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Role and species–specific expression of colon T cell homing receptor GPR15 in colitis

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Abstract

Lymphocyte recruitment maintains intestinal immune homeostasis but also contributes to inflammation. The orphan chemoattractant receptor GPR15 mediates regulatory T cell homing and immunosuppression in the mouse colon. We show that GPR15 is also expressed by mouse T_H17 and T_H1 effector cells, and is required for colitis in a model that depends on their trafficking to the colon. In humans GPR15 is expressed by effector cells including pathogenic T_H2 cells in ulcerative colitis, but is not expressed by regulatory T (T_{reg}) cells. The T_H2 transcriptional activator GATA-3 and the T_{reg}–associated transcriptional repressor FOXP3 robustly bind human, but not mouse, *GPR15* enhancer sequences, correlating with expression. Our results highlight species differences in GPR15 regulation, and suggest it as a potential therapeutic target for colitis.

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Author contributions.

JP, EOH, AHa, ECB, LPN performed exploratory gene profiling that identified GPR15 as a candidate colon homing receptor. LPN performed experiments and wrote the manuscript. HH and AE performed mouse T cell polarization and short–term homing experiments. JP identified the mouse *Gata3* mutation and JP and TTD characterized it. AHe and MRG performed ChIP–Seq and ChIP–qPCR. GML and RGJ initiated and supervised the ChIP work. RGJ performed ChIP, analyzed ChIP–Seq data and wrote parts of the manuscript. ECB initiated the project, analyzed data, provided overall supervision and wrote the manuscript. AHa supervised the project and wrote the manuscript.

Accession codes. GEO Accession codes for published ChIP–Seq data are provided in online Supplementary Table 2.

Competing interests

The authors declare no competing interests.

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Recruitment of lymphocytes from the circulation is a tissue– and cell–specific process that is mediated by adhesion and chemoattractant receptors[1,2]. The interaction of lymphocyte adhesion receptors with and their ligands on vascular endothelium allows endothelial capture of blood borne lymphocytes and mediates lymphocyte rolling, but arrest and firm adhesion of rolling cells, as well as subsequent diapedesis, require the engagement of lymphocyte receptors of the chemoattractant GPCR family[3]. Signaling via these chemoattractant receptors triggers rapid integrin-dependent lymphocyte adhesion on the endothelium, and activates and drives programs of lymphocyte motility. Chemoattractant receptors thus play critical roles in the recruitment of lymphocyte subsets from the blood, and they direct the trafficking of lymphocyte subsets in both homeostatic and inflammatory states[3,4]. As examples, CCR7 helps target naive lymphocytes and subsets of memory and effector cells to lymph nodes and tertiary lymphoid tissues in chronic inflammation; CCR10, CCR8 and CCR4 participate selectively though not exclusively in skin homing by memory/effector T cells; and CCR9 serves as a specific T cell and plasmablast homing receptor for the small intestines[5]. Interestingly, CCR10 also targets IgA plasmablast homing to mucosal tissues including the bronchial tree and the colon, but is not expressed by gut homing T cells: CCR10 expression on T cells is largely mutually exclusive with that of the integrin intestinal homing receptor $\alpha_4\beta_7$ (ref. 5), which is required for efficient lymphocyte homing to the intestines through its recognition of the mucosal vascular addressin MAdCAM1 (refs. 3,6). Although inflammatory chemokines and their receptors may participate[7,8], whether colon effector and memory T cells have specific chemoattractant trafficking receptors analogous to skin and small intestinal T cell–selective chemokine receptors has remained unclear.

GPR15 is an orphan GPCR and an HIV co–receptor that is structurally related to known lymphocyte trafficking receptors[9,10]. A recent study has implicated this receptor in colon homing of T_{reg} cells in the mouse[11], but its role in effector T cell trafficking and function is not known. Here we examine the expression and function of GPR15 on effector T cells in mouse and man. Our results show that GPR15 is important for effector as well as regulatory T cell localization to the mouse colon and implicate GPR15-dependent effector cell recruitment in murine colitis. We also describe substantial differences in GPR15 expression in the human, with expression by T_H2 cells but not T_{reg} cells in the normal and inflamed human colon. We relate the expression of GPR15 by human T_H2 cells and by mouse, but not human T_{reg} cells, to inter–species differences in binding of transcriptional regulators to *Gpr15* enhancer sequences.

Results

Activated colon CD4$^+$ T cells express GPR15

Using *Gpr15*–GFP knock–in mice[11], we confirmed that GFP$^+$ CD4$^+$ are most frequent among T cells in colon lamina propria (LP), as compared to small intestine (SI) and peripheral lymphoid tissues. They were infrequent among the intraepithelial lymphocytes of the colon or SI, and absent in naive CD4$^+$ T cells of all tissues (Supplementary Fig. 1a;[11]). GFP$^+$ CD4$^+$ T cells in the colon display an activated phenotype, characterized by high expression of activation antigens CD44, CD69 and CD25, and low expression of CD45RB

and CD62L (Supplementary Fig. 1b); and they express the integrin homing receptor $\alpha_4\beta_7$ for intestinal vascular adhesion ligand MAdCAM1 but lack the SI trafficking receptor CCR9 (Supplementary Fig. 1b). Consistent with a prior report[11], under homeostatic conditions most, although not all, colon LP GFP$^+$ CD4$^+$ T cells express markers of T_{reg} differentiation, including Foxp3, CD25, GITR and Helios, and lack effector cytokine expression (Supplementary Fig. 1c).

Gpr15 mediates CD4$^+$ T_{EM} accumulation in the colon

To assess the importance of GPR15 to effector/memory T cells (T_{EM}) in different tissues, we reconstituted irradiated mice with a mixture of allotype–marked *Gpr15*$^{gfp/gfp}$ (*Gpr15*–knockout; KO) and *Gpr15*$^{gfp/+}$ (*Gpr15*–heterozygous; het) bone marrow cells, allowing *Gpr15*–KO and *Gpr15*-het lymphocytes to compete for niches within a common host environment. *Gpr15*–KO CD4$^+$ repopulated the colon LP with significantly lower efficiency than their *Gpr15*–het counterparts (Supplementary Fig. 2a,b and Fig. 1a). In contrast, GPR15 deficiency had no effect on the reconstitution of CD4$^+$ T cells in lymphoid tissues or SI LP; nor did it alter colon naive CD4$^+$ T cell frequency (Fig. 1a). Importantly, T_{EM} cell (CD45RB^{lo}, CD44^{hi}, Foxp3$^-$) accumulation was reduced to the same extent as that of T_{reg} cells (CD25^{high}, Foxp3$^+$; Fig. 1a). This accumulation is likely mediated by GPR15–dependent recruitment, because GPR15 effects colon homing of *in vitro*–generated T_{EM} (Supplementary Fig. 3), T_{reg} cells as well as transduced T cells[11]; however, effects on retention, survival or proliferation cannot be excluded. These results demonstrate an important role for GPR15 in long–term T_{EM} as well as T_{reg} reconstitution and homeostasis in the mouse colon.

We next evaluated GPR15 involvement in colon antigen–specific CD4$^+$ T cell generation. Allotype–marked, ovalbumin (OVA)–specific (DO11.10) *Gpr15*–KO or *Gpr15*–het splenic CD4$^+$ T cells were transferred into recipient mice that were immunized intra–rectally 24 h later with ovalbumin (OVA) and cholera toxin (Supplementary Fig. 2c), a procedure that selectively induces T effector cell responses[12]. Activated T cells arising in the colon or draining mesenteric lymph node (MLN) then home via the blood to the colon[13]. OVA–specific *Gpr15*–het T cells were more efficient than their *Gpr15*–KO counterparts at populating the recipient mice colons (Fig. 1b and Supplementary Fig. 2d). This difference was not due to differences in the overall OVA response between the het and KO T cells, as indicated by similar antigen–specific proliferation in the MLN of FTY720–treated mice[14], in which lymphocyte exit from lymph nodes (MLN) is controlled (Supplementary Fig. 2e and Fig. 1c). Thus GPR15 supports the accumulation of antigen–reactive CD4$^+$ effector T cells in the colon.

