Sphingosine-1-phosphate receptor-1 (S1P₁) is expressed by lymphocytes, dendritic cells, and endothelium and modulated during inflammatory bowel disease

Thangaraj Karuppuchamy[1,2], **En-hui Behrens**[1,2], **Pedro González-Cabrera**[3], **Gor Sarkisyan**[3], **Lauren Gima**[1,2], **Joshua D. Boyer**[1,2], **Giorgos Bamias**[4], **Paul Jedlicka**[5], **Marisol Veny**[1,2], **David Clark**[1,2], **Robert Peach**[6], **Fiona Scott**[6], **Hugh Rosen**[3], and **Jesús Rivera-Nieves**[1,2]

[1]Inflammatory Bowel Disease Center, Division of Gastroenterology, University of California San Diego, La Jolla, CA

[2]VA San Diego Healthcare System, San Diego, CA

[3]Department of Chemical Physiology, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, CA

[4]Academic Department of Gastroenterology, Laikon Hospital, Athens, Greece

[5]Department of Pathology, University of Colorado Health Sciences, Aurora, CO

[6]Receptos Inc. 3033 Science Park Road, La Jolla, CA

Abstract

The sphingosine-1-phosphate receptor-1 (S1P₁) agonist ozanimod ameliorates ulcerative colitis, yet its mechanism of action is unknown. Here we examine the cell subsets that express S1P₁ in intestine using S1P₁-eGFP mice, the regulation of S1P₁ expression in lymphocytes after administration of DSS, after colitis induced by transfer of CD4⁺CD45RBʰⁱ cells and by crossing a mouse with TNF-driven ileitis with S1P₁-eGFP mice. We then assayed the expression of enzymes that regulate intestinal S1P levels, and the effect of FTY720 on lymphocyte behavior and S1P₁ expression. We found that not only T and B cells express S1P₁, but also dendritic (DC) and endothelial cells. Furthermore, chronic but not acute inflammatory signals increased S1P₁ expression, while the enzymes that control tissue S1P levels in mice and humans with IBD were uniformly dysregulated, favoring synthesis over degradation. Finally, we observed that FTY720 reduced T cell velocity and induced S1P₁ degradation and retention of naïve but not effector T cells. Our data demonstrate that chronic inflammation modulates S1P₁ expression and tissue S1P levels and suggests that the anti-inflammatory properties of S1PR agonists might not be solely due

Correspondence: Jesús Rivera-Nieves M.D., Inflammatory Bowel Disease Center, Division of Gastroenterology, University of California San Diego, 9500 Gilman Drive Bldg. BRF-II Rm. 4A32, San Diego, California 92093-0063, Office: 858.534.5495, Fax: 858.246.1788, jriveranieves@ucsd.edu.

to their lymphopenic effects, but also due to potential effects on DC migration and vascular barrier function.

INTRODUCTION

Antibodies that target lymphocyte traffic (i.e. natalizumab, vedolizumab) are effective therapeutics for IBD[1–3], yet they are expensive to produce and administer. Novel drugs that may be administered orally have the potential to become widely used alternatives. A recent trial of the selective $S1P_1$ agonist (ozanimod) in patients with ulcerative colitis (UC)[4] has met all primary and secondary endpoints, while a new phase 2 trial in patients with Crohn's will soon begin recruiting subjects. Yet, little is known regarding the potential mechanisms of action of this drug, or its cellular targets in the intestine, either in UC or Crohn's.

S1P is a pleiotropic sphingolipid metabolite with diverse physiological and immunological functions.[5, 6] The concentration gradient of S1P between tissues (low) and blood (high) regulates $S1P_1$-mediated lymphocyte egress from thymus and lymph nodes to circulation.[7] S1P signals through five G-protein-coupled receptors ($S1P_{1-5}$).[8, 9] $S1P_1$, originally known as endothelial differentiation gene-1 (Edg-1) inhibits angiogenic sprouting and enhances cell-to-cell adhesion by regulating VE-cadherin at endothelial junctions during embryogenesis.[10] In adult vertebrates, it regulates vascular and lymphatic permeability, astrocyte proliferation, neuronal protection[11], lymphocyte egress and marginal B cell migration in secondary lymphoid organs[12, 13] heart rate[14], endothelial integrity[15] and ischemia-reperfusion injury.[16] Thus, there is a vast array of possibilities for the mechanisms of action of compounds that bind and signal through this receptor.

Herein we used preclinical IBD models and intestinal biopsies from patients with IBD to investigate the role of the S1P pathway on the pathogenesis of the disease. First, we analyzed the expression of $S1P_1$ on cells isolated from the intestine and mesenteric lymph nodes (MLN) and assessed the modulation of $S1P_1$ under conditions of acute and chronic inflammation. Second, we analyzed the expression of key enzymes that regulate S1P levels in mouse models and in patients with IBD. Finally, we assessed the effect of the non-selective agonist FTY720 on lymphocyte behavior and $S1P_1$ expression.

RESULTS

Lymphocytes, dendritic and endothelial cells (EC) of intestinal lamina propria and MLN express $S1P_1$

To identify the cellular targets for $S1P_1$-selective agonists we assessed the surface expression of $S1P_1$ on T and EC using commercial antibodies. $S1P_1$ expression was not different, compared with isotype on freshly isolated cells (Fig. S1) or after culture for 24 hours in FBS/S1P-free media to allow receptor resensitization (data not shown). We then assessed $S1P_1$ expression on cells from intestinal LP and MLN isolated from $S1P_1$-eGFP mice[17]. $S1P_1$ signal was readily observed on CD4, CD8, B cells, DC and EC ($CD45^{neg}CD31^+$). Cells from C57BL6/J mice (without eGFP: $eGFP^{neg}$) served as controls (Fig. 1A–D). Immunohistochemistry confirmed the expression of $S1P_1$ on intestinal EC (which exhibited

the strongest signal), within the villous microvasculature and submucosal vessels (Fig. 1D1). The dimmer signal of T cells and DC was not visible under the same conditions. High endothelial venules (HEV) and lymphatics in the periphery of B cell follicles of MLN showed the strongest signal (Fig. 1D2), whereas the signal from lymphocytes was undetectable. Overall, the observed expression pattern was consistent with a role of $S1P_1$ on the regulation of key cellular elements known to control such as cell traffic to and from the intestine, gastrointestinal associated lymphoid tissue and peripheral circulation.

Naïve and central memory CD8$^+$T cells and subsets of effector T cells from intestine and MLN predominantly express $S1P_1$

Naive ($CD44^{neg}CD62L^+$) and CM ($CD44^+CD62L^+$) CD4$^+$T cells in ileal LP and MLN showed higher median fluorescent intensity (MFI) for $S1P_1$ compared with effector ($CD44^+CD62L^-$) CD4$^+$T cells. Similarly, naïve and CM CD8$^+$T cells showed higher $S1P_1$ MFI than effectors. However, CD8$^+$T cells from LP and MLN showed higher $S1P_1$ MFI than CD4$^+$T cells isolated from the same tissue (Fig. 2A–C). In line with these *ex vivo* findings, we observed reduced $S1P_1$ expression in splenocytes from $S1P_1$-eGFP mice following *in vitro* activation with PMA/ionomycin or antibodies against CD3/CD28 (Fig. S2). Thus, our results show that naïve and CM CD8$^+$T cells isolated from MLN exhibit the highest expression of $S1P_1$.

Activated mucosal dendritic cells show high $S1P_1$ expression

We then examined $S1P_1$ expression on immature and activated DC subsets, based on their co-stimulatory molecule expression (i.e. CD40, CD80, CD86) (Fig. 2D–F). Activated DCs ($CD40^+$, $CD80^+$, $CD86^+$) in ileal LP and MLN of WT/$S1P_1$-eGFP mice showed significantly higher MFI for $S1P_1$, compared with those immature DC with low costimulatory molecule expression. These results demonstrate that the state of DC maturation correlates with the expression of $S1P_1$.

