LETTER

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

Chris Schiering[1]†*, Thomas Krausgruber[1]*, Agnieszka Chomka[1], Anja Fröhlich[2], Krista Adelmann[1], Elizabeth A. Wohlfert[3]†, Johanna Pott[4], Thibault Griseri[1], Julia Bollrath[1], Ahmed N. Hegazy[1], Oliver J. Harrison[4], Benjamin M. J. Owens[1], Max Löhning[2], Yasmine Belkaid[3], Padraic G. Fallon[5] & Fiona Powrie[1]

FOXP3$^+$ regulatory T cells (T_{reg} cells) are abundant in the intestine, where they prevent dysregulated inflammatory responses to self and environmental stimuli. It is now appreciated that T_{reg} cells acquire tissue-specific adaptations that facilitate their survival and function[1]; however, key host factors controlling the T_{reg} response in the intestine are poorly understood. The interleukin (IL)-1 family member IL-33 is constitutively expressed in epithelial cells at barrier sites[2], where it functions as an endogenous danger signal, or alarmin, in response to tissue damage[3]. Recent studies in humans have described high levels of IL-33 in inflamed lesions of inflammatory bowel disease patients[4–7], suggesting a role for this cytokine in disease pathogenesis. In the intestine, both protective and pathological roles for IL-33 have been described in murine models of acute colitis[8–11], 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 T_{reg} cells, where it promotes T_{reg} function and adaptation to the inflammatory environment. IL-33 signalling in T cells stimulates T_{reg} responses in several ways. First, it enhances transforming growth factor (TGF)-β_1-mediated differentiation of T_{reg} cells and, second, it provides a necessary signal for T_{reg}-cell accumulation and maintenance in inflamed tissues. Strikingly, IL-23, a key pro-inflammatory cytokine in the pathogenesis of inflammatory bowel disease, restrained T_{reg} 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-33 and IL-23 may be a key controller of intestinal immune responses.

To identify potential tissue-specific modulators of colonic T_{reg} cells, we compared the messenger RNA expression profiles of mesenteric lymph node and colonic T_{reg} cells. We identified *St2* (also known as *Il1rl1*), the transcript coding for the IL-33 receptor[12], as one of the top differentially upregulated genes in colonic T_{reg} cells (Fig. 1a, b). Flow-cytometric analysis confirmed selective enrichment of ST2$^+$ T_{reg} 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$^+$ T_{reg} cells are a heterogeneous population containing thymus-derived T_{reg} cells as well as peripherally generated Helios$^-$ T_{reg} cells (Fig. 1e)[13]. A significant proportion of intestinal Foxp3$^+$ T_{reg} cells co-express the transcription factor GATA3 (refs 14–16), and GATA3 is known to regulate ST2 expression in T_H2 cells[17]. Indeed, ST2 expression was largely restricted to GATA3-expressing colonic T_{reg} cells (Fig. 1e) and selective ablation of GATA3 in Foxp3-expressing cells, using *Gata3$^{fl/fl}$-Foxp3-cre* mice[15], caused a marked reduction of ST2 protein levels (Fig. 1f).

Given that ST2$^+$ T_{reg} cells are prominent in the colon, we postulated that IL-33 may modulate *in vitro* induced (i)T_{reg}-cell differentiation. To test this, we sort-purified naive CD4$^+$ T cells from *Foxp3^{gfp}* reporter mice and activated them in the presence of TGF-β_1. Notably, both *Gata3* and *St2* expression were induced under iT_{reg}-differentiation conditions (Extended Data Fig. 1). Addition of IL-33 to iT_{reg} 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-β_1 (Fig. 2a). The presence of IL-33 in iT_{reg} cultures did not affect induction of T_H2 cytokines or expression of T_H1- and T_H17-associated transcription factors *Tbx21* and *Rorc* (Extended Data Fig. 1), suggesting that IL-33 preferentially regulates Foxp3 expression. Thus, our data indicate

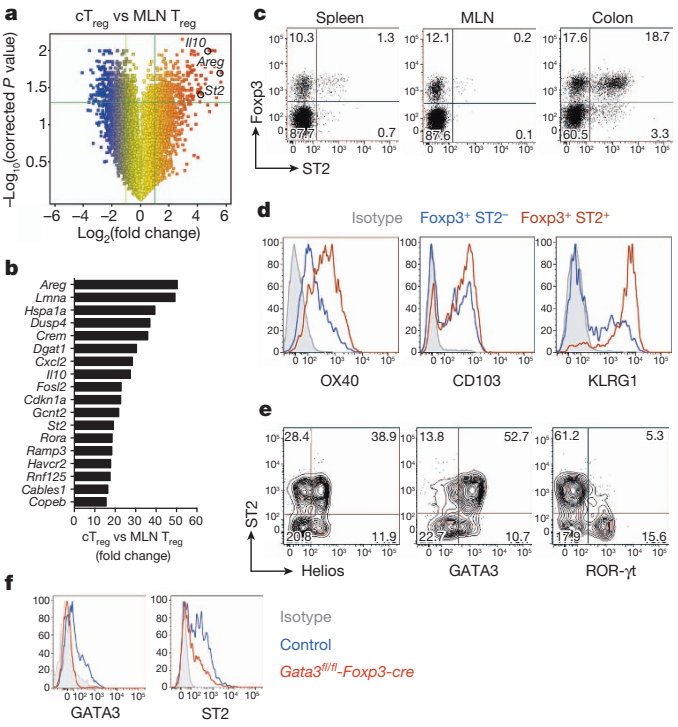

Figure 1 | ST2-expressing T_{reg} cells are enriched in the colon. **a**, Change in gene expression in colonic (c)T_{reg} cells versus mesenteric lymph node (MLN) T_{reg} cells ($n = 3$ per group) presented as volcano plot. **b**, Top differentially upregulated transcripts in colonic T_{reg} versus MLN T_{reg} cells. **c**, ST2 protein expression on T_{reg} cells from indicated organs. **d**, Phenotypic analysis of ST2$^-$ or ST2$^+$ colonic T_{reg} cells. **e**, Expression of transcription factors in colonic T_{reg} cells. **f**, Representative histograms gated on colonic T_{reg} cells from control or *Gata3$^{fl/fl}$-Foxp3-cre* mice.

[1]Translational Gastroenterology Unit, Nuffield Department of Clinical Medicine, Experimental Medicine Division, John Radcliffe Hospital, University of Oxford, Oxford OX3 9DU, UK. [2]Experimental Immunology, Department of Rheumatology and Clinical Immunology, Charité — University Medicine Berlin, and German Rheumatism Research Center (DRFZ), D-10117 Berlin, Germany. [3]Program in Barrier Immunity and Repair, Mucosal Immunology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda, Maryland 20892, USA. [4]Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK. [5]Trinity Biomedical Sciences Institute, Trinity College Dublin, Pearse Street, Dublin 2, Ireland. †Present addresses: Division of Molecular Immunology, MRC National Institute for Medical Research, Mill Hill, London NW7 1AA, UK (C.S.); Department of Microbiology and Immunology, School of Medicine and Biomedical Sciences, University at Buffalo (SUNY), Buffalo, New York 14214-3000, USA (E.A.W.).
*These authors contributed equally to this work.