Gpr15 required for CD45RB^{hi} T cell transfer colitis

Having shown that GPR15 contributes to colon effector T cell recruitment and accumulation, we next assessed its importance in colon inflammation. We used a well–established model of colitis, the CD45RB^{hi} CD4$^+$ T cell transfer model[15], in which colitis is dependent on effector T cell expression of intestinal trafficking receptors[16,17]. In the absence of T_{reg} cells, transferred T cells develop into pathogenic effector T cells that home to the colon and induce disease[15]. *Rag2*$^{-/-}$ mice that received naive *Gpr15*–KO T cells were

protected from developing colitis (Fig. 2), consistent with a critical function for GPR15 on pathogenic effector T cell colon trafficking. Inhibition of colitis by T_{reg} cells in this model does not require colon homing[18], and accordingly, GPR15–deficient T_{reg} cells and WT T_{reg} cells prevented or delayed colitis in a comparable manner (Fig. 2). In the acute DSS–induced colitis model, where innate immunity plays a predominant role and colitis is not dependent on T cell colon homing[19,20], as well as the acute TNBS model, *Gpr15*–KO and WT mice were similarly susceptible to intestinal inflammation. (Supplementary Fig. 4). The inefficiency of *Gpr15*–deficient cells to induce colitis in the $CD45RB^{hi}$ transfer model thus reveals a role of GPR15 in the colon trafficking of pathogenic proinflammatory T cells.

Colitis in the naive T cell transfer model depends on the generation of pathogenic IFN–γ- and IL17–producing T_H1 and T_H17 effector $CD4^+$ cells[21-23]. To determine whether a deficiency in generation or localization of such effector cells might underlie or contribute to the reduced ability of KO T cells to induce colitis, we transferred allotype–marked naive *Gpr15*–KO and het $CD4^+$ T cells together into *Rag2*$^{-/-}$ mice and assessed effector T cell presence in recipient tissues 2 weeks later, prior to clinical colitis onset (Supplementary Fig. 5a). Compared to GFP^+ *Gpr15*–KO T cells, GFP^+ *Gpr15*–het (GPR15–expressing) T cells preferentially accumulated in the colon of *Rag2*$^{-/-}$ recipients, consistent with a prominent role for the receptor in effector T cell localization (Fig. 3a). On the other hand, GPR15 deficiency had no significant effect on the frequency of T_H1 and T_H17 effector T cells among the donor populations (Fig. 3b and Supplementary Fig. 5b-d), or on the fraction of proliferating T cells as indicated by the extent of BrdU incorporation (Fig. 3c), or on that of apoptotic $CD4^+$ T cells as indicated by Annexin V staining (data not shown). In related *Rag2*$^{-/-}$ transfer studies (Supplementary Fig. 5e), we found that GFP–expressing subsets of colon–localized *Gpr15*–KO and het derived cells also displayed similar cytokine, Foxp3 and Ki67 expression (Supplementary Fig. 5f). These data suggest that the failure of GPR15–deficient T cells to induce colitis is not due to alterations in T cell activation or production of proinflammatory cytokines.

GPR15 expression on human $CD4^+$ T cells

To determine the relevance of these mouse studies to humans, we assessed GPR15 expression by colon $CD4^+$ T cells in ulcerative colitis (UC; a "T_H2–like" disease in which inflammation is limited to the colon[24]), in Crohn's disease (typically a T_H1 and T_H17 disease[25]), and in colons from non–UC patients that included healthy or normal margins of cecal or colorectal adenocarcinomas. Surprisingly, GPR15 was highly enriched among UC colon T_H2 cells ($IL\text{-}5^+$ or $IL\text{–}13^+$; Fig. 4a). Few IFN–γ^+ or $IL\text{–}17^+$ effector T cells expressed the receptor (Fig. 4a) and, in contrast to the mouse, there was little or no GPR15 expression on T_{reg} cells ($CD127^{lo}$, $Foxp3^+$, $CD4^+$) from either UC or non–UC colons (Fig. 4, Supplementary Fig. 6). T_{reg} cells lacked GPR15 expression whether resting or activated (Supplementary Fig. 6b). Consistent with this differential expression *in vivo*, GPR15 is preferentially induced on *in vitro*–generated human T_H2 effector cells ($IL\text{–}4^+$), but not on T_{reg} cells under conventional polarizing conditions (Fig. 5a; $Foxp3^+$ cells are $CD127^{lo}$; not shown). In contrast, GPR15 expression was observed in mouse cells under T_{reg} and T_H17 cells (Fig. 5b; and see[11]), but not T_H2–polarizing conditions (Fig. 5b). These results

highlight GPR15 expression by pathogenic T cells in colitis, and reveal dramatic differences in the patterns of cell type GPR15 expression in humans versus mice.

Binding of GATA–3 to *GPR15* enhancer region

GATA–3 is the master regulator of T_H2 cell differentiation and cytokine expression[26] and it interacts with Foxp3 to regulate T_H2 versus T_{reg} gene expression[27-29] cell. In an attempt to identify mechanisms underlying T cell subset– and species–specific GPR15 regulation, we analyzed GATA–3 binding at the human and mouse loci using previously generated ChIP–Seq data[30,31] (Fig. 6a,b). We found that GATA–3 bound to a highly conserved site downstream of *GPR15* in humans, but not in mice (site marked "Downstream" in Fig. 6a and b). In contrast, GATA–3 bound to a site upstream of *GPR15* close to *CLDND1* in humans and *Cldn25* in mice (marked "Upstream" in Fig. 6a,b). The significance of these interactions was confirmed using model–based analysis of ChIP–Seq (MACS), which identifies significant numbers of sequence reads indicative of transcription factor binding sites[32]. MACS identifies both the upstream and downstream GATA–3 binding sites in humans with p values of 2.6×10^{-101} and 1.1×10^{-63}, respectively (83 and 111 sequence reads, respectively), but only the upstream *Cldn25–I* associated upstream site was significant in mouse ($p = 6 \times 10^{-37}$ with 80 reads compared to only 9 reads for the downstream site, determined as not significant by MACS). A GATA–3 binding site within the *IL4* CNS2 was identified as significant in both human and mouse (1.7×10^{-85} with 83 reads and 5.1×10^{-33} with 97 reads, respectively). This species–specific binding of GATA–3 to the downstream site was confirmed by ChIP–qPCR, with binding to the upstream site and to the *IL4* CNS2 element remaining constant between human and mouse (Fig. 6c). Consistent with this species–specific binding, consensus GATA–3 sites could be identified at this site in human but not in the mouse (Supplementary Fig. 7). Notably, a tandem GATA–3-STAT–6 motif is conserved in multiple species, but contains an A–to–G nucleotide difference in the mouse (Supplementary Fig. 7), a mutation known to attenuate GATA–3 binding[33] (and TTD and JP, unpublished).

Active chromatin marks characterize this region in the GPR15–expressing ENCODE cell line GM12878 (Supplementary Fig. 7), whereas repressive chromatin modifications are observed in cell lines that do not express *GPR15*. We therefore hypothesized that the downstream site is an enhancer, active in human T_H2 cells. To test this, we performed ChIP–Seq for the active enhancer mark H3K27ac and the transcriptional initiation mark H3K4me3 in *in vitro* polarized human T_H1 and T_H2 cells (Fig. 6a). We found that the site was indeed associated with H3K27ac and more so in T_H2 than T_H1 cells, mirroring the expression of *GPR15*. The upstream site and the *GPR15* gene were also associated with greater amounts of H3K4me3 in T_H2 cells. In contrast, no H3K4me3 is present at the downstream site or at the *GPR15* gene in mouse (Fig. 6b), consistent with the lack of GATA–3 binding. We conclude that GATA–3 exhibits differential binding to an enhancer at the *GPR15* locus between human and mouse and that this contributes to altered patterns of enhancer activation and GPR15 expression in T_H2 cells between the species.

Foxp3 is responsible for the repression of T effector genes in T_{reg} cells[34]. We therefore considered that differential binding of this factor could be responsible for the differential

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expression of GPR15 between mouse and human T_{reg} cells. ChIP–Seq for FOXP3 in human and mouse T_{reg} cells identified significant binding to the *GPR15* enhancer in human but not mouse (Supplementary Fig. 8). In human T_{reg} cells, the *GPR15* enhancer was one of the most significant binding sites, with *p* values between 1.7×10^{-11} and 1.7×10^{-80} and in the top 1% of sites in 4 different experiments. By contrast, in replicate experiments in mouse, the *p* values for Foxp3 binding to the *Gpr15* enhancer were 1.4×10^{-4} and 2.6×10^{-4}, below the default MACS significance threshold of 10^{-5} and only within the top 13% or 23% of all sites with $p < 10^{-3}$. Therefore, the low expression of GPR15 in human T_{reg} cells may reflect efficient binding and repression by FOXP3.

