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Actions of a picomolar short-acting $S1P_1$ agonist in $S1P_1$-eGFP knock-in mice

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Abstract

Sphingosine 1-Phosphate Receptor 1 ($S1P_1$) plays a critical role in lymphocyte recirculation and is a clinical target for treatment of multiple sclerosis. By generating a short-duration $S1P_1$ agonist and mice where fluorescently tagged $S1P_1$ replaces wild-type receptor, we elucidate physiological and agonist-perturbed changes in expression of $S1P_1$ at a subcellular level *in vivo*. We demonstrate differential downregulation of $S1P_1$ on lymphocytes and endothelia following agonist treatment.

$S1P_1$ (*Edg1*) is a high-affinity G protein-coupled receptor (GPCR) for the zwitterionic lysophospholipid Sphingosine 1-Phosphate (S1P)[1] and is essential for vascular development[2,3] and integrity[4], and modulates both lymphocyte development and recirculation[5]. Selective agonists of $S1P_1$ induce effective lymphocyte sequestration of both T and B cells within the lymph node[6,7], and have been investigated as potential therapeutics in transplantation[8] and multiple sclerosis. FTY720 (fingolimod)[9], a prodrug whose phosphorylated form is an agonist of $S1P_{1,3-5}$, but is thought to strongly downmodulate $S1P_1$ expression[10], has recently received regulatory approval as the first oral treatment of relapsing, remitting multiple sclerosis[11–13].

Here we have generated both a short-acting, low picomolar agonist of $S1P_1$ and a mouse model where, rather than modulating $S1P_1$ expression[10,14–16], enhanced green fluorescent protein fused to $S1P_1$ protein ($S1P_1$-eGFP) replaces wild-type $S1P_1$ in order to better understand both normal and agonist-perturbed expression of $S1P_1$ (Detailed methods are

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Author Contributions

S.C. and H.R. designed the experiments and wrote the manuscript. S.C. generated the $S1P_1$-eGFP knockin mice and performed the *in vivo* experiments and flow cytometric analysis. P.G. and N.N performed biochemical experiments. G.S. performed two-photon experiments. M.S. performed *in vitro* characterization of RP-001 and W146 competition. L.H. and A.Y. synthesized RP-001. B.S. and F.S. performed *in vitro* characterization of RP-001.

Competing Financial Interests

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shown in Supplementary Methods). Utilization of mice expressing $S1P_1$-eGFP enabled detection of $S1P_1$ protein *ex vivo* by both quantitative biochemical and flow cytometric analysis at high resolution, circumventing limitations of antibody detection and demonstrating differential regulation of $S1P_1$ in lymphocytes and endothelial cells following short-duration agonist treatment[17]. Lymphocytes exhibited rapid, sustained downregulation, whereas endothelia demonstrated slower loss of $S1P_1$. Additionally, mice expressing $S1P_1$-eGFP allowed *in vivo* visualization of changes in subcellular localization of $S1P_1$-eGFP using two-photon microscopy.

While exploring the chemical space around the allosteric $S1P_1$ agonist, CYM-5442 (**1**)[7], we noticed that adding S1P-like headgroup interactions enhanced potency by 100-fold and shifted ligand binding into an orthosteric binding mode, leading to the short-acting agonist RP-001 (**2**) (Fig. 1a, Supplementary Results, Supplemental Scheme 1). RP-001 activated $S1P_1$ *in vitro* with an EC50 of 9pM, while having little activity on $S1P_2$-$S1P_4$ and only moderate affinity for $S1P_5$ (Supplemental Table 1). No differences were observed between the racemate and the resolved S- enantimoer across all five S1P receptors, demonstrating no chiral preference. RP-001, in contrast to CYM-5442, was competitive with the selective $S1P_1$ antagonist W146 (**3**) (Fig. 1b). RP-001 induced sustained signaling while in internalized vesicles, as reported for FTY720 (**4**)[18] and in contrast to the natural ligand S1P (**5**) (Fig. 1c). It also induced dose-dependent internalization and polyubiquitination of $S1P_1$ *in vitro* similar to other $S1P_1$ agonists[7] (Supplemental Fig. 2). *In vivo*, RP-001 caused dose-dependent rapid lymphopenia with an EC50 of 0.03 mg/kg, where maximal lymphocyte sequestration from blood occured within 2 hours and returned to untreated levels by 8 hours following treatment with 0.3mg/kg (Figs. 1d,e). The short duration of lymphopenia and short half life rendered RP-001 unsuitable for clinical use, but allowed examination of changes in $S1P_1$ expression during both induction and recovery of lymphopenia, an ability unique to such a short-acting agonist.