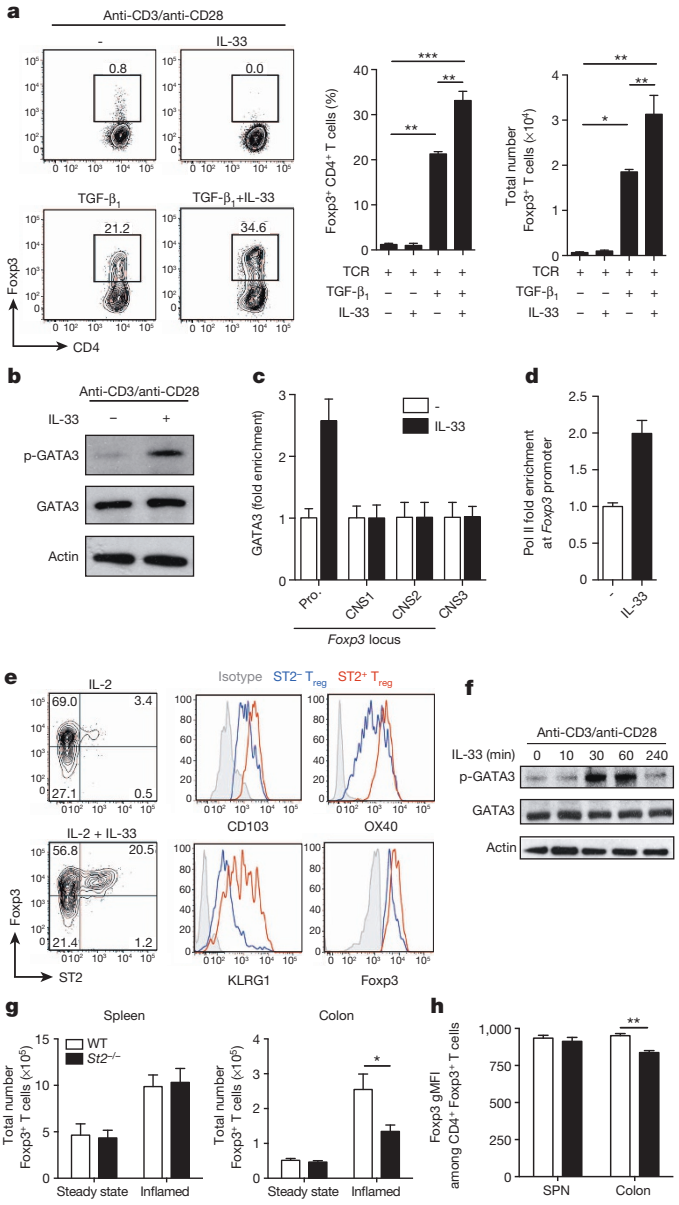

Figure 2 | Effects of IL-33 on iT$_{reg}$ and thymus-derived T$_{reg}$ cells. **a**, Naive CD4$^+$ T cells were cultured with anti-CD3/CD28 plus the indicated cytokines and the frequencies and absolute numbers of Foxp3$^+$ T cells were determined 3 days later (mean ± standard error of the mean (s.e.m.) of three independent experiments). **b**, Naive CD4$^+$ T cells were cultured for 48 h with anti-CD3/CD28 plus TGF-β_1, followed by stimulation with IL-33 for 45 min. Blots are representative of two independent experiments. p, phosphorylated. **c**, **d**, Cells were cultured and stimulated as in **b** and recruitment of GATA3 or RNA Pol II to the indicated regions was assessed by ChIP-qPCR. Data are from one experiment representative of two (mean ± standard deviation (s.d.)). Pro., promoter. −, no IL-33 added. **e**, Representative plots of T$_{reg}$ cells cultured with anti-CD3/CD28 plus indicated cytokines and analysed after 3 days. Data are representative of three independent experiments. **f**, T$_{reg}$ cells were cultured with anti-CD3/CD28 for 24 h followed by stimulation with IL-33. Blots are representative of three independent experiments. **g**, Mixed chimaeras were generated containing wild-type (WT) and *St2*$^{-/-}$ bone marrow cells. Reconstituted mice were analysed at steady state or 2 weeks after infection with *H. hepaticus* and anti-IL-10R treatment (inflamed). Absolute numbers of wild-type or *St2*$^{-/-}$ T$_{reg}$ cells in steady state ($n = 3$) and inflamed ($n = 6$) hosts (mean ± s.e.m.). **h**, Analysis of Foxp3 expression in T$_{reg}$ cells in spleen (SPN) and colon from inflamed chimaeric hosts presented as geometric mean fluorescence intensity (gMFI). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ as calculated by one-way analysis of variance (ANOVA) with Bonferroni post-test or paired Student's *t*-test.

that the alarmin IL-33 is a novel cofactor in TGF-β_1-mediated iT$_{reg}$ generation.

GATA3 is highly expressed in ST2$^+$ T$_{reg}$ cells (Fig. 1e) and IL-33 has been shown to activate GATA3 in T$_H$2 cells[17,18] as well as in innate lymphoid cells[19]. Consistent with this notion, we observed serine phosphorylation of GATA3 upon acute stimulation of iT$_{reg}$ 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 iT$_{reg}$ cells, we performed chromatin immunoprecipitation (ChIP) followed by quantitative polymerase chain reaction (PCR). Acute stimulation of iT$_{reg}$ 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 T$_H$2 cells, GATA3 has been shown to promote *St2* gene expression by binding to an enhancer element located 12 kilobases upstream of the *St2* transcription start site[17]. Consistent with this, we detected recruitment of GATA3 to the *St2* enhancer upon acute stimulation of iT$_{reg}$ 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 iT$_{reg}$ cells through direct transcriptional regulation of the *St2* locus, providing an amplification loop for further enhancement of iT$_{reg}$-cell differentiation.

Next we focused on thymus-derived T$_{reg}$ cells, which constitute a significant proportion of ST2$^+$ colonic T$_{reg}$ cells (Fig. 1e). In line with published reports[20,21], administration of recombinant IL-33 led to a significant increase in the frequency and total number of splenic T$_{reg}$ cells (Extended Data Fig. 3a, b) and these IL-33-elicted T$_{reg}$ 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 T$_{reg}$ cells but not in T effector cells (Extended Data Fig. 3e). To examine whether IL-33 acts directly on T$_{reg}$ 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*$^{-/-}$ T$_{reg}$ cells was significantly impaired (Extended Data Fig. 3f), suggesting that IL-33 acts directly on thymus-derived T$_{reg}$ cells to promote their proliferation and accumulation *in vivo*. This is further supported by the finding that sort-purified splenic T$_{reg}$ 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 T$_{reg}$ cells with IL-33 induced serine phosphorylation of GATA3 (Fig. 2f), further demonstrating that IL-33 acts directly on thymus-derived T$_{reg}$ cells.

To assess the impact of IL-33 on the T$_{reg}$ response during intestinal inflammation, we induced chronic colitis by infection with *Helicobacter hepaticus* and administration of an IL-10R blocking antibody[22] (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 colonic 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 receptor[23] and is increased in patients with active inflammatory bowel disease (IBD)[6,24], 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*$^{-/-}$ T$_{reg}$ cells in the colon but not the spleen was significantly impaired during the peak of intestinal inflammation (Fig. 2g). In addition, colonic *St2*$^{-/-}$ T$_{reg}$ cells expressed lower amounts of Foxp3 protein on a per cell