Subset of CD4 from MLN coexpresses high levels of $S1P_1$ and gut homing molecules

Effector T cells that have the ability to recirculate are critical for the maintenance of intestinal inflammation in IBD.[18] As gut tropism is mediated through the expression of intestinal-selective cell adhesion molecules, we hypothesized that if $S1P_1$ participates in the recruitment of pro-inflammatory, effector T cells, then it should be co-expressed with these gut-specific factors. To test our hypothesis, we analyzed $S1P_1$ expression on effector ($CD62L^{neg}CD44^+$) CD4 and CD8 subsets that expressed the critical gut homing molecules CCR9 and integrin β7. We found no significant differences in expression of $S1P_1$ on either CD4 or CD8 effectors that were localized within the ileal LP. However, in the MLN the CD4 subset with the highest expression of integrin β7 and CCR9 also showed the highest expression for $S1P_1$ (Fig. S3). In contrast, only a much smaller percentage of the CD8 within the MLN coexpressed high levels of $S1P_1$, integrin β7 and/or CCR9. This suggests that there might be differences in the recirculation potential of CD4 and CD8 effectors.

Acute inflammatory signals did not modulate S1P$_1$ expression on T cells

We induced colitis in S1P$_1$-eGFP mice by the administration of DSS in drinking water. Colitis was confirmed by weight loss, shortening of colon length at necropsy and histologically evident inflammation after 7 days (Fig. 3A–D). We did not observe significant differences in S1P$_1$ expression between CD4$^+$T and CD8$^+$T lymphocytes isolated from the colon, MLN or blood of colitic animals compared with untreated controls (Fig. 3E, F). Thus, S1P$_1$ expression on T cells is not readily responsive to acute inflammatory signals.

Surface expression of S1P$_1$ on mucosal effector CD4$^+$T cells is increased in mice with chronic colitis

Colitis was induced by transfer of Treg-depleted CD4$^+$CD45RB^{hi} T cells, isolated from S1P$_1$-eGFP mice into Rag1$^{-/-}$ mice. Controls were co-transferred with regulatory T cell-enriched CD45RB^{lo} cells. The development of colitis was indicated by weight loss at 3 weeks after cell transfer and confirmed by significant changes in the histological scores of mice transferred with CD4$^+$CD45RB^{hi} cells compared with controls (Fig. 4A–C). The frequency of CD4$^+$T cells increased in colonic LP, MLN and blood of mice with chronic colitis (Fig. 4D, E) which showed higher MFI for S1P$_1$ in all lymphoid compartments, compared with those co-transferred with CD4$^+$CD45RB^{lo} cells (Fig. 4F). Thus, unlike acute DSS colitis, S1P$_1$ expression on effector T cells is modulated by chronic inflammatory signals.

S1P$_1$ expression increased on T cells isolated from mice with chronic ileitis

To further examine the role of chronic inflammation on S1P$_1$ expression in mice with an intact immune system, we generated TNF ARE/S1P$_1$-eGFP mice (referred to as TNF ARE) by crossing heterozygous TNF ARE (TNF $^{ARE/+}$/S1P$_1$$^{wt/wt}$) with S1P$_1$$^{eGFP/eGFP}$ (referred to as WT) mice. The intensity of S1P$_1$ expression was higher only on naïve CD4$^+$T cells and naïve and central memory CD8$^+$T cells isolated from the ileal LP of TNF ARE/S1P$_1$-eGFP mice. By contrast in the MLN all CD8 subsets (naïve, effector, CM) as well as naive and effector CD4$^+$T cells showed significantly higher MFI for S1P$_1$ compared with uninflamed mice (Fig. 5A, B). There was a significant increase in the number of CD11c^{hi}/MHCII^{hi} DC in ileal LP, MLN and spleen (***$p < 0.001$; *$p < 0.05$ and *$p < 0.05$, respectively) of TNF ARE/S1P$_1$-eGFP mice compared with WT mice (Fig. 5C) and the expression of S1P$_1$ was significantly higher in DC from all compartments compared with those of uninflamed WT counterparts.

Endothelial S1P$_1$ expression was also increased in TNF ARE mice, where there were numerous S1P$_1$-expressing microvessels throughout the intestinal submucosa and muscularis (Fig. 5D). Increased S1P$_1$ eGFP signal likely reflects the altered vascular density in the inflamed ileal mucosa of TNF ARE mice (Video S1, S2) and suggest that S1P$_1$ is modulated by chronic inflammatory signals not only on T cells, but also on EC.

The enzymatic pathways that control tissue S1P levels are similarly dysregulated in intestinal tissues from mouse models and human IBD

We then compared the mRNA expression of critical enzymes that phosphorylate sphingosine (kinases: Sphk1, Sphk2), dephosphorylate S1P (phosphatases: Sgpp1, Sgpp2), degrade S1P

(lyase, Sgpl1), as well as of the transporter that transfers S1P from intracellular to extracellular compartments (Spns2) in uninflamed and inflamed intestine from mice and humans. We observed a uniform pattern of dysregulation, in which mRNA expression of inducible sphingosine kinase-1 was upregulated, while kinase-2 was downregulated. Both phosphatases were often downregulated, while mRNA for the transporter was increased in most preclinical models and human IBD (Fig. 6). This pattern of dysregulation suggests alterations in the S1P gradient between intestine, lymph nodes and blood, which may serve as a retention signal within intestine.

FTY720 decreases the velocity of MLN T cells and differentially alters the proportion of circulating lymphocytes in ileitic mice

To examine the effects of S1PR agonists on lymphocyte behavior within MLN, we imaged MLN explants of TNF ARE mice that had received T cells from DsRed mice, before and after administration of FTY720-P. T cell velocity decreased after addition of FTY720-P within MLN (Fig. 7A) and the directionality of the cell movement was also altered.

TNF ARE/$S1P_1$-eGFP mice treated with FTY720 for 6 weeks exhibited peripheral lymphopenia, with lower number of blood $CD4^+$T cells (**$p<0.01$), $CD8^+$T cells (**$p<0.01$) and $B220^+$ cells (***$p<0.001$) compared with vehicle-treated controls (data not shown). The lymphopenic effect was greater on circulating naïve and central memory CD4 and CD8 than on effectors (Fig. 7B). In addition, the MFI for $S1P_1$ on $CD4^+$T cells; $CD8^+$T cells and $B220^+$ and endothelial cells were significantly lower in TNF ARE/$S1P_1$-eGFP mice after FTY720 treatment, compared with vehicle-treated controls (Fig. 7C). As the GFP tag is at the carboxy terminus, the decreased $S1P_1$ signal is not due to internalization but due to $S1P_1$ degradation. Taken together, these results show that S1PR agonists alter cell behavior within the MLN, predominantly decrease naïve and CM T cell subsets from circulation and induce degradation of the receptor on lymphocytes and EC.

DISCUSSION

The dual efficacy of alpha-4 integrin blockade (Natalizumab) in multiple sclerosis (MS) and Crohn's has set a precedent for parallel trafficking mechanisms between these immune-mediated diseases. The therapeutic efficacy of S1PR agonists in MS and UC might represent the next example of shared pathogenetic mechanisms. FTY720, a small-molecule agonist of $S1P_{1,3,4,5}$ was the first oral drug for the treatment of MS,[19] while ozanimod, an $S1P_1$-selective agent, has shown efficacy in patients with UC.[4] Thus, it is worthwhile to examine the role of the S1P pathway during immune cell traffic to the chronically inflamed intestine. Here, we show that naïve and central memory and subsets of gut homing effectors (particularly $CD8^+$) T cells, activated DC and EC express $S1P_1$. Although acute inflammatory signals did not increase $S1P_1$ expression on T cells, chronic inflammatory signals upregulated $S1P_1$, not only on T cells, but also on endothelium. We found a very similar pattern of dysregulation of the enzymes that control tissue S1P levels in inflamed mouse and human intestine, with induction of S1P synthesis and suppression of degradation, which suggests that S1P levels in the intestine are altered. FTY720 promoted $S1P_1$

degradation and predominantly depleted naïve and central memory T cells from circulation, while effector T cells mobilized to the periphery.

In the past, even the basic characterization of cell subsets that express S1P receptors has been challenging, as these receptors internalize upon ligand binding, where they may be degraded or recirculate back to the cell surface.[20] Technical aspects are further complicated by the lack of reliable reagents. Indeed, a commercially available anti-$S1P_1$ antibody did not detect differences in antibody binding on any cell type.

