Stability and function of regulatory T cells expressing the transcription factor T-bet

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Adaptive immune responses are tailored to different types of pathogens through differentiation of naïve CD4 T cells into functionally distinct subsets of effector T cells (T helper 1 (T_{H1}), T_{H2}, and T_{H17}) defined by expression of the key transcription factors T-bet, GATA3, and RORγt, respectively1. Regulatory T (Treg) cells comprise a distinct anti-inflammatory lineage specified by the X-linked transcription factor Foxp3 (refs 2, 3). Paradoxically, some activated Treg cells express the aforementioned effector CD4 T cell transcription factors, which have been suggested to provide Treg cells with enhanced suppressive capacity4-8. Whether expression of these factors in Treg cells—as in effector T cells—is indicative of heterogeneity of functionally discrete and stable differentiation states, or conversely may be readily reversible, is unknown. Here we demonstrate that expression of the T_{H1}-associated transcription factor T-bet in mouse Treg cells, induced at steady state and following infection, gradually becomes highly stable even under non-permissive conditions. Loss of function or elimination of T-bet-expressing Treg cells—but not of T-bet expression in Treg cells—resulted in severe T_{H1} autoimmune disease. Conversely, following depletion of T-bet− Treg cells, the remaining T-bet+ cells specifically inhibited T_{H1} and CD8 T cell activation consistent with their co-localization with T-bet+ effector T cells. These results suggest that T-bet+ Treg cells have an essential immunosuppressive function and indicate that Treg cell functional heterogeneity is a critical feature of immunological tolerance.

Whether Treg cells expressing the T_{H1}-associated transcription factor T-bet represent a stable sub-lineage of cells with unique function or a transient activation state remains unknown. To address this question, we assessed the stability of T-bet expression in Treg cells using a novel Tbx21^{tdTomato-T2A-creERT2} knock-in allele combined with the R26Y recombination and Foxp3^{ Thy1.1} reporters. The resulting Tbx21^{RFP-creERT2} mice showed a range of red fluorescent protein (RFP) expression and CreERT2 activity, which faithfully reflected endogenous T-bet protein levels in major lymphocyte subsets (Fig. 1a, Extended Data Fig. 1a, b). RFP+ Treg cells comprised between 30–70% of CD4^{+}CD62L^{lo} effector Treg cells in lymphoid organs and non-lymphoid tissues; interestingly, intestinal Treg cells exhibited prevalent co-expression of T-bet and ROR-γt, but not T-bet and GATA3 (Extended Data Fig. 1d–i).

Three weeks after tamoxifen administration we found—in contrast to a previous report7—that the vast majority of both yellow fluorescent protein (YFP)-labelled Treg and effector CD4 T cells continued to express RFP (Fig. 1b, c, Extended Data Fig. 1j). The percentage of YFP+ cells expressing RFP was similarly high at three and seven months after tamoxifen administration, although percentages of YFP+ cells declined, indicating that continual T-bet+ cell recruitment into the T-bet− subset balances out cell turnover over time (Fig. 1b, c, Extended Data Fig. 1j). Indicative of intrinsic stability of T-bet− Treg cells typical of a differentiated cell state, treatment of Tbx21^{RFP-creERT2} mice with tamoxifen 3 weeks before infection with the helminth *Nippostrongylus brasiliensis* did not result in loss of RFP expression among YFP+ Treg (or effector CD4) T cells despite robust T_{H1} activation and cytokine production in the spleens and lungs of infected mice (Fig. 1d, Extended Data Fig. 3a, data not shown).

The presence of small percentages of YFP+ RFP− Treg cells 3 weeks after gavage (Fig. 1b, c) suggested that some Treg cells might have experienced transient unstable T-bet expression at the time of tamoxifen administration. Such a scenario would reconcile the above result with an earlier study.6 Indeed, in Tbx21^{RFP-creERT2} mice we observed RFPlo Treg cells that lacked the T-bet-dependent expression of chemokine receptor CXCR3, in addition to RFPloCXCR3+ Treg cells (Extended Data Fig. 2a). The former exhibited slightly lower CD44 and slightly higher CD62L expression than the latter and RNA sequencing (RNA-seq) analysis suggested that CD44 RFPloCXCR3− Treg cells were differentiation intermediates between CD44 RFP+CXCR3− cells and CD44 RFPloCXCR3+ Treg cells. Notably, populations of RFPloCXCR3− and YFP+RFP− Treg cells were also observed within the CD4 non-Treg cell population (Extended Data Fig. 2a). Thus, the observed instability of a low level of T-bet expression is not unique to Treg cells but is indicative of the gradual process of peripheral T cell effector differentiation.

In addition to steady state cues, T_{H1}-polarizing infection can drive increases in T-bet+ Treg cells.10 To determine whether infection expands T-bet+ Treg cells present at steady state, or rather induces T-bet expression in T_{H1}-treg cells, we administered tamoxifen to Tbx21^{RFP-creERT2} mice 3 weeks before challenge with the intracellular bacteria *Listeria monocytogenes*. Upon *L. monocytogenes* challenge, RFP+ Treg and effector CD4 T cell subsets increased markedly; however, YFP+ Treg cells did not (yielding a decreased YFP+/RFP− ratio) (Fig. 2a, b, Extended Data Fig. 3b). This pattern was indicative of de novo differentiation of T-bet+ cells from T-bet− Treg precursors in parallel with differentiation of T_{H1} cells. Following transfer, both CD4^+CD62L^{lo} RFP+ and CD4^+RFP−CXCR3− Treg cells upregulated RFP in response to *L. monocytogenes* infection (Extended Data Fig. 3c). Notably, upon *L. monocytogenes* infection, preformed T-bet+ Treg cells tagged with YFP prior to infection increased expression of T-bet and CXCR3, but not IL-10, an important suppressor molecule11. The latter was demonstrated by fate mapping experiments in Tbx21^{RFP-creERT2}J10GFP/WT mice, which revealed no increase in IL-10 (eGFP+) among YFP+ Treg cells, whereas bulk T-bet (RFP+) IL-10+ cells increased around threefold (Extended Data Fig. 3d–g). Similar results were obtained during lymphocytic choriomeningitis virus infection (data not shown).

