The alarmin IL-33 promotes regulatory T-cell function in the intestine

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FOXP3+ regulatory T cells (Treg cells) are abundant in the intestine, where they prevent dysregulated inflammatory responses to self and environmental stimuli. It is now appreciated that Treg cells acquire tissue-specific adaptations that facilitate their survival and function1; however, key host factors controlling the Treg response in the intestine are poorly understood. The interleukin (IL)-1 family member IL-33 is constitutively expressed in epithelial cells at barrier sites2, where it functions as an endogenous danger signal, or alarmin, in response to tissue damage2. Recent studies in humans have described high levels of IL-33 in inflamed lesions of intestinal inflammatory bowel disease patients3,4, suggesting a role for this cytokine in disease pathogenesis. In the intestine, both protective and pathologicales for IL-33 have been described in murine models of acute colitis5–7, but its contribution to chronic inflammation remains ill defined. Here we show in mice that the IL-33 receptor ST2 is preferentially expressed on colonic Treg cells, where it promotes Treg function and adaptation to the inflammatory environment. IL-33 signalling in T cells stimulates Treg responses in several ways. First, it enhances transforming growth factor (TGF)-β-mediated differentiation of Treg cells and, second, it provides a necessary signal for Treg-cell accumulation and maintenance in inflamed tissues. Strikingly, IL-23, a key pro-inflammatory cytokine in the pathogenesis of inflammatory bowel disease, restrained Treg responses through inhibition of IL-33 responsiveness. These results demonstrate a hitherto unrecognized link between an endogenous mediator of tissue damage and a major anti-inflammatory pathway, and suggest that the balance between IL-23 and IL-33 may be a key controller of intestinal immune responses.

To identify potential tissue-specific modulators of colonic Treg cells, we compared the messenger RNA expression profiles of mesenteric lymph node and colonic Treg cells. We identified St2 (also known as Il1rl1), the transcript coding for the IL-33 receptor, as one of the top differentially upregulated genes in colonic Treg cells (Fig. 1a, b). Flow-cytometric analysis confirmed selective enrichment of ST2+ Treg cells in the colon (Fig. 1c) and these cells expressed high levels of the activation markers KLRG1, CD103 and OX40 (Fig. 1d). Analysis of Helios expression revealed that ST2+ Treg cells are a heterogeneous population containing thymus-derived Treg cells as well as peripherally generated Helios+ Treg cells (Fig. 1e)11. A significant proportion of intestinal Foxp3+ Treg cells co-express the transcription factor GATA3 (refs 14–16), and GATA3 is known to regulate ST2 expression in Th2 cells17. Indeed, ST2 expression was largely restricted to GATA3-expressing colonic Treg cells (Fig. 1e) and selective ablation of GATA3 in Foxp3-expressing cells, using Gata3fl/flFoxp3cre mice18, caused a marked reduction of ST2 protein levels (Fig. 1f).

Given that ST2+ Treg cells are prominent in the colon, we postulated that IL-33 may modulate in vitro induced (i)Treg-cell differentiation.

To test this, we sort-purified naive CD4+ T cells from Foxp3cre reporter mice and activated them in the presence of TGF-β1. Notably, both Gata3 and St2 expression were induced under iTreg-differentiation conditions (Extended Data Fig. 1). Addition of IL-33 to iTreg cultures significantly increased both the percentage and total number of Foxp3-expressing cells but had no effect on Foxp3 expression in the absence of TGF-β1 (Fig. 2a). The presence of IL-33 in iTreg cultures did not affect induction of Th12 cytokines or expression of Th17-associated transcription factors Tbx21 and Rorc (Extended Data Fig. 1), suggesting that IL-33 preferentially regulates Foxp3 expression. Thus, our data indicate

Figure 1 | ST2-expressing Treg cells are enriched in the colon. a, Change in gene expression in colonic (c)Treg cells versus mesenteric lymph node (MLN) Treg cells (n = 3 per group) presented as volcano plot. b, Top differentially upregulated transcripts in colonic Treg versus MLN Treg cells. c, ST2 protein expression on Treg cells from indicated organs. d, Phenotypic analysis of ST2+ or ST2- colonic Treg cells. e, Expression of transcription factors in colonic Treg cells. f, Representative histograms gated on colonic Treg cells from control or Gata3fl/flFoxp3-cre mice.