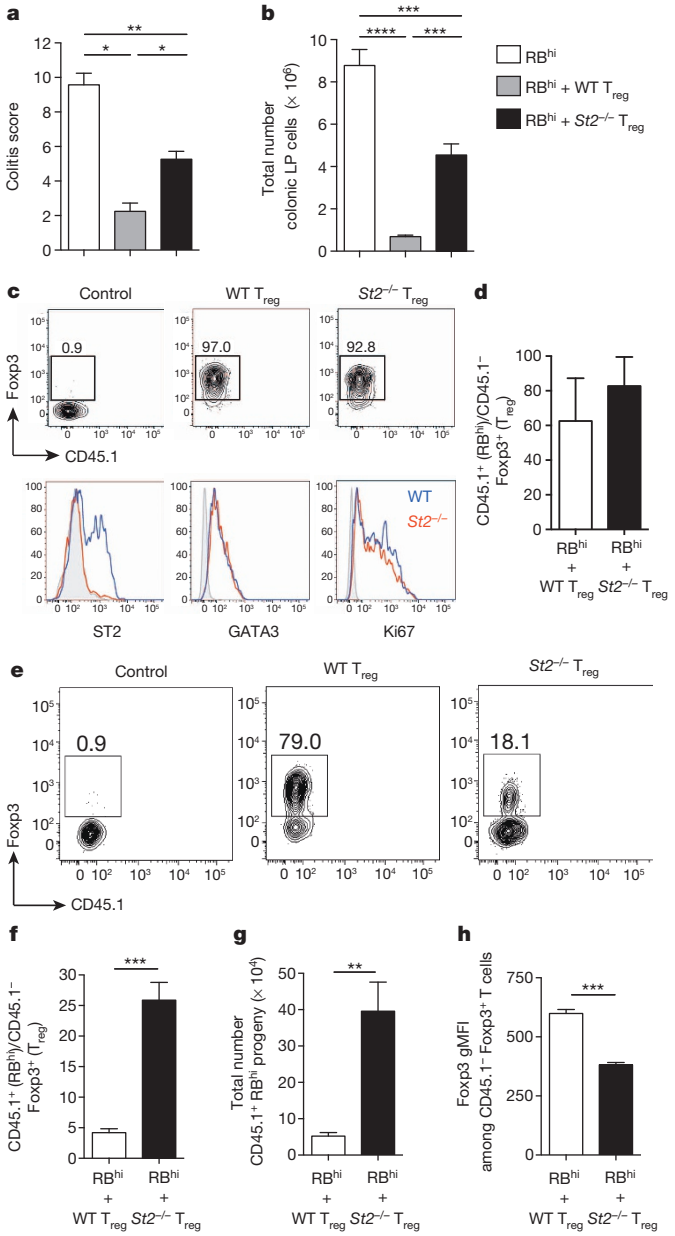

Figure 3 | IL-33 promotes T_{reg}-cell stability and function *in vivo*. **a**, C57BL/6 *Rag1*$^{-/-}$ mice were injected with CD45.1$^+$ naive T cells alone (RB^{hi}; $n = 4$) or in combination with wild-type (WT; $n = 4$) or *St2*$^{-/-}$ ($n = 6$) CD45.1$^-$ T_{reg} cells. Mice were killed 6–8 weeks 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 *Rag1*$^{-/-}$ mice were injected as in **a** and killed at 2 weeks post-injection. Representative plots are gated on colonic T_{reg}-cell progeny (CD45.1$^-$). **d**, Ratio of RB^{hi} T-cell progeny (CD45.1$^+$) to wild-type or *St2*$^{-/-}$ Foxp3$^+$ T_{reg}-cell progeny (CD45.1$^-$) in the colon ($n = 5$ per group) from mice in **c** (mean ± s.e.m.). **e**, C57BL/6 *Rag1*$^{-/-}$ mice were injected as in **a** and killed at 8 weeks post-injection. Representative plots are gated on colonic T_{reg}-cell progeny (CD45.1$^-$). **f**, Ratio of RB^{hi} T-cell progeny (CD45.1$^+$) to wild-type or *St2*$^{-/-}$ T_{reg}-cell progeny (CD45.1$^-$) in the colon from mice in **e** (mean ± s.e.m.). **g**, Absolute numbers of RB^{hi} T-cell progeny (CD45.1$^+$) in the colon from mice in **e** (mean ± s.e.m.). **h**, Analysis of Foxp3 expression in colonic Foxp3$^+$ CD45.1$^-$ T_{reg} cells presented as gMFI (mean ± s.e.m.). Results are representative of two independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ as calculated by one-way ANOVA with Bonferroni post-test or Student's *t*-test.

basis as compared to their wild-type counterparts (Fig. 2h). Together, these observations indicate that the alarmin IL-33 acts in a cell-intrinsic manner to promote the tissue-specific accumulation and stability of the T_{reg} phenotype in the intestine under inflammatory conditions. Furthermore, high levels of soluble ST2 during chronic intestinal inflammation may represent a mechanism to further perpetuate pathogenic responses by limiting IL-33-driven T_{reg} accumulation.

We next sought to compare the suppressive capacity of wild-type and *St2*$^{-/-}$ T_{reg} cells. *St2*$^{-/-}$ T_{reg} cells inhibited T-cell proliferation to the same extent as wild-type T_{reg} cells *in vitro* (Extended Data Fig. 5) and addition of IL-33 did not enhance wild-type T_{reg} suppressor function. We then tested the ability of *St2*$^{-/-}$ T_{reg} cells to protect from colitis induced by adoptive transfer of naive CD4$^+$ T cells. Interestingly, ST2 was highly expressed on wild-type T_{reg} cells upon T-cell transfer, pointing towards a potential role of ST2 in modulating T_{reg} function in this model (Extended Data Fig. 6a). Indeed, *St2*$^{-/-}$ T_{reg} cells were significantly impaired in their ability to prevent colonic inflammation and cellular infiltration (Fig. 3a, b), demonstrating that IL-33 signalling in T_{reg} cells is important for their suppressive function *in vivo*. Analysis of wild-type or *St2*$^{-/-}$ T_{reg} 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$^+$ RB^{hi} progeny) to T_{reg} cells (CD45.1$^-$ Foxp3$^+$ T_{reg} progeny) was also similar (Fig. 3d), suggesting that IL-33 signalling in T_{reg} 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*$^{-/-}$ T_{reg} cells contained a significantly lower proportion of Foxp3$^+$ 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_{reg} cells and the total number of T effector cells (CD45.1$^+$ RB^{hi} progeny) was markedly increased in recipients of *St2*$^{-/-}$ T_{reg} cells (Fig. 3f, g). Importantly, ST2-deficient T_{reg} 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_{reg}-cell function[25–27] and our data indicate that IL-33 signalling in T_{reg} cells contributes to the maintenance of Foxp3 expression under inflammatory stress, enabling T_{reg} cells to compete in the inflammatory niche and to control the intestinal effector T-cell response.

Next we sought to integrate our observations with existing pro-inflammatory pathways. IL-23 is a pivotal mediator of intestinal inflammation and polymorphisms in the *IL23R* locus are associated with increased susceptibility to IBD in humans[28]. We previously showed that IL-23 promotes intestinal inflammation in part through inhibition of iT_{reg}-cell differentiation[29,30]. However, the mechanism by which IL-23 blocked iT_{reg} generation remained undefined. Interestingly, whole transcriptome analysis of IL-23 target genes in colonic effector CD4$^+$ T cells revealed that IL-23 inhibits expression of *Gata3* and *St2* (Extended Data Fig. 7). On the basis of this observation we hypothesized that IL-23 might limit T-cell responsiveness to IL-33. Indeed, the cofactor activity of IL-33 on TGF-β_1-mediated Foxp3 induction *in vitro* was completely abrogated in the presence of IL-23 (Fig. 4a). Notably, addition of IL-23 prevented induction of *Gata3* and *St2* mRNA under iT_{reg} differentiation conditions (Fig. 4b), resulting in reduced ST2 protein expression (Fig. 4c). Consequently, acute stimulation of IL-23-exposed iT_{reg} cells with IL-33 did not lead to recruitment of GATA3 to the ST2 enhancer (Extended Data Fig. 8). We observed a similar role for IL-23 in limiting T_{reg} ST2 expression during bacterially driven intestinal inflammation (Extended Data Fig. 9). Collectively, our data indicate that IL-23 inhibits iT_{reg} differentiation by regulating T-cell responsiveness to IL-33.

We previously showed that IL-23 restrains T_{reg} cells *in vivo* because naive T-cell transfer into *Il23a*$^{-/-}$ *Rag1*$^{-/-}$ recipients resulted in increased T_{reg}-cell differentiation[29]. Therefore, we hypothesized that enhanced responsiveness to IL-33 may contribute to increased iT_{reg} differentiation in *Il23a*$^{-/-}$ *Rag1*$^{-/-}$ hosts. To test this, we transferred wild-type or *St2*$^{-/-}$ naive T cells into *Il23a*$^{-/-}$ *Rag1*$^{-/-}$ hosts and monitored iT_{reg}-cell generation. Indeed, ST2-deficient T cells were significantly impaired in their ability to differentiate into T_{reg} cells (Fig. 4d) and this correlated with a significant increase in intestinal pathology (Fig. 4e). Importantly,