A parallel genetic approach was taken to the system-wide analysis of physiological $S1P_1$ function. To avoid the pitfalls of gene deletion, or the use of receptors with altered signaling properties, we generated $Edg1^{eGFP/eGFP}$ mice by inserting the coding sequence for eGFP directly 3′ to the coding sequence of *Edg1*, leading to expression of $S1P_1$-eGFP as a fusion protein under normal regulatory mechanisms (Fig. 2a, Supplemental Fig. 3a–c). We observed Mendelian ratios of wild-type, heterozygous, homozygous mice, and generated hemizygous mice possessing only a single copy of the $Edg1^{eGFP}$ allele (Supplemental Fig. 3d), demonstrating that $S1P_1$-eGFP was fully functional. Direct in-gel fluorescence and immunoblotting for eGFP from homogenized tissues showed that $S1P_1$-eGFP was abundantly expressed across tissues, with highest expression in brain and lung (Fig. 2b, Supplemental Fig. 4a). We observed roughly equal amounts of both wild-type $S1P_1$ and $S1P_1$-eGFP protein in heterozygous mice by using an antibody specific to a carboxyl-terminal portion of the $S1P_1$ protein on immunoprecipitated brain tissues (Fig. 2c), and found that $S1P_1$-eGFP was N-glycosylated *in vivo* (Fig. 2d), suggesting normal surface export.

Wild-type and homozygous mice exhibited indistinguishable sequestration of T and B cells from the blood two hours after treatment with 0.3mg/kg RP-001 (Fig. 2e). Antagonism of

$S1P_1$ by the selective antagonist W146 causes significant leakage of fluid into the lungs[4]. Homozygous knock-in mice showed similar lung leakage two hours following treatment with 3 mg/kg W146, with no difference in the basal lung leakage between wild-type and homozygous animals (Fig. 2f). The equivalent effects of $S1P_1$ activation and inactivation in *Edg1*$^{eGFP\text{-}eGFP}$ mice and wild- type mice indicate that $S1P_1$-eGFP substitutes fully for wild-type $S1P_1$ *in vivo*.

The role of $S1P_1$ in lymphocyte egress is proposed to be due to $S1P_1$ expression on lymphocytes[19] and/or lymphatic sinus endothelial cells[20]. $S1P_1$-eGFP was expressed on CD4 and $CD8^+$ T cells as well as $CD19^+$ B cells within the lymph node (Fig. 3a), and was expressed on mature thymocytes (Supplemental Fig. 5). The $eGFP^{hi}$ population found in $CD8^+$ T cells results from accumulation of cleaved eGFP in peripheral $CD8^+$ T cells, which may indicate unique regulation of $S1P_1$ in $CD8^+$ T cells (Supplemental Fig. 4e,f). $S1P_1$-eGFP was expressed on lymphocytes similarly in both blood and secondary lymphoid tissues, indicating a lack of substantial receptor degradation even in S1P rich blood (Supplemental Fig. 6). Contrary to lymphocytes, $S1P_1$-eGFP was not expressed on myeloid-lineage cells within the spleen, as cells from *Edg1*$^{eGFP/eGFP}$ and *Edg1*$^{+/+}$ mice exhibited indistinguishable fluorescence intensities. (Supplemental Fig. 7b).