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that the alarmin IL-33 is a novel cofactor in TGF-β1-mediated iTreg generation.

GATA3 is highly expressed in ST2+ Treg cells (Fig. 1e) and IL-33 has been shown to activate GATA3 in Treg cells as well as in innate lymphoid cells26. Consistent with this notion, we observed serine phosphorylation of GATA3 upon acute stimulation of iTreg cells with IL-33 (Fig. 2b). The Foxp3 locus contains putative GATA3-binding sites within its promoter and intragenic conserved noncoding sequences (CNSs) 1–3 (ref. 14). To investigate whether IL-33 influences the binding of GATA3 to any of these elements in iTreg cells, we performed chromatin immunoprecipitation (ChIP) followed by quantitative polymerase chain reaction (PCR). Acute stimulation of iTreg cells with IL-33 induced GATA3 recruitment to the Foxp3 promoter but not CNS1, 2 or 3 (Fig. 2c). In addition, RNA polymerase II (Pol II) was recruited to the Foxp3 promoter upon IL-33 stimulation (Fig. 2d), suggesting that IL-33 directly regulates Foxp3 expression through activation and recruitment of GATA3 to the Foxp3 promoter. In Treg cells, GATA3 has been shown to promote St2 gene expression by binding to an enhancer element located 12 kilobases upstream of the St2 transcription start site17. Consistent with this, we detected recruitment of GATA3 to the St2 enhancer upon acute stimulation of iTreg cells with IL-33 and this correlated with RNA Pol II enrichment at the St2 promoter (Extended Data Fig. 2). Thus, in addition to its role in Foxp3 induction, IL-33 also promoted its own receptor expression in iTreg cells through direct transcriptional regulation of the St2 locus, providing an amplification loop for further enhancement of iTreg-cell differentiation.

Next we focused on thymus-derived Treg cells, which constitute a significant proportion of ST2+ colonic Treg cells (Fig. 1e). In line with published reports20,21, administration of recombinant IL-33 led to a significant increase in the frequency and total number of splenic Treg cells (Extended Data Fig. 3a, b) and these IL-33-elicited Treg cells expressed higher levels of Foxp3 and ST2 (Extended Data Fig. 3c, d). Further analysis of the proliferation marker Ki67 showed that IL-33 induced proliferation in splenic Treg cells but not in T effector cells (Extended Data Fig. 3e). To examine whether IL-33 acts directly on Treg cells, we injected IL-33 into chimaeric mice containing a mixture of wild-type and ST2−/− haematopoietic cells. In this setting, the proliferative capacity of ST2−/− Treg cells was significantly impaired (Extended Data Fig. 3f), suggesting that IL-33 acts directly on thymus-derived Treg cells to promote their proliferation and accumulation in vivo. This is further supported by the finding that sort-purified splenic Treg cells cultured in the presence of IL-33 expressed higher levels of ST2, showed a more activated phenotype and expressed increased amounts of Foxp3 protein (Fig. 2e). In addition, acute stimulation of T-cell antigen receptor (TCR)-activated splenic Treg cells with IL-33 induced serine phosphorylation of GATA3 (Fig. 2f), further demonstrating that IL-33 acts directly on thymus-derived Treg cells.