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We next assessed the persistence and recall response of T-bet+ Treg cells induced by L. monocytogenes infection. To preferentially label infection-induced T-bet+ cells, tamoxifen was administered at the peak of the primary L. monocytogenes response (days 7 and 9). Mice were assessed 8 weeks later, at which time the percentage of splenic and liver Treg cells that were RFP+ had returned to roughly pre-infection levels (Fig. 2c, d). Given the turnover rate of T-bet+ cells (Fig. 1c), we reasoned that by day 60 after infection YFP+ cells would be relatively enriched for infection-induced Treg cells compared to the bulk RFP+ cell pool. Reinfection increased bulk RFP+ Treg and effector CD4 T cells and even more prominently increased the corresponding cell subsets tagged with YFP (Fig. 2d, Extended Data Fig. 3h–j). On day 65 after primary infection, more than 90% of YFP+ Treg cells continued to express T-bet, as did uninfected control cells (Fig. 2e). Furthermore, mice infected with L. monocytogenes that were administered tamoxifen on days 37 and 39 after resolution of the primary response and re-infected on day 60 exhibited a parallel increase in bulk RFP+ and YFP+ Treg cell subsets on day 65, suggesting cells that acquired T-bet expression during primary infection remained T-bet-positive and expanded upon reinfection (Fig. 2f). Together, these studies demonstrate that bacterial

**Figure 1** Stable T-bet expression in a subset of peripheral Treg cells. a, Splenic cells in Tbx21RFP-creERT2 mice 3 weeks after tamoxifen gavage on days −2 and 0. Numbers on graph indicate the mean. Data are mean ± s.e.m. b, Schematic of tamoxifen administration to Tbx21RFP-creERT2 mice (top) and flow cytometry (bottom) of splenic CD4 Thy1.1+ and Thy1.1− cells. c, Upper, RFP+ (left axis, squares) and YFP+ (right axis, circles) Treg cells. Lower, percentage of RFP+ of YFP+ Treg cells 3 weeks (white symbols), 3 months (grey symbols), and 7 months (black symbols) after tamoxifen gavage. d, Upper, schematic of tamoxifen treatment and N. brasiliensis infection. Lower, percentage of RFP+ among YFP+ Treg cells in mice challenged with PBS (white circles) and N. brasiliensis (Nb; black circles); (bottom, right) RFP expression in Treg (shaded histograms) or YFP+ Treg (open histograms) cells from spleens of mice challenged with PBS (black) or N. brasiliensis (red). Data are mean. Two-tailed t-test (NS, not significant). All data are representative of 2 experiments, n ≥ 3 mice per each group.

**Figure 2** Stable differentiation of T-bet+ Treg cells in response to L. monocytogenes infection. a, Schematic of experiment shown in b combining tamoxifen (TX) treatment and L. monocytogenes (Lm) infection in Tbx21RFP-creERT2 mice. b, Percentage of RFP+, YFP+, and YFP+/RFP+ ratio in CD4+ Thy1.1+ (left) and Thy1.1− (right) cells in spleens and livers of mice challenged with PBS and L. monocytogenes. c, Schematic of experiments shown in d, e and f; 1° and 2° indicate primary and secondary challenge, respectively. d, Data presented as in b. e, Percentage of RFP+/ YFP+ Treg cells. f, Data presented as in b. Bars, mean. Two-tailed t-test (**P < 0.001, *P < 0.01, and *P < 0.05, respectively; NS, not significant). All data are representative of ≥ 2 experiments, n ≥ 4 mice per group each.
infection caused de novo differentiation of T-bet+ Treg cells into stable T-bet+ cells uniquely suited for reactivation under conditions that drove their initial acquisition of T-bet.

Although the stability of T-bet+ Treg cells suggested a particular function presumably imparted by T-bet itself, we found that 12-week-old Foxp3–/–Tbx21RFP+ mice were clinically indistinguishable from littermate controls, consistent with previous studies. Foxp3+/+Tbx21RFP+ mice did exhibit mild T1 and (but not CD8 T cell) activation, indicating that T-bet expression in Treg cells moderately potentiated suppression of T1 autoimmunity (Extended Data Fig. 4a).

We considered the possibility that T-bet deficiency might not fully impair the function of T-bet+ Treg cells. As Treg cell suppressor function requires continuous expression of the Foxp3 gene, we ablated Foxp3 in T-bet+ Treg cells using a novel Tbx21RFP–/Tomato–T2A–cre allele (Extended Data Fig. 5a). Loss of Foxp3 expression in T-bet+ Treg cells in 8-week-old Tbx21RFP+/Flxop3WT mice resulted in deceased weight gain, lymphadenopathy, T cell activation, and marked immune infiltration in the lung; with age, loss of hair pigmentation and rectal prolapse were evident (Fig. 3a–d, Extended Data Fig. 5).

Indicative of T1-type inflammation, the majority of expanded effector CD4 and CD8 T cells in Tbx21RFP+/Flxop3WT mice expressed RFP (Fig. 3e–g, Extended Data Fig. 5). Additionally, IFN- and IL-2 but neither IL-4 nor IL-17 production by T cells were increased compared to controls (Fig. 3g, h, Extended Data Fig. 5). Antibiotic treatment did not mitigate autoimmunity in Tbx21RFP+/Flxop3WT mice, excluding microbial antigens as the drivers of T1 inflammation (Fig. 3b, data not shown). We considered whether induction of a robust non-T1 immune response in Tbx21RFP+/Flxop3WT mice might reveal a potential function for T-bet+ Treg cells in its control. However, the T12 response to N. brasiliensis infection was not increased in Tbx21RFP+/Flxop3WT mice.
Diphtheria toxin per group. NS, not significant). Data are representative of 2 experiments, with CD45.2
deerich mice with a 1:1 mix of either CD45.1
similarly unleash TH1 inflammation, we generated bone marrow chi-
ablation of T-bet+ Treg cells results in T11 immune activation. Bone marrow chimeric mice were injected with 0.5 μg
dipheria toxin (DT) on day 0, then treated daily with 0.1 μg of dipheria
toxin until day 15. a, Weight loss in the indicated mice. b, Flow cytometry of splenic CD4 (top) and Treg (bottom) cells in the indicated mice. c, Activation status of CD45.1+ and CD45.2+ Treg cell compartments in spleens of indicated mice. d, e, T cell activation (d) and cytokine production (e) in control (white circles) and T-bet-depleted (black circles) chimeras. Data are mean ± s.e.m. Two-tailed t-test (\(P < 0.01, * P < 0.05; NS, not significant). Data are representative of 2 experiments, n ≥ 6 mice per group.

compared to control mice, in contrast to the exacerbated T112 response observed upon pan-Treg-cell depletion during helminth infection\(^{15,16}\) (Extended Data Fig. 6). Notably, whereas CXCR3+ Treg cells were signi-
cantly depleted neither total nor effector Treg cell numbers were
diminished, and analysis of Tbx21RFP+R26y of CD4+ and CD8+ T cells were simultaneously ablated, impli-
cating the latter in driving disease (Extended Data Fig. 7d, e).

