsible federal health authorities of the state of Hamburg, Germany (the Behörde für Gesundheit und Verbraucherschutz). Mice were sacrificed by cervical dislocation under deep CO2 narcosis. Two cohorts with five WT and five healthy Δ/Δ animals each were infected on day 0 by injection of 1000 S. ratti larvae subcutaneously into the footpad. Cohort 2 was sacrificed 6 days after infection and intestinal parasite count, stool PCR, and flow cytometry (Treg frequency, cytokine restimulation) were performed. Cohort 2 was sacrificed 30 days after infection; stool collection on day 6, 8, 10, 14 followed by stool-PCR; flow cytometry (Treg frequency, cytokine restimulation) on day 14. To count the number of adult parasitic females in the gut, the small intestine was flushed slowly with tap water to remove feces, sliced open longitudinally and incubated at 37 °C for 3 h in a Petri dish with tap water. The released adult females were collected by centrifugation for 5 min at 1200 rpm and counted. To quantify the release of S. ratti larvae by infected mice, the feces of individual mice was collected over 24 h periods and DNA from representative 200 mg samples was extracted as described. Two hundred nanograms of DNA was used as a template for qPCR specific for S. ratti 28S and 18S rDNA. The qPCR was performed on 384 well optical plates (Corning) using the Bio-Rad CFX connectivity™ thermal cycler equipped with the BioRad CFX manager software. Blood was collected from infected mice at the indicated time points and allowed to coagulate for 1 h at RT. Serum was collected after centrifugation at 10,000g for 10 min at RT and stored at −20 °C for further analysis. Strongyloides-specific IgM in the serum was quantified by ELISA, as described. Serum concentration of IgG was quantified using the IgG Enzyme-Linked immunosorbent assay (ELISA) kit according to the manufacturer’s recommendations. In flow cytometry experiments, spleens were collected, red blood cells lysed and samples stained as described previously. For cytokine restimulation experiments, single-cell suspensions were incubated with cell stimulation cocktail plus transport inhibitor for 6 h at 37 °C followed by intracellular cytokine staining. To measure secreted IL-9 levels, mesenteric LN-derived cells were mashed and incubated with anti-CD3 antibody (1 µg/mL) for 72 h at 37 °C with 1 × 105 cells per well. IL-9 levels were quantified using the IL-9 ELISA kit according to the manufacturer’s recommendations.

Methylation of the TSDR. Genomic DNA of sorted cell populations was purified according to manufacturer’s guidelines using the DNeasy Blood and Tissue Kit (Qiagen). DNA quantity and concentration were measured with a NanoDrop® spectrophotometer. Bisulfite-conversion was performed using the EpiTect Bisulfite Conversion Kit (Qiagen). Barcode-labeled primers for the Foxp3 CNS2 (TSDR) were used to generate PCR amplicons from bisulfite-converted DNA. The PCR amplicons were separated by agarose gel and purified using a Quick Gel Extraction Kit (Life Technologies). PCR amplicons were processed on a GS Junior Sequencer (Roche). Sequence reads were aligned to the B6-conversed mouse genome and genotyped.

In vitro Treg suppression assay. First, we isolated MHCII-positive antigen-presenting cells (CD19−CD25−Foxp3−Foxp3+GM-CSF−MHCII−CD90.1−CD90.2−) as well as CD4−positive T-responder cells (CD4+CD25−Foxp3+GM-CSF+MHCII−CD90.1+CD90.2−) from Foxp3−Foxp3+GM-CSF+MHCII+CD90.1+CD90.2− T-responder cells were then labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) at 1 µM concentration in 10 ml cell culture medium for 15 min at RT, followed by several washing steps. Next, Treg (CD4−CD25+CD45+Foxp3−Foxp3+GM-CSF−MHCII−CD90.1+CD90.2−) and Tcon (CD4+CD25−Foxp3+YFP−MHCII−CD90.1−CD90.2−) cells from WT and affected Δ/Δ mice were isolated by FACS and serially diluted. In each well, 50,000 T-responder cells, 100,000 MHCII-positive APCs, and serially diluted Treg or Tcon, cells were added. To stimulate APC-driven T-responder cell proliferation, 2 µg/mL anti-CD3 antibody was added and serially diluted Treg or Tcon, cells were added. To stimulate APC-driven T-responder cell proliferation, 2 µg/mL anti-CD3 antibody was added and cells incubated for 5 days at 37 °C, followed by re-staining for flow cytometric analysis of CFSE-dye dilution in T-responder cells.

