

Published in final edited form as:

Nature. 2014 September 25; 513(7519): 564–568. doi:10.1038/nature13577.

The Alarmin IL-33 Promotes Regulatory T Cell Function in the Intestine

Chris Schiering^{#1,6}, Thomas Krausgruber^{#1}, Agnieszka Chomka¹, Anja Fröhlich⁴, Krista Adelmann¹, Elizabeth A. Wohlfert^{2,7}, Johanna Pott³, Thibault Griseri¹, Julia Bollrath¹, Ahmed N. Hegazy¹, Oliver J. Harrison³, Benjamin M.J. Owens¹, Max Löhning⁴, Yasmine Belkaid², Padraic G. Fallon⁵, and Fiona Powrie¹

¹Translational Gastroenterology Unit, Nuffield Department of Clinical Medicine, Experimental Medicine Division, John Radcliffe Hospital, University of Oxford, Oxford OX3 9DU, UK ²Program 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, USA ³Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford, OX1 3RE, United Kingdom ⁴Experimental Immunology, Department of Rheumatology and Clinical Immunology, Charité – University Medicine Berlin, and German Rheumatism Research Center (DRFZ), Berlin, Germany ⁵Trinity Biomedical Sciences Institute, Trinity College Dublin, Pearse Street, Dublin, Ireland

These authors contributed equally to this work.

Abstract

Foxp3⁺ regulatory T 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 function¹; however, key host factors controlling the T_{reg} response in the intestine are poorly understood. 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 following tissue damage³. Recent studies in humans have described high levels of IL-33 in inflamed lesions of inflammatory bowel disease (IBD) patients^{4–7} suggesting a role for this cytokine in the pathogenesis of IBD. In the intestine, both protective and pathologic roles for IL-33 have been described in murine models of acute colitis^{8–11} but its

Users may view, print, copy, and download text and data-mine the content in such documents, for the purposes of academic research, subject always to the full Conditions of use: http://www.nature.com/authors/editorial_policies/license.html#terms

Correspondence should be addressed to F.P. (fiona.powrie@path.ox.ac.uk).

⁶Current address: Division of Molecular Immunology, MRC National Institute for Medical Research, Mill Hill, London, UK.

⁷Current address: Department of Microbiology and Immunology, School of Medicine and Biomedical Sciences, University at Buffalo (SUNY), Buffalo, NY.

Author contributions

C.S. and T.K. planned experiments and analyzed the data. C.S., T.K. and F.P. wrote the paper. A. C., A. F., K.A., O. H., A. 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.

The authors declare no competing financial interests.