RNA-seq analysis revealed that 561 genes, including Tbx21, Cxcr3, Gamb, Ebi3, Fgl2, and Il11, were more highly expressed in CD44RFP+ Treg cells compared to CD44RFP− Treg cells (Extended Data Fig. 2c). Expression of this gene set was increased upon loss of Foxp3 in ex-Treg cells, suggesting that Foxp3 opposes the transcriptional signature of T-bet+ Treg cells to prevent full T11 differentiation\(^{16}\) (Extended Data Fig. 4b). Notably, the T11+ associated chemokine receptor CCR5 and adhesion molecule (1 integrin (CD29) were expressed in T-bet+ Treg cells independently of T-bet (Extended Data Fig. 4c, d) indicating that some functional redundancy of homing molecules may in part explain the mild phenotype of Foxp3\()^{\text{RFP} + }\)Tbx21\()^{\text{RFP} + }\) mice. Moreover, we found that the TCR repertoires of CD44\(\text{RFP} + \)CXCR3+ (T-bet+ and CD44\(\text{RFP} - \)CXCR3+ (T-bet−) Treg subsets in DO11.10 TCR\(\text{RFP} + \) mice were distinct, suggesting that antigenic specificity of T-bet+ Treg cells may also contribute to distinct localization and suppressor capacity, as recent studies revealed TCR-dependent spatial proximity of Treg and IL-2-producing self-reactive T cells\(^{19}\) (Extended Data Fig. 4e).
Therefore, we sought to determine the relative spatial positioning of T-bet+ and T-bet− Treg and effector T cells in secondary lymphoid organs of Tbx21\#\textsuperscript{RFP-cre}\# mice. Immunofluorescence imaging revealed pronounced preferential proximity of CD4\#TH\#T-bet+ versus CD4\#TH\#T-bet− Treg cells to CD4\#TH\#T-bet+ T\_\text{H}1 and CD8 T cells (Fig. 3i-k, Extended Data Fig. 8a-d). In contrast, CD4\#TH\#T-bet− Treg cells were no nearer to T-bet− CD4 effectors than were CD4\#TH\#T-bet− Treg cells (Fig. 3j, Extended Data Fig. 8c). Notably, the CD4\#TH\#T-bet− Treg cells remaining in Tbx21\#\textsuperscript{RFP-cre}Foxp3\# mice were no nearer to T\_\text{H}1 or CD8 T cells than were CD4\#TH\#RFP− Treg cells in healthy Tbx21\#\textsuperscript{RFP-cre}Foxp3\# mice (Extended Data Fig. 8e-f). This result suggests that failure of non-T-bet+ Treg cells to approximate T\_\text{H}1 cells may at least in part account for their inability to suppress T\_\text{H}1 inflammation.

Lastly, to complement T-bet+ Treg cell ‘loss-of-function’ experiments we sought to selectively eliminate T-bet− Treg cells. We generated a Foxp3\#\textsuperscript{GDTRE} allele by inserting a loxP-flanked IRES-DTREGFP DNA sequence into the 3′ UTR of the Foxp3 gene (Extended Data Fig. 9a) and generated Foxp3\#\textsuperscript{GDTRE}Tbx21\#\textsuperscript{RFP-creERT2}\# mice (Fig. 5a). After 9 days of diphtheria toxin treatment, Treg cells in mice pre-treated with tamoxifen (day −5 and −3) were present in undiminished percentages and were exclusively T-bet+ and CXCXR3+ (Fig. 5a–c). Compared to vehicle (oil)-treated mice, tamoxifen-treated Foxp3\#\textsuperscript{GDTRE}Tbx21\#\textsuperscript{RFP-creERT2}\# mice displayed robustly suppressed CD8 T cell activation and selective suppression of IFN\#\gamma\# production by CD4 and CD8 T cells, but unrestrained T\_\text{H}2 and T\_\text{H}17 cytokine production (Fig. 5d–f). T-bet− Treg cells similarly suppressed pre-established T\_\text{H}1, but not T\_\text{H}2 or T\_\text{H}17, activation induced by depletion of Treg cells before tamoxifen treatment (Extended Data Fig. 9b–g). Selective T\_\text{H}1 suppression was not simply a feature of activated Treg cells rebounding after depletion, as partial depletion and recovery of Treg cells in Foxp3\#\textsuperscript{DTR}\# mice resulted in prominently inhibited T\_\text{H}2 responses (Extended Data Figs 9h–l, 10).

Our studies suggest that T-bet expression in Treg cells denotes a differentiated cell state with unique T-bet-dependent and -independent gene expression and TCR specificity, capable of driving potent immunosuppression limited to circumstances of T\_\text{H}1 and CD8 T cell activation. It is possible that GATA3- and ROR\#\textsuperscript{c}T-expressing Treg cells may play analogous roles in suppression of T\_\text{H}2 and T\_\text{H}17 responses. Such division of anti-inflammatory labour among Treg cells, arising at steady state and during infection, may enable focused regulation of specific T helper cell responses without incurring undesired bystander suppression.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions A.G.L. and A.Y.R. conceived the study, designed experiments, and wrote the manuscript. A.G.L. generated mice, performed experiments, and analysed data. A.M. designed and performed immunofluorescence experiments and A.M. and S.F. analysed the data. S.H. assisted with some experiments. B.M. performed TCR sequencing studies. E.V.P. and D.M.C. performed TCR sequencing analysis. A.G.L. and R.E.N. analysed and generated sequence into the 3′ UTR. B.M. performed TCR sequencing studies. E.V.P. and D.M.C. performed TCR sequencing analysis. A.G.L. designed and performed experiments, and wrote the manuscript.

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**METHODS**

**Animals.** Tbx21	extsuperscript{tdTomato-T2A-creERT2} mice were generated by insertion of a targeting construct into the Tbx21 locus by homologous recombination in embryonic stem cells on the C57BL/6 background; the targeting construct was generated by inserting sequence containing exons 2–5 of the Tbx21 gene from BAC RP23-237M14 (BACPAC Resources Center) into a plasmid backbone containing a PGK promoter driving expression of diphtheria toxin A subunit (DTA) followed by BGHpa-A sequence (modified PL452 plasmid). A SalI restriction enzyme site was simultaneously engineered into the Tbx21 3’ UTR between the stop codon and the polyadenylation site. The Clontech Infusion HD Cloning system was used to generate in the pUC19 plasmid backbone sequence (in order from 5’ to 3’) encephalomyocarditis virus IRES; tandem dimer (td) Tomato; T2A self-cleaving peptide from Thosea asigna virus; Cre recombinase fused to the oestrogen receptor ligand binding domain (ER); followed by a frt site-flanked PGK-Neomycin resistance gene (NEO)-BGHpa-A cassette. The IRES-tdTdT-T2A-creERT2-rtf-NEO-BGHpa-A-rtf sequence was PCR-amplified and inserted into the SalI site in the Tbx21 3’ UTR in the modified PL452 plasmid. The resulting plasmid was linearized with the restriction enzyme NotI before electroporation into embryonic stem cells. Tbx21	extsuperscript{tdTomato-T2A-creERT2} mice were generated similarly, with Cre recombinase containing a nuclear localization sequence replacing the CreERT2 sequence. Tbx21	extsuperscript{tdTomato-T2A-creERT2} and Tbx21	extsuperscript{tdTomato-T2A-creERT2} mice were bred to FLPeR mice to excise the NEO cassette and backcrossed to C57BL/6 mice to remove the FLPeR allele.