The ability to detect total $S1P_1$-eGFP expression allowed us to examine the effect that RP-001 had on $S1P_1$-eGFP expression. $S1P_1$-eGFP expression on lymphocytes in the lymph node was significantly decreased two hours following treatment with 0.1 mg/kg RP-001, suggesting degradation of $S1P_1$-eGFP (Fig. 3a, Supplemental Table 2). Lymphocytic $S1P_1$-eGFP did not fully recover to untreated levels by eight hours after treatment, despite restoration of normal lymphocyte recirculation, indicating that decreased lymphocytic $S1P_1$ is not the driving force behind maintenance of lymphocyte sequestration by RP-001. 0.2mg/kg FTY720 treatment also induced $S1P_1$-eGFP degradation on lymphocytes corresponding with sequestration of T and B lymphocytes from the blood, which partially recovered by 48 hours following treatment (Supplemental Fig. 8a). Due to the carboxyl-terminal location of eGFP, we were unable to distinguish by flow cytometry whether $S1P_1$-eGFP was on the cell surface or internalized, reinforcing the need for widely available extracellular $S1P_1$ antibodies, the development of which continue to be troublesome.

We examined whether RP-001 was able to induce changes in the subcellular localization of $S1P_1$-eGFP on lymphocytes by sucrose gradient density centrifugation, similar to previous work with COS7 cells expressing tagged $S1P_1$ *in vitro*[21]. $S1P_1$-eGFP on lymphocytes from untreated mice was found predominantly in Caveolin-1-rich (Cav1) fractions, with significant $S1P_1$-eGFP being found in fractions that colocalized with the early endosomal marker EEA-1 (Fig. 3b), demonstrating significant receptor reserve in Cav1-rich fractions as well as constitutive activation and internalization into endosomal compartments. Following treatment of mice for 2 hours with 0.1 mg/kg or 1 mg/kg RP-001, lymphocytic Cav1-associated fractions demonstrated no significant loss of $S1P_1$-eGFP, but endosomal fractions exhibited a loss of $S1P_1$-eGFP, suggesting lysosomal sorting and degradation of $S1P_1$-eGFP (Fig. 3c, Supplemental Fig. 9). Cav1-associated $S1P_1$-eGFP could potentially be heterogeneous, consisting of both cell-surface and internalized $S1P_1$-eGFP, which is likely shifted by RP-001 treatment.

We next investigated $S1P_1$-eGFP expression on endothelial cells within the lymph node by flow cytometry following enzymatic digestion[22], finding that both $CD31^+GP38^-$ blood and $CD31^+GP38^+$ lymphatic vessels were very abundant in $S1P_1$-eGFP (Fig. 3d). $S1P_1$-eGFP was readily observed on both lymphatic and blood endothelial cells within the lymph node by confocal microscopy, however lymphocytes were largely below the level of detection (Supplemental Fig. 10). Expression of $S1P_1$-eGFP on high endothelial venules (HEVs) was highly expressed on the luminal side of the cell, ideally situated for sensing changes in S1P levels in the blood. Treatment of mice with RP-001 caused changes in $S1P_1$-eGFP expression on both blood and lymphatic endothelial cells within the lung, demonstrating a small loss of $S1P_1$- eGFP two hours following 0.1mg/kg RP1138 treatment, but substantial degradation eight hours following treatment, when no agonist was present in circulation (Fig. 3e, Supplemental Table 3).