TCR sequencing. Single cell suspensions from spleen and lymph nodes (combined axillary, cervical, brachial) from individual WT vs. Δ/Δ mice were established, and red blood cells lysed. Treg cells (CD3+CD4+CD8+CD45+Foxp3−YFP+) were pre-enriched with CD25-magnetic bead-based purification and sorted via FACS. Genomic DNA was isolated with the DNeasy blood and tissue kit according to manufacturer’s instructions and measured on nanodrop photometer. Five hundred nanograms of qDNA per individual mouse were shipped to Adaptive Biotechnologies (Seattle, WA) for TCR sequencing. Data were analyzed with online tools provided by Adaptive Biotechnologies.

Active caspase-3 assay and intracellular cytokine secretion. For measurement of active caspase-3, single-cell suspensions were resuspended in FCS-containing cell culture medium (Gibco) and 1 µL of Red-DEVD-FMK (abcam) was added to each well in a 96-well tissue culture plate. Samples were incubated for 60 min at 37 °C, followed by washing with supplied wash buffer and surface antibody staining. As a positive control, splenocytes were incubated for 5 min at 42 °C. To measure intracellular cytokines, splenocytes, or lymph node-derived cell suspension were resuspended in FCS-containing cell culture medium. For stimulation, cells received 1X PMA-Ionomycin cocktail plus transport inhibitor, whereas controls received 1X transport inhibitor only cocktails (eBiosciences). Samples were incubated for 6-8 h at 37 °C, followed by surface and intracellular staining with Foxp3 Fix-perm staining mix.

Detection of phosphorylated Stat5 or Stat6. Spleen-derived single-cell suspensions were resuspended in FCS-containing cell culture medium (Gibco) and 1 µL of Red-DEVD-FMK (abcam) was added to each well in a 96-well tissue culture plate. Samples were incubated for 60 min at 37 °C, followed by washing with supplied wash buffer and surface antibody staining. As a positive control, splenocytes were incubated for 5 min at 42 °C. To measure intracellular cytokines, splenocytes, or lymph node-derived cell suspension were resuspended in FCS-containing cell culture medium. For stimulation, cells received 1X PMA-Ionomycin cocktail plus transport inhibitor, whereas controls received 1X transport inhibitor only cocktails (eBiosciences). Samples were incubated for 6-8 h at 37 °C, followed by surface and intracellular staining with Foxp3 Fix-perm staining mix.

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manufacturer’s instructions with the following modifications: The adapter-ligated double-stranded cDNA (10 µL) was amplified using NEBNext Multiplex Oligos for Illumina Multiplex Set v2 (8, 25 µM per primer) and the HiSeq2000 PCR Master Mix (New England Biolabs) and 15 cycles of PCR. Final libraries were validated using Agilent 2100 Bioanalyzer (Agilent Technologies) and Qubit fluorometer (Invitrogen), normalized and pooled in equimolar ratios. 30 bp single-read sequencing was performed on the Illumina HiSeq 2000 v4 according to the manufacturer’s protocol.

Mapping of RNA seq data, statistical evaluation, and plotting. For all samples, low-quality bases were removed with Fastx_quality_filter from the FASTX Toolkit 0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/index.html) with 90% of the reads needing a quality phred score >20. Homertools 4.758 were used for PolyA-tail option for validated using Agilent 2100 Bioanalyzer (Agilent Technologies) and Qubit.