Antibodies that target alpha-4 integrins, such as vedolizumab and natalizumab ameliorate IBD by interfering with the traffic of antigen-experienced gut-homing effector T cells, which are recruited to the intestine at postcapillary venules. Yet effector cells lack L-selectin (CD62L), which has been linked to $S1P_1$ expression.[21] Interestingly, we observed that although L-selectin-expressing naïve and central memory $CD4^+T$ and $CD8^+T$ cells expressed $S1P_1$, there were subsets of effectors $CD4^+$ and $CD8^+$ within the MLN that also exhibited high $S1P_1$ expression. Interference with recruitment of gut-homing effector (rather than naïve) T cells at postcapillary venules is most in line with the mechanism of action of natalizumab and vedolizumab. Consistent with a potential role for $S1P_1$ at this level, we observed $S1P_1$-expressing microvessels localized to submucosal areas. In MLN $S1P_1$ was predominantly observed within HEV-like structures within germinal centers and surrounding T cell zones of MLN, consistent with its known role on naïve T cell traffic. This pattern of expression within key cellular mediators of leukocyte recruitment to intestine and associated lymphoid tissues suggests that the S1PR agonists might act though additional mechanisms, beyond naïve T cell retention within thymus and lymph nodes.

S1P plays a role on the migration of DC from skin and lung to draining lymph nodes[22, 23] and that of mature bone marrow DCs via $S1P_3$.[6, 24] Activated DCs migrate to lymph nodes to initiate T cell and B cell responses, based upon the cues obtained from the environment. CD80 (B7.1) and CD86 (B7.2) expressed by activated DCs are critically important for initiation of T cell responses, [25] as well as the CD40/CD40L pathway which also participates in T cell priming and differentiation [26], thus expression of these molecules is reflective of their state of maturation. While within T cells, the naïve subset were predominant expressors of $S1P_1$, it is the activated DCs that had the most $S1P_1$ expression. This pattern is consistent with a potential role for $S1P_1$ on the migration of activated DC from LP to MLN, enabling critical encounters with naïve T cells. Modulation of DC-T cell encounters represents an additional potential point of control for S1PR agonists in IBD.

In the gut, $S1P_1$ downregulation is required for the establishment of tissue residence, particularly of CD8 subsets.[21] However, the clinical evidence suggests that there is also a pathogenically relevant recirculating T cell pool, as blockade of α4β7-MAdCAM-1 interactions offers clear therapeutic benefit in IBD.[1, 2] While the differences in $S1P_1$ expression on subsets of gut-homing molecule-expressing effectors ($CD44^+/CD62L^{neg}$) within intestine were negligible, within the MLN, the majority of the CD4 with high surface expression of CCR9 and integrin β7 also had the most total $S1P_1$, suggesting that these cells might be poised to recirculate. By contrast, within the CD8, those that exhibited high $S1P_1$ had the least surface gut homing receptors. This might account for differences in the relative

trafficking abilities of T cells, with the majority of effector CD4 but only a minority of $CD8^+$ effectors being poised to recirculate.

The present study provides insight into the regulation of $S1P_1$ expression by inflammatory signals. On the one hand, chemical injury to the colonic mucosa, which induces an acute inflammatory response and is primarily mediated by innate immunity, did not affect $S1P_1$ expression on colonic T cells, despite the presence of severe acute inflammation. On the other hand, chronic inflammatory signals demonstrate a strong inductive effect on effector $CD4^+$T cell $S1P_1$ expression, following development of chronic colitis induced by transfer of Treg-depleted $CD4^+$T cells. However, this more representative model of human IBD still lacks the complexity of an intact immune system, as $Rag^{-/-}$ mice are devoid of functional CD8 and B cells, even after CD4 reconstitution. TNF ARE mice bear a deletion of the AAUU-rich region of the TNF gene and develop chronic Crohn's-like transmural ileitis and arthritis[27] that lasts throughout the animal's lifetime. By crossing TNF ARE mice with $S1P_1$-eGFP mice we were able to comprehensively assess $S1P_1$ expression on relevant intestinal immune cell subsets. [9, 28] We observed increased T cell recruitment into the ileal LP and MLN of TNF ARE/$S1P_1$-eGFP mice and $S1P_1$ expression increased on several $CD4^+$ and $CD8^+$ subsets within inflamed ilea and most T cell subsets isolated from MLN. $S1P_1$ was similarly upregulated on DC isolated from ilea, MLN and spleen of inflamed mice and $S1P_1$-expressing microvessels were abundant in inflamed ilea of TNF ARE/$S1P_1$-eGFP mice, particularly in the region of postcapillary venules. The role of S1P on vascular integrity is well-known and genetic deletion of $S1P_1$ results in colonic vascular fragility.[29, 30] It is conceivable that administration of S1PR agonists may exert functional effects at the level of the postcapillary venules, such as tightening the barrier or downregulating endothelial integrin ligands (e.g. MAdCAM-1, VCAM-1). Indeed, most effective IBD therapies are known to act through more than a single mechanism.

Further control of the S1P system takes place at the level of enzymatic regulation of its tissue concentration. Once again, chronic inflammatory signals appear to be important determinants, as we observed an almost uniform pattern of expression in both preclinical models and patients with IBD. Different from the effects on $S1P_1$ expression, enzyme dysregulation was observed even during acute colitis. In particular, the inducible sphingosine kinase-1 (Sphk1) and the intra- to extracellular S1P transporter, Spinster homolog 2 (Spns2) were upregulated, whereas the degrading enzymes (S1P lyase1: sgpl1 and phosphatases: sgpp1, 2) were downregulated. Cyster and colleagues had shown that just reducing lyase activity increased tissue S1P levels, thus this expression pattern likely results in alterations of the tissue to blood S1P gradient,[7] which may promote lymphocyte retention within inflamed tissues. As expected, FTY720 induced marked lymphopenia and induced $S1P_1$ degradation on lymphocytes and EC, predominantly altered the percentage of naïve and central memory $CD4^+$ and $CD8^+$ T cells, while unexpectedly increasing the percentage of effector T cells in peripheral blood. We speculate that functional antagonists such as FTY720 and ozanimod that induce $S1P_1$ degradation may therefore promote escape from S1P-mediated retention and mobilization of critical gut homing $CD4^+$ effectors. Once in circulation the lack of survival signals may result in apoptosis of pathogenic gut homing T cells and decreased inflammation.

FTY720 sequesters lymphocytes within lymph nodes, presumably preventing them from reaching sites of inflammation in immune-mediated diseases.[11, 12] Previous studies had shown that FTY720 prevented inflammation in the DSS-induced and the CD4+CD62L+ T cell transfer model of colitis.[31] It also ameliorated IL-10−/−[32], CD45RB[hi33], and oxazolone colitis[34] but not TNBS-induced colitis.[35] An antibody against S1P$_1$ prevented T cell chemotaxis towards S1P[36] arguing for the popular T cell centric mechanism of action of S1PR agonists.

Although ozanimod's selectivity for S1P$_1$ may be advantageous over FTY720, a previous trial of another S1P$_1$-selective agonist (KRP-203) was discontinued early due to a lack of clinical response in UC (www.novctrd.com). KRP-203 had previously shown to attenuate chronic colitis in IL-10 deficient mice,[37] a model considered to be representative of human UC. It is tempting to speculate that this might be due to inherent differences in the compound's pharmacological properties or due to small sample size or premature assessment of trial endpoints. The anti-inflammatory effects of all anti-trafficking strategies improve with time, perhaps due to the long lives of effector lymphocytes, while most clinical trial endpoints continue to be assessed between 6–8 weeks, likely based on our experience with TNF inhibitors.

In conclusion, based on the expression of S1P$_1$ and the functional effects of S1PR agonists on lymphocytes, DC and endothelium we envision a tripartite mechanism of action for these family of drugs. Such mechanism may combine the retention of naïve T cells at secondary lymphoid organs with the mobilization of effector T cells from intestine and subsets of activated DC to inductive sites, as well as the modification of endothelial barrier function, with a resultant net effect of attenuation of intestinal inflammation.

METHODS

Mice

Edg-1[eGFP/eGFP] mice, hereafter referred to as S1P$_1$-eGFP mice, developed as described[17] were used as controls and for the induction of acute colitis by DSS. Rag1−/− mice (B6.129S7-Rag1[tm1Mom]/J), DsRed mice (005441; 00Tg(CAG-DsRed*MST) 1Nagy) were purchased from Jackson (Bar Harbor, ME). The B6.129S-Tnf[tm2Gkl]/Jarn (TNF ARE) strain was previously described.[27, 38] TNF ARE mice were crossed with S1P$_1$-eGFP^{+/+} transgenic mice[17] to generate TNF ARE [ARE/+]/S1P$_1$-eGFP^{+/+} mice. Institutional Animal Care and Use Committees (IACUC) of the University of California San Diego and The Scripps Research Institute approved all the animal procedures.