Referenced accessions

Gene Expression Omnibus
GSE58164

contribution to chronic inflammation remains ill defined. Here we show that the IL-33 receptor ST2 is preferentially expressed on colonic T_{reg} (cT_{reg}) cells, where it promotes T_{reg} function and adaptation to the inflammatory environment. IL-33 signaling into T cells stimulates T_{reg} responses in several ways. Firstly, it enhances transforming growth factor- β 1 (TGF- β ₁) mediated differentiation of T_{reg} cells and secondly, it provides a necessary signal for T_{reg} accumulation and maintenance in inflamed tissues. Strikingly, IL-23, a key pro-inflammatory cytokine in the pathogenesis of IBD, 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 cT_{reg} cells, we compared the mRNA expression profiles of mesenteric lymph node (MLN) and cT_{reg} cells. We identified *St2*, the transcript coding for the IL-33 receptor¹², as one of the top differentially upregulated genes in cT_{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 regulatory (tT_{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 GATA3¹⁴⁻¹⁶ and GATA3 is known to regulate ST2 expression in Th2 cells¹⁷. Indeed, ST2 expression was largely restricted to GATA3-expressing cT_{reg} cells (Fig. 1e) and selective ablation of GATA3 in Foxp3-expressing cells, using *Gata3^{fl/fl}-Foxp3-Cre* mice¹⁵, 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 T_{reg}-cell (iT_{reg}) differentiation. To test this, we sort-purified naïve 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 percent 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 Th2 cytokines or expression of Th1 and Th17-associated transcription factors *Tbx21* and *Rorc* (Extended Data Fig. 1), suggesting that IL-33 preferentially regulates Foxp3 expression. Thus, our data indicate that the alarmin IL-33 is a novel co-factor 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 Th2 cells¹⁸ as well as innate lymphoid cells¹⁹. 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 (CNS) 1-3¹⁴. 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 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 Th2 cells, GATA3 has been shown to promote *St2* gene expression by binding to an enhancer element located 12-kb upstream of the *St2* transcription start site¹⁷. 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⁺ cT_{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-elicited T_{reg} cells expressed higher levels of Foxp3 and ST2 (Extended Data Fig. 3c,d). Further analysis of proliferation marker Ki67 showed IL-33 induced proliferation in splenic T_{reg} cells but not T effector cells (Extended Data Fig. 3e). To examine if IL-33 acts directly on T_{reg} cells we injected IL-33 into chimeric mice containing a mixture of WT and *St2*^{-/-} hematopoietic 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 tT_{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 TCR-activated splenic T_{reg} cells with IL-33 induced serine phosphorylation of GATA3 (Fig. 2f) further demonstrating that IL-33 acts directly on tT_{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 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 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 (sST2), which is produced primarily by colonic stromal cells (Extended Data Fig. 4b, e-f). sST2 is thought to limit IL-33 bioavailability by acting as a decoy receptor²³ and is increased in patients with active IBD^{6,24} suggesting that the chronic inflammatory tissue environment may antagonize IL-33 activity. Despite high levels of sST2, analysis of chimeric 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 levels of Foxp3 protein on a per cell basis as compared to their WT 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 sST2 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 WT and *St2*^{-/-} T_{reg} cells. *St2*^{-/-} T_{reg} cells inhibited T cell proliferation to the same extent as WT T_{reg} cells *in vitro* (Extended Data Fig. 5) and addition of IL-33 did not enhance WT T_{reg} suppressor function. We then tested the ability of *St2*^{-/-} T_{reg} to protect from colitis induced by adoptive transfer of naïve CD4⁺ T cells. Interestingly, ST2 was highly expressed on WT T_{reg} cells upon T cell transfer pointing toward 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 signaling into T_{reg} cells is important for their suppressive function *in vivo*. Analysis of WT or *St2*^{-/-} T_{reg} cells two weeks post transfer, prior to 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 signaling in T_{reg} cells is dispensable for their ability to expand and index with effector cells in the lymphopenic host at this time point. By contrast, analysis at 8 weeks post-transfer showed that 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 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 function²⁵⁻²⁷ and our data indicate that IL-33 signaling 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 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 humans²⁸. 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). Based on this observation we hypothesized that IL-23 might limit T cell responsiveness to IL-33. Indeed, the co-factor activity of IL-33 on TGF-β₁-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 naïve T cell transfer into *Il23a*^{-/-}*Rag1*^{-/-} recipients resulted in increased T_{reg} differentiation²⁹. 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 WT or *Stat2*^{-/-} naïve T cells into *Il23a*^{-/-}*Rag1*^{-/-} hosts and monitored iT_{reg} 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, ST2-deficiency had minor effects on *in vivo* differentiation of Th1, Th2 or Th17 cells (Fig. 4f) further supporting the notion that the increased colitogenic potential of *Stat2*^{-/-} CD4⁺ T cells is a consequence of deficient iT_{reg} differentiation rather than dysregulated effector T cell responses. In summary, 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 signaling 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 tT_{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 GATA3¹⁶ 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 signaling, 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 tT_{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 signaling through this pathway plays 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.

Method Summary

A description of the *in vitro* assays and details of *in vivo* models can be found in the full Methods.

Materials and Methods

Mice

Wild-type C57BL/6, congenic B6.SJL-*Cd45.1*, C57BL/6 *Il23r*^{-/-}, C57BL/6 *Rag1*^{-/-}, C57BL/6 *Il23a*^{-/-}*Rag1*^{-/-} and *Foxp3*^{sgfp} 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 i.p. injected with recombinant IL-33 (1 µg / injection; Biolegend) for 5 consecutive days and sacrificed 24 h after the last injection. *Gata3*^{fl/fl}-*Foxp3*^{Cre} mice were kept at the NIH and experiments were performed at the NIH. Spleen, MLN and bone marrow from C57BL/6 *St2*^{-/-} mice were obtained from Padraic Fallon (Trinity College Dublin, Ireland) or Max Loehning (Charité – University Medicine Berlin, Germany). 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. Exclusions 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 WT (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 naïve T cell transfer colitis 4×10⁵ CD4⁺CD25⁻CD45RB^{hi} T cells were injected i.p. into *Rag1*^{-/-} or *Il23a*^{-/-}*Rag1*^{-/-} recipients. In co-transfer experiments 2×10⁵ CD4⁺CD25⁻CD45RB^{hi} T cells from each source were mixed and injected i.p. into *Rag1*^{-/-} hosts. For T_{reg} mediated protection from colitis 4×10⁵ CD4⁺CD25⁻CD45RB^{hi} T cells and 2×10⁵ CD4⁺CD25⁺ T_{reg} cells were mixed and injected i.p. into *Rag1*^{-/-} hosts³³. Mice were sacrificed at indicated time points or sacrificed when weight loss approached 20% of the original body weight at the start of the experiment.

Induction of *Helicobacter hepaticus*/anti-IL-10R colitis

Mice were fed 1×10⁸ cfu *Helicobacter hepaticus* by oral gavage with a 22G curved needle on day 0, day 1 and day 2 of the experiment. In addition mice received 1mg of an anti-IL-10R blocking antibody by i.p. injection once weekly starting at the day of *Helicobacter 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 hematoxylin and eosin, and inflammation was scored in a blinded fashion using a previously described scoring system³⁴.