To assess the impact of IL-33 on the Treg response during intestinal inflammation, we induced chronic colitis with infection with Helicobacter hepaticus and administration of an IL-10R blocking antibody22 (Extended Data Fig. 4a). We detected an increase in IL-33 protein levels in colon explant cultures and its expression kinetics mirrored that of IL-23, which is essential for the development of intestinal inflammation in this model (Extended Data Fig. 4b). Consistent with its pattern of expression, IL-33 protein levels were elevated in colon intestinal epithelial cells isolated from the inflamed gut (Extended Data Fig. 4c, d). Interestingly, the onset of intestinal pathology correlated with a marked increase of soluble ST2, which is produced primarily by colonic stromal cells (Extended Data Fig. 4b, e, f). Soluble ST2 is thought to limit IL-33 bioavailability by acting as a decoy receptor23 and is increased in patients with active inflammatory bowel disease (IBD)24, suggesting that the chronic inflammatory tissue environment may antagonize IL-33 activity. Despite high levels of soluble ST2, analysis of chimaeric mice showed that accumulation of ST2−/− Treg cells in the colon but not the spleen was significantly impaired during the peak of intestinal inflammation (Fig. 2g). In addition, colonic ST2−/− Treg cells expressed lower amounts of Foxp3 protein on a per cell
IL-33 promotes T<sub>reg</sub>-cell stability and function in vivo. a, C57BL/6 Rag<sup>1−/−</sup> mice were injected with CD45.1<sup>+</sup> naive T cells alone (RB<sup>hi</sup>; n = 4) or in combination with wild-type (WT; n = 4) or St2<sup>−/−</sup> (n = 6) CD45.1<sup>+</sup> T cells. Mice were killed 6–8 weeks after transfer and colitis scores are shown (mean ± s.e.m.). b, Absolute numbers of colon lamina propria (LP) cells from mice in a (mean ± s.e.m.). c, C57BL/6 Rag<sup>1−/−</sup> mice were injected as in a and killed at 2 weeks post-injection. Representative plots are gated on colonic T<sub>reg</sub>-cell progeny (CD45.1<sup>+</sup>). d, Ratio of RB<sup>hi</sup>-T cell progeny (CD45.1<sup>+</sup>) to wild-type or St2<sup>−/−</sup> Foxp3<sup>+</sup> T<sub>reg</sub>-cell progeny (CD45.1<sup>+</sup>) in the colon (n = 5 per group) from mice in c (mean ± s.e.m.). e, C57BL/6 Rag<sup>1−/−</sup> mice were injected as in a and killed at 8 weeks post-injection. Representative plots are gated on colonic T<sub>reg</sub>-cell progeny (CD45.1<sup>+</sup>). f, Ratio of RB<sup>hi</sup>-T cell progeny (CD45.1<sup>+</sup>) to wild-type or St2<sup>−/−</sup> T<sub>reg</sub>-cell progeny (CD45.1<sup>+</sup>) in the colon from mice in e (mean ± s.e.m.). g, Absolute numbers of RB<sup>hi</sup>-T cell progeny (CD45.1<sup>+</sup>) in the colon from mice in e (mean ± s.e.m.). h, Analysis of Foxp3 expression in colonic Foxp3<sup>+</sup> CD45.1<sup>+</sup> T<sub>reg</sub> cells presented as gMFI (mean ± s.e.m.). Results are representative of two independent experiments.

**Discussion**

IL-33 promotes intestinal inflammation in part through inhibition of iTreg-cell differentiation<sup>29,30</sup>. However, the mechanism by which IL-23 prevents intestinal inflammation is associated with increased susceptibility to IBD in humans<sup>28</sup>. We previously showed that IL-23 restrains Treg cells and inhibited T<sub>reg</sub>-cell function<sup>26,30</sup>. We next sought to compare the suppressive capacity of wild-type and St2<sup>−/−</sup> T<sub>reg</sub> cells. St2<sup>−/−</sup> T<sub>reg</sub> cells inhibited T-cell proliferation to the same extent as wild-type T<sub>reg</sub> cells<sup>26</sup>. Interestingly, ST2 was highly expressed on wild-type T<sub>reg</sub> cells upon T-cell transfer, pointing towards a potential role of ST2 in modulating T<sub>reg</sub> function in this model (Extended Data Fig. 6a). Indeed, St2<sup>−/−</sup> T<sub>reg</sub> cells were significantly impaired in their ability to prevent colonic inflammation and cellular infiltration (Fig. 3a, b), demonstrating that IL-33 signalling in T<sub>reg</sub> cells is important for their suppressive function in vivo. Analysis of wild-type or St2<sup>−/−</sup> T<sub>reg</sub> cells 2 weeks after transfer, before the onset of intestinal pathology, showed similar proliferative capacity and Foxp3 expression between groups (Fig. 3c). The ratio of T effector cells (CD45.1<sup>+</sup> RB<sup>hi</sup> progeny) to T<sub>reg</sub> cells (CD45.1<sup>+</sup> Foxp3<sup>+</sup> T<sub>reg</sub> progeny) was also similar (Fig. 3d), suggesting that IL-33 signalling in T<sub>reg</sub> cells is dispensable for their ability to expand and index with effector cells in the lymphopenic host at 2 weeks after transfer. By contrast, analysis at 8 weeks after transfer showed that the progeny of St2<sup>−/−</sup> T<sub>reg</sub> cells contained a significantly lower proportion of Foxp3<sup>+</sup> cells and expressed significantly less Foxp3 on a per cell basis, suggesting that they had lost Foxp3 expression (Fig. 3e, h). Under these circumstances the ratio of T effector/T<sub>reg</sub> cells and the total number of T effector cells (CD45.1<sup>+</sup> RB<sup>hi</sup> progeny) was markedly increased in recipients of St2<sup>−/−</sup> T<sub>reg</sub> cells (Fig. 3f, g). Importantly, ST2-deficient T<sub>reg</sub> cells did not themselves acquire the capacity to produce inflammatory cytokines (Extended Data Fig. 6c). Perturbations of Foxp3 expression have been shown to affect T<sub>reg</sub>-cell function<sup>25,27</sup> and our data indicate that IL-33 signalling in T<sub>reg</sub> cells contributes to the maintenance of Foxp3 expression under inflammatory stress, enabling T<sub>reg</sub> cells to compete in the inflammatory niche and to control the intestinal effector T-cell response.