Tissue fixation, paraffin embedding & histological scoring

Tissues were prepared as described and severity of inflammation assessed in a blinded fashion by a pathologist (Paul Jedlicka). [39, 40]

Cell isolation

Spleen, MLN and lamina propria (LP) mononuclear cells were isolated as previously described.[9]

Flow cytometry

Cells from indicated compartments were suspended in 1X phosphate buffered saline (PBS) with 2% fetal bovine serum (FBS), supplemented with Fc block and stained with anti-mouse antibodies against $S1P_1$ (713412) (R&D Systems, MN, USA); CD4 (RM4-5), CD62L (MEL-14), CD44 (IM7), CD11b (M1/70), CD31 (MEC13.3), CD80 (16-10A1), CD40 (3/23), β7 (FIB504), CD45RB (C363-16A) (Biolegend, San Diego, CA), CD8 (53-6.7), B220 (RA3-6B2), CD45 (3-F11), MHCII (M5/114.15.2), CD11c (N418), CD86 (GL1), CD103 (2E7), CCR9 (eBioCW-1.2) (eBioscience, San Diego, CA) or corresponding isotype controls. Cells were washed twice and fixed in BD stabilizing fixative (BD Biosciences, San Jose, CA). Flow cytometry analyses were performed using a Cytek DxP8 (Cytek, Fremont, CA). Data was analyzed using FLOWJO software (Tree Star Inc.).

RNA extraction, cDNA synthesis, and real-time PCR

Total RNA was isolated using RNeasy Kit (Qiagen, Valencia, CA, USA). RNA (500 ng) was reverse-transcribed with a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA). Real-time qRT-PCR assays for SPHK1 (Hs00184211_m1), SPHK2 (Hs00219999_m1), SGPL1 (Hs00393700_m1), SGPP1 (Hs00229266_m1), SGPP2 (Hs00544786_m1), SPNS2 (Hs01390449_g1), Sphk1 (Mm00448841_g1), Sphk2 (Mm00445021_m1), Sgpl1 (Mm00486079_m1), Sgpp1 (Mm00473016_m1), Sgpp2 (Mm01158866_m1) and Spns2 (Mm01249324_m1) were performed using TaqMan Universal Master Mix (Applied Biosystems) with GAPDH/gapdh as endogenous controls. Relative gene expression was calculated using the comparative C_T (C_T) quantitation method with Applied BioSystems StepOne Software v2.3.

Immunohistochemistry and imaging

Tissues were fixed in Z-fix (Anatech, LTD) and placed into 30% sucrose/PBS for 48 h at 4°C. Five to seven μm OCT frozen sections were permeabilized, blocked in 5% normal goat serum-PBST, washed and incubated overnight at 4°C with anti-GFP (Abcam, ab6556) primary at 1:100 diluted in PBST-1% normal goat serum. Sections were washed and incubated with secondary (Life Technologies; 1:1,000) and DAPI nuclear stain (Life Technologies, 1:10,000) prior to mounting on Vectashield (Vector Laboratories). Images were obtained using an Olympus BX51 microscope with MetaMorph (Molecular devices, CA) software.

Inflamed and normal ilea of TNF ARE/$S1P_1$-eGFP mice and $S1P_1$-eGFP controls were imaged. Freshly prepared ileal sections were adhered (VetBondTM, 3M) to a reservoir of 95% O_2/5% CO_2 superfused RPMI, were imaged by 2-photon microscopy as reported.[41, 42] Image volumes of 600 μm × 600 μm × 150 μm were obtained with a Leica SP5 confocal microscope and processed with Imaris (Bitplane).

Induction of acute colitis by dextran sulfate sodium

10-week-old $S1P_1$-eGFP mice received 3% dextran sulfate sodium (DSS) in the drinking water for 7 days. Weight loss was monitored daily and tissues were collected at day 7.

Two-photon intravital microscopy

Cells were isolated from lymph nodes and spleen of DsRed mice, sorted using Dynabeads Mouse Pan T (11443D, ThermoScientific) according to the manufacturer's protocol and injected (5×10^6 T cells) retro-orbitally. Cell behavior was analyzed 16 hours later in MLN explants by 2-photon microscopy as reported[41, 42], before and after FTY720-P.

FTY720 treatment studies

FTY720 (3 mg/kg) or vehicle (control) was administered intraperitoneally to 10- to 12-week-old TNF ARE/$S1P_1$-eGFP mice every other day for 6 weeks.

CD45RB^{hi} adoptive transfer colitis studies

$CD4^+$ splenocytes from $S1P_1$-eGFP mice were enriched using magnetic beads (Miltenyi Biotec, San Diego, CA), according to the manufacturer's protocol. $CD4^+CD45RB^{hi}$/$CD25^{neg}$ cells and $CD4^+CD45RB^{lo}$ cells were FACS-sorted and 2×10^5 $CD4^+CD45RB^{hi}$ cells transferred into 8-week-old $Rag1^{-/-}$ mice. Control mice additionally received 1×10^5 $CD4^+CD45RB^{lo}$ cells. Tissues were harvested once the colitic group lost 10% of their initial body weight.

Statistical Analysis

Results are expressed as mean ± SEM unless otherwise indicated. Data were analyzed using GraphPad Prism 5 (GraphPad Software, Inc.). Significant differences between individual groups were calculated using two-tailed unpaired *t* test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Feagan BG, et al. Vedolizumab as induction and maintenance therapy for ulcerative colitis. N Engl J Med. 2013; 369:699–710. [PubMed: 23964932]

2. Sandborn WJ, et al. Vedolizumab as induction and maintenance therapy for Crohn's disease. N Engl J Med. 2013; 369:711–721. [PubMed: 23964933]

3. Ghosh S, et al. Natalizumab for active Crohn's disease. N Engl J Med. 2003; 348:24–32. [PubMed: 12510039]

4. Sandborn W, et al. 445 The TOUCHSTONE Study. A Randomized, Double-Blind, Placebo-Controlled Induction Trial of an Oral S1P Receptor Modulator (RPC1063) in Moderate to Severe Ulcerative Colitis. Gastroenterology. 148:S-93.

5. Zhang H, Desai NN, Olivera A, Seki T, Brooker G, Spiegel S. Sphingosine-1-phosphate, a novel lipid, involved in cellular proliferation. J Cell Biol. 1991; 114:155–167. [PubMed: 2050740]

6. Eigenbrod S, Derwand R, Jakl V, Endres S, Eigler A. Sphingosine kinase and sphingosine-1-phosphate regulate migration, endocytosis and apoptosis of dendritic cells. Immunol Invest. 2006; 35:149–165. [PubMed: 16698674]

7. Schwab SR, Pereira JP, Matloubian M, Xu Y, Huang Y, Cyster JG. Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. Science. 2005; 309:1735–1739. [PubMed: 16151014]

8. Sanchez T, Hla T. Structural and functional characteristics of S1P receptors. J Cell Biochem. 2004; 92:913–922. [PubMed: 15258915]

9. Collins CB, et al. CD44 deficiency attenuates chronic murine ileitis. Gastroenterology. 2008; 135:1993–2002. [PubMed: 18854186]

10. Gaengel K, et al. The sphingosine-1-phosphate receptor S1PR1 restricts sprouting angiogenesis by regulating the interplay between VE-cadherin and VEGFR2. Dev Cell. 2012; 23:587–599. [PubMed: 22975327]

11. Choi JW, et al. FTY720 (fingolimod) efficacy in an animal model of multiple sclerosis requires astrocyte sphingosine 1-phosphate receptor 1 (S1P1) modulation. Proc Natl Acad Sci U S A. 2011; 108:751–756. [PubMed: 21177428]

12. Mandala S, et al. Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. Science. 2002; 296:346–349. [PubMed: 11923495]

13. Cinamon G, Zachariah MA, Lam OM, Foss FW Jr, Cyster JG. Follicular shuttling of marginal zone B cells facilitates antigen transport. Nat Immunol. 2008; 9:54–62. [PubMed: 18037889]

14. Sanna MG, et al. Sphingosine 1-phosphate (S1P) receptor subtypes S1P1 and S1P3, respectively, regulate lymphocyte recirculation and heart rate. J Biol Chem. 2004; 279:13839–13848. [PubMed: 14732717]