**Fxo3	extsuperscript{DTR}** mice were similarly generated by insertion of a targeting construct into the Fxo3 locus by homologous recombination in embryonic stem cells on the C57BL/6 background; the targeting construct was generated by inserting sequence containing exons 8–13 of the Fxo3 gene from a 30.8-kb cosmid containing the Foxp3 gene from a 30.8-kb cosmid containing the Foxp3 gene into the plasmid backbone containing a PGK promoter driving expression of diphtheria toxin A subunit (DTA) followed by BGHpa-A sequence (modified PL452 plasmid). The Clontech Infusion HD Cloning system was used to generate in the pUC19 plasmid backbone sequence containing (in order from 5’ to 3’) a loxp site; encephalomyocarditis virus IRES; diphtheria toxin receptor (DTR) enhanced green fluorescent protein (eGFP) fusion protein; a triple SV40 5’ UTR in the modified PL452 backbone. The resulting plasmid was linearized with the restriction enzyme NotI before electroporation into embryonic stem cells. Tbx21	extsuperscript{tdTomato-T2A-creERT2} mice were generated similarly, with Cre recombinase containing a nuclear localization sequence replacing the CreERT2 sequence. Tbx21	extsuperscript{tdTomato-T2A-creERT2} and Tbx21	extsuperscript{tdTomato-T2A-creERT2} mice were bred to FLPeR mice to excise the NEO cassette and backcrossed to C57BL/6 mice to remove the FLPeR allele.

Fxo3	extsuperscript{Thy1.1, R26Y, Fxo3, Foxp3	extsuperscript{KDO}, RorcGFP, Fxo3	extsuperscript{FPP-foxp3, IL-10	extsuperscript{GFP}}, and Tbx21	extsuperscript{mi} mice have been previously described.

**Generation of bone marrow chimaeric mice.** Tbx21	extsuperscript{RFP-creE82} and Foxp3	extsuperscript{WT} mice were bred to FLPeR mice to excise the NEO cassette and backcrossed to C57BL/6 mice to remove the FLPeR allele. Foxp3	extsuperscript{Thy1.1, R26Y, Fxo3, Foxp3	extsuperscript{KDO}, RorcGFP, Fxo3	extsuperscript{FPP-foxp3, IL-10	extsuperscript{GFP}}, and Tbx21	extsuperscript{mi} mice have been previously described.

**Isolation of cells.** For analysis of TFP-labelled CD4 T cells in Tbx21	extsuperscript{FPP-foxp3E82} mice, CD4 T cells in spleens and lymph nodes were enriched using the Dynabeads CD4 Positive Isolation Kit (microtainer). To isolate lymphocytes from tissues, tissues were euthanized and immediately perfused with 20 ml PBS. Small and large intestines were removed, flushed with PBS and Peyer’s patches were removed. Subsequently, 0.5-cm-long fragments of intestines were washed in PBS and incubated in PBS supplemented with 5% fetal calf serum, 1% l-glutamine, 1% penicillin–streptomycin, 10 mM HEPES, 1 mM dithiothreitol, and 1 mM EDTA for 15 min. Samples were washed and incubated in digest solution (RP1 supplemented with 5% fetal calf serum, 1% l-glutamine, 1% penicillin–streptomycin, 10 mM HEPES, 1 mM mg	extsuperscript{1} collagenase, and 1 U ml	extsuperscript{1} DNase I) for 10 min twice. After filtering through a 100-μm strainer, cells were resuspended in 35% Percoll to eliminate debris. Lymphocytes from livers and lungs were isolated by 50–60 min incubation in digest solution, filtered through 100-μm strainers, and after debris removal in 35% Percoll, purified by centrifugation (1,000g, 7.5 min) over a step-wise 44%–67% Percoll gradient at 4°C.

**Nippostrongylus brasiliensis and Listeria monocytogenes infections.** N. brasiliensis was maintained by passage in 9–10-week-old male Wistar rats as previously described.

**Cell transfer experiments.** For cell transfer experiments, pooled spleens and lymph nodes were enriched for CD4 T cells using the Dynabeads CD4 Positive Isolation Kit. Cells were FACS-sorted on an Aria II cell sorter (BD Biosciences), washed 3 times in PBS, resuspended in 200 μl PBS, and transferred into recipients via retro-orbital injection.

**Flow cytometric analysis.** Cells were stained with LIVE/DEAD Fixable Yellow Dead Cell Stain (Molecular Probes) and the following antibodies purchased from eBioscience, BioLegend, BD Biosciences, Tombo, or obtained from the NIH tetracer control: anti-CD4 (RM4-5, Biolegend 105484), anti-CD8a (SHI10, BD Biosciences 564297), anti-TCR-γ (H57-597, eBioscience 47-9612), and anti-CD69 (H60-244, BD Biosciences 566782). The following antibodies were purchased from eBioscience, BioLegend, BD Biosciences, Tombo, or obtained from the NIH tetracer control: anti-CD4 (RM4-5, Biolegend 105484), anti-CD8a (SHI10, BD Biosciences 564297), anti-TCR-γ (H57-597, eBioscience 47-9612), and anti-CD69 (H60-244, BD Biosciences 566782). For flow cytometric analysis, cells were FACS-sorted on an Aria II cell sorter (BD Biosciences), washed 3 times in PBS, resuspended in 200 μl PBS, and transferred into recipients via retro-orbital injection.

**generation of bone marrow chimaeric mice.** Tbx21	extsuperscript{RFP-creE82} and Foxp3	extsuperscript{WT} mice were lethally irradiated with 650 Gy. The following day, bone marrow was isolated from femurs of donor mice and depleted of T cells and B cells via staining with biotinylated anti-Thy-1.2 and anti-Ter119 antibodies followed by magnetic bead negative selection. 5 × 10⁶ total T-cell-depleted bone marrow cells were transferred into recipient mice via retro-orbital injection.