The generation of mice expressing $S1P_1$-eGFP also allowed us to directly observe $S1P_1$ expression *in vivo* using two-photon microscopy. Initial experiments confirmed our *ex vivo* findings, demonstrating expression on different types of endothelial cells within the lymph node and relatively little expression on lymphocytes other than motile $eGFP^{hi}$ $CD8^+$ T cells (Supplemental Fig. 11a, Supplemental Video 1). Treatment of mice with 0.1mg/kg RP-001, a dose that causes significant lymphocyte sequestration, did not lead to internalization of $S1P_1$- eGFP on endothelial cells within the lymph node within 80 minutes (Fig. 3f, Supplemental Fig. 11b). By explanting lymph nodes and incubating them in the presence of vehicle alone (Supplemental Video 2) or 250nM RP-001, a concentration 10 fold higher than the C_{max} of RP- 001 *in vivo* at 0.1mg/kg, we observed rapid ligand-induced internalization of $S1P_1$-eGFP on endothelial cells (Supplemental Fig. 3c, Supplemental Video 3), indicating that while higher concentrations of RP-001 could achieve full receptor occupancy on endothelial cells and cause significant internalization of $S1P_1$, this was not required for induction of lymphopenia.

Clarifying $S1P_1$ expression also allows insights into how $S1P_1$ agonist treatment can affect the pathogenesis of both multiple sclerosis[23] and influenza virus infection[24,25] by allowing direct analysis of $S1P_1$ expression on the variety of cell types that are involved in these pathologies, and how this expression changes following treatment with $S1P_1$ modulatory drugs. The study of the complex regulation of $S1P_1$ requires a full set of methodologies to provide a clearer view of the multiple roles that $S1P_1$ can play in many physiological systems. $Edg1^{eGFP/eGFP}$ mice, together with continued optimization of $S1P_1$ modulatory compounds, can help validate proposed roles of $S1P_1$, find novel roles for $S1P_1$ in physiological and pathophysiological systems, and aid in the clinical treatment of diseases such as multiple sclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Okamoto H, et al. J Biol Chem. 1998; 273:27104–27110. [PubMed: 9765227]
2. Liu Y, et al. J Clin Invest. 2000; 106:951–961. [PubMed: 11032855]
3. Allende ML, Proia RL. Biochim Biophys Acta. 2002; 1582:222–227. [PubMed: 12069832]
4. Sanna MG, et al. Nat Chem Biol. 2006; 2:434–441. [PubMed: 16829954]
5. Matloubian M, et al. Nature. 2004; 427:355–360. [PubMed: 14737169]
6. Sanna MG, et al. J Biol Chem. 2004; 279:13839–13848. [PubMed: 14732717]
7. Gonzalez-Cabrera PJ, et al. Mol Pharmacol. 2008; 74:1308–1318. [PubMed: 18708635]
8. Salvadori M, et al. Am J Transplant. 2006; 6:2912–2921. [PubMed: 17061999]
9. Mandala S, et al. Science. 2002; 296:346–349. [PubMed: 11923495]
10. Graler MH, Goetzl EJ. FASEB J. 2004; 18:551–553. [PubMed: 14715694]
11. O'Connor P, et al. Neurology. 2009; 72:73–79. [PubMed: 19122034]
12. Comi G, et al. Mult Scler. 2010; 16:197–207. [PubMed: 20028707]
13. Kappos L, et al. N Engl J Med. 2010; 362:387–401. [PubMed: 20089952]
14. Allende ML, Dreier JL, Mandala S, Proia RL. J Biol Chem. 2004; 279:15396–15401. [PubMed: 14732704]
15. Liu G, et al. Nat Immunol. 2009; 10:769–777. [PubMed: 19483717]
16. Wang W, Huang MC, Goetzl EJ. J Immunol. 2007; 178:4885–4890. [PubMed: 17404269]
17. Thangada S, et al. J Exp Med. 2010; 207:1475–1483. [PubMed: 20584883]
18. Mullershausen F, et al. Nat Chem Biol. 2009; 5:428–434. [PubMed: 19430484]
19. Grigorova IL, et al. Nat Immunol. 2009; 10:58–65. [PubMed: 19060900]
20. Wei SH, et al. Nat Immunol. 2005; 6:1228–1235. [PubMed: 16273098]
21. Igarashi J, Michel T. J Biol Chem. 2000; 275:32363–32370. [PubMed: 10921915]
22. Pham TH, et al. J Exp Med. 2010; 207:17–27. S11–14. [PubMed: 20026661]
23. Van Doorn R, et al. Glia. 2010; 58:1465–1476. [PubMed: 20648639]
24. Marsolais D, et al. Mol Pharmacol. 2008; 74:896–903. [PubMed: 18577684]
25. Marsolais D, et al. Proc Natl Acad Sci U S A. 2009; 106:1560–1565. [PubMed: 19164548]