Discussion

GPR15 is an HIV co–receptor and an orphan GPCR with structural homology to known chemokine receptors. Recently, GPR15 was shown to be expressed by colon T_{reg} cells, and to contribute to their homing to and function in the colon. Our studies reveal that GPR15 is also an effector/memory $CD4^+$ T cell receptor with a role in $CD4^+$ T_{EM} cell homing and colitis pathogenesis, whose expression is differentially regulated in $CD4^+$ T cell subsets between humans and mice. We show that GPR15 expression is enriched on human T_H2 colon cells, but in striking contrast to the mouse, most human colon T_{reg} cells (whether in IBD or non–IBD human colons) fail to express GPR15. We identified mutations that disrupt GATA–3 binding and GPR15–enhancer activation in the mouse enhancer region, and described differences in GATA–3 and FOXP3 binding to the enhancer in mice versus humans that may underlie the species–specific regulation of GPR15 on effector versus regulatory T cell subsets. Moreover, ChIP–Seq data indicate that FOXP3, a repressor of T_H2–associated genes in T_{reg} cells, binds with high efficiency to the human but not mouse enhancer in T_{reg} cells, correlating with inhibition of GPR15 expression in human regulatory T cells.

Our results are consistent with, but extend, those of another study[11]. They used transfected naive lymphocytes to show convincingly that GPR15 functions as a colon homing receptor, and demonstrated its role in T_{reg} cell localization and function in the colon as well. Our results confirm the receptor's role in T_{reg} cell localization in the mouse, but also demonstrate a similarly important role in T_{EM} cell homing and accumulation in the large intestine. Although GPR15 participation as a T cell trafficking receptor for the colon is now established, additional roles for the receptor in cell retention or survival are not excluded, nor is its involvement in homing to other epithelial surfaces[35]. Expression of GPR15 on T_{reg}cells was previously shown to be required for transferred T_{reg} cells to prevent mortality and severe colitis associated with *Citrobacter rodentium*[11], a widely accepted model of infectious colitis with properties similar to the colitis caused by human pathogen enteropathogenic *E. coli*. We, however, employed the widely used $CD45RB^{hi}$ $CD4^+$ T cell transfer colitis model that does not require T_{reg} cell homing to the colon for disease control. T_{reg} cell suppression of colitis in the $CD45RB^{hi}$ $CD4^+$ T model does not appear to require gut homing: β7–deficient T_{reg} cells are fully capable of suppressing disease[18], whereas T_{reg} cell suppression of colitis in this model requires T_{reg} cell migration to lymph nodes; CCR7–deficient T_{reg} cells were less efficient at preventing colitis as compared to CCR7–sufficient T_{reg} cells[36]. In contrast, the pathogenic T cells in the $CD45RB^{hi}$ $CD4^+$ T cell transfer model

do require intestinal trafficking receptors for colitogenesis: colitis in this model is inhibited by antibody blockade of the gut homing receptor α4β7 or by genetic deficiency of integrin β[17,37]; and we showed that it is also inhibited by GPR15 deficiency on transferred T cells. β7 integrin deficiency does not alter the course of DSS–induced colitis[19,20], correlating with the lack of effect of GPR15 deficiency, and likely due to the non–critical role of T cells in the acute chemically–induced models. Together, the results highlight the importance of GPR15 expression for T cell localization and function in the colon. GPR15–dependent T cell homing can mediate immune pathogenesis in T effector–cell driven colitis when T_{EM} cell activity is required locally in the colon, but it can contribute to immune regulation in models in which T_{reg} cell responses are important locally in the gut wall. In this context, we note that the CD45RB^{hi} CD4^{+} T cell transfer model has been used to identify and validate antibody inhibitors of intestinal lymphocyte trafficking that have led to effective therapeutics for human colitis[17,38,39].

These considerations may have significant implications for translation of mouse studies to the human. Our data show that patterns of GPR15 expression are quite different in man. In sharp contrast to the mouse, few if any human colon T_{reg} cells isolated from patients with colon cancer (normal adjacent tissue), ulcerative colitis, or Crohn's disease expressed the receptor. The highest frequency of GPR15 expression was found on T_H2 effector T cells, an important pathogenic T_{EM} cell population in the colon of ulcerative colitis patients[24,40,41]. Notably, *in vitro*–generated human, but not mouse T_H2 cells, expressed GPR15. Although it is not feasible to carry out functional studies of the orphan receptor in humans, GPR15 is highly conserved in mammalian species including man. Thus our results suggest that GPR15 may help target pathogenic T_H2 cells to the colon in man, but is probably less important in man than in mouse for the homing and function of regulatory T cells in the gut wall.

We identified an active GPR15 enhancer region with marked differences in sequence and cell type specific transcription factor binding and chromatin marks in human and mouse. The human enhancer comprises canonical GATA–3 binding motifs that are conserved in multiple species but missing in the mouse. GATA–3 plays key roles in T_H2 cell differentiation and cytokine expression[26]. GATA–3 ChIP–Seq data[30,31] and targeted ChIP-qPCR revealed that GATA–3 binds to the 3′ (downstream) *GPR15* enhancer in human but not mouse T_H2 cells, whereas binding to a site upstream at *CLDND1/Cldn25* and to the established *IL4 CNS2* locus is conserved between the two species. The active enhancer mark H3K27ac and the transcriptional initiation mark H3K4me3 in human T_H1 and T_H2 cells confirmed the increased activity of the enhancer and increased expression of *GPR15* in human T_H2 cells. Unlike in the human, H3K4me3 is not observed at *Gpr15* or its enhancer in mouse T_H2 cells. We conclude from these experiments that GATA–3 only binds, and the enhancer is only activated, in human T_H2 cells and not in mouse T_H2 cells.

We also found that Gpr15 expression in T_{reg} cells is species–specific, with expression suppressed in human cells. Potentially explaining this, ChIP–Seq for Foxp3, a transcriptional regulator necessary for repression of T effector cell genes in T_{reg} cells[42,43] demonstrates significant binding to the GPR15 enhancer only in human and not mouse T_{reg} cells. Foxp3 is thought to bind DNA with co–factors, including GATA–3[29], thus the reduced binding of Foxp3 to the Gpr15 enhancer in mouse T_{reg} cells may be due to differences in

GATA motifs and loss of GATA–3 binding. Additional factors are also likely to be involved in the differential expression of GPR15 between human and mouse T cell subsets, including those responsible for the increased expression in mouse T_H17 cells.

Although differences in transcription factor binding between species have been reported[44], to our knowledge, the differential binding of GATA–3 at GPR15 between human and mouse is the first example in which defined differences in transcription factor binding have been associated with species differences in targeting mechanisms of functionally distinct T cell populations, differences expected to result in altered patterns of local (T_H–cell derived) cytokine production and thus mechanisms of pathogenesis. Manipulation of the enhancer sequences in T cells *in vitro* and *in vivo* will be required to elucidate the specific consequences of the species differences in GATA–3 binding shown here.

In conclusion, our studies show that GPR15 can control the colon localization of T effector cells, and we demonstrate its key role in a model of colitis that requires effector cell homing to and function in the gut wall. Our studies also identify significant differences in T_{reg} versus T effector cell expression of the receptor between mice and humans, differences that suggest a predominant proinflammatory role for GPR15 in man. These species differences may fundamentally restrict the relevance of mouse models for the study of T_{reg} trafficking in colitis. Indeed, given its preferential expression by pathogenic effector cells in man, inhibition of GPR15 may present a therapeutic approach to controlling inflammatory T cell recruitment in ulcerative colitis.