**Flow cytometric analysis.** Cells were stained with LIVE/DEAD Fixable Yellow Dead Cell Stain (Molecular Probes) and the following antibodies purchased from eBioscience, BioLegend, BD Biosciences, Tombo, or obtained from the NIH tetracer control: anti-CD4 (RM4-5, Biolegend 105484), anti-CD8a (SHI10, BD Biosciences 564297), anti-TCR-γ (H57-597, eBioscience 47-9612), and anti-CD69 (H60-244, BD Biosciences 566782). The following antibodies were purchased from eBioscience, BioLegend, BD Biosciences, Tombo, or obtained from the NIH tetracer control: anti-CD4 (RM4-5, Biolegend 105484), anti-CD8a (SHI10, BD Biosciences 564297), anti-TCR-γ (H57-597, eBioscience 47-9612), and anti-CD69 (H60-244, BD Biosciences 566782). For flow cytometric analysis, cells were FACS-sorted on an Aria II cell sorter (BD Biosciences), washed 3 times in PBS, resuspended in 200 μl PBS, and transferred into recipients via retro-orbital injection.

**Isolation of cells.** For analysis of TFP-labelled CD4 T cells in Tbx21	extsuperscript{FPP-foxp3E82} mice, CD4 T cells in spleens and lymph nodes were enriched using the Dynabeads CD4 Positive Isolation Kit (microtainer). To isolate lymphocytes from tissues, tissues were euthanized and immediately perfused with 20 ml PBS. Small and large intestines were removed, flushed with PBS and Peyer’s patches were removed. Subsequently, 0.5-cm-long fragments of intestines were washed in PBS and incubated in PBS supplemented with 5% fetal calf serum, 1% l-glutamine, 1% penicillin–streptomycin, 10 mM HEPES, 1 mM dithiothreitol, and 1 mM EDTA for 15 min. Samples were washed and incubated in digest solution (RP1 supplemented with 5% fetal calf serum, 1% l-glutamine, 1% penicillin–streptomycin, 10 mM HEPES, 1 mM mg	extsuperscript{1} collagenase, and 1 U ml	extsuperscript{1} DNase I) for 10 min twice. After filtering through a 100-μm strainer, cells were resuspended in 35% Percoll to eliminate debris. Lymphocytes from livers and lungs were isolated by 50–60 min incubation in digest solution, filtered through 100-μm strainers, and after debris removal in 35% Percoll, purified by centrifugation (1,000g, 7.5 min) over a step-wise 44%–67% Percoll gradient at 4°C.
realignment was performed using the Genome Analysis Toolkit (GATK). For each sample, raw count of reads per gene was measured using R, and DESeq2 R package was used to perform differential gene expression among different conditions. A cutoff of 0.05 was set on the obtained P values (that were adjusted using Benjamini–Hochberg multiple testing correction) to get the significant genes of each comparison.

**TCR sequencing and data analysis.** In brief, following isolation of CD4⁺ T cells from spleens and lymph nodes of DO11.10 TCR3 transgenic Tcrα+Foxp3⁺/− mice using the Dynabeads CD4 Positive Isolation Kit (Invitrogen), CD4⁺CXCR3⁺ and CD4⁺CXCR3⁻ eGFP(Foxp3)⁺ Treg and eGFP effector CD4 T cells were FACS sorted and stored in Trizol. TCR sequencing and data analyses were performed as previously described. Pearson's correlation of clonotype frequencies for the shared TCR clones was used for the generation of the dendrogram.

**Microscopy.** Confocal imaging was done using standard conditions. In brief, mice were perfused in PLP buffer. Lymph nodes and spleens were excised, fixed for 1 h at room temperature in 4% paraformaldehyde, and dehydrated at 4°C in sucrose (30% in PBS). Tissues were snap-frozen in OCT compound (Sakura Tissue-Tek). 10 μm tissue sections were cut and fixed with Acetone for 20 min at −20°C, rehydrated in PBS and blocked with 10% normal donkey serum, in PBS with 0.3% Triton X-100, followed by overnight antibody staining at 4°C in a humidified chamber. After antibody staining nuclei were stained with 5 μM Draq7 (Abcam) for 20 min at room temperature. Sections were imaged in Prolong Diamond mounting media (Life Technologies). All images were acquired using a confocal microscope (LSM880; Carl Zeiss) with a 40× oil immersion objective. Images were processed and analysed using ImageJ software (version 2.0.0-rc-54/1.51h). Nearest neighbour analysis was performed using MATLAB program (software R2016b, MathWorks) to calculate nearest cell distance are provided in the Supplementary Information.

**Statistical analysis.** All statistical analyses (excluding RNA-seq and TCR sequence analyses, described above) were performed using GraphPad Prism 6 software. Differences between individual groups were analysed for statistical significance using the unpaired or paired two-tailed t test. *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant. The Kolmogorov–Smirnov test is used to determine the significance between the distributions of signature genes and the rest of expressed genes. One-way ANOVA is used to compare the means of three or more samples. No statistical method was used to predetermine sample size. The number of mice used in each experiment to reach statistical significance was determined on the basis of preliminary data. No animals were excluded from the analyses. No methods of randomization were used to allocate animals into experimental groups. No blinding was used. Data met assumptions of statistical methods used and variance was similar between groups that were statistically compared.

**Code availability.** The colocalization program (Image) software, 2.0.0-rc-54/1.51h, National Institutes of Health) used to find cell positions and the MATLAB program (software R2016b, MathWorks) to calculate nearest cell distance are provided in the Supplementary Information.

