The lysophosphatidylserine receptor GPR174 constrains regulatory T cell development and function

Michael J. Barnes,1,2 Chien-Ming Li,3 Ying Xu,1,2 Jinping An,1,2 Yong Huang,3 and Jason G. Cyster1,2

1Howard Hughes Medical Institute, 2Department of Microbiology and Immunology, and 3Department of Bioengineering and Therapeutic Sciences, University of California, San Francisco, San Francisco, CA 94143

Regulatory T cell (T reg cell) numbers and activities are tightly calibrated to maintain immune homeostasis, but the mechanisms involved are incompletely defined. Here, we report that the lysophosphatidylserine (LysoPS) receptor GPR174 is abundantly expressed in developing and mature T reg cells. In mice that lacked this X-linked gene, T reg cell generation in the thymus was intrinsically favored, and a higher fraction of peripheral T reg cells expressed CD103. LysoPS could act in vitro via GPR174 to suppress T cell proliferation and T reg cell generation. In vivo, LysoPS was detected in lymphoid organ and spinal cord tissues and was abundant in the colon. Gpr174−/− mice were less susceptible to experimental autoimmune encephalomyelitis than wild-type mice, and GPR174 deficiency in T reg cells contributed to this phenotype. This study provides evidence that a bioactive lipid, LysoPS, negatively influences T reg cell accumulation and activity through GPR174. As such, GPR174 antagonists might have therapeutic potential for promoting immune regulation in the context of autoimmune disease.

GPR174 is one of four GPCRs known to be activated by the bioactive lipid lysophosphatidylserine (LysoPS; Inoue et al., 2012). Phospholipase A1 and A2 enzymes can catalyze the generation of LysoPS by hydrolyzing phosphatidylserine (PS) at the sn-1 or sn-2 position, respectively, resulting in the removal of an acyl chain (Grzelczyk and Gendaszewska-Darmach, 2013). The enzyme ABHD16a also exhibits PS lipase activity, and Abhd16a deficiency results in reduced LysoPS levels in vivo (Kamat et al., 2015). LysoPS species vary by acyl chain length and saturation, among which the 16:0, 18:0, and 18:1 isoforms are the most abundant in brain, heart, kidney, and lung tissues (Blankman et al., 2013). PS-PLA1, ABHD6, and ABHD12 can catalyze the degradation of LysoPS, and genetic deficiencies in the latter two enzymes have been linked to metabolic syndrome and inflammatory neurodegenerative disease, respectively (Sato et al., 1997; Blankman et al., 2013;...
RESULTS AND DISCUSSION

Enriched GPR174 and LysoPS receptor expression in T reg cells

Our initial interest in GPR174 stemmed from an effort to identify GPCRs involved in regulating lymphocyte transit through lymphoid organs (Pham et al., 2008). Quantitative PCR analysis of the mRNA expression levels of 353 non-odorant GPCRs (Regard et al., 2008) in naïve T and B cells identified Gpr174 (previously known as Fksg79) as one of the most abundantly expressed transcripts (not depicted). To characterize the role of this X-linked gene in the immune system, we replaced the single coding exon with a cassette encoding an "in-frame" dTomato allele (Fig. 1 A). Analysis of dTomato expression in Gpr174−/− male mice (Fig. 1, B–D) confirmed high levels of GPR174 expression in naïve T and B cells (Fig. 1, B and C), and dTomato expression patterns were similar to Gpr174 mRNA expression levels (Fig. 1, D and E). Naïve T and B cell numbers and lymphoid tissue organization were normal in Gpr174−/− mice (not depicted). In LN transit assays (Pham et al., 2008), no differences in trafficking between wild-type and Gpr174−/− T or B cells were detected (not depicted). Further characterization of dTomato expression showed abundant GPR174 expression in CD25+CD4+T cells, populations that are highly enriched in naïve T reg cells, Thomas et al., 2013). Roles for LysoPS in suppressing T cell proliferation in vitro (Bellini and Bruni, 1993) and activating mast cells (Martin and Lagunoff, 1979) have been described, but the mechanisms whereby it mediates these effects and its importance in vivo remain unclear.

The first LysoPS receptor to be deorphanized was GPR34, an X-linked GPCR that is most abundantly expressed in microglia, capable of coupling to Gαi-containing heterotrimerics, and protective in the central nervous system (CNS) against Cryptococcus neoformans infection–induced pathology (Liebscher et al., 2011; Kitamura et al., 2012). Subsequently, three other GPCRs, GPR174, P2RY10, and P2RY10-L, were identified as selective and high-affinity LysoPS receptors using an in vitro screening approach (Inoue et al., 2012). These three receptors are all closely linked on the X chromosome, abundantly expressed by many immune cell types, and capable of signaling via Gz12/Gz13-containing heterotrimeric G proteins; GPR174 has also been suggested to have Gαs affinity (Sugita et al., 2013). Functions for these three receptors in the immune system have not yet been described.