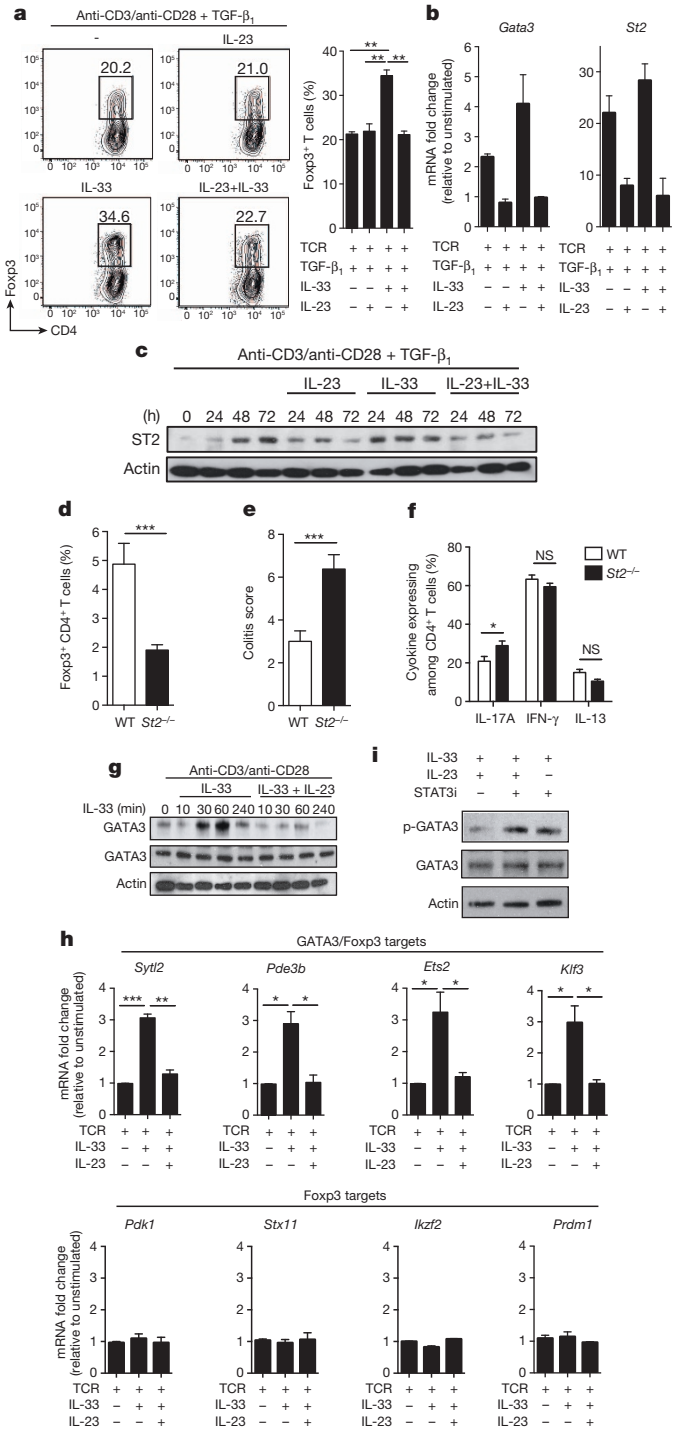

Figure 4 | IL-23 inhibits the effects of IL-33 on T_{reg} cells. **a**, Naive $CD4^+$ T cells were cultured with anti-CD3/CD28 plus TGF-β_1, as well as the indicated cytokines, and the frequencies of $Foxp3^+$ T cells were determined 3 days later (mean ± s.e.m. of three independent experiments). **b**, 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 as indicated. Representative blots of two independent experiments are shown. **d**, C57BL/6 *Il23a*$^{-/-}$ *Rag1*$^{-/-}$ mice were injected with $CD45RB^{hi}$ wild-type (WT; $n = 9$) or *St2*$^{-/-}$ ($n = 10$) T cells. Mice were killed 6–8 weeks after transfer and frequencies of $Foxp3^+$ $CD4^+$ T cells in colon are shown (mean ± s.e.m.). **e**, Colitis scores for mice in **d** (mean ± s.e.m.). **f**, Expression of the indicated cytokines by colonic $CD4^+$ T cells from mice in **d** (mean ± s.e.m.). **g**, T_{reg} 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**, T_{reg} 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 for 45 min in the presence or absence of IL-23 (mean ± s.e.m. of three independent experiments). **i**, T_{reg} 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.

ST2 deficiency had minor effects on *in vivo* differentiation of T_H1, T_H2 or T_H17 cells (Fig. 4f), further supporting the notion that the increased colitogenic potential of *St2*$^{-/-}$ $CD4^+$ T cells is a consequence of deficient iT_{reg} differentiation rather than dysregulated effector T-cell responses. Our data strongly suggest that IL-33 is a major factor responsible for driving iT_{reg} differentiation in the absence of IL-23.

Finally, we investigated whether IL-23 can interfere with ST2 signalling in T_{reg} cells. Interestingly, sort-purified $ST2^+$ T_{reg} cells from the colon expressed detectable levels of *Il23r* (Extended Data Fig. 10). Indeed, exposure of TCR-activated thymus-derived T_{reg} cells to IL-23 completely abolished IL-33-mediated GATA3 phosphorylation (Fig. 4g). Furthermore, IL-33 preferentially induced genes co-regulated by Foxp3 and GATA3 (ref. 16), and this was completely abrogated in the presence of IL-23 (Fig. 4h). Addition of a specific inhibitor of STAT3, the main transcription factor downstream of IL-23 signalling, reversed this inhibitory effect of IL-23 (Fig. 4i). Together our data suggest that IL-23 inhibits ST2 signal transduction and expression of a distinct set of GATA3-regulated genes in thymus-derived T_{reg} cells.

Our results identify a new function for IL-33 as an important link between inflammation-driven tissue damage and the local intestinal T_{reg}-cell response. We show that colonic T_{reg} cells are poised to respond to the release of IL-33 upon tissue damage through selective expression of ST2 and that signalling through this pathway has an essential role in their capacity to adapt to the inflammatory tissue environment and restrain intestinal inflammation. The ability of IL-33 to amplify regulatory networks in response to tissue injury may represent a more general mechanism by which alarmins limit immune-mediated damage to self at barrier tissues. Strikingly, IL-23 limits this regulatory mechanism through inhibition of T_{reg}-cell responsiveness to IL-33, suggesting that the balance between IL-23 and IL-33 may be a major determinant of the outcome of intestinal immune responses.

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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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-*Cd45.1*, C57BL/6 *Il23r*$^{-/-}$, C57BL/6 *Rag1*$^{-/-}$, C57BL/6 *Il23a*$^{-/-}$ *Rag1*$^{-/-}$ and *Foxp3*^{gfp} reporter mice[31,32] were bred and maintained under specific pathogen-free conditions in accredited animal facilities at the University of Oxford. Where indicated, mice were intraperitoneally (i.p.) injected with recombinant IL-33 (1 μg per injection; Biolegend) for 5 consecutive days and killed 24 h after the last injection. *Gata3*$^{fl/fl}$-*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. Loehning. 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. Wherever 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 (CD45.2^{+}), *Il23r*$^{-/-}$ or *St2*$^{-/-}$ mice was mixed at a 1:1 ratio with bone marrow taken from B6.SJL-*Cd45.1* mice and injected intravenously into gamma-irradiated (5.5 Gy, 550 rad) C57BL/6 *Rag1*$^{-/-}$ recipients and chimaeras were used in experiments >8 weeks after injection.

T-cell transfer colitis. For naive T-cell transfer colitis, 4×10^5 CD4^{+} CD25^{-} CD45 RB^{hi} T cells were injected i.p. into *Rag1*$^{-/-}$ or *Il23a*$^{-/-}$ *Rag1*$^{-/-}$ recipients. In co-transfer experiments, 2×10^5 CD4^{+} CD25^{-} CD45 RB^{hi} T cells from each source were mixed and injected i.p. into *Rag1*$^{-/-}$ hosts. For T$_{reg}$-mediated protection from colitis, 4×10^5 CD4^{+} CD25^{-} CD45 RB^{hi} T cells and 2×10^5 CD4^{+} CD25^{+} T$_{reg}$ cells were mixed and injected i.p. into *Rag1*$^{-/-}$ hosts[33]. 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 1×10^8 colony-forming units (c.f.u.) *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-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[29].

Isolation of leukocyte subpopulations and flow cytometry. Cell suspensions from spleen, MLN, and the lamina propria were prepared as described previously[34] and first incubated with anti-CD16/CD32 (eBioscience) to prevent nonspecific binding. Single-cell suspensions were stained with antibodies against CD4, CD25, TCR-β, CD45.1, CD45.2, CD103, OX40, IL-17A, IFN-γ, IL-13, Foxp3, GATA3, Helios (all from eBioscience), ROR-γt, Ki67 (BD Biosciences), CD45RB, KLRG1 (Biolegend) and anti-ST2 conjugated to biotin (mdBioproducts). Intracellular staining was performed as follows. Cells were restimulated for 4 h as previously described[35], washed and incubated with anti-CD16/CD32. Cells were washed and stained for surface markers as indicated earlier and fixed in eBioscience Fix/Perm buffer, followed by permeabilization in eBioscience permeabilization buffer for 1 h in the presence of antibodies. Cells were acquired with a BD LSRII and analysis was performed with FlowJo (Tree Star) software.