Isolation of leukocyte subpopulations and flow cytometry

Cell suspensions from spleen, MLN, and the lamina propria were prepared as described previously³⁵ 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 γ t, Ki67, (BD Biosciences) and CD45RB, KLRG1 (all from Biolegend) and anti-ST2 conjugated to biotin (mdBioproducts). Intracellular staining was performed as follows: cells were restimulated for 4 h as previously described³⁶, washed and incubated with anti-CD16/CD32. Cells were washed and stained for surface markers indicated above 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⁻ naïve CD4⁺ T cells were sort-purified from *Foxp3^{gfp}* reporter mice. Cells were plated at 2×10^5 cells/well in flat bottomed 96 well plates coated with anti-CD3 (5 μ g/ml) in RPMI (Invitrogen) containing 10% FCS, 2 mM l-glutamine, 0.05 mM 2-mercaptoethanol, and 100 U/ml each of penicillin and streptomycin (complete media). Antibodies and cytokines were added at the following concentrations, anti-CD28 (2 μ g/ml), TGF- β 1 (R&D, 500pg/ml), IL-2 (Peprotech, 1U/ml), IL-23 (R&D, 20ng/ml), IL-33 (Biolegend, 1 ng/ml). Cells were harvested at indicated times, lysed for use for qPCR, western blot or analyzed 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/well in flat bottomed 96 well plates coated with anti-CD3 (5 μ g/ml) in complete media and anti-CD28 (2 μ g/ml), IL-2 (Peprotech, 1U/ml) or IL-33 (Biolegend, 1 ng/ml) were added at the start of the culture. Cells were analyzed after 3 days by flow cytometry. For acute stimulation, 2×10^5 cells/well were plated in 96 well plates coated with anti-CD3 (5 μ g/ml) in complete media and soluble anti-CD28 (2 μ g/ml) and cultured for 24 h. Cells were then stimulated with IL-33 (10 ng/ml) 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 WT 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 LN cells from *St2^{-/-}* mice. T responder cells were labeled with Violet cell trace and plated together with the sorted Tregs at ratios of 1:1 and 1:3 together with irradiated APCs in the presence of anti-CD3 (1 μ g/ml). IL-33 was added at 30 ng/ml. After 4 days proliferation of T responder cells was measured by flow cytometry.

Colon explant cultures

Organ explants were prepared as previously described³⁶ and cultured overnight in complete media. Cytokine levels in the supernatants were determined by 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 harvested and washed twice in PBS containing 2% BSA. Cells were plated at 10×10^6 cells/ml in complete DMEM containing 40 µg/ml Gentamycin (Sigma). After 24–48 hours, 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 for 48 hours and the concentration of sST2 in supernatants was determined by ELISA (R&D Systems).

qPCR and microarray preparation

RNA was extracted according to the manufacturers' protocol (RNeasy, Qiagen) and cDNA 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 primers from³⁷. For soluble ST2 the following primers were used: sST2 forward 5'-tcgaaatgaaagtccagca -3' and sST2 reverse 5'-tgtgtgagggacactcctac -3' Samples were assayed in duplicate on a Bio-Rad CFX96 RT-qPCR machine and differences were calculated using the $2^{\Delta C(t)}$ 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 hybridised to MouseWG-6 v2.0 Expression BeadChip (Illumina). Hybridisations 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 was normalised using 75% percentile shift normalisation 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 described³⁸. Equal amounts of protein were resolved by SDS-PAGE and analyzed 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 30min before cytokine stimulation.

Chromatin immunoprecipitation

ChIP assays were performed as described³⁸ with antibody to GATA3 (L50-823, BD Pharmingen) or RNA polymerase II (sc-899; Santa Cruz). The immunoprecipitated DNA fragments were then analyzed by real-time PCR with SYBR Premix Ex Taq II master mix (Takara Bio) and the following primers: locus encoding St2 enhancer, 5'-gcccaaccacaacagcagatggggaaa -3' and 5'-actgagatcctgccttgcttcct -3'; locus encoding St2 promoter, 5'-tgccctccttggaaggcttggt -3' and 5'-agtgcaggaggggcatggagatga. Primers for analyzing binding to CNS1, CNS2, CNS3 and promoter locus of Foxp3 were from²⁵. Data were analyzed using Rotogene 6000 software (Corbett Research Ltd). 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 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 $P \leq 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

C. S., K. A., A. C and F. P are supported by the Wellcome Trust. FP is also supported by Fondation Louis Jeantet. A.N.H is supported by a European Molecular Biology Organization (EMBO) long-term fellowship (ALTF 116-2012). PGF is supported by Science Foundation Ireland. M.L. and A.F. are supported by Volkswagen Foundation (Lichtenberg Program) and BMBF (e:Bio/T-Sys). B.M.J.O is supported by an Oxford-UCB Pharma Postdoctoral Fellowship.

We thank all members of the Oxford Translational Gastroenterology Unit for assistance and support. We are grateful to Helen Ferry and Kate Alford for essential flow cytometry support and the staff of the University of Oxford for excellent animal care. We are also grateful to Dilair Baban (Wellcome Trust Center for Human Genetics, Oxford, UK) for conducting microarray hybridizations.