**Data availability.** The RNA-seq data that support the findings of this study have been deposited in the NIH SRA database with the accession code SRP102941.
Extended Data Figure 1 | Analysis of T-bet+ cells in Tbx21<sup>RFP-creERT2</sup> reporter mice. a, Targeting strategy for the Tbx21 locus. b, T-bet protein levels in immune cells in Tbx21<sup>RFP-creERT2</sup> mice. c, T-bet protein levels in Tbx21<sup>RFP-creERT2</sup> mice gavaged with tamoxifen on days −2 and 0 and analysed 3 weeks later. d, Flow cytometry of RFP expression in T<sub>reg</sub> and non-T<sub>reg</sub> CD4 T cells. e, Flow cytometry of splenic T<sub>reg</sub> cells. f, Percentage of CD4<sup>hi</sup>CD62<sup>lo</sup> among Thy1.1<sup>+</sup> (top) and RFP<sup>+</sup> among CD4<sup>hi</sup>CD62<sup>lo</sup>Thy1.1<sup>+</sup> (bottom) cells in Tbx21<sup>IDR-creERT2</sup> 3 weeks (white squares), 3 months (grey squares), and 7 months (black squares) after tamoxifen treatment. g, Flow cytometry of T-bet expression in GATA3<sup>+</sup> (blue gate, left, and histogram, right) T<sub>reg</sub> cells isolated from the large intestine lamina propria. h, Percentage RFP<sup>+</sup> cells among eGFP<sup>+</sup>CD4<sup>+</sup>Thy1.1<sup>+</sup> (open circles) and Thy1.1<sup>+</sup> (black circles) cells in Tbx21<sup>IDR-creERT2,ROR<sub>γ</sub>T<sup>CreER</sup>,WT<sup>+</sup></sup> mice. LN, lymph node; SI, small intestine; LI, large intestine. i, Flow cytometry of CD4 T cells in Tbx21<sup>IDR-creERT2,ROR<sub>γ</sub>T<sup>CreER</sup>,WT<sup>+</sup></sup> mice as quantified in h. j, Top, RFP<sup>+</sup> (left axis, squares) and YFP<sup>+</sup> (right axis, circles) effector CD4 T cells. Bottom, percentage of RFP<sup>+</sup> among YFP<sup>+</sup> effector CD4 T cells 3 weeks (white symbols), 3 months (grey symbols), and 7 months (black symbols) after tamoxifen gavage, as outlined in Fig. 1b. Data are mean ± s.e.m. All data are representative of ≥2 experiments, n ≥ 4 mice per group each.
Extended Data Figure 2 | T-betlo cells probably represent transient unstable intermediates in the differentiation of stable T-bethi Treg cells. a, Flow cytometry of the indicated cell subsets. b, CD44 and CD62L expression on RFP−CXCR3− (grey shaded histograms, squares), RFP+CXCR3− (black histograms, squares), and RFP−CXCR3+ (red histograms, squares) splenic CD4+Thy1.1+ cells. c, Differential gene expression between CD44hiRFP− and CD44hiRFPhiCXCR3+ Treg cells sorted from pooled spleens and lymph nodes of Tbx21RFP-creERT2 mice. All genes significantly up- (red) or downregulated (blue) are indicated. d, Expression of the 288 genes up- (≥1.5-fold; left) or 184 genes downregulated (<1.5-fold; right) in CD44hiRFP+CXCR3+ versus CD44hiRFP− cells. Genes with a mean expression value of <15 were excluded from the analysis. P, paired t-test; adjustments were made for multiple comparisons. e, CD44hiCD62LhiRFP−, CD44hiRFP+, CD44hiRFPhiCXCR3−, and CD44hiRFP+CXCR3+ cells sorted from pooled spleens and lymph nodes of Thy1.1+ mice. All data are representative of ≥2 experiments, n ≥ 2 mice per group each.
Extended Data Figure 3 | Fate mapping T-bet-expressing Treg cells during infectious challenge. a, Preferential expansion of CD44loCD62LhiRFP− versus CD44hiRFP+ CD4 effector T cells during N. brasiliensis infection. Flow cytometry analysis of splenic (top) and lung (bottom) CD4+Thy1.1+ cells from mice challenged with PBS (left) and N. brasiliensis (Nb, right). b, Flow cytometry of splenic CD4+Thy1.1+ (left) and Thy1.1− (right) cells of mice challenged with PBS (top) and L. monocytogenes (Lm, bottom), as indicated in Fig. 2a. Numbers indicate percentage of RFP− (left) and YFP+ (right) cells. c, Top, schematic of experiment. CD44hiCD62LhiRFP−, CD44hiRFP+, and CD44loRFPloCXCR3lo CD4+Thy1.1+ cells were FACS-sorted from pooled spleens and lymph nodes of Tbx21RFP-creERT2Il10eGFP/WT mice and transferred into lymphoreplete hosts one day before PBS or L. monocytogenes challenge. Bottom, flow cytometry of transferred populations (indicated on left) on day 9 in spleens of mice challenged with PBS (left) or L. monocytogenes (right). d, Representative histograms of RFP and CXCR3 expression on total CD4+Thy1.1+ (shaded histograms) or Th1.1+-YFP+ (open histograms) cells from spleens of mice challenged with PBS (black) or L. monocytogenes (red), as indicted in Fig. 2a. e–g, eGFP expression in PBS or L. monocytogenes challenged Tbx21RFP-creERT2Il10eGFP/WT mice. e, Schematic of tamoxifen (Tx) administration to Tbx21RFP-creERT2Il10eGFP/WT mice for data shown in f, g. f, Flow cytometry of Treg (top) and YFP (bottom) cells in spleens of PBS (left) and L. monocytogenes (right) treated mice. g, Left, percentage of RFP−eGFP+ and RFP+eGFP+ among Treg cells, as gated in f (top). Right, percentage of eGFP+ cells among YFP+ Treg cells, as gated in f (bottom). h, Schematic of L. monocytogenes reinfection in Tbx21RFP-creERT2Il10eGFP/WT mice for data shown in i, j; 1° and 2° indicate primary and secondary challenge, respectively. i, Flow cytometry of CD4+Thy1.1+ cells in Tbx21RFP-creERT2Il10eGFP/WT mice on day 65, treated as indicated above. j, Percentage of RFP eGFP+ and RFP+eGFP+ cells among Thy1.1+ cells, as gated in i. All data are representative of ≥2 experiments, n ≥ 2 mice per group each. Data are mean ± s.e.m. Two-tailed t-test (NS, not significant).
Extended Data Figure 4 | Features of T-bet<sup>+</sup> T<sub>reg</sub> cells. a, T cell activation, CXCR3 expression, and cytokine production in 12-week-old control Foxp3<sup>YFP-creTbx21<sup>WT/WT</sup></sup> and Foxp3<sup>YFP-creTbx21<sup>fl/fl</sup></sup> (white circles) and experimental Foxp3<sup>YFP-creTbx21<sup>fl/WT</sup></sup> (black circles) mice. Data are mean ± s.e.m. Two-tailed t-test (*P < 0.05; NS, not significant). Data are representative of three experiments, n ≥ 7 mice per group. 

b, Cumulative distribution function plot of the 561 genes up in Thy1.1<sup>+</sup> CD4<sup>+</sup>CD44<sup>hiRFPhiCXCR3</sup><sup>+</sup> versus CD44<sup>hiRFP</sup><sup>-</sup> cells in Tbx21<sup>RFP-creERT2</sup> mice compared to all genes differentially expressed in CD4<sup>+</sup>CD25<sup>+</sup> Treg cells from Tbx21<sup>RFP-creFoxp3<sup>WT</sup></sup> mice versus CD4<sup>+</sup>CD25<sup>lo</sup> ex-Treg cells from Tbx21<sup>RFP-creFoxp3<sup>fl</sup></sup> mice. 