T$_{reg}$-cell cultures. CD25^{-} CD62L^{+} CD44^{lo} GFP^{-} naive CD4^{+} T cells were sort-purified from *Foxp3*^{gfp} reporter mice. Cells were plated at 2×10^5 cells per well in flat-bottomed 96-well plates coated with anti-CD3 (5 μg ml^{-1}) in RPMI (Invitrogen) containing 10% FCS, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, and 100 U ml^{-1} each of penicillin and streptomycin (complete media). Antibodies and cytokines were added at the following concentrations: anti-CD28 (2 μg ml^{-1}), TGF-β$_1$ (R&D; 500 pg ml^{-1}), IL-2 (Peprotech; 1 U ml^{-1}), IL-23 (R&D; 20 ng ml^{-1}), IL-33 (Biolegend; 1 ng ml^{-1}). Cells were harvested at indicated times, lysed for use for qPCR, western blot or analysed by flow cytometry. GFP^{+} CD4^{+} T$_{reg}$ cells were sort-purified from *Foxp3*^{gfp} reporter mice and cells were plated at 2×10^5 cells per well in flat-bottomed 96-well plates coated with anti-CD3 (5 μg ml^{-1}) in complete media and anti-CD28 (2 μg ml^{-1}), IL-2 (Peprotech; 1 U ml^{-1}) or IL-33 (Biolegend; 1 ng ml^{-1}) were added at the start of the culture. Cells were analysed after 3 days by flow cytometry. For acute stimulation, 2×10^5 cells per well were plated in 96-well plates coated with anti-CD3 (5 μg ml^{-1}) in complete media and soluble anti-CD28 (2 μg ml^{-1}) and cultured for 24 h. Cells were then stimulated with IL-33 (10 ng ml^{-1}) for the indicated time and used for qPCR or western blot.

T$_{reg}$-cell suppression assay. CD25^{+} CD4^{+} T$_{reg}$ cells were sorted by flow cytometry from wild-type and *St2*$^{-/-}$ mice. CD25^{-} CD62L^{+} CD44^{-} CD4^{+} T cells (responder cells) were sorted from *St2*$^{-/-}$ mice. Antigen presenting cells (APCs) were isolated by magnetic bead separation from splenocytes and lymph node cells from *St2*$^{-/-}$ mice. T responder cells were labelled with Violet cell trace and plated together with the sorted T$_{reg}$ 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 was measured by flow cytometry.

Colon explant cultures. Organ explants were prepared as previously described[35] and cultured overnight in complete media. Cytokine levels in the supernatants were determined by enzyme-linked immunosorbent assay (ELISA) IL-23 and IL-33 (eBioscience) and soluble ST2 (R&D), and concentrations were normalized to the weight of the explants.

Isolation and culture of colonic stromal cells. Colonic tissue was digested and cells from the 30:40% Percoll gradient interface were harvested and washed twice in PBS containing 2% BSA. Cells were plated at 10×10^6 cells ml^{-1} in complete DMEM containing 40 μg ml^{-1} gentamycin (Sigma). After 24–48 h, non-adherent cells were removed by vigorous washing, and media replaced. Adherent intestinal stromal cells were cultured for 10 days until confluent. Cells were then plated at 1×10^6 cells ml^{-1} for 48 h and the concentration of soluble ST2 in supernatants was determined by ELISA (R&D Systems).

qPCR and microarray preparation. RNA was extracted according to the manufacturers' protocol (RNeasy, Qiagen) and complementary DNA synthesis was performed using Superscript III reverse transcription and Oligo dT primers (both from Invitrogen). qPCR reactions were performed using TaqMan Gene Expression Assays and normalized to HPRT (all from Applied Biosystems). Alternatively, qPCR reactions were performed using SYBR green PCR SensiMix (Quantace) with previously described primers[16]. For soluble ST2 the following primers were used: soluble ST2 forward 5′-TCGAAATGAAAGTTCCAGCA-3′ and soluble ST2 reverse 5′-TGTGTGAGGGACACTCCTTAC-3′. Samples were assayed in duplicate on a Bio-Rad CFX96 RT–qPCR machine and differences were calculated using the $2^{\Delta Ct}$ method.

For microarray gene expression analysis, RNA was extracted using the RNAqueous-Micro Total RNA Isolation Kit or RiboPure RNA Purification Kit (both Ambion). For RNA amplification and labelling the TargetAmp2-Round Biotin-aRNA Amplification Kit 3.0 (Epicentre, Illumina) or the Illumina TotalPrep RNA Amplification Kit (Ambion) were used and RNA quality was assessed using the Agilent 2100 Bioanalyzer and only samples with RNA integrity number (RIN) values above 7 were used for further processing. Biotinylated cRNA was hybridized to MouseWG-6 v.2.0 Expression BeadChip (Illumina). Hybridizations to BeadChips and data acquisition were performed by the microarray core facility at the Wellcome Trust Center for Human Genetics, Oxford. 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 cut-off was set to 0.05.

Total protein extracts and immunoblot analysis. Total protein extracts were prepared as described[36]. Equal amounts of protein were resolved by SDS–PAGE and analysed with anti-pGATA3 (ab61052; Abcam), anti-total GATA3 (L50-823; BD Pharmingen), anti-ST2 (101001B; mdBioproducts), anti-actin (A5541; Sigma) or anti-IL-33 (396118, R&D). For inhibition of STAT3 activity *in vitro*, STAT3 VI (10 mM; Santa Cruz) was added 30 min before cytokine stimulation.

ChIP. ChIP assays were performed as described[36] with antibody to GATA3 (L50-823; BD Pharmingen) or RNA polymerase II (sc-899; Santa Cruz). The immunoprecipitated DNA fragments were then analysed by real-time PCR with SYBR Premix Ex TaqII master mix (Takara Bio) and the following primers: locus encoding *St2* enhancer, 5′-GCCAACCACAACAGCAGATGGGGAAA-3′ and 5′-ACTGAGATCCTG CCTGGCTTCCCT-3′; locus encoding *St2* promoter, 5′-TGGCCTCCTTGGAAAG GCTTGGT-3′ and 5′-AGTGCAGGAGGGGCATGGAGATGA-3′. Primers for analysing binding to CNS1, CNS2, CNS3 and the promoter locus of *Foxp3* were as described previously[25]. Data were analysed using Vii7A qPCR software (Applied Biosystems). Results are normalized to input DNA and presented as fold enrichment relative to unstimulated cells.

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 $P \leq 0.05$.