15. Allende ML, Yamashita T, Proia RL. G-protein-coupled receptor S1P1 acts within endothelial cells to regulate vascular maturation. Blood. 2003; 102:3665–3667. [PubMed: 12869509]

16. Awad AS, et al. Selective sphingosine 1-phosphate 1 receptor activation reduces ischemia-reperfusion injury in mouse kidney. Am J Physiol Renal Physiol. 2006; 290:F1516–1524. [PubMed: 16403835]

17. Cahalan SM, et al. Actions of a picomolar short-acting S1P(1) agonist in S1P(1)-eGFP knock-in mice. Nat Chem Biol. 2011; 7:254–256. [PubMed: 21445057]

18. Hart AL, Ng SC, Mann E, Al-Hassi HO, Bernardo D, Knight SC. Homing of immune cells: role in homeostasis and intestinal inflammation. Inflamm Bowel Dis. 2010; 16:1969–1977. [PubMed: 20848507]

19. Brinkmann V, et al. Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. Nat Rev Drug Discov. 2010; 9:883–897. [PubMed: 21031003]

20. Spiegel S, Milstien S. The outs and the ins of sphingosine-1-phosphate in immunity. Nat Rev Immunol. 2011; 11:403–415. [PubMed: 21546914]

21. Skon CN, Lee JY, Anderson KG, Masopust D, Hogquist KA, Jameson SC. Transcriptional downregulation of S1pr1 is required for the establishment of resident memory CD8+ T cells. Nat Immunol. 2013; 14:1285–1293. [PubMed: 24162775]

22. Czeloth N, Bernhardt G, Hofmann F, Genth H, Förster R. Sphingosine-1-Phosphate Mediates Migration of Mature Dendritic Cells. J Immunol. 2005; 175:2960–2967. [PubMed: 16116182]

23. Gollmann G, et al. Sphingosine-1-phosphate receptor type-1 agonism impairs blood dendritic cell chemotaxis and skin dendritic cell migration to lymph nodes under inflammatory conditions. Int Immunol. 2008; 20:911–923. [PubMed: 18495625]

24. Maeda Y, Matsuyuki H, Shimano K, Kataoka H, Sugahara K, Chiba K. Migration of CD4 T cells and dendritic cells toward sphingosine 1-phosphate (S1P) is mediated by different receptor subtypes: S1P regulates the functions of murine mature dendritic cells via S1P receptor type 3. J Immunol. 2007; 178:3437–3446. [PubMed: 17339438]

25. Lenschow DJ, et al. CD28/B7 regulation of Th1 and Th2 subsets in the development of autoimmune diabetes. Immunity. 1996; 5:285–293. [PubMed: 8808683]

26. MacDonald KP, Munster DJ, Clark GJ, Dzionek A, Schmitz J, Hart DN. Characterization of human blood dendritic cell subsets. Blood. 2002; 100:4512–4520. [PubMed: 12393628]

27. Kontoyiannis D, Pasparakis M, Pizarro TT, Cominelli F, Kollias G. Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. Immunity. 1999; 10:387–398. [PubMed: 10204494]

28. Collins CB, et al. Flt3 ligand expands CD103+ dendritic cells and FoxP3+ T regulatory cells, and attenuates Crohn's-like murine ileitis. Gut. 2012; 61:1154–1162. [PubMed: 22068168]

29. Hatoum OA, Miura H, Binion DG. The vascular contribution in the pathogenesis of inflammatory bowel disease. Am J Physiol Heart Circ Physiol. 2003; 285:H1791–1796. [PubMed: 14561675]

30. Montrose DC, et al. S1P(1) localizes to the colonic vasculature in ulcerative colitis and maintains blood vessel integrity. J Lipid Res. 2013; 54:843–851. [PubMed: 23296878]

31. Deguchi Y, et al. The S1P receptor modulator FTY720 prevents the development of experimental colitis in mice. Oncol Rep. 2006; 16:699–703. [PubMed: 16969482]

32. Mizushima T, et al. Therapeutic effects of a new lymphocyte homing reagent FTY720 in interleukin-10 gene-deficient mice with colitis. Inflamm Bowel Dis. 2004; 10:182–192. [PubMed: 15290910]

33. Fujii R, et al. FTY720 suppresses CD4+CD44highCD62L- effector memory T cell-mediated colitis. Am J Physiol Gastrointest Liver Physiol. 2006; 291:G267–274. [PubMed: 16574986]

34. Daniel C, Sartory N, Zahn N, Geisslinger G, Radeke HH, Stein JM. FTY720 ameliorates Th1-mediated colitis in mice by directly affecting the functional activity of CD4+CD25+ regulatory T cells. J Immunol. 2007; 178:2458–2468. [PubMed: 17277153]

35. Radi ZA, Heuvelman DM, Masferrer JL, Benson EL. Pharmacologic evaluation of sulfasalazine, FTY720, and anti-IL-12/23p40 in a TNBS-induced Crohn's disease model. Dig Dis Sci. 2011; 56:2283–2291. [PubMed: 21374065]

36. Liao JJ, et al. Immunosuppressive human anti-lymphocyte autoantibodies specific for the type 1 sphingosine 1-phosphate receptor. FASEB J. 2009; 23:1786–1796. [PubMed: 19158154]

37. Song J, et al. A novel sphingosine 1-phosphate receptor agonist, 2-amino-2-propanediol hydrochloride (KRP-203), regulates chronic colitis in interleukin-10 gene-deficient mice. J Pharmacol Exp Ther. 2008; 324:276–283. [PubMed: 17898319]

38. McNamee EN, Masterson JC, Jedlicka P, Collins CB, Williams IR, Rivera-Nieves J. Ectopic lymphoid tissue alters the chemokine gradient, increases lymphocyte retention and exacerbates murine ileitis. Gut. 2013; 62:53–62. [PubMed: 22267601]

39. McNamee EN, et al. Novel model of TH2-polarized chronic ileitis: the SAMP1 mouse. Inflamm Bowel Dis. 2010; 16:743–752. [PubMed: 19856411]

40. Burns RC, Rivera-Nieves J, Moskaluk CA, Matsumoto S, Cominelli F, Ley K. Antibody blockade of ICAM-1 and VCAM-1 ameliorates inflammation in the SAMP-1/Yit adoptive transfer model of Crohn's disease in mice. Gastroenterology. 2001; 121:1428–1436. [PubMed: 11729122]

41. Sarkisyan G, Cahalan SM, Gonzalez-Cabrera PJ, Leaf NB, Rosen H. Real-time differential labeling of blood, interstitium, and lymphatic and single-field analysis of vasculature dynamics in vivo. Am J Physiol, Cell Physiol. 2012; 302:C1460–1468. [PubMed: 22357735]

42. Sarkisyan G, Gay LJ, Nguyen N, Felding BH, Rosen H. Host endothelial S1PR1 regulation of vascular permeability modulates tumor growth. Am J Physiol, Cell Physiol. 2014; 307:C14–24. [PubMed: 24740542]
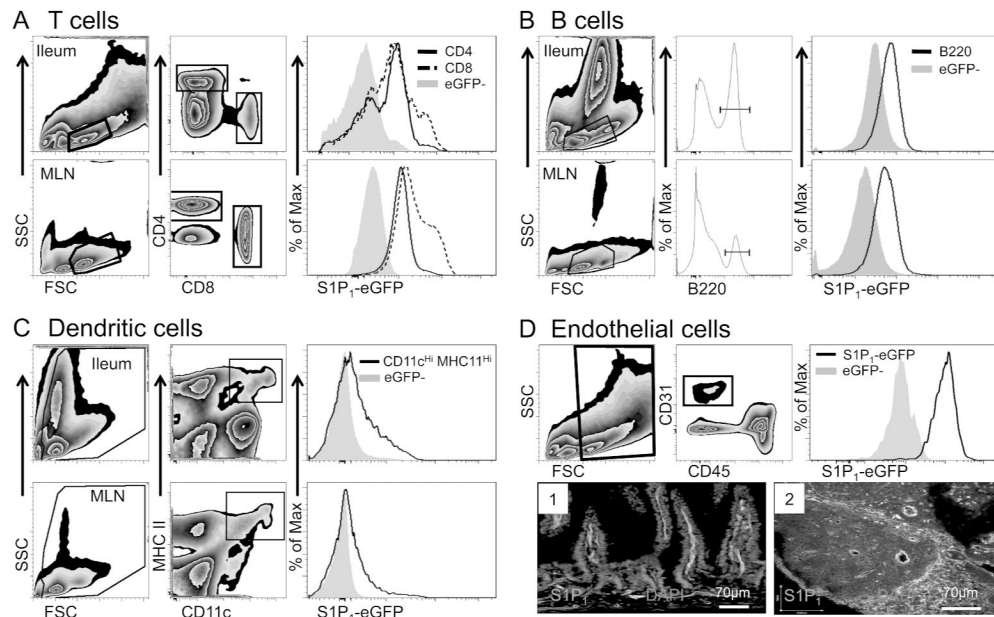
**Figure 1. Expression of S1P$_1$ on T, B, dendritic and endothelial cells**