P = 0.2 × 10<sup>-15</sup>, two-sample Kolmogorov–Smirnov test. 

c, Expression of CCR5 (top) and CD29 (bottom) in CD4<sup>+</sup>CD62L<sup>hi</sup> naive (blue histogram), CD4<sup>+</sup>CXCR3<sup>-</sup> (black histogram) and CD4<sup>+</sup>CXCR3<sup>+</sup> (red histogram) T<sub>reg</sub> (left) and CD4<sup>+</sup>Foxp3<sup>-</sup> (right) T cells from spleens of Foxp3<sup>YFP-creTbx21<sup>WT/WT</sup></sup> mice. 

d, Expression of CXCR3 (left), CCR5 (middle), and CD29 (right), gated on CD4<sup>+</sup> T cells in spleens of Foxp3<sup>YFP-creTbx21<sup>WT/WT</sup></sup> and Foxp3<sup>YFP-creTbx21<sup>fl/WT</sup></sup> mice. 

e, Dendrogram represents cluster analysis of TCR sequences in CD4<sup>+</sup>CXCR3<sup>+</sup> (red symbols) and CD4<sup>+</sup>CXCR3<sup>-</sup> (black symbols) T<sub>reg</sub> (right) and effector CD4 T (left) cells in spleens (white symbols) and lymph nodes (grey symbols) of DO11.10 TCR<sup>+</sup> Tcra<sup>−/−</sup> Foxp3 reporter mice. Sample preparation and statistical analyses are described in the Methods. Pearson’s correlation of clonotype frequencies for the shared TCR clones was used for the generation of the dendrogram.
Extended Data Figure 5 | Characterization of Tbx21<sup>RFP-cre</sup>Foxp3<sup>fl</sup> mice. 

**a.** Targeting strategy for the Tbx21 locus (top) and RFP expression in the indicated cell populations in spleens of homozygous Tbx21<sup>RFP-cre</sup> mice (bottom). 

**b.** Progressive loss of hair pigmentation in Tbx21<sup>RFP-cre</sup>Foxp3<sup>fl</sup> mice. 

**c.** RFP and YFP expression (upper) and CD44 and CD62L expression (lower) in the indicated splenic cell populations in Tbx21<sup>RFP-creR26</sup> mice. 

**d.** Activation and expansion of RFP<sup>+</sup> T cells in lymph nodes (top) and lungs (bottom) of the indicated mice. 

**e.** Cytokine production by CD4<sup>+</sup>Foxp3<sup>-</sup> and CD8<sup>+</sup>T cells in lungs of the indicated mice. 

**f.** Characterization of lymph node T<sub>reg</sub> cells. 

**g.** Percentages of ex-T<sub>reg</sub> cells in spleens, lymph nodes, and lungs. 

**h.** Top, flow cytometry of lymph node CD4<sup>+</sup>T cells, as quantified in **g**; numbers indicate the percentage of Foxp3<sup>-</sup>CD25<sup>+</sup>. Bottom, histogram showing expression of T<sub>reg</sub> cell signature molecules in CD4<sup>+</sup>Foxp3<sup>-</sup>CD25<sup>+</sup> cells in lymph nodes of Tbx21<sup>RFP-creF</sup>xop3<sup>WT</sup> (open grey histogram), Tbx21<sup>RFP-creF</sup>xop3<sup>WT</sup> (open red histogram), Tbx21<sup>RFP-creF</sup>xop3<sup>WT/WT</sup> (open blue histogram), and Tbx21<sup>RFP-creF</sup>xop3<sup>fl/WT</sup> (open black histogram) mice. CD4<sup>+</sup>Foxp3<sup>-</sup>CD25<sup>+</sup> cells from a Tbx21<sup>RFP-creF</sup>xop3<sup>WT</sup> (shaded grey histogram) mouse are shown as a point of reference. Data are mean ± s.e.m. Two-tailed t-test (**P < 0.01, ***P < 0.001, *P < 0.05, respectively; NS, not significant). Data represent the combined results from several experiments.
Extended Data Figure 6 | The Th2 response to *N. brasiliensis* is not exacerbated in Tbx21<sup>RFP</sup>-creFoxp3<sup>fl</sup> mice. *Tbx21<sup>RFP</sup>-creFoxp3<sup>fl</sup>* and *Tbx21<sup>RFP</sup>-creFoxp3<sup>WT</sup>* mice were infected with *N. brasiliensis* and analysed on day 9 after challenge. 

**a**, Flow cytometry of GATA3 expression in CD4<sup>+</sup> Foxp3<sup>-</sup>CD25<sup>-</sup> T cells in spleens (top) and lungs (bottom) of Tbx21<sup>RFP</sup>-creFoxp3<sup>WT</sup> (left) and Tbx21<sup>RFP</sup>-creFoxp3<sup>fl</sup> (right) mice. 

**b**, Quantification of data in a. *Tbx21<sup>RFP</sup>-creFoxp3<sup>WT</sup>* and *Tbx21<sup>RFP</sup>-creFoxp3<sup>fl</sup>* mice are indicated by grey and red circles, respectively. 