Online Methods

Animals

BALB/c, C57BL/6 and B6.SJL mouse strains were purchased from Jackson Laboratory and bred in house. *Gpr15*–GFP mice, generated as described[11], were backcrossed 10 times onto the C57BL/6 and BALB/c backgrounds prior to use in experiments, or for mating with B6.SJL and DO11.10 breeders to obtain CD45.1$^+$ and DO11.10 *Gpr15* Het (*Gpr15*$^{gfp/+}$) and KO (*Gpr15*$^{gfp/gfp}$) mice. *Rag2* constitutive knockout (*Rag2*$^{-/-}$) mice were obtained from Taconic and housed for at least 1 week prior to being used as recipients in colitis studies. Animals were maintained in accordance to National Institutes of Health guidelines and experiments were approved by Stanford University Institutional Animal Care and Use Committee.

Cell Isolation

Spleen, Peyer’s patches, mesenteric and peripheral (including inguinal, brachial and axillary) lymph nodes were mechanically dispersed through a stainless steel 200 μm wire mesh to yield single–cell suspensions in Hank’s buffered salt solution (HBSS; Mediatech, Inc.) containing 2% BCS. Splenocytes were incubated in Red Blood Cell Lysing buffer (Sigma–Aldrich) and the cells washed by centrifugation for 5–10 min at 300 *g*, 4 °C. Intraepithelial and lamina propria lymphocytes were isolated from the small intestine and colon as described previously[45]. After excision of the Peyer’s patches, the intestines were cut into ~0.5 cm pieces and cleansed in HEPES–buffered HBSS containing 2% BCS. To

harvest IEL, the tissues were incubated twice with 1 mM dithiothreitol at 37 °C for 20 min and the supernatant collected. To isolate LP lymphocytes, the tissue pieces were incubated in 2 mM EDTA for 15 min (× 2 rounds) followed by 3 rounds of collagenase–digestion at 37 °C (30–45 min each), and the supernatant was collected, pooled and washed. Centrifugation in a 30/70% percoll step–gradient produced enriched IEL or LP lymphocytes at the interface, and the cells were then washed, counted and stained for flow cytometry analysis.

Antibodies and Fluorescence–activated cytometry

The following antibodies were used for flow cytometry in the mouse studies: CD62L–APC (MEL-14), CD103–PE (M290) (BD Pharmingen); CD25–PE–Cy7 (PC61), Foxp3–PE, Foxp3–FITC, Foxp3–APC (FJK–16s), α4β7–APC (DATK32), CCR9–PE (CW–1.2), and mouse IgG2a K isotype control–PE (eBioscience); CD4–Alexa Fluor 700 (GK1.5), CD45RB–APC–Cy7 (C363-16A), CD44–Pacific Blue (IM7) (Biolegend). For staining human blood and tissues, these antibodies were used: rabbit anti–human GPR15 (Abcam), rabbit IgG isotype control (Sigma–Aldrich) and goat anti–rabbit–AF488 secondary antibody (Invitrogen) and goat anti–mouse AF488 (Invitrogen); IL–13–PE (JES10-5A2), IFN-γ-PE (B27), IL-17A–PE–Cy7 (BL168), CD45RO–APC–Cy7 (UCHL1) and CD25–AF700 (BC96) were purchased from Biolegend; Foxp3–APC (PCH101) from eBioscience; CD4–Pacific Blue (RPA–T4) and IL–5–PE (TRFK5) from BD Biosciences. The mouse anti–human GPR15 MAb (Clone 367902; R&D Systems) and corresponding mouse IgG2B isotype control (R&D Systems) were used only in side-by-side assessment of the GPR15 PAb, as described in below. Where indicated, cells were activated with phorbol myristate acetate (PMA; 50 ng/mL), ionomycin (1 μg/mL) in the presence of Brefeldin A (to prevent cytokine release into media) for 4 h at 37 °C prior to surface staining[46]. Staining of mouse and human Foxp3 and intracellular cytokine antibodies was performed using Foxp3 fixation and permeabilization solutions as recommended (eBioscience). Data was acquired on an LSRII or Fortessa cytometer (BD Biosciences) and analyzed with FlowJo software (FlowJo, LLC).

CD45RB^{hi} CD4^{+} T cell transfer model of colitis

CD4^{+} T cells were enriched from spleen of WT or *Gpr15* KO mice by negative selection using the CD4^{+} T cell Isolation Kit II (Miltenyi Biotec). The bead–enriched CD4^{+} T cells were stained and sorted by flow cytometry using the ARIA II or ARIA III cell sorter (BD Biosciences) to separate naive cells (CD4^{+}, CD45RB^{hi}, CD25^{lo}) and T_{reg} cells (CD4^{+}, CD45RB^{lo}, CD25^{hi}). Cells were washed with sterile HBSS, and 0.5 × 10^{6} naive CD4^{+} T cells alone or with 0.1 × 10^{6} T_{reg} cells were transferred i.v. into each *Rag2*$^{-/-}$ mouse. The mice were weighed weekly and assessed for weight loss, stool consistency, bloody stools and general well being. The mice were euthanized at week 6 (or sooner if moribund or weight loss exceeded 20%). Following euthanasia, spleen, MLN and colon were harvested and colon length and stool consistency assessed. For histologic assessment, the distal–most 1 cm section of colons was fixed in 10% buffered formalin for tissue sectioning and hematoxylin & eosin staining[47]. The histology was assessed and scored in a blinded fashion as previously described[48].

GPR15 expression in human colon samples

Colon resection samples were obtained from patients undergoing ulcerative colitis– or adenocarcinoma–indicated colectomies with approval by the Stanford University Institutional Review Board and obtaining informed patient consents. After removal of the serosa, mesentery fat and muscularis externa, lymphocytes were isolated from the lamina propria as described[49], and the cells activated and stained for flow cytometric analysis.

Chromatin IP and ChIP–Seq data analysis

Human and mouse GATA–3 ChIP–Seq has previously been published[30,31]. Human and mouse H4K4me3 ChIP–Seq data have also been published[50,51]. Mouse GATA–3 and Foxp3 and human Foxp3 ChIP–Seq data were downloaded as fastq files from GEO (accessions listed in Supplementary Table 2), aligned to mm9 or hg18 with bowtie2 (default settings), bigwig files generated for visualization in the UCSC genome browser as described[30] and binding sites identified with MACS (using input DNA as background, if available). ChIP for H3K27ac in *in vitro* polarized human T_H1 and T_H2 cells was performed as described[30] using the antibody ab4729 (Abcam). ChIP for GATA–3 was performed with the D16 antibody (Santa Cruz Biotechnologies, Inc.) in mouse T_H2 cells polarized as described[52]. ChIP for Foxp3 in mouse iT_{reg} cells is described in the Supplementary information. DNA enriched by GATA–3 ChIP was measured by quantitative PCR relative to input DNA and normalized to a control region at the *GPR15* locus that lacked GATA–3 binding. The primers used are listed in Supplementary Table 1.

Mixed bone marrow chimera

Bone marrow was extracted from femur and tibia of *Gpr15*–KO (*Gpr15*$^{gfp/gfp}$; CD45.2) and *Gpr15*–het (*Gpr15*$^{gfp/+}$; CD45.1) mice and red blood cells lysed. Cells were combined in equal ratios and injected into lethally irradiated F1 offspring of C57BL/6 × B6.SJL breeding (CD45.1, CD45.2) to generate experimental KO/Het mixed chimeras. In parallel, cells from *Gpr15*–het (CD45.2) and congenic *Gpr15*–het (CD45.1) mice were similarly transferred to generate Het/Het control chimeras. After 8–12 weeks, tissues from F1 recipients were harvested and prepared for analysis by flow cytometry. For each mouse, the ratio of $CD45.2^+$ $CD4^+$ to $CD45.1^+$ $CD4^+$ T cells recovered from each organ was calculated. Since bone marrow 'take' from different donors can vary between recipients, the CD45.2/CD45.1 ratio of T cells in different organs was normalized to that of $CD19^+$ B cells, and the relative ratio of cells in each tissue was additionally normalized to that of the spleen as described[53].

Stable GPR15 transfectant generation

Murine pre–B cell lymphoma L1.2 cells were transfected (Nucleofector™, Lonza Inc.) with a mammalian expression vector pCMV6–AC–GFP) containing the human *GPR15* cDNA with a C–terminal tGFP tag (Origene). G418–selected transfectants with highest levels of GFP expression were sorted for testing of antibody to human GPR15. The mouse *Gpr15* ORF was subcloned into the pCMV6–AC–GFP vector 3′ of the Kozak consensus sequence to similarly generate stable L1.2 transfectants.