We previously showed that IL-23 restrains T<sub>reg</sub> cells in vivo because naive T-cell transfer into Il23a<sup>−/−</sup> Rag<sup>1−/−</sup> recipients resulted in increased T<sub>reg</sub>-cell differentiation<sup>29</sup>. Therefore, we hypothesized that enhanced responsiveness to IL-33 may contribute to increased iT<sub>reg</sub> differentiation in Il23a<sup>−/−</sup> Rag<sup>1−/−</sup> hosts. To test this, we transferred wild-type or St2<sup>−/−</sup> naive T cells into Il23a<sup>−/−</sup> Rag<sup>1−/−</sup> hosts and monitored iT<sub>reg</sub> cell generation. Indeed, ST2-deficient T cells were significantly impaired in their ability to differentiate into T<sub>reg</sub> cells (Fig. 4d) and this correlated with a significant increase in intestinal pathology (Fig. 4e). Importantly,
IL-23 inhibits the effects of IL-33 on Treg cells. a. Naive CD4⁺ T cells were cultured with anti-CD3/CD28 plus TGF-β, as well as the indicated cytokines, and the frequencies of Foxp3⁺ T cells were determined 3 days later (mean ± s.e.m. of three independent experiments). b. Naive CD4⁺ T cells were cultured with anti-CD3/CD28 plus the indicated cytokines for 48 h. Data are from one experiment representative of two (mean ± s.d.). c. Naive CD4⁺ T cells were cultured with anti-CD3/CD28 and the mRNA expression of the indicated genes was measured after stimulation with IL-33 for 45 min in the presence or absence of IL-23 (mean ± s.e.m. of three independent experiments). d. Treg cells were cultured with anti-CD3/CD28 for 24 h and the mRNA expression of two independent experiments are shown. e. Treg cells were cultured in the presence of anti-CD3/CD28 for 24 h and the mRNA expression of the indicated genes was measured after stimulation with IL-33 in the presence or absence of IL-23. Representative blots of two independent experiments are shown. f. Expression of the indicated cytokines by colonic CD4⁺ T cells from mice in d (mean ± s.e.m.). g. Treg cells were cultured with anti-CD3/CD28 for 24 h followed by stimulation with IL-33 in the presence or absence of IL-23. Representative blots of two independent experiments are shown. h. Expression of the indicated cytokines is from one experiment representative of two (mean ± s.e.m.). i. Treg cells were cultured with anti-CD3/CD28 for 24 h and representative blots of two independent experiments are shown. p, phosphorylated. *P < 0.05, **P < 0.01, ***P < 0.001 as calculated by one-way ANOVA with Bonferroni post-test or Student’s t-test. NS, not significant.