Herein, we report that LysoPS is abundant in the thymus, peripheral lymphoid tissues, CNS, and colon, and that T reg cell homeostasis is altered in mice that lack the LysoPS receptor GPR174. In the thymus, T reg cells from Gpr174−/− mice accumulated, and in the periphery, they showed increased CD103 expression; both phenotypes occurred in a cell-intrinsic manner. Furthermore, in the experimental autoimmune encephalomyelitis (EAE) model of CNS autoimmunity, GPR174-deficient T reg cells could limit immunopathology.
compared with naive CD4\(^+\) T cells (Fig. 1, B and C). These Gpr174 gene expression patterns were confirmed by quantitative RT-PCR analysis of sorted cell populations from wild-type mice (Fig. 1 E). We observed that elevated Gpr174 expression correlated with Foxp3 expression in both immature T reg (Foxp3\(^+\)CD25\(^-\)) and mature T reg (Foxp3\(^+\)CD25\(^+\)) CD4 SP thymocytes, whereas levels were not elevated in Foxp3\(^+\)CD25\(^+\) thymic T reg cell precursors (Fig. 1 E). In contrast, when we differentiated naive CD4\(^+\) T cells in Th1 or Th17 polarizing conditions, reduced GPR174-dTomato expression was observed compared with cells cultured in Th0 conditions (Fig. 1 D).

To characterize how T cells might sense LysoPS, we measured mRNA expression levels of the four known LysoPS receptors. Similar to Gpr174, its homologue P2ry10 also showed elevated mRNA levels in Foxp3\(^+\) T reg cells and thymocytes; however, unlike Gpr174, expression in double-positive (DP) thymocytes was also elevated (Fig. 1 E). Transcripts encoding P2ry10 were less abundant and not enriched in T reg cells or thymocytes. Gpr34 transcripts were detectable in Foxp3\(^+\) T reg cells, although their levels appeared to be at least an order of magnitude lower than those encoding Gpr174 or P2ry10 (Fig. 1 E).

**Role of GPR174 in T reg cell accumulation and homeostasis**

Because T reg cells expressed high amounts of LysoPS receptors, we characterized the effects of GPR174 deficiency on these cells. Initially, we took advantage of the linkage of Gpr174 to the X chromosome, for which one copy is subject to random inactivation in female cells. Therefore, approximately half of the hematopoietic cells in female Gpr174\(^{-/-}\) mice express the Gpr174\(^{-/-}\)-dTomato allele. Intriguingly, we consistently detected an increased frequency of dTomato\(^+\) cells among CD25\(^-\) CD4 SP thymocytes (Fig. 2 A), suggesting that the fitness of T reg cells might be greater when GPR174 is absent. To quantify T reg cell abundance in Gpr174\(^{-/-}\) mice, we measured Foxp3 expression in the thymus, secondary lymphoid organs, and colon lamina propria. The frequency of Gpr174\(^{-/-}\) Foxp3\(^+\) CD4 SP cells was significantly increased in the thymus, the site where most T reg cells develop, and in the colon lamina propria, where both thymus-derived and peripherally induced T reg cells can accumulate in microbiota-colonized mice (Fig. 2 B; Bollrath and Powrie, 2013). In contrast, T reg cell frequencies were unaffected in the spleen and LNs (Fig. 2 B), where extrinsic factors that include γ\(_c\)-cytokines govern the niche size. Therefore, GPR174 deficiency appeared to favor T reg cell accumulation in specific tissues.

T reg cells can be subdivided into different subsets that have been associated with specialized regulatory functions (Campbell and Koch, 2011). To assess whether GPR174 deficiency favored the accumulation of a T reg cell subset, we analyzed a panel of cell surface molecules associated with T reg cell differentiation. No changes in T reg cell expression of CCR7, CD25, CD44, CD62L, CXCXR3, CXCXR6, or E- or P-selectin were detected in the thymus, spleen, or LN of Gpr174\(^{-/-}\) mice (not depicted). However, more Gpr174\(^{-/-}\) T reg cells expressed CD103, a marker associated in some contexts with augmented in vitro and in vivo suppressive activity and trafficking to nonlymphoid tissues (McHugh et al., 2002; Huhne et al., 2004; Suffia et al., 2005; Campbell and Koch, 2011; Geuken et al., 2011). This effect was not
observed in Gpr174−/Y thymic T reg cells but could be observed in peripheral lymphoid tissues, suggesting that GPR174 constrains the expression of CD103 by T reg cells after they egress from the thymus (Fig. 2 C). The levels and frequencies of Helios expression, which have been associated with thymus-derived T reg cells, were similar in T reg cells from wild-type and Gpr174−/Y mice (Fig. 2 C), suggesting that GPR174 deficiency did not bias thymus-derived versus peripherally induced T reg cell accumulation under homeostatic conditions.