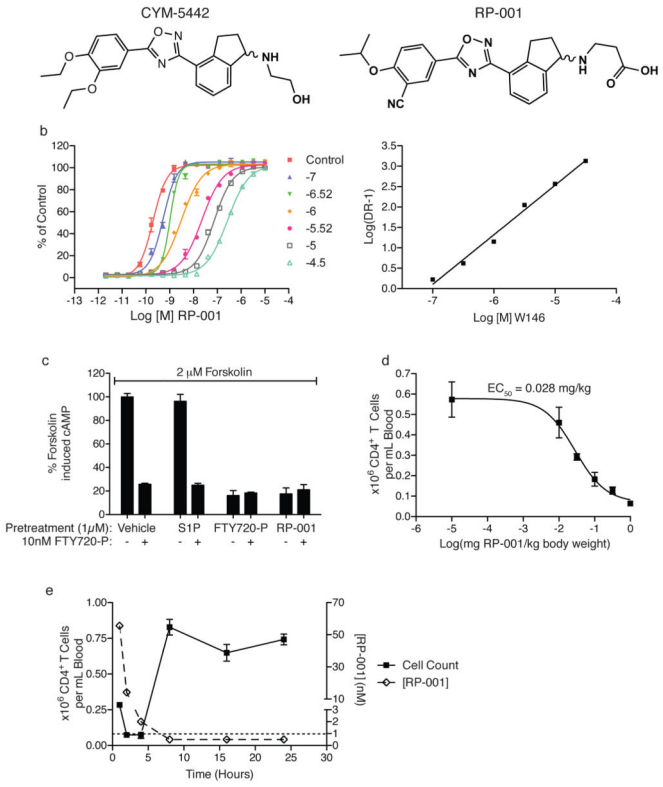

Figure 1. RP-001 is an orthosteric, short-duration $S1P_1$ selective agonist

(a) Chemical structures of CYM-5442 and its derivative, RP-001. **(b)** Left graph represents dose- response curves of RP-001 activation of $S1P_1$, expressed as a percent of S1P-induced activation. Right graph is a Schild Plot demonstrating competition between RP-001 and the $S1P_1$ antagonist W146. **(c)** RP-001 induces sustained $S1P_1$ signaling in internalized vesicles. $S1P_1$ CRE-β-lactamase expressing CHO-K1 cells were pretreated with 1μM of the indicated compounds for 1 hour, washed, and rested for 5 hours. Cells were then treated with 2μM forskolin with or without 10nM FTY720 as indicated, and cyclic AMP induced beta lactamase expression was detected by cleavage of CC4-AM. **(d)** RP-001 elicits dose-dependent lymphopenia of $CD4^+$ T cells. **(e)** RP-001 induces acute lymphopenia with rapid recovery to untreated levels. Graph for **d** and **e** display number of $CD4^+$ T cells per mL of blood, while **e** additionally displays concentration of RP-001 found in the blood as quantified by mass spectrometry with a dashed line. The bottom horizontal line represents the lower limit of detection of RP-001 (1nM). All intravital experiments are representative of at least 3 experiments, 3–4 mice per group per experiment and are presented as mean ±SEM.