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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Figure 4 | IL-23 inhibits the effects of IL-33 on Treg cells. a. Naive CD4⁺ T cells were cultured with anti-CD3/CD28 plus TGF-β, as well as the indicated cytokines, and the frequencies of Foxp3⁺ T cells were determined 3 days later (mean ± s.e.m. of three independent experiments). b. Naive CD4⁺ T cells were cultured with anti-CD3/CD28 plus the indicated cytokines for 48 h. Data are from one experiment representative of two (mean ± s.d.). c. Naive CD4⁺ T cells were cultured with anti-CD3/CD28 and the mRNA expression of the indicated genes was measured after stimulation with IL-33 for 45 min in the presence or absence of IL-23 (mean ± s.e.m. of three independent experiments). d. Treg cells were cultured with anti-CD3/CD28 for 24 h and the mRNA expression of two independent experiments are shown. e. Treg cells were cultured in the presence of anti-CD3/CD28 for 24 h and the mRNA expression of the indicated genes was measured after stimulation with IL-33 in the presence or absence of IL-23. Representative blots of two independent experiments are shown. f. Expression of the indicated cytokines by colonic CD4⁺ T cells from mice in d (mean ± s.e.m.). g. Treg cells were cultured with anti-CD3/CD28 for 24 h followed by stimulation with IL-33 in the presence or absence of IL-23 (mean ± s.e.m. of three independent experiments). h. Treg cells were cultured with anti-CD3/CD28 for 24 h and the mRNA expression of two independent experiments are shown. p, phosphorylated. *P < 0.05, **P < 0.01, ***P < 0.001 as calculated by one-way ANOVA with Bonferroni post-test or Student’s t-test. NS, not significant.

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Author Contributions C.S. and T.K. planned experiments and analysed the data. C.S., T.K. and F.P. wrote the paper. A.C., F.A., K.A., O.J.H., A.N.H., E.A.W., T.G., J.B., B.M.J.O. and J.P. performed particular experiments. M.L., Y.B. and P.G.F. provided essential materials and were involved in data discussions.

Author Information Microarray data have been deposited in the Gene Expression Omnibus under accession number GSE58164. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to F.P. (fiona.powrie@path.ox.ac.uk).
METHODS

Mice. Wild-type C57BL/6, congenic B6.SJL-Gdf5.1, C57BL/6 H2br+/−, C57BL/6 Rag1−/−, C57BL/6 Rag2−/−, Rag2−/− reporter mice and Foxp3-gfp reporter mice were bred and maintained under specific pathogen-free conditions in accredited animal facilities at the University of Oxford. Where indicated, mice were intrauterine (i.p.) injected with recombinant IL-33 (1 μg per injection; Biolegenda) for 5 consecutive days and killed 24 h after the last injection. Gata3 GFP, Foxp3-cre mice were kept at the National Institutes of Health (NIH) and experiments were performed at the NIH. Spleen, MLN and bone marrow from C57BL/6 St2−/− mice were obtained from P. Fallon or M. Loening. All procedures were conducted in accordance with the UK Scientific Procedures Act of 1986. Mice were negative for Helicobacter spp. and other known intestinal pathogens, were age and sex-matched and more than 6 weeks old when first used. Both female and male mice were used in experiments. Where possible, preliminary experiments were performed to determine requirements for sample size, taking into account resources available and ethical, reductionist animal use. Generally, each mouse of the different experimental groups is reported. Exclusion criteria such as inadequate staining or low cell yield due to technical problems were pre-determined. Animals were assigned randomly to experimental groups. Each cage contained animals of all the different experimental groups.

Generation of mixed bone marrow chimaeras. Bone marrow isolated from wild-type (C57BL/6 J), H2br/− or St2−/− mice was mixed at a 1:1 ratio with bone marrow taken from B6.SJL-Gdf5.1 mice and injected intravenously into gamma-irradiated (5.5 Gy, 250 rad) St2−/− recipient recipients and chimaeras were used in experiments 8 weeks after injection.

T-cell transfer colitis. For naive T-cell transfer colitis, 4 × 10^4 CD45.2+ CD25− CD45 RBhi T cells were injected i.p. into Rag1−/− or H2br/− Rag1−/− recipients. In co-transfer experiments, 2 × 10^4 CD45.2+ CD45 RBhi T cells from each source were mixed and injected i.p. into Rag1−/− hosts. For Treg-mediated protection from colitis, 4 × 10^4 CD45.2+ CD45 RBhi T cells and 2 × 10^4 CD45.1+ CD25− Treg cells were mixed and injected i.p. into Rag1−/− hosts. Mice were killed at indicated time points or killed when weight loss approached 20% of the original body weight at the start of the experiment.