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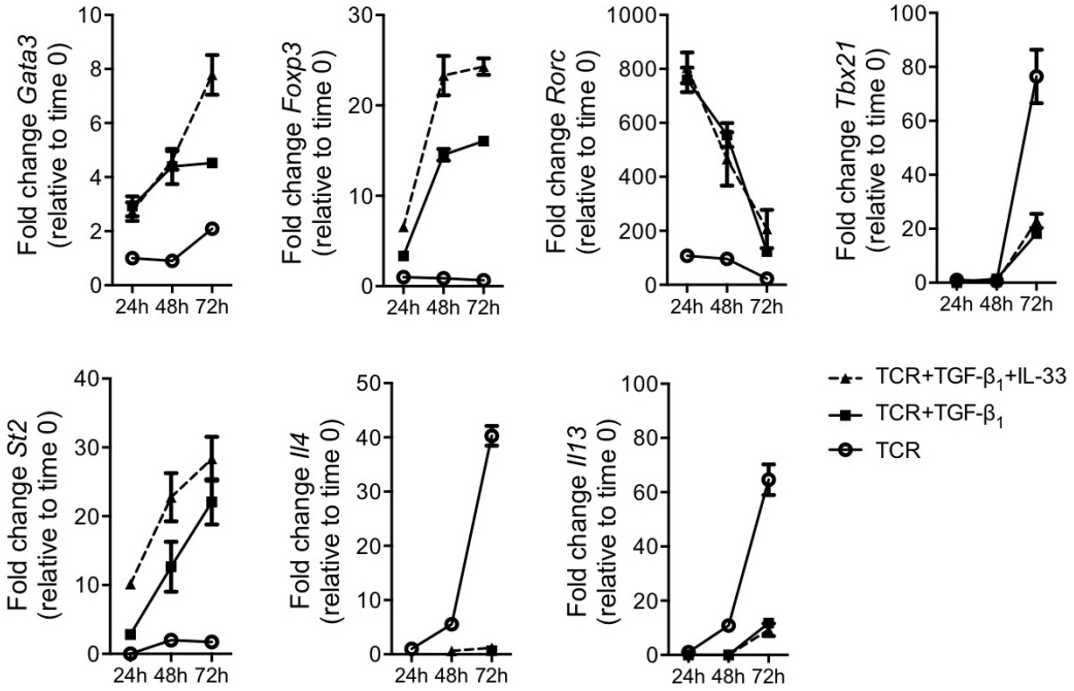
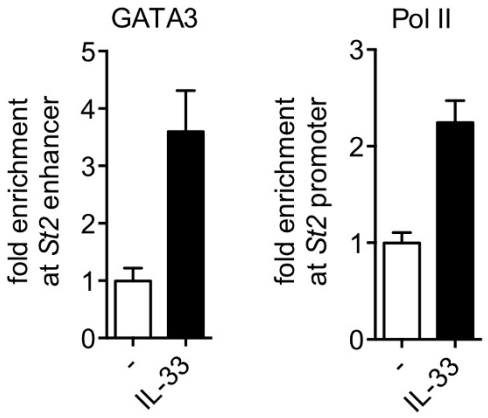
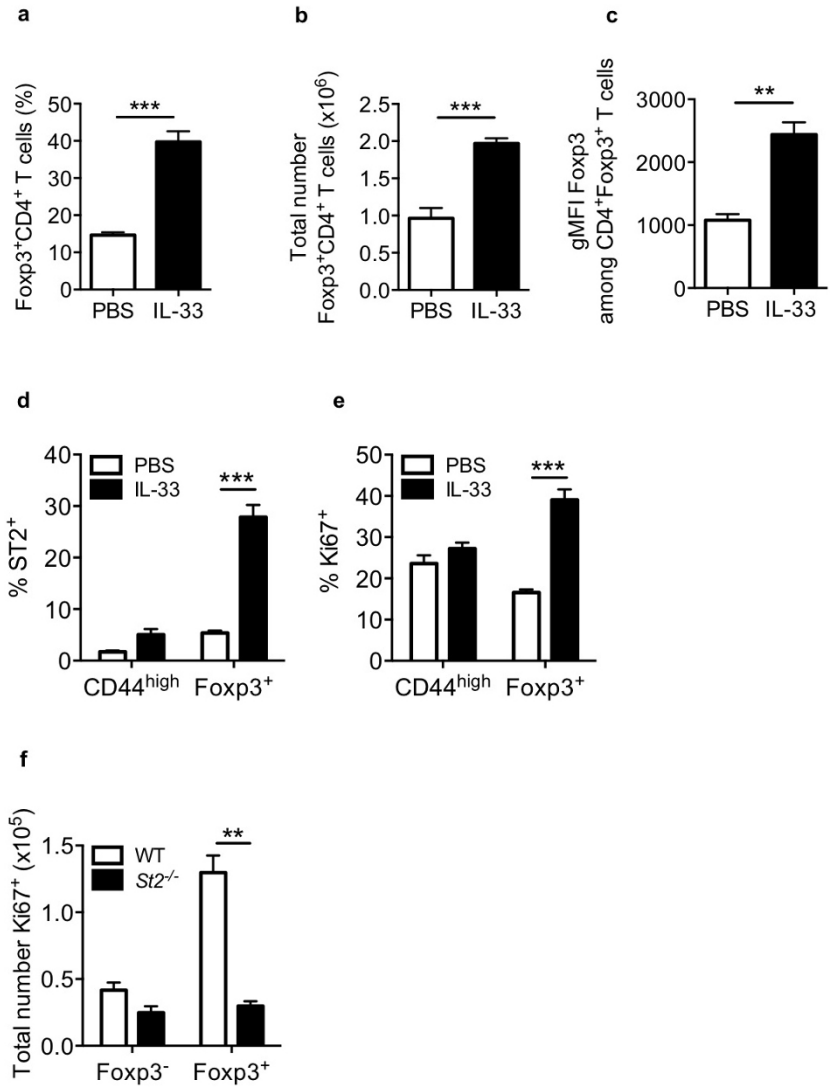


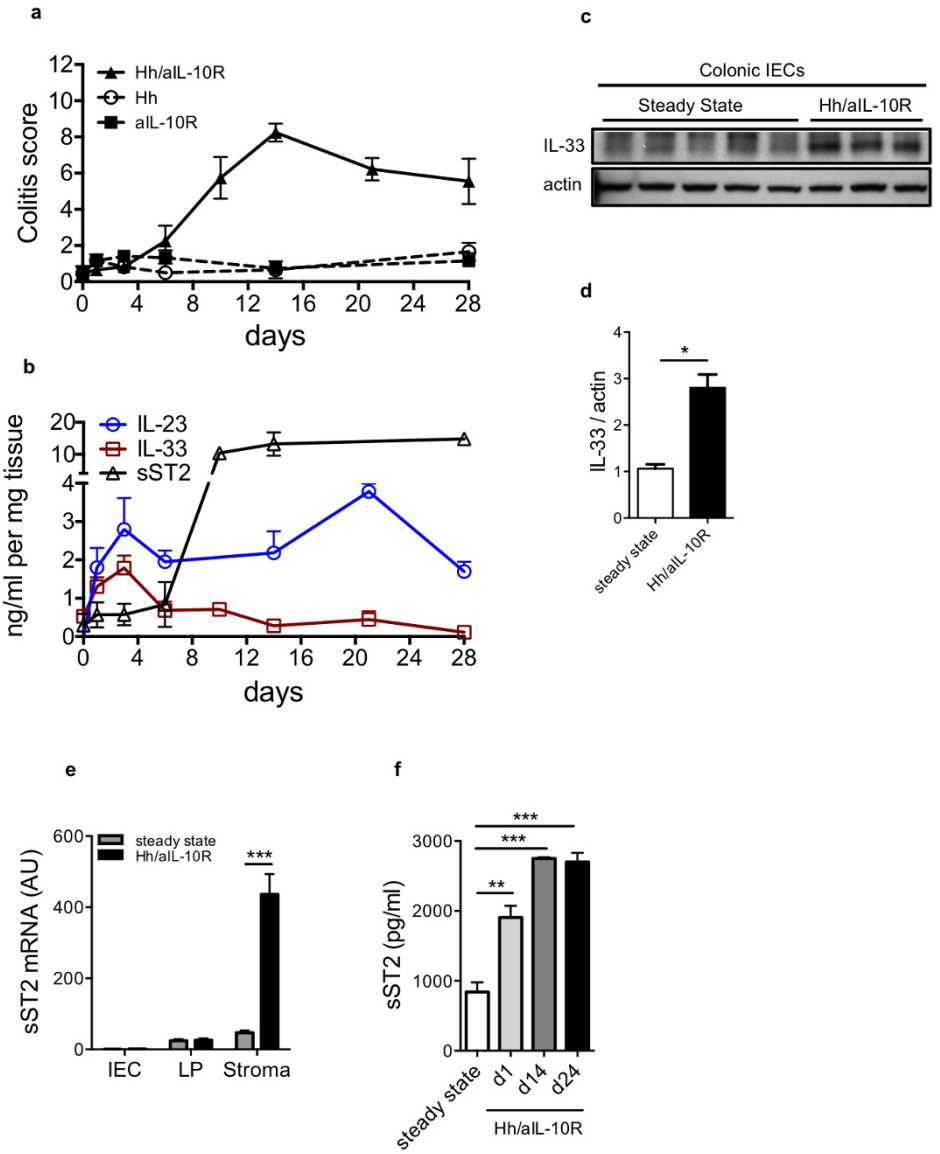
Extended Data Figure 1 | Effects of IL-33 on gene expression during iT_{reg} differentiation. **a**, Sort-purified $CD25^{-}$ $CD62L^{+}$ $CD44^{lo}$ GFP^{-} naive $CD4^{+}$ T cells from *Foxp3*^{gfp} reporter mice were stimulated with anti-CD3/anti-CD28 in the presence of indicated cytokines. mRNA expression 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.