References

1. Burzyn D, Benoist C, Mathis D. Regulatory T cells in nonlymphoid tissues. *Nature immunology*. 2013; 14:1007–1013. [PubMed: 24048122]
2. Pichery M, et al. Endogenous IL-33 is highly expressed in mouse epithelial barrier tissues, lymphoid organs, brain, embryos, and inflamed tissues: in situ analysis using a novel IL-33-LacZ gene trap reporter strain. *Journal of immunology*. 2012; 188:3488–3495.
3. Palmer G, Gabay C. Interleukin-33 biology with potential insights into human diseases. *Nature reviews. Rheumatology*. 2011; 7:321–329. [PubMed: 21519352]
4. Beltran CJ, et al. Characterization of the novel ST2/IL-33 system in patients with inflammatory bowel disease. *Inflammatory bowel diseases*. 2010; 16:1097–1107. [PubMed: 20014018]
5. Kobori A, et al. Interleukin-33 expression is specifically enhanced in inflamed mucosa of ulcerative colitis. *J Gastroenterol*. 2010; 45:999–1007. [PubMed: 20405148]

6. Pastorelli L, et al. Epithelial-derived IL-33 and its receptor ST2 are dysregulated in ulcerative colitis and in experimental Th1/Th2 driven enteritis. *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107:8017–8022. [PubMed: 20385815]
7. Seidelin JB, et al. IL-33 is upregulated in colonocytes of ulcerative colitis. *Immunol Lett*. 2010; 128:80–85. [PubMed: 19913053]
8. Oboki K, et al. IL-33 is a crucial amplifier of innate rather than acquired immunity. *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107:18581–18586. [PubMed: 20937871]
9. Duan L, et al. Interleukin-33 ameliorates experimental colitis through promoting Th2/Foxp3(+) regulatory T-cell responses in mice. *Molecular medicine*. 2012; 18:753–761. [PubMed: 22426954]
10. Grobota P, Doser K, Falk W, Obermeier F, Hofmann C. IL-33 attenuates development and perpetuation of chronic intestinal inflammation. *Inflammatory bowel diseases*. 2012; 18:1900–1909. [PubMed: 22508383]
11. Sedhom MA, et al. Neutralisation of the interleukin-33/ST2 pathway ameliorates experimental colitis through enhancement of mucosal healing in mice. *Gut*. 2013; 62:1714–1723. [PubMed: 23172891]
12. Schmitz J, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity*. 2005; 23:479–490. [PubMed: 16286016]
13. Thornton AM, et al. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *Journal of immunology*. 2010; 184:3433–3441.
14. Wang Y, Su MA, Wan YY. An essential role of the transcription factor GATA-3 for the function of regulatory T cells. *Immunity*. 2011; 35:337–348. [PubMed: 21924928]
15. Wohlfert EA, et al. GATA3 controls Foxp3(+) regulatory T cell fate during inflammation in mice. *J Clin Invest*. 2011; 121:4503–4515. [PubMed: 21965331]
16. Rudra D, et al. Transcription factor Foxp3 and its protein partners form a complex regulatory network. *Nature immunology*. 2012; 13:1010–1019. [PubMed: 22922362]
17. Guo L, et al. IL-1 family members and STAT activators induce cytokine production by Th2, Th17, and Th1 cells. *Proceedings of the National Academy of Sciences of the United States of America*. 2009; 106:13463–13468. [PubMed: 19666510]
18. Maneechotesuwan K, et al. Regulation of Th2 cytokine genes by p38 MAPK-mediated phosphorylation of GATA-3. *Journal of immunology*. 2007; 178:2491–2498.
19. Furusawa JI, et al. Critical Role of p38 and GATA3 in Natural Helper Cell Function. *Journal of immunology*. 2013; 191:1818–1826.
20. Turnquist HR, et al. IL-33 expands suppressive CD11b+ Gr-1(int) and regulatory T cells, including ST2L+ Foxp3+ cells, and mediates regulatory T cell-dependent promotion of cardiac allograft survival. *Journal of immunology*. 2011; 187:4598–4610.
21. Wasserman A, et al. Interleukin-33 augments Treg cell levels: a flaw mechanism in atherosclerosis. *The Israel Medical Association journal: IMAJ*. 2012; 14:620–623. [PubMed: 23193783]
22. Kullberg MC, et al. IL-23 plays a key role in *Helicobacter hepaticus*-induced T cell-dependent colitis. *The Journal of experimental medicine*. 2006; 203:2485–2494. [PubMed: 17030948]
23. Hayakawa H, Hayakawa M, Kume A, Tominaga S. Soluble ST2 blocks interleukin-33 signaling in allergic airway inflammation. *The Journal of biological chemistry*. 2007; 282:26369–26380. [PubMed: 17623648]
24. Diaz-Jimenez D, et al. Soluble ST2: a new and promising activity marker in ulcerative colitis. *World journal of gastroenterology: WJG*. 2011; 17:2181–2190. [PubMed: 21633527]
25. Zheng Y, et al. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature*. 2010; 463:808–812. [PubMed: 20072126]
26. Wan YY, Flavell RA. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. *Nature*. 2007; 445:766–770. [PubMed: 17220876]
27. Kitoh A, et al. Indispensable role of the Runx1-Cbfbeta transcription complex for in vivo-suppressive function of FoxP3+ regulatory T cells. *Immunity*. 2009; 31:609–620. [PubMed: 19800266]