(A, B) Flow cytometry gating strategy (left panels) and S1P$_1$-eGFP signal of CD4$^+$T, CD8$^+$T and B cells isolated from ileal LP and MLN of S1P$_1$-eGFP mice. Representative histograms (right panels) from indicated cell subsets. (C) Flow cytometry analyses of DC (CD11c^{hi} MHC II^{hi}) gated as shown, isolated from ileal LP and MLN of S1P$_1$-eGFP mice. Representative histograms. (D) Flow cytometry analyses of endothelial cells (CD31$^+$CD45^{neg}) isolated from ileal LP (upper panels) of S1P$_1$-eGFP mice. Immunohistochemical localization of S1P$_1$ in ileum (D1) and on high endothelial venules and lymphatics of MLN (D2). Representative plots and images from three or more independent experiments, n 3 or more mice/group.
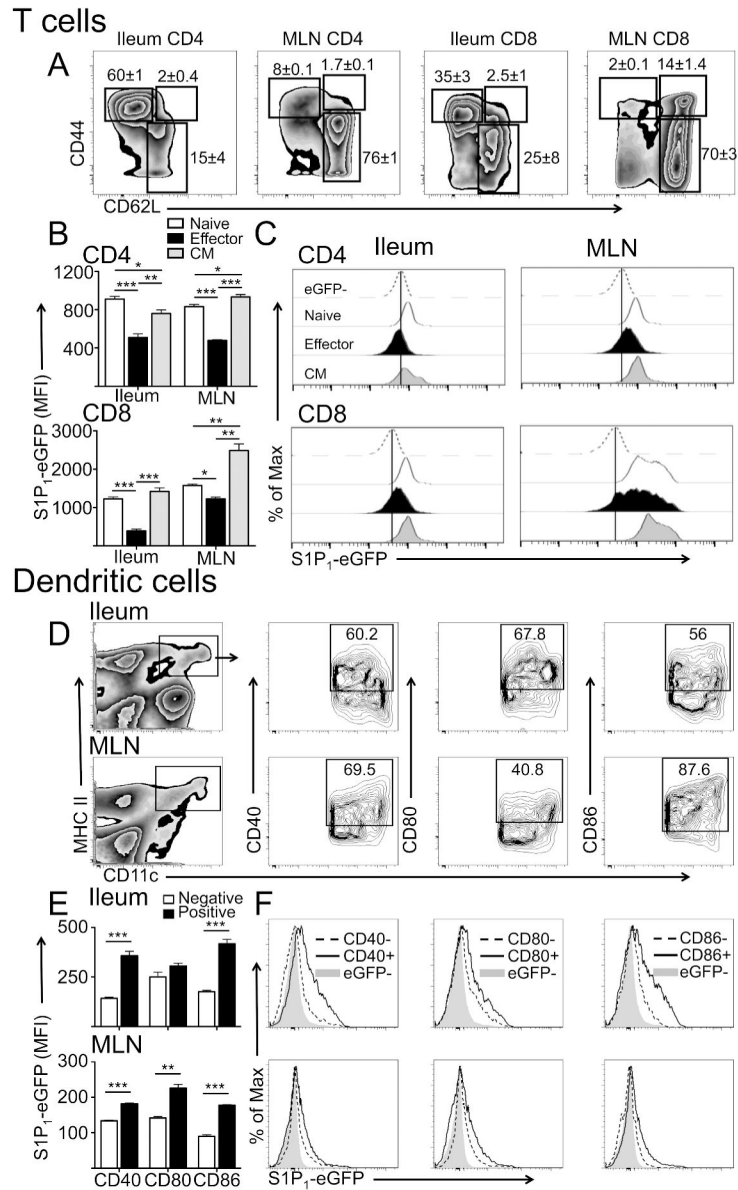
Figure 2. Expression of S1P$_1$ is higher on naïve, central memory CD8$^+$T cells and activated dendritic cells