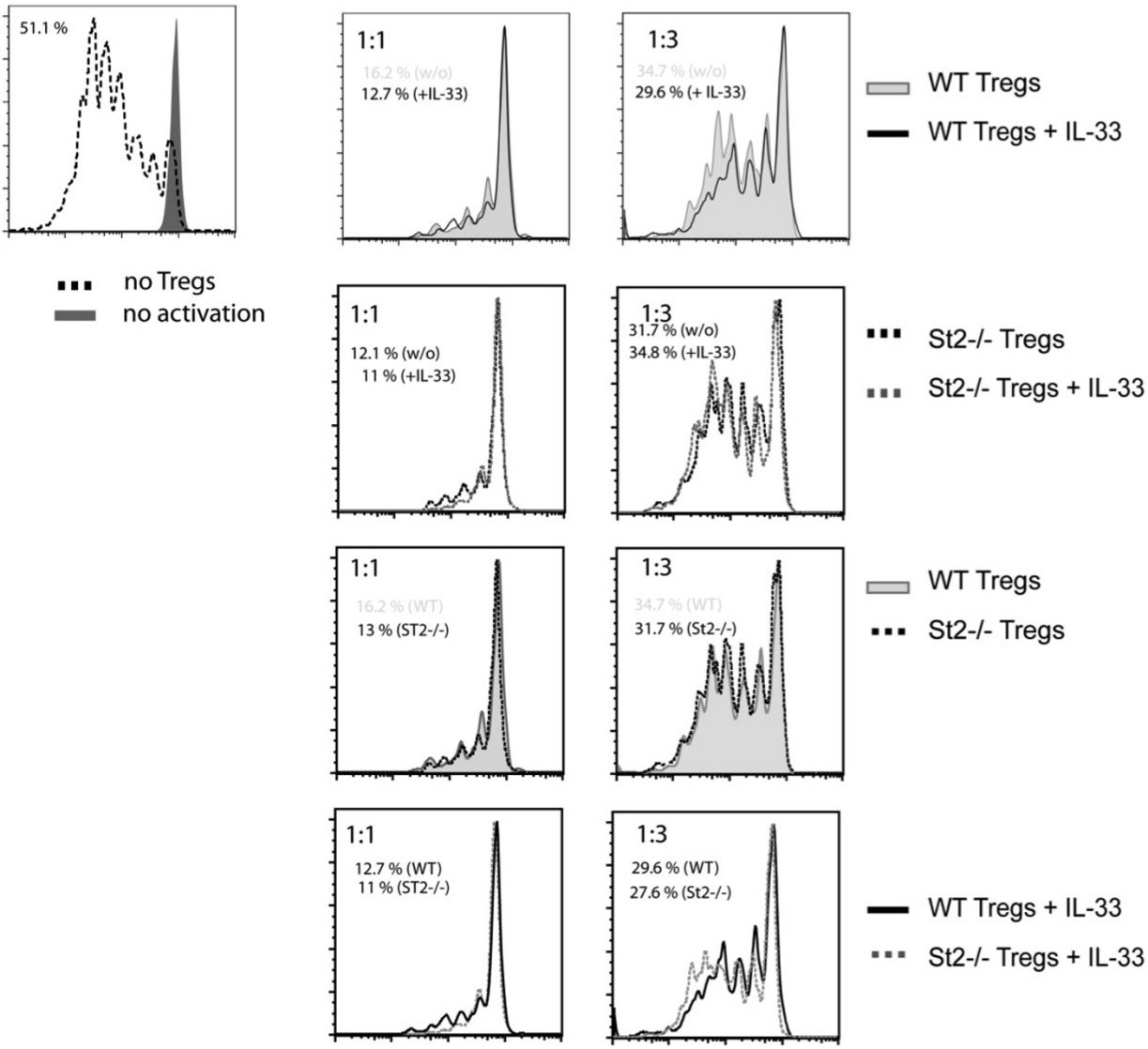
Extended Data Figure 2 | IL-33 directly regulates ST2 expression. Sort-purified $CD25^{-}$ $CD62L^{+}$ $CD44^{lo}$ GFP^{-} naive $CD4^{+}$ T cells from *Foxp3*^{gfp} reporter mice were cultured with anti-CD3/anti-CD28 in the presence of TGF-β_1 followed by acute stimulation with IL-33 for 45 min. Shown 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.).



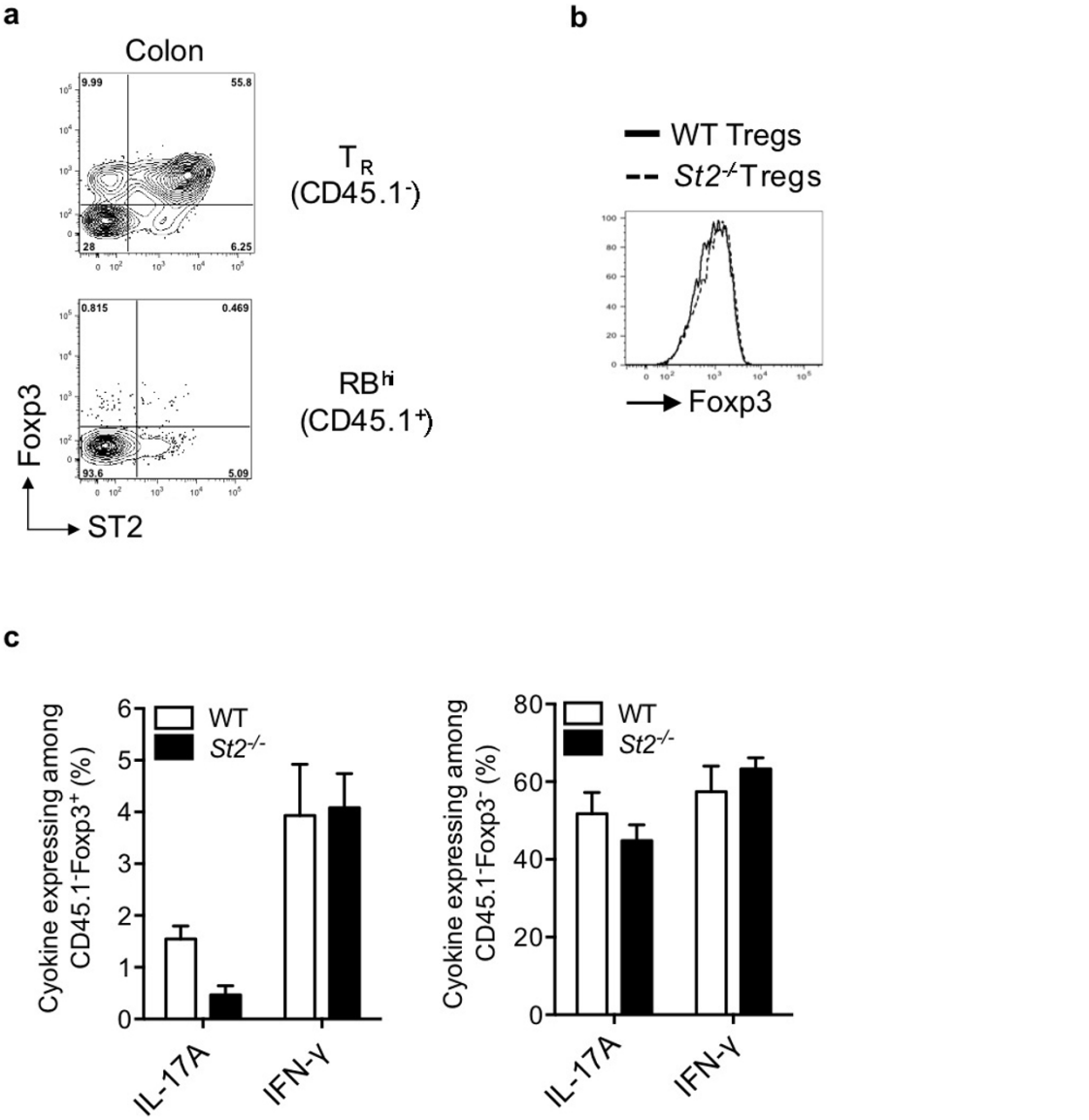
Extended Data Figure 3 | IL-33 acts directly on thymus-derived T_{reg} cells to promote their proliferation and accumulation *in vivo*. **a**–**e**, *Foxp3*^{gfp} reporter mice were injected intraperitoneally (i.p.) with recombinant IL-33 (1 μg per mouse per day) for 5 days. **a**, Frequencies of TCR-β$^+$ CD4$^+$ Foxp3$^+$ splenic thymus-derived T_{reg} cells. **b**, Absolute numbers of TCR-β$^+$ CD4$^+$ Foxp3$^+$ splenic T_{reg} cells. **c**, gMFI of Foxp3 among TCRβ$^+$ CD4$^+$ Foxp3$^+$ splenic T_{reg} cells ($n = 10$ for PBS and $n = 14$ for IL-33). **d**, Frequencies of ST2$^+$ cells among splenic CD44^{hi} T cells or Foxp3$^+$ T_{reg} cells. **e**, Frequencies of Ki67$^+$ cells among splenic CD44^{hi} T cells or Foxp3$^+$ T_{reg} 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 2.5×10^6 wild-type (WT; CD45.1$^+$) and 2.5×10^6 *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.