28. Maloy KJ, Powrie F. Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature*. 2011; 474:298–306. [PubMed: 21677746]
29. Izcue A, et al. Interleukin-23 restrains regulatory T cell activity to drive T cell-dependent colitis. *Immunity*. 2008; 28:559–570. [PubMed: 18400195]
30. Ahern PP, et al. Interleukin-23 drives intestinal inflammation through direct activity on T cells. *Immunity*. 2010; 33:279–288. [PubMed: 20732640]
31. Fontenot JD, et al. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity*. 2005; 22:329–341. [PubMed: 15780990]
32. Bettelli E, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature*. 2006; 441:235–238. [PubMed: 16648838]
33. Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *The Journal of experimental medicine*. 2000; 192:295–302. [PubMed: 10899916]
34. Izcue A, et al. Interleukin-23 restrains regulatory T cell activity to drive T cell-dependent colitis. *Immunity*. 2008; 28:559–570. [PubMed: 18400195]
35. Uhlig HH, et al. Characterization of Foxp3+CD4+CD25+ and IL-10-secreting CD4+CD25+ T cells during cure of colitis. *Journal of immunology*. 2006; 177:5852–5860.
36. Hue S, et al. Interleukin-23 drives innate and T cell-mediated intestinal inflammation. *The Journal of experimental medicine*. 2006; 203:2473–2483. [PubMed: 17030949]
37. Rudra D, et al. Transcription factor Foxp3 and its protein partners form a complex regulatory network. *Nature immunology*. 2012; 13:1010–1019. [PubMed: 22922362]
38. Krausgruber T, et al. IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses. *Nature immunology*. 2011; 12:231–238. [PubMed: 21240265]