H. hepaticus infection and anti-IL-10R treatment. Mice were fed 1 × 10^6 colony-forming units (cfu) H. hepaticus by oral gavage with a 22 G curved needle on day 0, day 1 and day 2 of the experiment. In addition mice received 1 mg of an anti-CD3 (5 mg ml−1) and anti-IL-10R blocking antibody by i.p. injection once weekly starting at the day of H. hepaticus infection.

Histological assessment of intestinal inflammation. Proximal, mid, and distal colon samples were fixed in buffered 10% formalin solution. Paraffin-embedded sections were cut (5 mm) and stained with haematoxylin and eosin, and inflammation was scored in a blinded fashion using a previously described scoring system. Histological assessment of intestinal inflammation. H. hepaticus anti-IL-10R blocking antibody by i.p. injection once weekly starting at the day of H. hepaticus infection.

Microarray analysis. Microarray analysis was performed using GeneSpring GX12 software (Agilent). Data were normalized using 75% percentile shift normalization algorithm and baseline transformed to the median of all samples. Statistical significance was determined using an unpaired t-test followed by Benjamini-Hochberg false discovery rate multiple testing correction. P value cutoff was set to 0.05.

Total protein extracts and immunoblot analysis. Total protein extracts were prepared as described. Equal amounts of protein were resolved by SDS-PAGE and analysed with anti-GATA3 (ab10652; Abcam), anti-total GATA3 (GATA3, 1:5000; BD Pharmingen), anti-STAT3 (rabbit polyclonal, R&D Systems, 1:1000). Immunoblotting was performed using GeneSpring GX12 software (Agilent). Data were normalized using 75% percentile shift normalization algorithm and baseline transformed to the median of all samples. Statistical significance was determined using an unpaired t-test followed by Benjamini-Hochberg false discovery rate multiple testing correction. P value cutoff was set to 0.05.

Statistical analysis. Where appropriate, Student’s t-test was used. For the comparison of more than two groups a one-way ANOVA followed by a Bonferroni multiple comparison test was performed. All statistical analysis was calculated in Prism (GraphPad). Differences were considered to be statistically significant when P ≤ 0.05.