**c**, Numbers of eosinophils in lungs of the indicated mice. 

**d**, Cytokine production by CD4<sup>+</sup> Foxp3<sup>-</sup> and CD8 T cells in spleens and lungs of the indicated mice. Data are mean ± s.e.m. Two-tailed t-test ( *P* < 0.05; NS, not significant). Data represents 1 experiment, *n* ≥ 5 mice per group.
Extended Data Figure 7 | Distinguishing the drivers of autoimmunity in the absence of T-bet+ Treg cells. a–c, Ex-Treg cells are no more pathogenic than effector CD4 T cells. a, CD4+CD25+(Treg) cells were sorted from lymph nodes of Tbx21RFP-CreFoxp3WT mice, and CD4+CD25− (effector) and CD4+CD25lo (ex-Treg) cells were sorted from lymph nodes of Tbx21RFP-CreFoxp3fl mice for transfer into Tcrb−/−Tcrd−/−mice. Intraacellular staining for Foxp3 demonstrates purity of cell populations. b, Weights of Tcrb−/−Tcrd−/−mice receiving CD4+CD25+(white squares), CD4+CD25− (black squares), and CD4+CD25lo (grey squares) cells. c, Percentages and numbers of the indicated T cell populations in spleens of mice analysed on day 62 after transfer. d, e, T-bet+ effector αβT cells drive disease upon ablation of T-bet+ Treg cells. Lethally irradiated Tcrb−/−Tcrd−/−mice were reconstituted with a 1:1 mix of CD45.2+Tbx21RFP-Cre/WT R26DTT:Foxp3KO, CD45.1+Foxp3WT, or CD45.2+TcrbKO T-cell depleted bone marrow cells. Mice were injected with 0.5 μg diphtheria toxin (DT) on day 0, then treated daily with 0.1 μg diphtheria toxin for 22 days before analysis. d, Weight loss in Tbx21RFP-Cre/WT R26DTT:Foxp3KO (red line) versus Tbx21RFP-Cre/WT R26DTT:Foxp3WT (black line) versus Tbx21RFP-Cre/WT R26DTT:Foxp3KO (blue line) reconstituted mice. e, Representative flow cytometry of splenic cell populations (indicated on right) in chimaeric mice (as indicated above). All data represent 1 experiment, n ≥ 3 mice per group.
Extended Data Figure 8 | Co-localization of T-bet\(^+\) Treg and T-bet\(^+\) effector T cells in vivo. \(a, b\), Representative images (left) and insets (right) of lymph node sections from Tbx21\(^{RFP, cr}\) mice with CD4 \((a)\) or CD8 \((b)\) in green, RFP in red, Foxp3 in blue, and CD44 in grey. In inset, arrowheads indicate CD4\(^+\)CD44\(^{hi}\)RFP\(^+\)Foxp3\(^+\) \((a)\) or CD8\(^+\)CD44\(^{hi}\)RFP\(^+\) \((b)\) cells and arrows indicate CD4\(^+\)CD44\(^{hi}\)RFP\(^+\)Foxp3\(^+\) cells. \(c, d\), Quantification of nearest distances between Treg cells and CD4 \((c)\) and CD8 \((d)\) T cells, as shown in \(a, b\). Foxp3\(^+\) denotes CD4\(^+\)CD44\(^{hi}\)Foxp3\(^+\); Foxp3\(^-\) \((c)\) denotes CD4\(^+\)CD44\(^{hi}\)Foxp3\(^-\) and CD8\(^+\) \((d)\) denotes CD8\(^+\)CD44\(^{hi}\)RFP\(^+\). \(e, f\), Quantification of nearest distances between Treg and non-Treg CD4 \((e)\) and CD8 \((f)\) T cells in spleens of Tbx21\(^{RFP, cr}\)Foxp3\(^{WT}\) and Tbx21\(^{RFP, cr}\)Foxp3\(^{fl}\) mice. Genotypes of mice are indicated above plots; cell types being analysed are shown below plots, as in \(c, d\). Bars indicate mean. \(P\) values were calculated using a two-tailed \(t\)-test \((e, d)\) or one-way ANOVA \((e, f)\) (**\(P < 0.01\), ***\(P < 0.001\), \(P < 0.05\); NS, not significant). Data are representative of multiple imaged sections from \(\geq 2\) mice.
Extended Data Figure 9 | T-bet+ Treg cells suppress pre-established Th1 but not Th2 or Th17 activation induced by depletion of Treg cells.

a. Targeting strategy for the Foxp3 locus. b. Schematic for experiment shown in c-g depleting all Treg cells and subsequently depleting all or only non-T-bet-expressing Treg cells in Foxp3<sup>DTR</sup>Tbx21<sup>RFP-creERT2</sup> mice. c. Flow cytometry of splenic CD4 T cells in the indicated mice treated with tamoxifen or oil, as indicated. d-g, Percentages of Treg cells (d) and activation status of (e) and cytokine production by (f, g) splenic CD4<sup>+</sup>Foxp3<sup>-</sup> and CD8 T cells in tamoxifen-treated Foxp3<sup>DTR</sup>Tbx21<sup>RFP-creERT2</sup> mice (open circles), mock oil-treated Foxp3<sup>DTR</sup>Tbx21<sup>RFP-creERT2</sup> (black circles), and tamoxifen-treated Foxp3<sup>DTR</sup>Tbx21<sup>RFP-creERT2</sup> (grey circles) mice. h-1, Treg cells rebounding post depletion in DT-treated Foxp3<sup>DTR</sup>Tbx21<sup>RFP-creERT2</sup> mice efficiently suppress Th2 responses. h. Left, schematic for control experiment shown in i-1. Right, flow cytometry of splenic CD4 T cells in mice treated with high dose diphtheria toxin (DT<sup>hi</sup>, 1 μg per mouse), low dose diphtheria toxin (DT<sup>lo</sup>, 0.0625 μg per mouse), and PBS. Group 1 (control); group 2 (depletion without Treg cell recovery); group 3 (depletion with partial recovery); group 4 (depletion with full recovery). i-1, Percentages of Treg cells (i) and activation status of (j) and cytokine production by (k, l) splenic CD4<sup>+</sup>Foxp3<sup>-</sup> and CD8 T cells in the indicated groups of mice. Data are mean ± s.e.m. Two-tailed t-test (***P < 0.001, **P < 0.01, *P < 0.05; NS, not significant). Data are representative of ≥ 1 experiment, n ≥ 4 mice per group.
Extended Data Figure 10 | T<sub>reg</sub> cells rebounding post transient depletion efficiently suppress T<sub>H2</sub> and T<sub>H17</sub> responses. a, Experimental schematic. Mice were treated with tamoxifen (tx) or oil (to additionally control for potential effects of tamoxifen) on days −5 and −3 and received PBS on days 0, 1, 3, 5, 7 (control); 1μg diphtheria toxin (DThi) on days 0, 1, 3, 5, and 7 (no T<sub>reg</sub> cell recovery); 0.062μg diphtheria toxin (DTlo) on days 0, 1, 3, 5, and 7 (partial T<sub>reg</sub> cell recovery); or 0.062μg diphtheria toxin (DTlo) on day 0 and PBS on days 1, 3, 5, and 7 (full T<sub>reg</sub> cell recovery). Mice were analysed on day 9. b, Flow cytometry analysis of CD4 T cells in spleens of the indicated groups of mice. c–e, Percentages of T<sub>reg</sub> cells (c) and CD4<sup>+</sup>Foxp3<sup>-</sup> and CD8 T cell activation (d) and cytokine production (e) in spleens of the indicate mice (group 1, open circles; group 2, black circles; group 3, dark grey circles; group 4, light grey circles). Data are mean ± s.e.m. Two-tailed t-test (***P < 0.001, **P < 0.01; NS, not significant). Data represent the combined results from two experiments, n ≥ 3 mice per group.
Corrigendum: Stability and function of regulatory T cells expressing the transcription factor T-bet

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In this Letter, author Alejandra Mendoza was incorrectly spelled Alejandra Medoza. The original Letter has been corrected online.