Analysis of rabbit polyclonal antibody to human GPR15

To determine the specificity of the polyclonal GPR15 antibody (Abcam) used throughout the study, L1.2 transfectants (1×10^6 cells) of human (or mouse) *GPR15* were incubated with 0.5 or 1 μg of the GPR15 polyclonal antibody for 30 min, followed by 30 min with PE–donkey anti–rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.; Supplementary Fig. 9a). The specific staining of the polyclonal antibody was also tested side–by–side with a monoclonal antibody to human GPR15 (Clone 367902; R&D Systems) on freshly isolated human colon cells (Supplementary Fig. 9b,c), and the two antibodies produced similar staining.

DSS–induced colitis

Two months old gender–matched *Gpr15*–KO and wild–type BALB/c mice were given drinking water containing 4.5% DSS (w/v; dextran sulfate sodium salt, 36,000-50,000 Da; MP Biomedicals) beginning day 0 (ref.[54]). The mice were weighed daily and assessed for the presence of bloody stool and general well being. They were euthanized on day 9.

TNBS–induced colitis

Two months old gender–matched *Gpr15*–KO and wild–type BALB/c mice were rectally instilled with 2 mg of TNBS (2,4,6–Trinitrobenzenesulfonic acid solution; Sigma–Aldrich) in 100 μL of 40% ethanol on day 0 (ref.[54]). The mice were assessed daily for weight loss, bloody stool and general well being, and they were euthanized on day 4.

Co–transfer of *Gpr15* KO and WT naive $CD4^+$ T cells into *Rag2*$^{-/-}$ mice

Similar to the cell transfer model of colitis, splenocytes from *Gpr15*$^{gfp/gfp}$ (*Gpr15*–KO; CD45.2) or *Gpr15*$^{gfp/+}$ (*Gpr15*–het; CD45.1, CD45.2) mice were sorted by flow cytometry to enrich naive $CD4^+$ T cells of >90% purity. The cells were combined at a 1:1 ratio, and a total of 1×10^6 cells were i.v. injected into each *Rag2*$^{-/-}$ recipient. After 2 weeks, lymphocytes from MLN, spleen and colon were isolated, and cells activated for 4 h prior to staining with surface markers and intracellular cytokines. Where indicated, GFP^+ $CD4^+$ T cells were sorted from pooled MLN or colon, activated and stained to assess GPR15 expression among effector/memory $CD4^+$ T cells. For data analysis, normalization to spleen is used to control for variability between mice in injected cell numbers. The spleen lacks high endothelial venules, and unlike the other tissues examined, it is empirically less affected by homing or trafficking effects, making it a preferable (and commonly employed) internal reference tissue.

In vitro–polarized T helper cells

Naive $CD62–L^+$ $CD4^+$ T cells were enriched (Miltenyi Biotec) from human buffy coats and seeded into 96–well plates (0.2×10^6 cells/well, pre–coated with 1 μg/mL anti–CD3 antibody; clone HIT3a). To generate T_H1 cells, IFN–γ (10 ng/mL) and recombinant IL–12 (5 ng/mL) were added. To polarize T_H2 cells, IL–4 (10 ng/mL) and anti–IL–12 antibody (10 μg/mL) were used; for T_H17 cells, IL–1β, IL–23, IL–6, TGF–β (10 ng/mL each) and 10 μg/mL of anti–IFN–γ and anti–IL–4 antibodies; for T_{reg}cells, 5 ng/mL TGF–β. Cells were cultured for 5–6 days in the presence of IL–2 (10 ng/mL) and soluble anti–CD28 (1 μg/mL;

clone CD28.2). Cells were washed, activated for 4 hours and stained with surface and intracellular markers as described above. Mouse cells from spleen of *Gpr15*$^{gfp/+}$ mice were subjected to similar polarizing conditions as described previously[55-57]. Cytokines and antibodies were purchased from Peprotech Inc. or eBioscience.

Competitive short–term homing of effector CD4$^+$ T cells

Bead–enriched CD4$^+$ T cells (MACS kit; Miltenyi Biotec) from spleen of allotype–marked *Gpr15*–het and KO mice were cultured under T_H17–polarizing conditions using plate bound anti–CD3 mAb (1 μg/mL; clone 145–2C11), soluble anti–CD28 mAb (1 μg/mL; clone 37.51), rmIL–2 (20 ng/mL), rmIL–6 (20 ng/mL), rhTGF–β1 (5 ng/mL) and retinoic acid (100 pM) as previously described[11]. After 3 days, cells were washed and i.v. injected into normal hosts. After 8–10 h, host lymphocytes were assessed for presence of donor cells.

Antigen–specific T cell accumulation

Splenocytes (25 × 10^6) from DO11.10, *Gpr15*–hetor DO11.10, *Gpr15*–KO were i.v. injected into Balb/c (Thy–1.1) mice. After 24 h, anesthetized recipients were treated intra–rectally with ovalbumin from chicken egg white (500 μg; Sigma–Aldrich) and cholera toxin (10 μg; Sigma–Aldrich) in a 150 μL volume. At day 5–post treatment, recipient tissues were collected and lymphocytes prepared for analysis by flow cytometry.

Realtime–PCR of human colon cells

Quantitative PCR was performed on sorted human colon lymphocytes from 1 UC and 2 non–UC samples to verify *GPR15* and *FOXP3* transcript levels in GPR15$^+$ (Abcam) cells. GPR15$^+$ subset (CD4$^+$, CD45RO$^+$, GPR15^{hi}) contained <10% CD127^{lo}, CD25^{hi} cells CD4$^+$ T cells. Total RNA was prepared using RNeasy Mini kit (Qiagen), and cDNA using GoScript reverse transcription system (Promega). Quantitative PCR was performed with SYBR Green PCR Master Mix and ABI–StepOnePlus Real–Time PCR system (Applied Biosystems) using commercially available primer sets (GPR15, VHPS–3771; FOXP3, VHPS–3398; IL2RA, VHPS–4553; GAPDH, VHPS–3541; RealTimePrimers.com).

Realtime–PCR of ChIP DNA

The enrichment of DNA by GATA–3 ChIP was quantified relative to input DNA using primers listed in Supplementary Table 1.

Blockade of lymphocyte egress with FTY720

To determine whether GPR15 is induced in the MLN, CFSE–stained (1 μM; Life Technologies) lymphocytes from *Gpr15*–het DO11 (Thy–1.1, Thy–1.2) and KO DO11 (Thy–1.2) spleens were i.v. injected in equal proportion (15 × 10^6 total cells) into Thy–1.1 Balb/c mice. After 24 h, mice were treated intrarectally with OVA (500 μg) and CT (10 μg) mixture. At indicated time, mice were i.p. injected with FTY720 or vehicle (PBS) and their tissues collected. Stock solutions of FTY720 (Sigma–Aldrich; 10 mg/mL ethanol) were diluted with PBS to 100 μg/mL, and i.p. injected at 1 mg/kg.

Comparative sequence analyses

We analyzed a highly conserved non–coding region downstream of mouse, chimpanzee, monkey, rat and human *GPR15*. The conserved region is located ~23kb and 15kb 3′ of *GPR15* in human and mouse, respectively. The human sequence is within a region (chr3:98,272,711–98,276,110 in Hg19) highlighted in the ENCODE/BROAD histone modification track (UCSC genome browser) as an active promoter region in the *GPR15*–expressing B lymphoblastoid cell line GM12878. DNAase hypersensitivity marks from human T_H2 cells, as well as histone modification comparisons with GM12878 and non *GPR15*–expressing H1–hESC embryonic stem cells, HepG2 hepatocyte line, HUVEC umbilical vein endothelial cells, HMEC microvascular endothelial cells, HSMM smooth muscle myoblasts, NHEK epidermal keratinocytes and NHLF lung fibroblasts were from the UCSC genome browser and the ENCODE project consortium. To verify the A–to–G mutation in the mouse GATA–3 consensus sequence adjacent to the conserved STAT6 binding motif, we surveyed mouse SNP databases (http://www.sanger.ac.uk/sanger/Mouse_SnpViewer/rel-1303) and found no variation in this nucleotide, indicating that it is a species– (not strain–) specific variant.