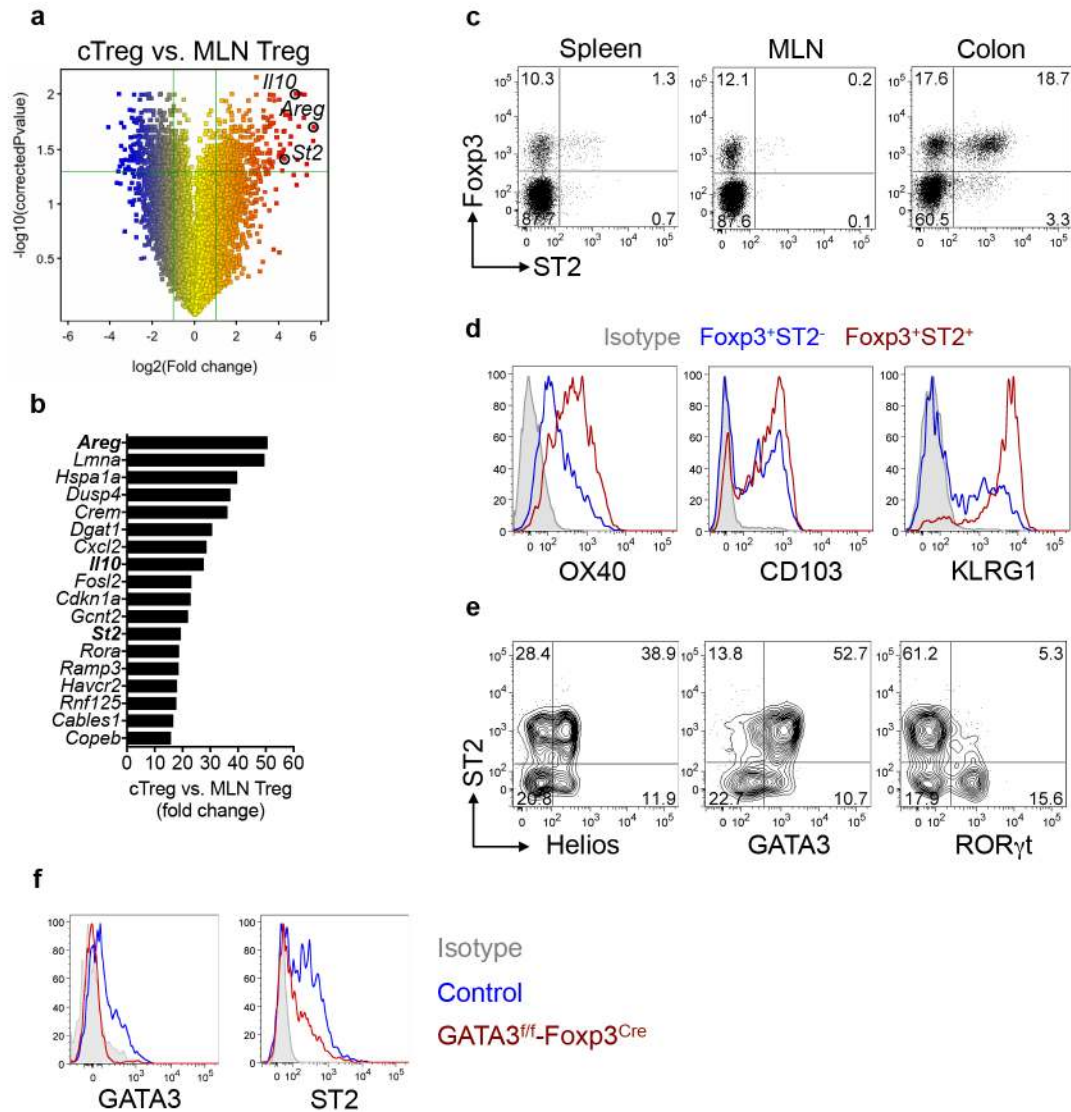


Figure 1. ST2-expressing T_{reg} cells are enriched in the colon

a, Change in gene expression in cT_{reg} cells vs. MLN T_{reg} cells ($n=3$ per group) presented as volcano plot. **b**, Top differentially upregulated transcripts in cT_{reg} vs. MLN T_{reg} cells. **c**, ST2 protein expression on T_{reg} cells from indicated organs. **d**, Phenotypic analysis of ST2⁻ or ST2⁺ cT_{reg} cells. **e**, Expression of transcription factors in cT_{reg} cells. **f**, Representative histograms gated on cT_{reg} cells from control or *Gata3^{fl/fl}-Foxp3-Cre* mice.

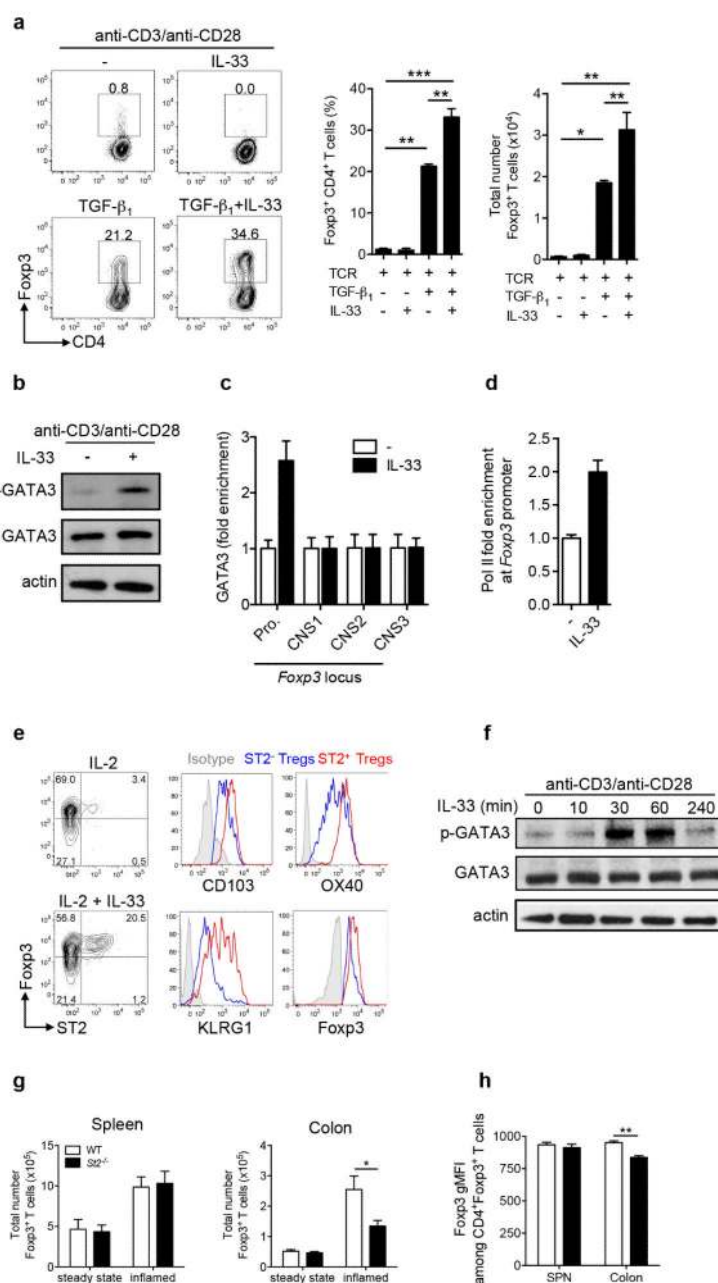


Figure 2. Effects of IL-33 on iT_{reg} and tT_{reg} cells

a, Naïve CD4⁺ T cells were cultured with anti-CD3/CD28 plus indicated cytokines and the frequencies and absolute numbers of Foxp3⁺ T cells determined 3 days later (mean \pm s.e.m. of three independent experiments). **b**, Naïve CD4⁺ T cells were cultured for 48h with anti-CD3/CD28 plus TGF- β_1 followed by stimulation with IL-33 for 45 minutes. Blots are representative of two independent experiments. **c-d**, Cells were cultured and stimulated as in (b) and recruitment of GATA3 or RNA polymerase II (Pol II) to the indicated regions assessed by CHIP-qPCR. Data are from one experiment representative of two (mean \pm s.d.).