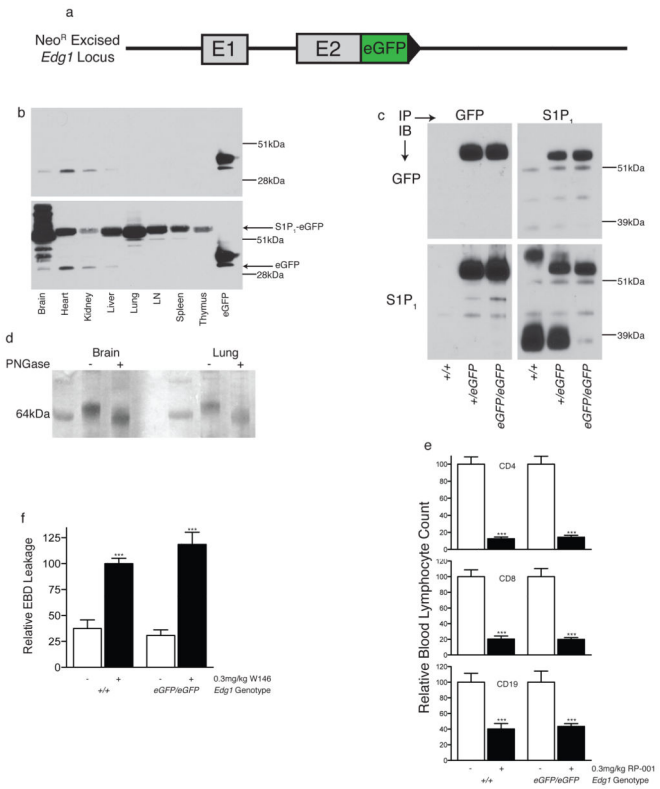

Figure 2. Expression and function of $S1P_1$-eGFP in *Edg1*$^{eGFP/eGFP}$ mice

(a) Schematic for the final locus for mice expressing *Edg1-eGFP* from the *Edg1* locus. E1 and E2 represent exons 1 and 2 of *Edg1*, triangle represent loxP recombination site **(b)** $S1P_1$ is broadly expressed across many tissues by Western blot for eGFP from tissues listed under non- denaturing conditions. **(c)** $S1P_1$-eGFP is expressed similarly to wild-type $S1P_1$. Brain lysates were immunoprecipitated with either an antibody specific to the carboxyl-terminus of $S1P_1$ or an antibody specific to GFP. These lysates were separated by SDS-PAGE, transferred onto membrane, and incubated with antibodies specific to either $S1P_1$ or GFP. **(d)** $S1P_1$-eGFP is N- Glycosylated *in vivo*. Homogenized brain or lung tissues from *Edg1*$^{eGFP/eGFP}$ mice were immunoprecipitated with an antibody specific to GFP then incubated with or without PNGase F. The smaller size of $S1P_1$-eGFP following incubation with PNGase F indicates loss of N-linked sugars from $S1P_1$-eGFP. **(e)** T and B lymphocytes from *Edg1*$^{eGFP/eGFP}$ and *Edg1*$^{+/+}$ mice are sequestered following $S1P_1$ agonist RP-001 treatment. Lymphocyte counts were taken two hours after intraperitoneal injection of either 0.3mg/kg RP-001 or vehicle. **(f)** Enhancement of vascular leakage following $S1P_1$ antagonism. Graphs represent leakage of Evans' Blue Dye into the lungs 1 hour after treatment with 3mg/kg W146 or vehicle intraperitoneally, standardized to wild-type mice treated with W146. Graphs in **e** and **f** represent mean +/− SEM for 2 pooled experiments, 3–4 mice per group per experiment. *p<0.05, **p<.01, ***p<0.001 for **e** and **f** using unpaired t-test.