Foxp3 ChIP–Seq in mouse iT_{reg} cells

$CD4^+$ T cells from spleens and lymph nodes of 4– to 10–wk–old mice were purified by CD4 positive selection (Miltenyi Biotec) followed by sorting of naive $CD4^+$, $CD25^-$, $CD62L^{hi}$, $CD44^{lo}$ cells using a FACSAria II (BD Biosciences). Cells were activated by plate–bound anti–CD3 and anti–CD28 (both 10 μg/ml; clones 145–2C11 and 37.51, respectively; Bio X Cell). iT_{reg} cells were generated by culturing in recombinant human TGF–β1 (33 ng/ml) and IL–2 (20 ng/ml; R&D Systems) for 7 days. Following activation, cross–linking, and sonication[30], Foxp3–bound genomic DNA was isolated from whole cell lysate using a mix two antibodies (5 μg each); Santa Cruz S–31738 and eBioscience FJK–16. Libraries were constructed and sequenced as described[30].

Statistical analysis

Prism software (GraphPad Software) was used for statistical analyses and statistical tests employed are indicated in the figure legends, with p values of <0.05 considered as significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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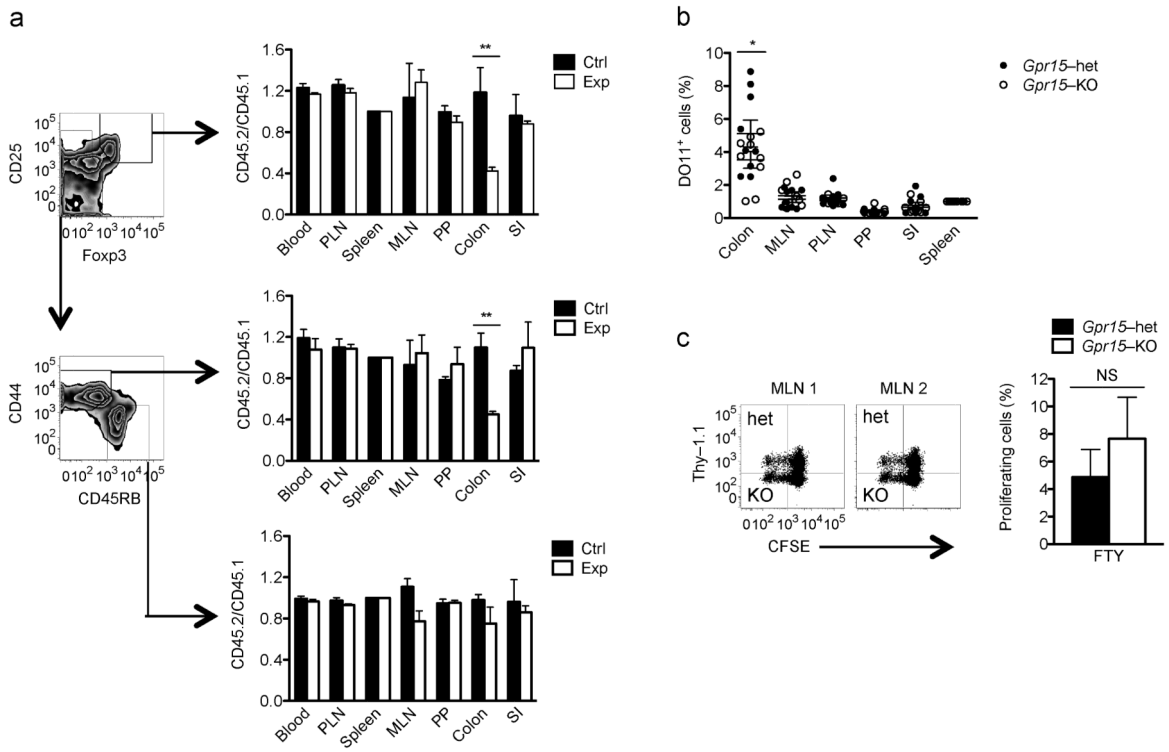

Figure 1.

GPR15 mediates accumulation of T_{EM} and T_{reg} cells in the colon. **(a)** *Gpr15*–KO (CD45.2) or het (CD45.2) bone marrow cells were combined with *Gpr15*-het (CD45.1) cells and transferred into irradiated hosts (CD45.1 × CD45.2 F1) to generate experimental (Exp) or control (Ctrl) chimeras, respectively, as in Supplementary Figure 2a. Ratios of *Gpr15*–KO to *Gpr15*–het derived cells (CD45.2/CD45.1) among T_{reg} cells ($CD4^+$, $CD25^{hi}$, $Foxp3^+$; top), T_{reg}–excluded T_{EM} cells ($CD4^+$, $Foxp3^-$, $CD44^{hi}$, $CD45RB^{lo}$; middle), and naive $CD4^+$ T cells ($CD4^+$, $Foxp3^-$, $CD44^{lo}$, $CD45RB^{hi}$; bottom) in Exp mixed bone marrow chimeras (white bars); ratios of *Gpr15*- het to het cells (CD45.2/CD45.1) in control chimeras (black bars). Peripheral lymph nodes (PLN), mesenteric lymph node (MLN), Peyer's patches (PP), small intestine (SI). $n = 3$, mean + SEM, $^{**}p < 0.01$ (2–way ANOVA, Bonferonni posttest). Representative of 3 experiments. **(b)** Splenocytes from DO11.10 *Gpr15*–het or DO11.10 *Gpr15*–KO were i.v. injected into Balb/c (Thy–1.1) mice. After 24 h, recipients were treated intra-rectally with ovalbumin (OVA) and cholera toxin (CT). Accumulation of OVA-specific $CD4^+$ T cells in tissues was assessed 5 days later, as in Supplementary Figure 2c. The percent antigen–specific ($DO11^+$) of *Gpr15*-het or KO $CD4^+$ T_{EM} cells ($CD44^{hi}$, $CD45RB^{lo}$) 5 days after antigen exposure was normalized to the ratio in the spleen. Data from 9 mice from 3 independent experiments are shown. Mean ± SEM, $^{*}p < 0.05$ (2–way ANOVA, Bonferroni posttest). **(c)** Similar proliferation rate of antigen–reactive *Gpr15*–het and *Gpr15*–KO cells. CFSE–labeled splenocytes from *Gpr15*–het (Thy–1.1, Thy–1.2) and KO (Thy–1.2) DO11 mice were co–injected i.v. in equal proportion into congenic non–DO11 hosts (Thy–1.1) on day 0. Recipients were treated with OVA and CT per rectum on day 1, FTY720 or PBS i.p on day 3, and tissues were collected on day 5, as in

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Supplementary Figure 2e. **(d)** Recipient MLN gated on donor $DO11^+$ $CD4^+$ T cells. $n = 3$; mean + SEM. Not significant (NS).

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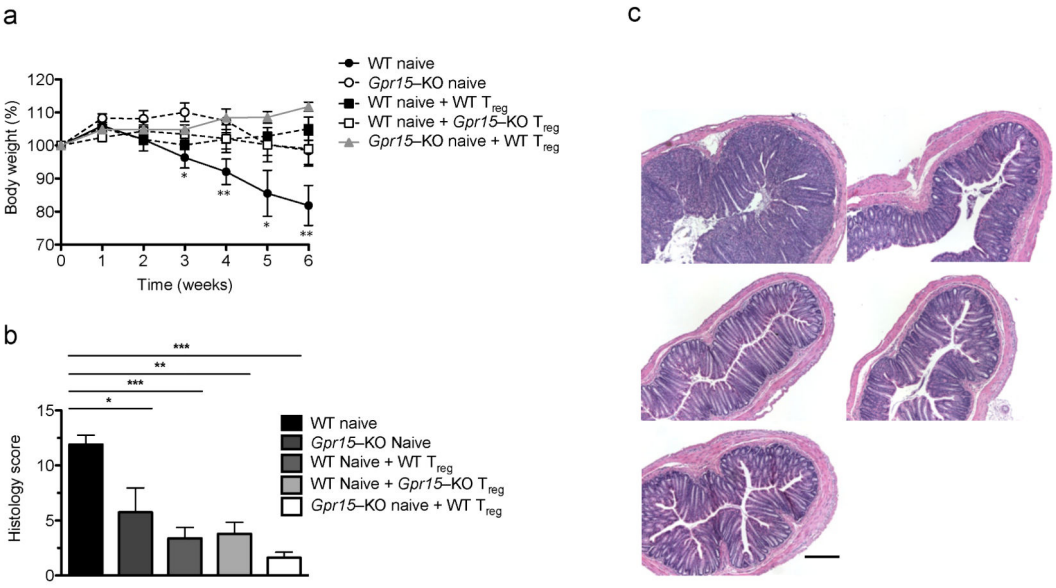

Figure 2.