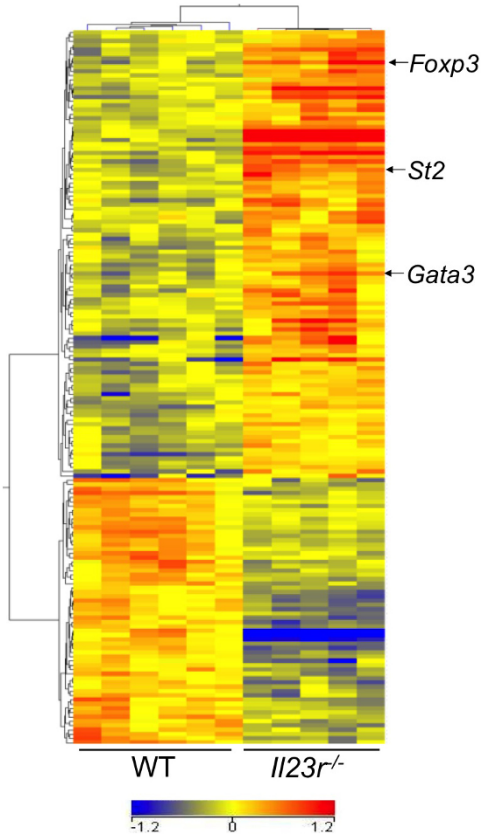
Extended Data Figure 4 | Kinetics of IL-33 and IL-23 during *H. hepaticus* and anti-IL-10R colitis. **a**–**f**, C57BL/6 mice were infected by oral gavage with ~10^8 colony-forming units (c.f.u.) *H. hepaticus* on 3 consecutive days with concomitant i.p. injections of a blocking anti-IL-10R monoclonal 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.



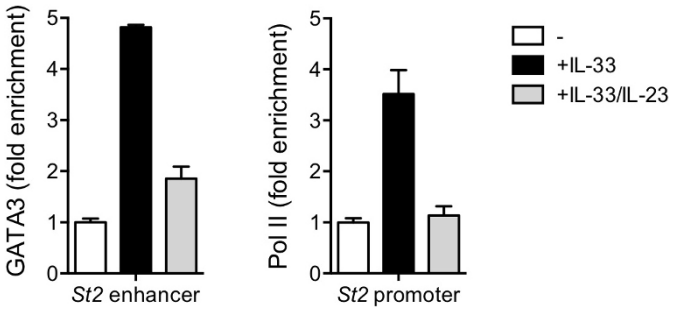
Extended Data Figure 5 | The *in vitro* suppressive function of *St2*$^{-/-}$ T_{reg} cells is not impaired. $CD25^+ CD4^+$ T_{reg} cells were sorted by flow cytometry from wild-type (WT) and *St2*$^{-/-}$ mice. $CD25^- CD62L^+ CD44^- CD4^+$ T cells (responder cells) and antigen presenting cells (APCs) were sorted from *St2*$^{-/-}$ mice. T responder cells were labelled with Violet cell trace and plated together with the sorted T_{reg} 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 (T_{resp}) was measured by flow cytometry. Data are representative of two independent experiments.



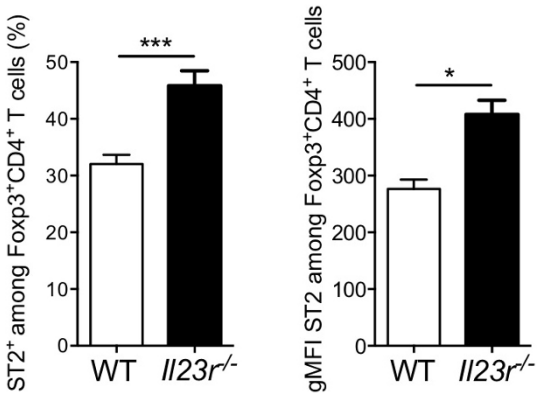
Extended Data Figure 6 | ST2 is preferentially expressed on T_{reg}-cell progeny. **a**, C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4×10^5 CD4$^+$ CD25$^-$ CD45 RB^{hi} CD45.1$^+$ naive T cells in combination with 2×10^5 wild-type (WT) CD4$^+$ CD25$^+$ CD45.1$^-$ T_{reg} cells. Mice were killed at 6–8 weeks after transfer. Representative plots of the colon gated on progeny of CD4$^+$ CD45 RB^{hi} cells (CD45.1$^+$) or wild-type T_{reg} (CD45.1$^-$) cells are shown. **b**, Histogram overlay of Foxp3 expression by wild-type (solid line) or *St2*$^{-/-}$ (dotted line) CD4$^+$ CD25$^+$ T_{reg} cells before injection into C57BL/6 *Rag1*$^{-/-}$ mice. **c**, C57BL/6 *Rag1*$^{-/-}$ mice were injected as in **a** and frequencies of cytokine-producing colonic T_{reg} progeny (CD45.1$^-$) among Foxp3$^-$ or Foxp3$^+$ cells are shown. Wild type ($n = 4$) and *St2*$^{-/-}$ ($n = 6$); error bars represent mean ± s.e.m.



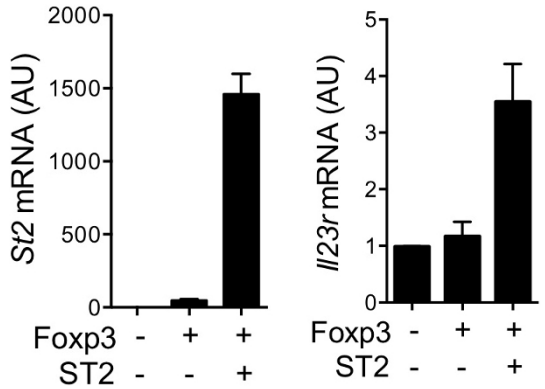
Extended Data Figure 7 | *St2* is an IL-23 target gene in intestinal CD4$^+$ T cells. C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with a 1:1 mixture of 2×10^5 wild-type (WT; CD45.1$^+$) and 2×10^5 *Il23r*$^{-/-}$ (CD45.2$^+$) CD4$^+$ CD25$^-$ CD45 RB^{hi} T cells or 2×10^5 wild-type (CD45.1$^+$) and 2×10^5 wild-type (CD45.2$^+$) CD4$^+$ CD25$^-$ CD45 RB^{hi} 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 $CD25^{-}$ $CD62L^{+}$ $CD44^{lo}$ GFP^{-} naive $CD4^{+}$ T cells from *Foxp3*^{gfp} reporter mice were cultured with anti-CD3/anti-CD28/ TGF-β_1 +/− 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*$^{-/-}$ (CD45.1$^-$) bone marrow cells ($n = 5$). Reconstituted mice were infected by oral gavage with $\sim 10^8$ 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 T_{reg} 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 *Foxp3^{gfp}* reporter mice. AU, arbitrary units. Error bars represent the mean ± s.e.m. from three independent experiments.