(A) Gating strategy for naïve (CD44$^-$CD62L$^+$), effector (CD44$^+$CD62L$^-$) and central memory (CM: CD44$^+$CD62L$^+$) CD4$^+$ and CD8$^+$ T cells isolated from ileal LP and MLN of S1P$_1$-eGFP mice. (B, C) MFI and representative histograms for S1P$_1$ expression by naïve, effector and CM CD4$^+$ and CD8$^+$ T cells from indicated compartments. (D) Gating strategy for DC (CD11c^{high}/MHCII^{high}) isolated from the ileum and MLN of S1P$_1$-eGFP mice and gated further based on their costimulatory molecule (CD40, CD80 and CD86) expression. (E, F) MFI and representative histograms for S1P$_1$ expression by activated DC (CD40$^+$, CD80$^+$ and CD86$^+$), compared with that of CD40$^-$, CD80$^-$ and CD86$^-$ DC. *p<0.05, **p<0.01, ***p<0.001 by two-tailed t test. Data are shown as mean ± SEM, n=3 mice/group and representative histograms from three independent experiments.
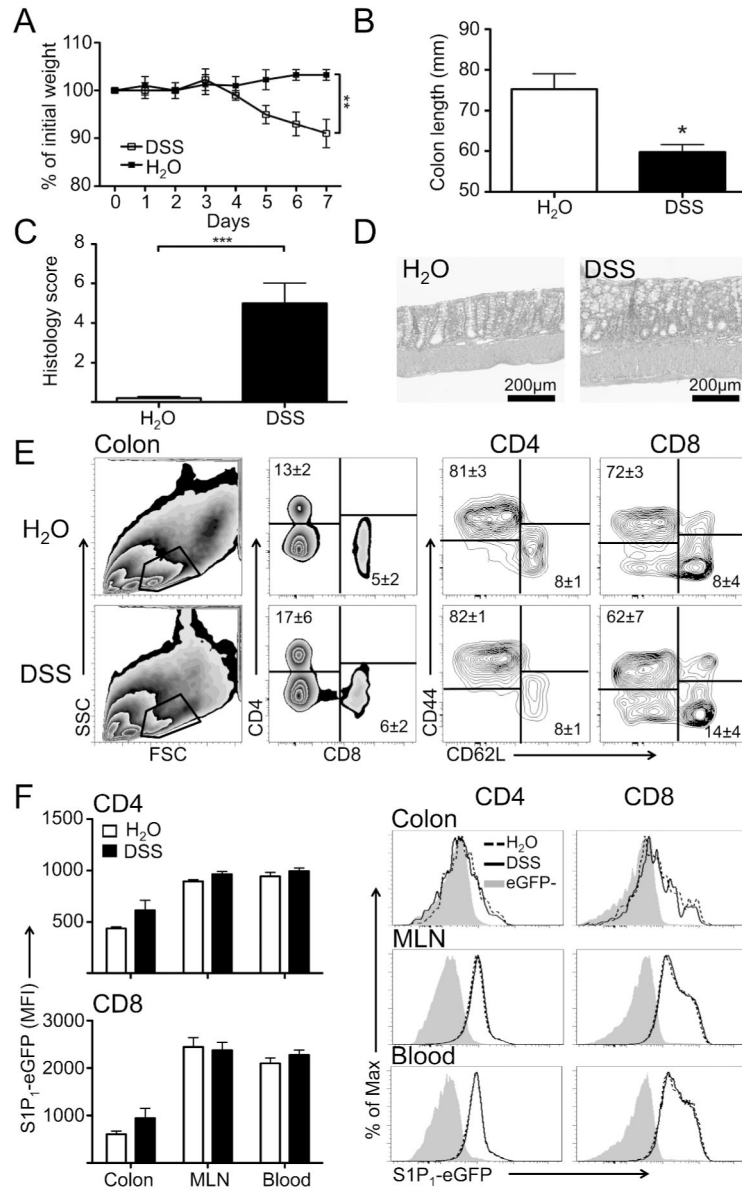
**Figure 3. Acute inflammation did not modulate the expression of S1P$_1$ on T cells**
S1P$_1$-eGFP mice were treated with 3% DSS or H$_2$O (vehicle) for 7 days. (A, B) Weight loss curves and shortening of colon length (mm) of mice receiving DSS or H$_2$O. (C) Histological inflammatory indices in colon of mice treated with DSS or H$_2$O. (D) Representative micrographs of colon of S1P$_1$-eGFP mice treated with DSS or H$_2$O. (E) Flow cytometric analysis of CD4^{+}T and CD8^{+}T cells isolated from colonic LP of S1P$_1$-eGFP mice treated with DSS or H$_2$O. (F) Bar graph represents MFI for S1P$_1$ expression by CD4^{+}T and CD8^{+}T cells from colonic LP, MLN and blood of S1P$_1$-eGFP mice treated with DSS or H$_2$O. Representative histograms. Scale bar, 200 μm. Shaded histograms are control for eGFP (eGFP-); line and dashed histograms are from DSS- and H$_2$O-treated mice respectively. Data are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 by two-tailed t test, n=5 mice/ group.
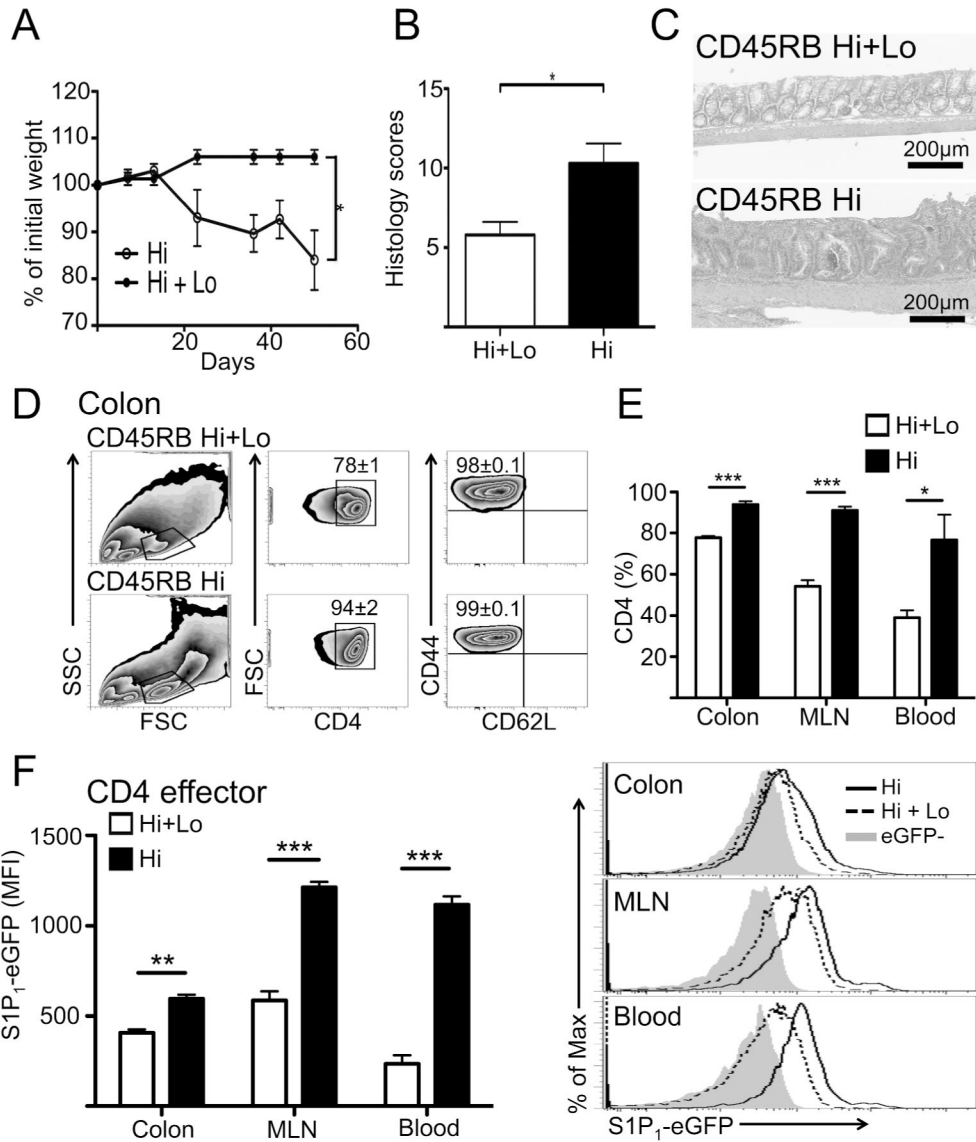
Figure 4. Chronic inflammation upregulated $S1P_1$ on adoptively transferred T cells after development of colitis

$CD4^+CD45RB^{hi}$ cells from $S1P_1$-eGFP mice were adoptively transferred into $Rag1^{-/-}$ mice. Control group was co-transferred with $CD4^+CD45RB^{lo}$ cells. (A) Weight loss curve suggests development of colitis after 3 weeks post cell transfer. (B, C) Histological assessment of colon confirmed development of colitis compared with co-transferred controls. (D) Gating strategy for effector $CD4^+$T cells isolated from colon of mice with and without colitis. (E) Percentage of effector CD4 in mice with and without colitis. (F) MFI for $S1P_1$ expression by effector $CD4^+$T cells in colon, MLN and blood in mice with and without colitis. Representative histograms. Data are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 by two-tailed *t* test, n=4 mice/group.
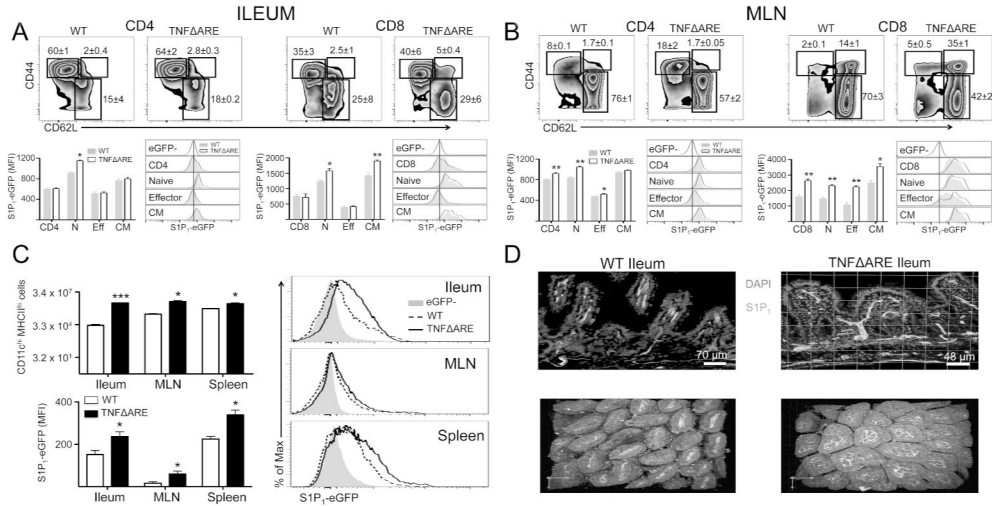
Figure 5. Chronic inflammation modulated S1P$_1$ expression on T, dendritic and endothelial cells of TNF ARE mice