GPR15–deficient naive T cells fail to induce colitis in the CD45RB^{hi} CD4^{+} T cell transfer model. **(a)** Weight loss of *Rag2*$^{-/-}$ mice that received either WT naive CD4^{+} T cells, *Gpr15*–KO naive CD4^{+} T cells, WT naive plus WT T_{reg} cells, WT naive plus KO T_{reg} cells, or KO naive plus WT T_{reg} cells. $n = 5$, mean + SEM, *p <0.05, **p <0.01 between WT naive (disease control) and *Gpr15* KO naive (2-way ANOVA, Bonferroni posttest). Representative of 3 experiments. **(b)** Clinical score for histological inflammation in colon. *p <0.05, **p < 0.01, ***p <0.001 (1–way ANOVA, Tukey's posttest). **(c)** H&E staining of distal colon section: WT naive (top left), *Gpr15*–KO naive (top right), WT naive + WT T_{reg} (middle left), WT naive + *Gpr15*–KO T_{reg} (middle right) and *Gpr15*–KO naive and WT T_{reg} (bottom). Scale bar denotes 10 μm.

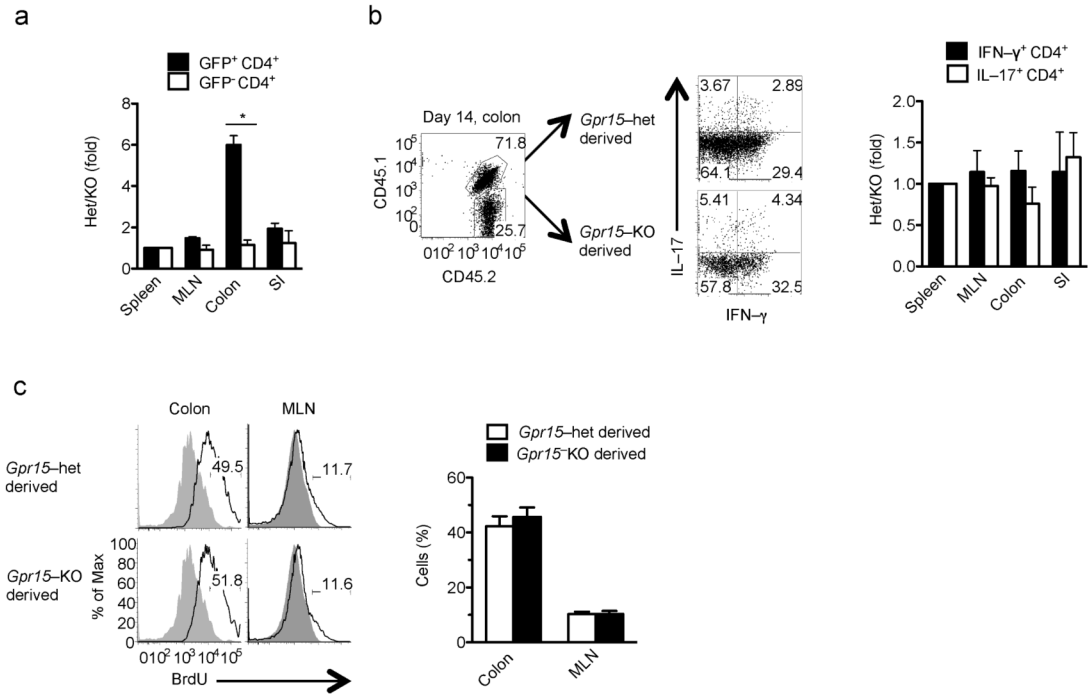

Figure 3.

GPR15 deficiency inhibits effector T cell accumulation in the colon, but not T_H1 or T_H17 differentiation. Naive CD45RB^{hi} CD4$^+$ T cells sorted from *Gpr15*–KO (CD45.2) or het (CD45.1, CD45.2) mice were co–injected into *Rag2*$^{-/-}$ recipients in equal proportion, as in Supplementary Figure 5a. **(a)** Ratios *Gpr15*–het to *Gpr15*–KO GFP$^+$ or GFP$^-$ CD4$^+$ T cells in recipient tissues after 2 weeks, normalized to those in the spleen. $n = 5$, mean + SEM, $^*p < 0.001$. (2–way ANOVA, Bonferroni posttest). **(b)** Similar percentage of *Gpr15*–het- and KO-derived IFN-γ and IL–17$^+$ CD4$^+$ T cells in recipient colon after 2 weeks (left). Ratio of *Gpr15*– het- to KO-derived IFN–γ$^+$ or IL–17$^+$ CD4$^+$ T cells, normalized to that of the spleen (right). Representative of 2 experiments. **(c)** BrdU incorporation 2 weeks after cell transfer as in (a); baseline (grey shade). $n = 3$; mean + SEM.

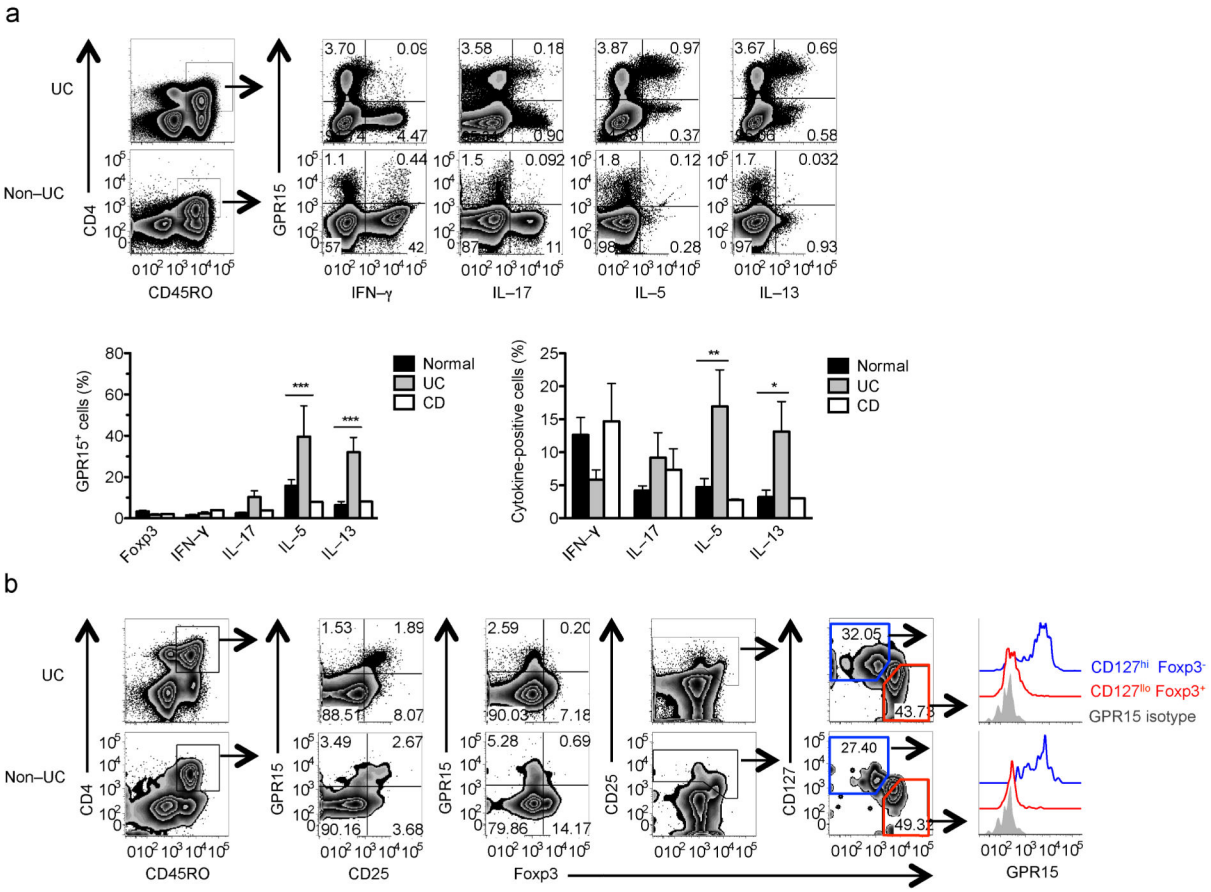

Figure 4.