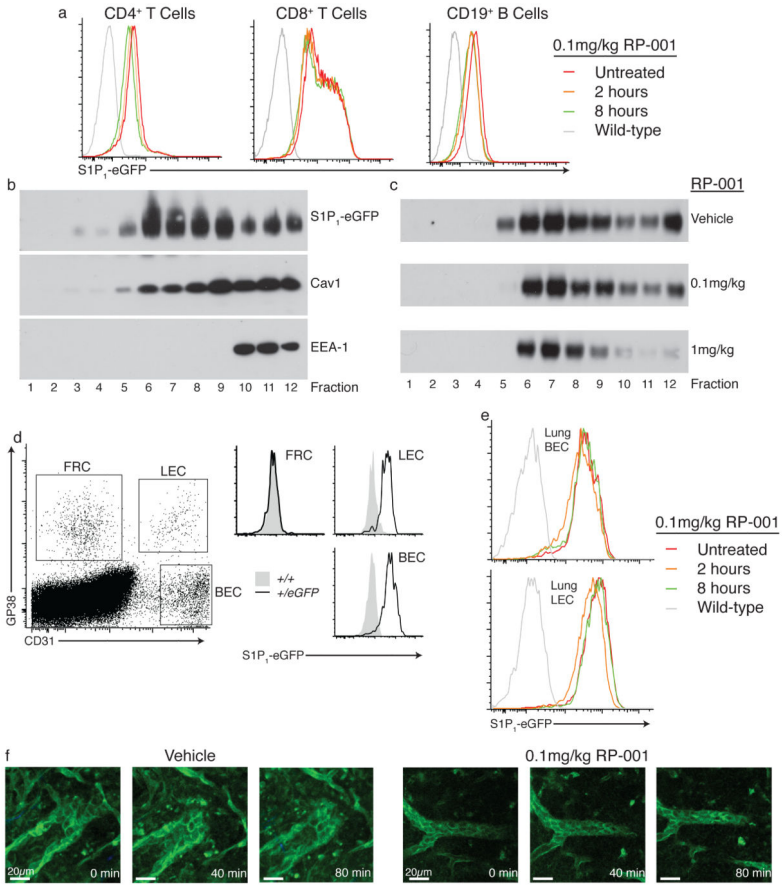

Figure 3. RP-001 causes changes in lymphcytic and endothelial $S1P_1$-eGFP expression and localization

(a) Flow cytometry histograms for eGFP fluorescence on specified lymphocyte populations isolated from lymph nodes of *Edg1*$^{eGFP/eGFP}$ (colored) and wild-type (gray) mice at indicated timepoints following IP injection of RP-001. **(b)** Lymphocytes were homogenized and mixed 1:1 with 90% sucrose. These lysates were placed in centrifuge tubes and overlaid sequentially with equal volumes of 35% sucrose followed by 5% sucrose. Following centrifugation at 100,000×g for 16 hours, twelve equal volume fractions were collected and Western blotted for GFP, Caveolin-1 (Cav1), Early Endosomal Antigen 1 (EEA-1). **c)** Equal numbers of lymphocytes were isolated and centrifuged as above. $S1P_1$-eGFP in Cav1-rich fractions is highly preserved following treatment with RP-001, but endosomal-associated $S1P_1$-eGFP is lost, particularly at 1mg/kg. **(d)** Left panel represents scatter plot of GP38 and CD31 expression on CD45.2$^-$ cells isolated from Collagenase/DNaseI digested lymph nodes. Right panels are histograms for eGFP fluorescence on blood endothelial cells (BECs), lymphatic endothelial cells (LECs), and fibroblast reticular cells (FRCs). **(e)** Flow cytometry histograms for eGFP from lung BECs and LECs from the same mice as in **a. (f)** Snapshots from intravital two-photon microscopy of lymph nodes treated as indicated. Internalization of $S1P_1$-eGFP is not detected at 0.1mg/kg at timepoints up to 80 minutes. Flow cytometry plots in **a** and **e** are representative of 3 experiments, 3 mice per group per experiment.