e, Representative plots of T_{reg} cells cultured with anti-CD3/CD28 plus indicated cytokines and analyzed after 3 days. Data are representative of three independent experiments. **f**, T_{reg} cells were cultured with anti-CD3/CD28 for 24h followed by stimulation with IL-33. Blots are representative of three independent experiments. **g**, Mixed chimaeras were generated containing WT and *St2*^{-/-} bone marrow cells. Reconstituted mice were analyzed at steady state or two weeks after infection with *Helicobacter hepaticus* and anti-IL-10R treatment (inflamed). Absolute numbers of WT 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 from inflamed chimaeric hosts presented as geometric mean fluorescence intensity (gMFI). **P*<0.05, ***P*<0.01 *** *P*<0.001 as calculated by 1way-ANOVA with Bonferroni post-test or paired Students' *t*-test.

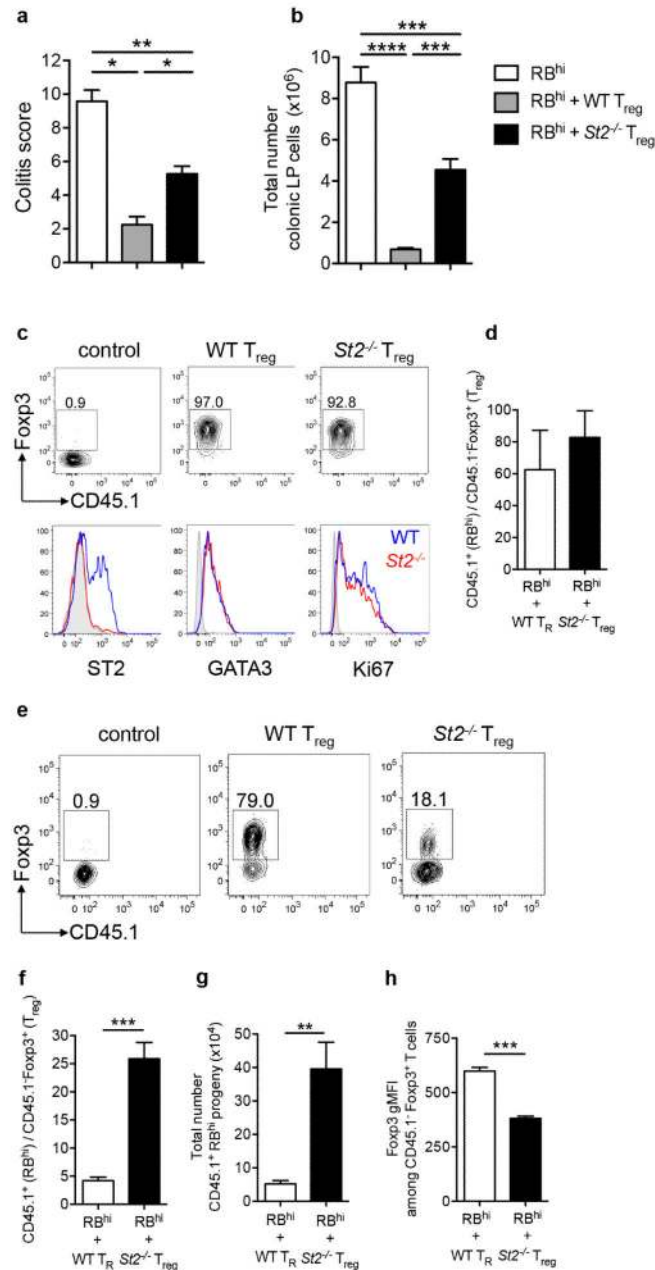


Figure 3. IL-33 promotes T_{reg} cell stability and function *in vivo*

a, C57BL/6 *Rag1*^{-/-} mice were injected with CD45.1⁺ naïve T cells alone (RB^{hi}, $n = 4$) or in combination with WT ($n = 4$) or *St2*^{-/-} ($n = 6$) CD45.1⁻ T_{reg} cells. Mice were sacrificed after 6-8 weeks post transfer and colitis scores are shown. **b**, Absolute numbers of colon lamina propria (LP) cells from mice in (a). **c**, C57BL/6 *Rag1*^{-/-} mice were injected as in (a) and sacrificed 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 WT or *St2*^{-/-} Fop3⁺ T_{reg} cell progeny (CD45.1⁻) in the colon ($n = 5$ per group) from mice in (c), (mean \pm s.e.m).

e, C57BL/6 *Rag1*^{-/-} mice were injected as in (a) and sacrificed 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 WT or *Sl2*^{-/-} 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 1way-ANOVA with Bonferroni post-test or Students' *t*-test.