Human proinflammatory CD4$^+$ T cells express GPR15 in the colon. **(a)** Expression of GPR15 on cytokine–producing CD45RO$^+$CD4$^+$ T cells from ulcerative colitis (UC) or non–UC colons (top). In UC colons, GPR15 is expressed on IL–5$^+$ and IL–13$^+$ CD45RO$^+$CD4$^+$ T cells (bottom). n = 5 UC specimens, 11 Non–UC or normal samples, and 2 Crohn's disease (CD) colons, which showed similar cytokine expression among GPR15$^+$ cells as normal samples. Mean + SEM; *p <0.05, **p < 0.01, ***p <0.001 (2–way ANOVA, Bonferroni posttest). Statistics not determined for CD samples. **(b)** Lower expression of GPR15 on T_{reg} cells (CD127^{lo}, Foxp3$^+$; red histogram) as compared to non–T memory CD4$^+$ T_{reg}, cells (CD127^{hi}, Foxp3$^-$; blue).

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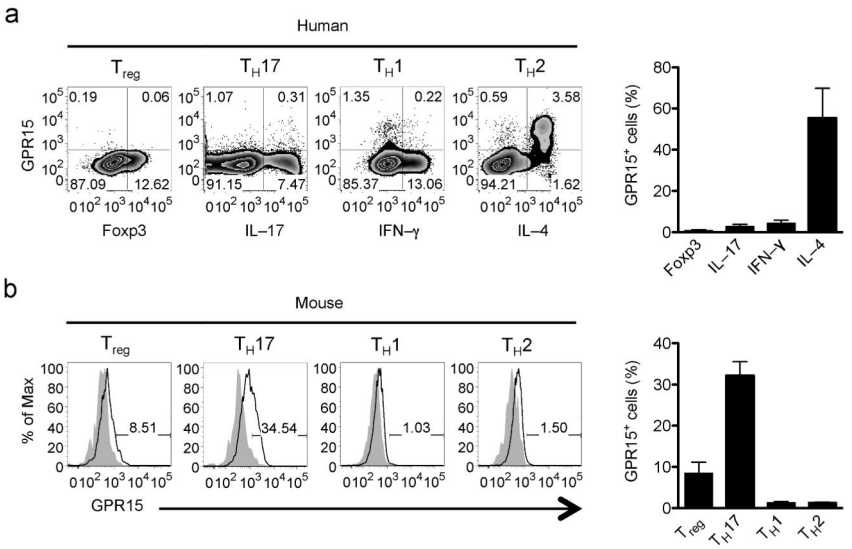

Figure 5.

Distinct patterns of GPR15 expression by *in vitro*–polarized human and mouse T cells. Naive $CD4^+$ T cells enriched from human PBMC **(a)** or mouse *Gpr15*–het spleen **(b)** were polarized as described in the online methods. GPR15 is expressed on human but not mouse *in vitro*–polarized T_H2 cells. Human cells are gated on $CD45RO^+$ $CD4^+$ T cells, and mouse cells are gated on $CD44^{hi}$ $CD4^+$ T cells. Combined results of 3 mouse and 4 human experiments; mean + SEM.

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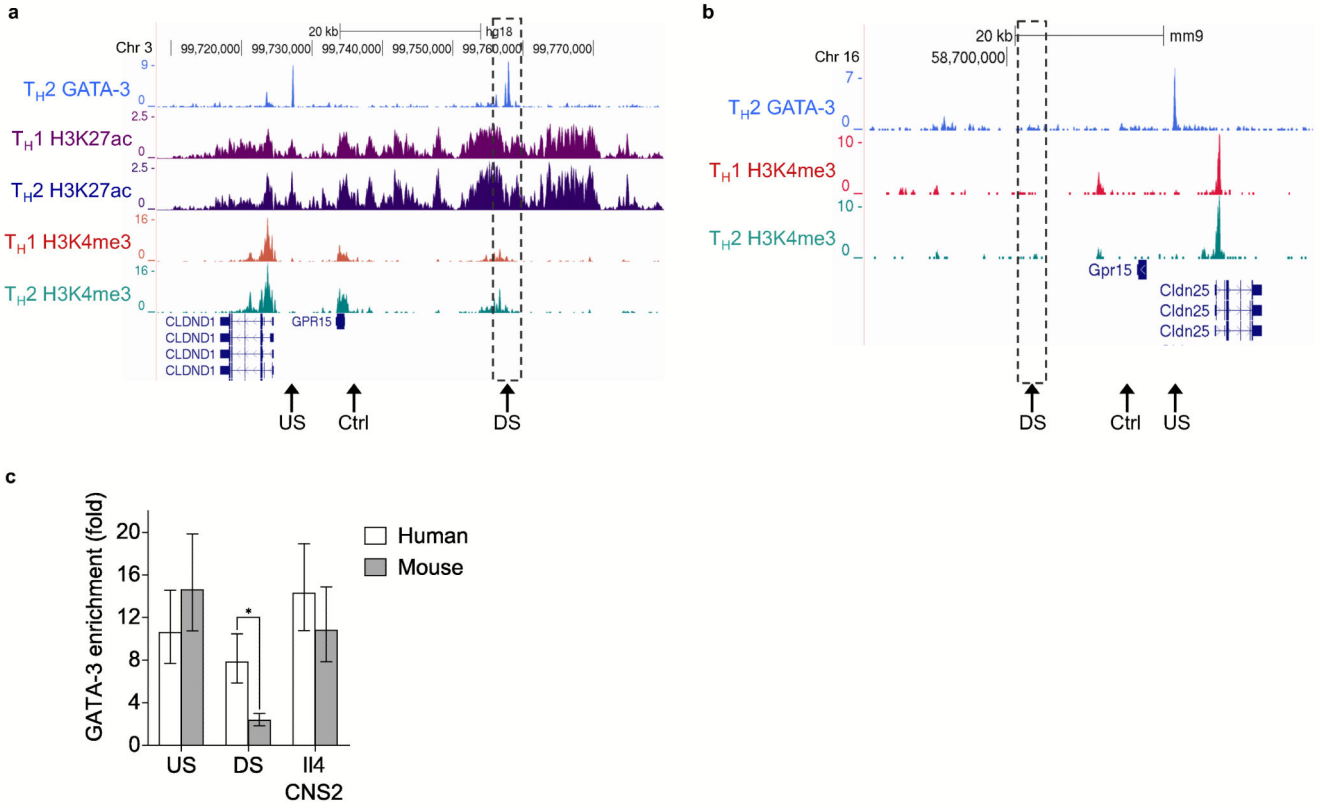

Figure 6.

Differential binding of GATA–3 to human and mouse *Gpr15* enhancer sequences. **(a)** ChIP–Seq data showing binding of GATA–3 at the human *GPR15* locus in T_H2 cells. The number of sequencing reads from ChIP–enriched DNA are plotted per million background–subtracted total reads and aligned with the Hg18 build of the human genome. Association with H3K4me3 and H3K27ac in human T_H1 and T_H2 cells are shown below. The position of the upstream enhancer is marked with a dashed box. The positions of qPCR primers used in (c) are marked by arrows. **(b)** As (a), except for Gata–3 and H3K4me3 at the mouse *Gpr15* locus (Mm9 build). **(c)** Enrichment of DNA corresponding to the human and mouse upstream and downstream GATA–3 binding sites marked in (a) and (b), relative to input DNA and a control non–bound region (also marked in (a) and (b)), measured by ChIP–qPCR. Mean and SD from triplicate wells from one ChIP per species. *$p < 0.05$, unpaired Student's *t*–test.

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