(A, B) Naïve (CD44$^-$CD62L$^+$), effector (CD44$^+$CD62L$^-$) and central memory (CM: CD44$^+$CD62L$^+$) CD4$^+$ and CD8$^+$ T cells were isolated from ileal LP and MLN of S1P$_1$-eGFP (WT) and TNF ARE/S1P$_1$-eGFP (TNF ARE) mice and gated as shown. Bar graph represents MFI for S1P$_1$ expression by total, naïve, effector and central memory CD4$^+$T and CD8$^+$T cells from ileum LP and MLN of TNF ARE/S1P$_1$-eGFP mice compared with S1P$_1$-eGFP mice. Representative histograms. (C) Absolute counts of DC (CD11c^{hi} MHCII^{hi} cells) from ileal LP, MLN and spleen of TNF ARE/S1P$_1$-eGFP mice compared with S1P$_1$-eGFP mice. MFI for S1P$_1$ expression by DC in indicated tissues of S1P$_1$-eGFP and TNF ARE/S1P$_1$-eGFP mice. Representative histograms. (D) Immunohistochemistry and three-dimensional reconstruction of ileum for S1P$_1$ expression in mice with and without ileitis. Data are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 by two-tailed t test, n=5 mice/group.
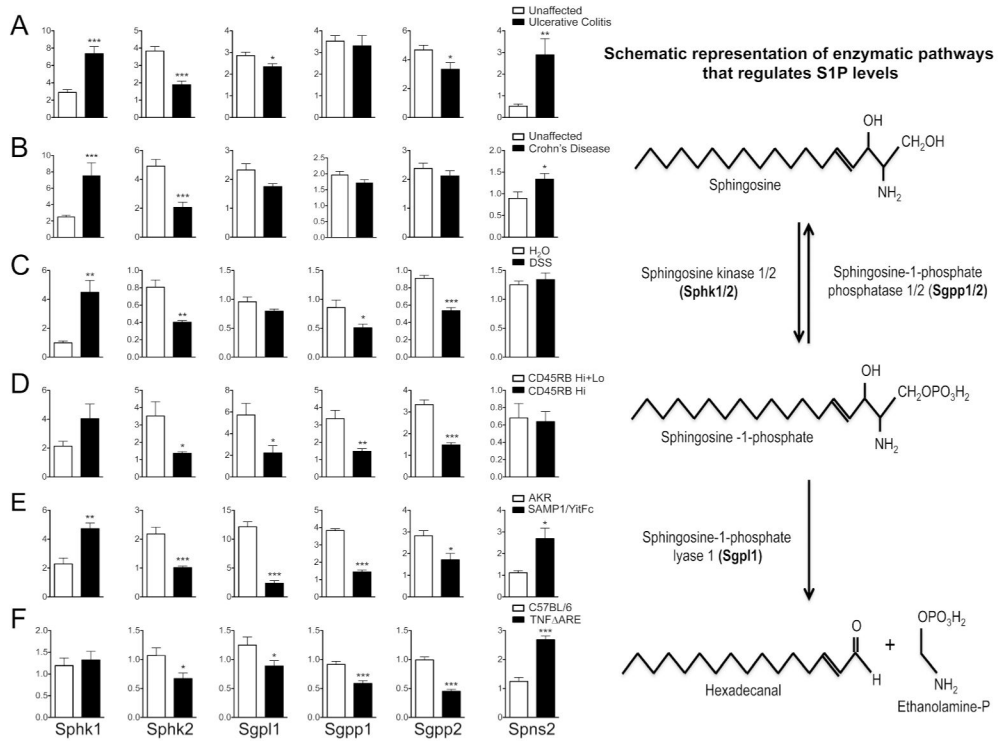
Figure 6. Inflammation alters the expression of enzymes that regulate tissue S1P levels

Analyses of mRNA expression of enzymes that control S1P levels (Sphk1, sphingosine kinase 1; Sphk2, sphingosine kinase 2; Sgpl1, sphingosine-1-phosphate lyase; SGPP1, sphingosine-1-phosphate phosphatase 1; SGPP2, sphingosine-1-phosphate phosphatase 2; Spns2 (Spinster homolog 2) was performed by real-time qRT-PCR on intestinal tissues from mice and humans with IBD, compared with respective normal controls,

(A) Patients with and without ulcerative colitis (n=8/group),

(B) Patients with and without Crohn's disease (n=8/group),

(C) Mice treated with DSS or vehicle (H$_2$O) (n=4/group),

(D) Mice with (CD45RB^{Hi}) or without (CD45RB^{Hi+Lo}) T cell transfer colitis (n=4–6/group),

(E) Uninflamed AKR and SAMP1/YitFc with spontaneous ileitis (n=7/group) and

(F) Normal C57BL/6 and ileitic TNF ARE (n=9/group). Data are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 by two-tailed *t* test.
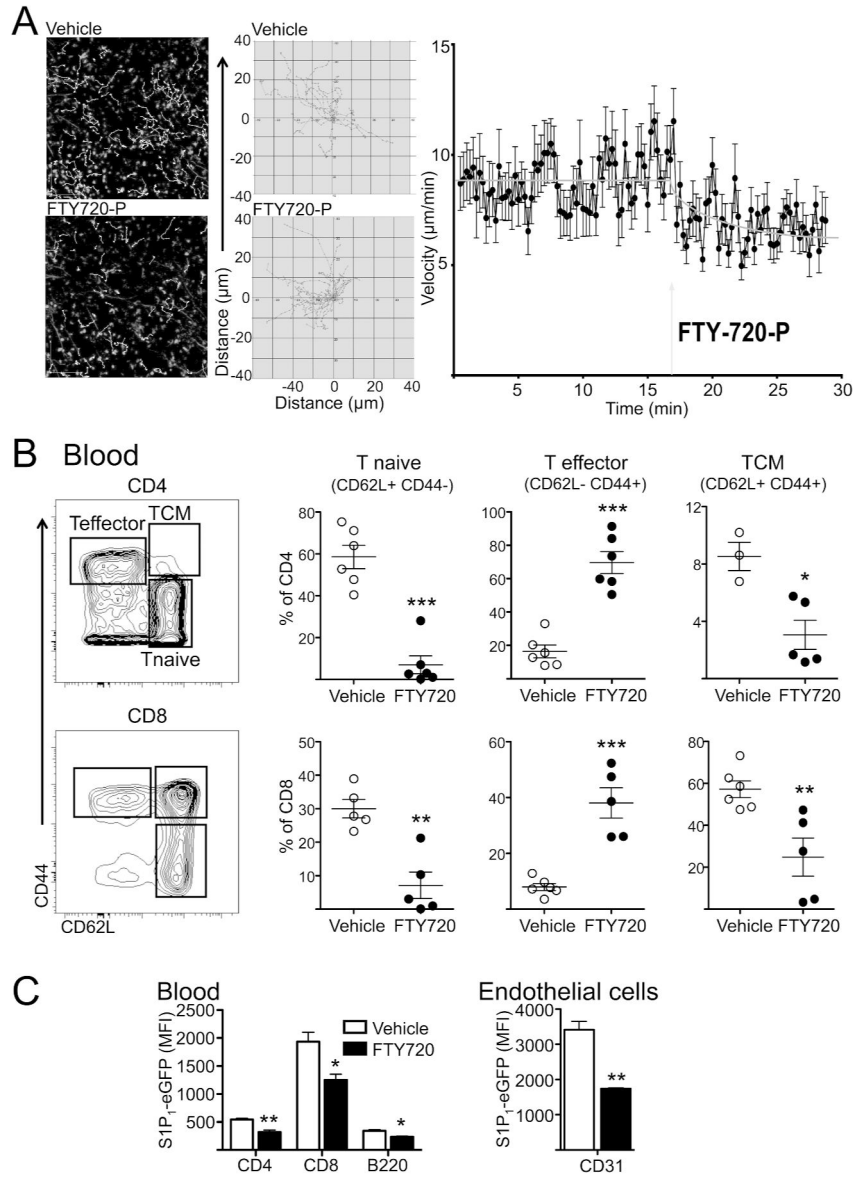
Figure 7. FTY720 reduced velocity of MLN lymphocytes, depleted naïve and central memory T cells from circulation and downregulated S1P$_1$ expression on T, B and endothelial cells in mice with chronic ileitis

(A) Sorted T cells from DsRed mice were transferred into TNF ARE mice. 16 hours later, the effect of FTY720-P on cell behavior was analyzed via intravital microscopy in MLN explants. Cell tracks from point of origin illustrate directional movement after FTY720-P or vehicle. Analyses show the effect of FTY720-P on cell velocity (data from 3 experiments, S.D.****p<0.0001).

(B) Percentage of circulating naïve, effector and central memory T cells in blood of TNF ARE/S1P$_1$-eGFP mice treated with FTY720 (3 mg/kg) or vehicle for 6 weeks.

(C) S1P$_1$ expression (MFI) of CD4$^+$T cells, CD8$^+$T cells and B cells (B220$^+$) from blood, endothelial cells (CD45$^-$ CD31$^+$) isolated from ileal LP of TNF ARE/S1P$_1$-eGFP mice

treated with FTY720 (3 mg/kg) or vehicle for 6 weeks. (Data from 3 independent experiments, n=5, *p<0.05, **p<0.01, ***p<0.001 by two-tailed t test)