In-vitro Treg differentiation with IL-4 and IL-33. Spleen and lymph-nodes were isolated and single-cell suspensions established. Treg cells were pre-purified using CD25+ magnetic bead staining and column-based isolation, followed by FACS-based sorting of CD4+CD25+ Fopx3-GFP+ Treg cells. 20,000 cells each were supplemented with either recombinant mouse IL-4 (500 ng/mL) plus recombinant mouse IL-33 (500 ng/mL) (IL4 + IL33 Treg group) or without additional cytokines (Ctrl Treg group). In addition, both Treg groups were cultured with 5000 U/mL IL-2 and anti-CD3 microbeads at 4:1 bead to cell ratio were added to each well. Cells were incubated with the respective cytokine mix for 6 days at 37 °C. medium was re-supplemented on day 4. On day 6, cells were counted using a flow cytometer and expression of ILT3 and Gata-3 was measured. Cells were washed three times to remove remaining cytokines and used for the in vitro DC differentiation assay and for the in vitro Treg polarization assay.

In vitro DC differentiation assay. Spleen and mesenteric LCs from donor animals were isolated and single-cell suspensions established. DCs were pre-purified using CD11c microbeads and column-based isolation, followed by FACS-based sorting of a MCHII+CD11c+ population. DCs were stimulated with LPS (100 ng/mL), and anti-CD3 (4 µg/mL) was added. In vitro expanded Treg cells were added at indicated ratio to the DC bulk and added to each well. Cells were incubated with the respective cytokine mix for 6 days at 37 °C. Cells were fixed, and DCs were analyzed after antibody surface and intracellular staining with the BD Fix/Perm Buffer kit.

Measurement of Gata-3 induction upon cytokine challenge. Spleen and lymph-node derived Treg cells (CD4+CD25+ Fopx3-YFP+) were isolated from WT and healthy Δ/Δ animals via FACS. 75,000 cells each were supplemented with IL-4 at different concentrations, along with 5000 U/mL IL-2, 20 µg/mL IL-12 blocking mAb, 20 µg/mL INFy-blocking antibody, and CD3/CD28 microbeads at 4:1 bead to cell ratio. Cells were incubated with the respective cytokine for 40 h at 37 °C. followed by surface and intracellular staining with the Fix/Perm Buffer kit.

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

Data availability
RNA microarray data, RNA sequencing data and ATAC-seqencing data that support the findings of the study have been deposited in the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi) under the accession number GSE119169. The source data underlying Figs. 1a, c, e, f, and Supplementary Figs. 1–4, a–f, 7a–d, 8a–d, 9b–f, and Supplementary Figs. 1–4 and 8a, b are provided as a Source Data file. All other data are available from the author upon reasonable request.

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References
1. Wildin, R. S. et al. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. Nat. Genet. 27, 18–20 (2001).
2. Brunkow, M. E. et al. Disruption of a new forhead/wing-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat. Genet. 26, 68–73 (2001).
3. Khattri, R., Cox, T., Yasyko, S. A. & Ramsdell, F. An essential role for Scurfin in CD4+ CD25+ T regulatory cells. Nat. Immunol. 4, 337–342 (2003).
4. Hori, S., Nomura, T. & Sakaguchi, S. Control of regulatory T cell development by the transcription factor Foxp3. Science 299, 1057–1061 (2003).
5. Myhill-Jones, J. et al. Protein-ligand binding analysis using the DeepMind AI platform. Nat. Methods 11, 835–837 (2014).
6. Ohkura, N. et al. T cell receptor stimulation-induced epigenetic changes and Foxp3 expression are independent and complementary events required for Treg cell development. *Immunity* **37**, 785–799 (2012).

7. Delacher, M. et al. Genome-wide DNA-methylation landscape defines spatiotemporal regulation of T cells in tissues. *Nat. Immunol.* **18**, 1160–1170 (2017).

8. Kitagawa, Y. et al. Guidance of regulatory T cell development by Satb1-dependent super-enhancer establishment. *Nat. Immunol.* **18**, 173–183 (2017).

9. Schmidt, C. et al. Lineage-specific DNA methylation in T cells correlates with histone methylation and enhancer activity. *Genome Res.* **19**, 1165–1174 (2009).

10. Feuerer, M. et al. Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. *Nat. Med.* **15**, 930–939 (2009).

11. Campbell, D. J. & Koch, M. A. Phenotypical and functional specialization of FoxP3+ regulatory T cells. *Nat. Rev. Immunol.* **11**, 119–130 (2011).