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Extended Data Figure 1 | Effects of IL-33 on gene expression during iTreg differentiation. a, Sort-purified CD25− CD62L− CD44lo GFP− naive CD4+ T cells from Foxp3gfp reporter mice were stimulated with anti-CD3/anti-CD28 in the presence of indicated cytokines. mRNA expression of indicated genes was measured at 24 h, 48 h and 72 h and presented as fold change over time 0. Data are representative of two independent experiments and show the mean ± s.d.
Extended Data Figure 2 | IL-33 directly regulates ST2 expression. Sort-purified CD25−CD62L−CD44lo GFP− naive CD4+ T cells from Foxp3gfp reporter mice were cultured with anti-CD3/anti-CD28 in the presence of TGF-β1 followed by acute stimulation with IL-33 for 45 min. Shown are the recruitment of GATA3 or RNA Pol II to the indicated regions of the gene encoding ST2 assessed by ChIP followed by qPCR. Results are normalized to those obtained with genomic DNA (input) and presented as fold enrichment relative to unstimulated cells. Data are from one experiment representative of two (error bars show s.d.).
Extended Data Figure 3 | IL-33 acts directly on thymus-derived Treg cells to promote their proliferation and accumulation in vivo. a–e, Foxp3 GFP reporter mice were injected intraperitoneally (i.p.) with recombinant IL-33 (1 μg per mouse per day) for 5 days. a, Frequencies of TCR-β+ CD4+ Foxp3+ splenic thymus-derived Treg cells. b, Absolute numbers of TCR-β+ CD4+ Foxp3+ splenic Treg cells. c, gMFI of Foxp3 among TCRβ+ CD4+ Foxp3+ splenic Treg cells (n = 10 for PBS and n = 14 for IL-33). d, Frequencies of ST2+ cells among splenic CD44hi T cells or Foxp3+ Treg cells. e, Frequencies of Ki67+ cells among splenic CD44hi T cells or Foxp3+ Treg cells (n = 5 for PBS and n = 8 for IL-33). f, Mixed bone marrow chimaeras were generated by irradiation of C57BL/6 Rag1-/- mice followed by intravenous (i.v.) injection of 2.5 × 10⁶ wild-type (WT; CD45.1+) and 2.5 × 10⁶ St2-/- (CD45.1-) bone marrow cells (n = 5). After reconstitution, mixed chimaeras were injected with recombinant IL-33 (1 μg per mouse per day) for 5 days and the absolute number of Ki67+ among TCR-β+ CD4+ Foxp3+ or TCRβ+ CD4+ Foxp3+ cells are shown. **P < 0.01, ***P < 0.001 as calculated by unpaired (wild-type mice) or paired (chimaeric mice), Student’s t-test.
Extended Data Figure 4 | Kinetics of IL-33 and IL-23 during H. hepaticus and anti-IL-10R colitis. a–f, C57Bl/6 mice were infected by oral gavage with $10^8$ colony-forming units (c.f.u.) H. hepaticus on 3 consecutive days with concomitant i.p. injections of a blocking anti-IL-10R monoclonal antibody (1 mg per week) starting on the day of the first infection. a, b, Colitis scores for the indicated groups H. hepaticus (Hh), anti-IL-10R (aIL-10R) or H. hepaticus and anti-IL-10R (Hh/aIL-10R) (a) and IL-33, IL-23 and soluble ST2 protein production (b) in colon explant cultures from H. hepaticus and anti-IL-10R treated mice were determined at the indicated time points (n = 4, data show the mean ± s.e.m.). c, Colonic intestinal epithelial cells (IECs) were isolated from steady state (n = 5) or colitic (n = 3) (day 10) mice. Total protein extracts from each time point were analysed by immunoblot and biological replicates are shown. d, Densitometric quantification of immunoblot in c (mean ± s.e.m.). e, mRNA expression analysis of IECs, lamina propria cells (LP) and stromal cells isolated from the colon at steady state or 10 days after induction of colitis (steady state, n = 5; colitic, n = 4; mean ± s.e.m.). f, Colonic stromal cells were isolated at the indicated time points, cultured and passaged and spontaneous soluble (s)ST2 protein production determined in supernatants after 48 h cultures. Stromal cells were uniformly CD45$^-$ EpCAM$^-$ . Data represent mean ± s.e.m. (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 as calculated by one-way ANOVA with Bonferroni post-test or Student’s t-test.
Extended Data Figure 5 | The in vitro suppressive function of St2−/− Treg cells is not impaired. CD25+CD4+ Treg cells were sorted by flow cytometry from wild-type (WT) and St2−/− mice. CD25+CD62L+CD44−CD4+ T cells (responder cells) and antigen presenting cells (APCs) were sorted from St2−/− mice. T responder cells were labelled with Violet cell trace and plated together with the sorted Treg cells at ratios of 1:1 and 1:3 together with irradiated APCs in the presence of anti-CD3 (1 μg ml−1). IL-33 was added at 30 ng ml−1. After 4 days, proliferation of T responder cells (Tres) was measured by flow cytometry. Data are representative of two independent experiments.
Extended Data Figure 6 | ST2 is preferentially expressed on Treg-cell progeny. a, C57BL/6 Rag1<sup>−/−</sup> mice were injected i.p. with 4 × 10<sup>5</sup> CD4<sup>+</sup> CD25<sup>−</sup> CD45 RB<sup>hi</sup>CD45.1<sup>+</sup> naive T cells in combination with 2 × 10<sup>5</sup> wild-type (WT) CD4<sup>+</sup> CD25<sup>−</sup>CD45.1<sup>2</sup>Treg cells. Mice were killed at 6–8 weeks after transfer. Representative plots of the colon gated on progeny of CD4<sup>+</sup> CD45 RB<sup>hi</sup> cells (CD45.1<sup>+</sup>) or wild-type Treg (CD45.1<sup>2</sup>) cells are shown.