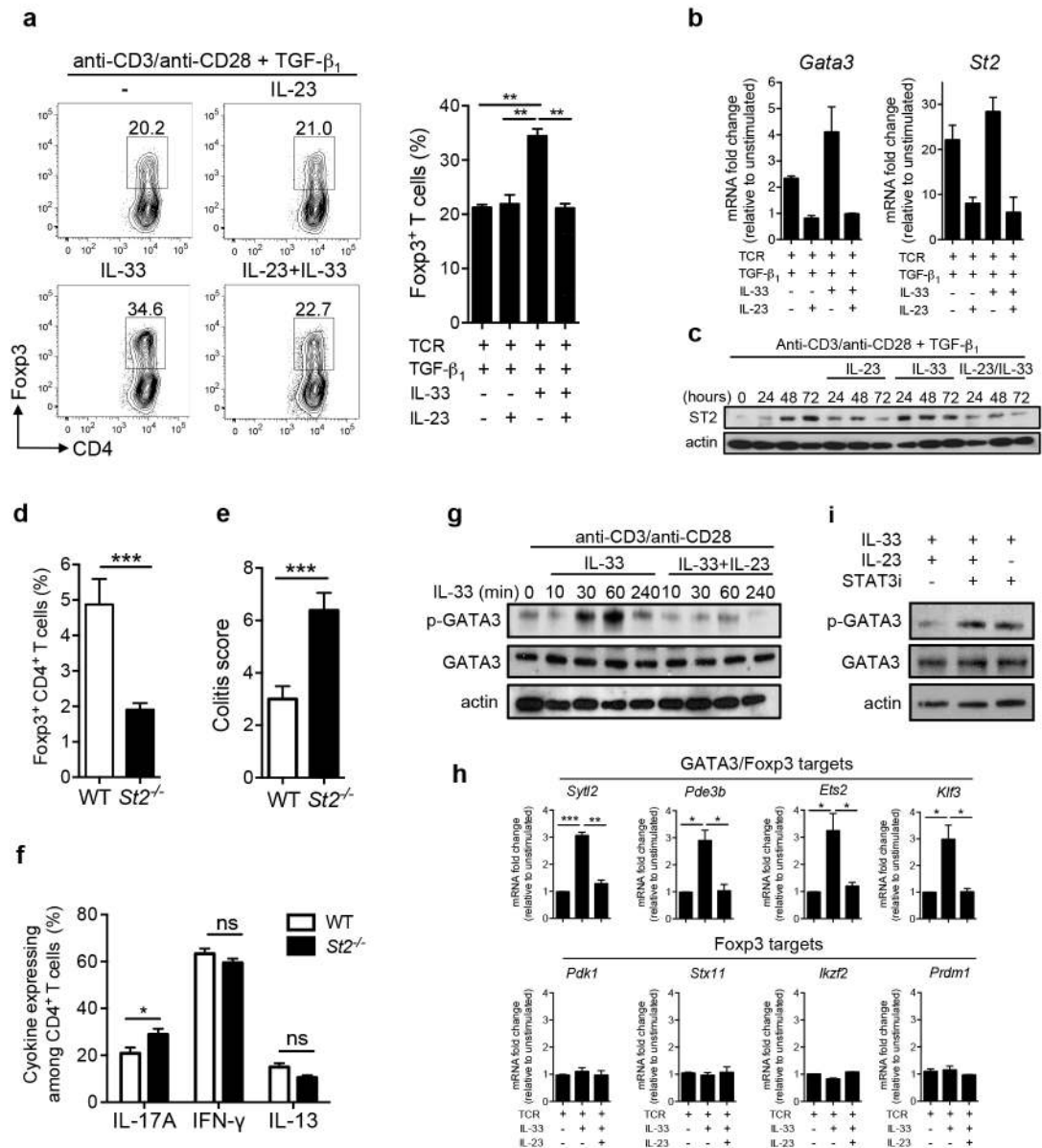


Figure 4. IL-23 inhibits the effects of IL-33 on T_{reg} cells

a, Naïve CD4⁺ T cells were cultured with anti-CD3/CD28 plus TGF- β_1 as well as indicated cytokines and the frequencies of Foxp3⁺ T cells determined after 3 days later (mean \pm s.e.m. of three independent experiments). **b**, Naïve CD4⁺ T cells were cultured with anti-CD3/CD28 plus indicated cytokines for 48h. Data are from one experiment representative of two (mean \pm s.d.). **c**, Naïve 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} WT ($n = 9$) or *St2*^{-/-} ($n = 10$) T cells. Mice were sacrificed after 6-8 weeks post transfer and frequencies of Foxp3⁺CD4⁺ T cells in colon are shown. **e**, Colitis scores for mice in (d). **f**, Expression of indicated cytokines by colonic CD4⁺ T cells from mice in (d).

g, T_{reg} cells were cultured with anti-CD3/CD28 for 24h 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 24h and mRNA expression of indicated genes measured following stimulation with IL-33 for 45 minutes in the presence or absence of IL-23 (mean \pm s.e.m. of three independent experiments). **i**, T_{reg} cells were cultured with anti-CD3/CD28 for 24h and representative blots of two independent experiments are shown. * P <0.05, ** P <0.01 *** P <0.001 as calculated by 1way-ANOVA with Bonferroni post-test or Students' t -test.