12. Koch, M. A. et al. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat. Immunol.* **10**, 595–602 (2009).

13. Zheng, Y. et al. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control Th1(2) responses. *Nature* **458**, 351–356 (2009).

14. Chaudhary, A. et al. CD4+ regulatory T cells control Th17 responses in a Stat3-dependent manner. *Science* **326**, 986–991 (2009).

15. Radtke, F., Fasnacht, N. & Macdonald, H. R. Notch signaling in the immune system. *Immunity* **32**, 14–27 (2010).

16. Tu, L. et al. Notch signaling is an important regulator of type 2 immunity. *J. Exp. Med.* **202**, 1037–1042 (2005).

17. Amsen, D., Averna, M., Mancuso, A. & Flavell, R. A. The different faces of Notch in T-helper-cell differentiation. *Nat. Rev. Immunol.* **9**, 116–124 (2009).

18. Amsen, D. et al. Direct regulation of Gata3 expression determines the T helper differentiation potential of Notch. *Immunity* **27**, 89–99 (2007).

19. Fang, T. C. et al. Notch directly regulates Gata3 expression during T helper 2 lineage development. *J. Immunol. Methods* **362**, 327–336 (2011).

20. Charbonnier, L. M., Wang, S., Georgiev, P., Seeber, A. & Ho, A. M. Notch controls Foxp3(+) regulatory T cell fate during differentiation in inflammation. *J. Clin. Invest.* **121**, 4503–4515 (2011).

21. Taghon, T. et al. Enforced expression of GATA-3 severely reduces human thymic cellularity. *J. Immunol.** **167**, 4468–4475 (2001).

22. Scripture-Adams, D. D. et al. GATA-3 dose-dependent checkpoints in early T helper 17 lineage impairs oral tolerance and promotes food allergy. *Immunity* **42**, 512–523 (2015).

23. Kurotaki, N. et al. Foxp3 expression are independent and complementary events required for Treg cell development. *Immunity* **10**, 595–602 (2009).

24. Blankenhaus, B. et al. Foxp3(-/-) regulatory T cells delay apoptosis of intestinal IECs. *FASEB J.* **25**, 1239–1247 (2011).

25. Polansky, J. K. et al. DNA methylation controls Foxp3 gene expression. *Immunity* **27**, 1090–1099 (2007).

26. Kilbride, S. M. et al. AMP-activated protein kinase mediates apoptosis in T helper 1 cells. *Immunity* **29**, 546–558 (2008).

27. Consiglio, A. et al. MAPK signaling link to apoptosis. *PLoS. Pathog.* **10**, e1003913 (2014).

28. Surh, C. D. & Sprent, J. Homeostasis of naive and memory T cells. *Nat. Immunol.* **8**, 1199–1208 (2007).

29. Soares, A. et al. Novel application of Ki67 to quantify antigen-specific responses in live cells. *Immunity* **36**, 558–356 (2009).

30. Foxwell, B. M., Beadling, C., Guschin, D., Kerr, I. & Cantrell, D. Interleukin-7 homeostasis and function during type 1 immunity. *Nat. Immunol.* **10**, 1654–1666 (2009).

31. Hsiao, H. W. et al. Deltex1 antagonizes HIF-1alpha and sustains the stability of regulatory T cells that affect metabolic parameters. *Nat. Med.* **15**, 930–939 (2009).

32. Roychoudhuri, R. et al. Bach2 represses effector programs to stabilize T cells. *Nat. Immunol.* **10**, 1213–1218 (2009).

33. de Hoon, M. J., Imoto, S., Nolan, J. & Miyano, S. Open source clustering software. *Bioinformaticas** **20**, 1453–1454 (2004).
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Author contributions

M.D., M.B., J.A. and M.F. designed experiments; M.D., Y.H., W.H., F.B., D.K., U.T., A.-C. H., S.B., D.W., A.B. and G.F. performed the experiments; R.M.S. provided material; M.D., C.S., M.B., W.H., C.I., A. H-W., T.H., H.J.G., M.R., J.A. and M.F. analyzed data; M.D. and M.F. wrote the manuscript.

Additional information

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