b, Histogram overlay of Foxp3 expression by wild-type (solid line) or ST2<sup>−/−</sup> (dotted line) CD4<sup>+</sup> CD25<sup>−</sup>T<sub>reg</sub> cells before injection into C57BL/6 Rag1<sup>−/−</sup> mice. c, C57BL/6 Rag1<sup>−/−</sup> mice were injected as in a and frequencies of cytokine-producing colonic T<sub>reg</sub> progeny (CD45.1<sup>2</sup>) among Foxp3<sup>+</sup> or Foxp3<sup>−</sup> cells are shown. Wild type (n = 4) and ST2<sup>−/−</sup> (n = 6); error bars represent mean ± s.e.m.
Extended Data Figure 7 | St2 is an IL-23 target gene in intestinal CD4⁺ T cells. C57BL/6 Rag1⁻/⁻ mice were injected i.p. with a 1:1 mixture of 2 × 10⁵ wild-type (WT; CD45.1⁺) and 2 × 10⁵ Il23r⁻/⁻ (CD45.2⁻) CD4⁺ CD25⁻ CD45RBhi T cells or 2 × 10⁵ wild-type (CD45.1⁺) and 2 × 10⁵ wild-type (CD45.2⁻) CD4⁺ CD25⁺ CD45 RBhi T cells. Mice were killed upon development of clinical signs of inflammation (~8 weeks). Viable TCR-β⁺ CD4⁺CD45.2⁺ wild-type or Il23r⁻/⁻ T cells were sort-purified from the colon and RNA extracted without further manipulation. Whole transcriptome gene expression analysis was performed using the Illumina Bead Array platform. Two-dimensional cluster analysis (hierarchical, Pearson uncentred, average linkage, unsupervised) of the 168 transcripts whose expression was significantly affected in the comparison of Il23r⁻/⁻ versus wild-type CD4⁺ T cells. Each row corresponds to a gene and each column to a sample (n = 6 for wild type and n = 5 for Il23r⁻/⁻). The 168 transcripts represent 147 unique genes, including genes with unknown function.
Extended Data Figure 8 | IL-23 inhibits IL-33-induced GATA3 recruitment to the St2 locus. Sort-purified CD25−CD62L+ CD44lo GFP− naive CD4+ T cells from Foxp3gfp reporter mice were cultured with anti-CD3/anti-CD28/TGF-β, +/− IL-23 for 48 h followed by acute stimulation with IL-33 for 45 min. Shown are the enrichment of GATA3 or RNA Pol II to the indicated regions of the gene encoding ST2 assessed by ChIP followed by qPCR. Results are normalized to those obtained with genomic DNA (input) and presented as fold recruitment relative to unstimulated cells. −, no IL-33 added. Data are from one experiment representative of two (error bars show s.d. of duplicate cultures).
Extended Data Figure 9 | IL-23 is a negative regulator of ST2 expression 
in vivo. Mixed bone marrow chimaeras were generated by irradiation of 
C57BL/6 Rag1−/− mice followed by i.v. injection of 2.5 × 10^6 wild-type 
(WT; CD45.1) and 2.5 × 10^6 Il23r−/− (CD45.2) bone marrow cells (n = 5). 
Reconstituted mice were infected by oral gavage with ~10^8 c.f.u. H. hepaticus 
on 3 consecutive days with concomitant i.p. injections of a blocking anti-IL-10R 
monoclonal antibody (1 mg per week) starting on the day of the first 
infection. Mice were killed at 2 weeks after infection. Frequencies of ST2+ and 
gMFI of ST2 among TCR-β+ CD4+ Foxp3+ colonic Treg cells are shown. 
*P < 0.05, ***P < 0.001 as calculated by paired Student’s t-test.
Extended Data Figure 10 | Il23r mRNA is expressed by ST2⁺ Foxp3⁺ T(reg) cells. mRNA expression of indicated genes in Foxp3⁻ ST2⁻, Foxp3⁺ ST2⁻ or Foxp3⁺ ST2⁺ populations sort-purified from the steady state colonic lamina propria (cLP) of Foxp3gfp reporter mice. AU, arbitrary units. Error bars represent the mean ± s.e.m. from three independent experiments.