The T reg cell–constraining influence of GPR174 was cell intrinsic, as Gpr174−/Y Foxp3+ CD4 SP thymocyte frequencies were significantly higher than those of Gpr174+/Y DP and CD4 SP thymocytes in mixed bone marrow chimeric mice (Fig. 2 D). The contribution of Gpr174−/Y cells to the naive T cell compartment matched the extent of reconstitution of other lineages, such as granulocytes that lack GPR174 expression (not depicted), consistent with GPR174 having a selective role in T reg cells. Under these competitive conditions, Gpr174−/Y-derived T reg cells maintained their overrepresentation in peripheral lymphoid organs (Fig. 2 D).

These data suggest that after wild-type or Gpr174−/Y T reg cell generation occurs in the thymus, the cells are equally competent to be maintained in the periphery. Moreover, the overall peripheral niche size available to T reg cells is not altered by GPR174 deficiency. The frequency of Gpr174−/Y-derived T reg cells expressing CD103 continued to be increased in mixed chimeras, indicating a cell-intrinsic bias for the accumulation of cells expressing this adhesion molecule (Fig. 2 E).

No differences in Foxp3 or CD103 expression were detected in control wild-type (congenic) mixed bone marrow chimeras (Fig. 2 E and not depicted). The gene expression profile of Gpr34 led us to consider whether this LysoPS receptor also modulated T reg cell development; however, we found equivalent frequencies of Gpr34−/Y and wild-type–derived Foxp3+ and Foxp3+CD103+ cells in the thymus and periphery of mixed bone marrow chimeras (not depicted).

To better characterize the development of thymic Gpr174−/Y T reg cells, we crossed Gpr174−/Y mice to animals expressing a Nur77-GFP transgene (Moran et al., 2011). Levels of Nur77 increase in positively selected thymic T reg cells and CD25+Foxp3+ T reg cell precursors in response to recent MHC class II–dependent signaling. Importantly, Nur77 levels are reduced in conditions that favor enhanced T reg cell development, such as in response to exogenous TNFR family–mediated co-stimulation (Mahmud et al., 2014). CD25+ CD4 SP thymocytes in Gpr174−/Y mice expressed lower levels of Nur77−GFP, consistent with a model in which GPR174 deficiency results in the exposure of developing thymocytes to T reg cell–favoring signals (Fig. 3 A). Additionally, we noted that CD25− CD4 SP thymocytes showed a very slight reduction in Nur77−GFP levels (Fig. 3 A). Because TNF receptor family members contribute to thymic T reg cell development and reduced Nur77−GFP abundance (Mahmud et al., 2014), we measured levels of glucocorticoid-induced TNFR family–related gene (GITR), OX40, and TNFR2 in the Foxp3+ and Foxp3− CD4 SP subsets and detected no differences between wild-type and Gpr174−/Y thymocytes (not depicted). In line with the accumulation of Gpr174−/Y thymic T reg cells, more Foxp3+, but not Foxp3− CD4 SP thymocytes expressed Ki-67, a marker of cells that have recently divided, compared with cells in wild-type littermates (Fig. 3 B). This effect was most obvious in the thymus of mice younger than 8 wk old (not depicted). Together, these data suggest that GPR174 signaling might intrinsically constrain thymic T reg cell proliferation.

LysoPS suppresses CD4+ T cell proliferation and T reg cell differentiation via GPR174

LysoPS was previously reported to suppress lymphocyte proliferation in human PBMC cultures (Bellini and Bruni, 1993). To test whether LysoPS could suppress mouse T cell proliferation, we cultured total LN cells with mitogenic soluble anti-CD3 mAb in the presence of 1 or 10 μM LysoPS. The proliferation of wild-type CD4+ T cells could be suppressed by LysoPS, and this effect was GPR174 dependent (Fig. 3 C).

Next, we sorted naive CD4+ T cells and measured the proliferation of co-cultured wild-type and Gpr174−/Y cells after stimulation with plate-bound anti-CD3 and anti-CD28 mAbs in the presence of exogenous IL-2. In this setting, LysoPS effectively suppressed the proliferation of wild-type, but not Gpr174−/Y cells, suggesting that LysoPS can directly inhibit T cell proliferation in vitro (Fig. 3 D). However, the in vivo proliferation of Gpr174−/Y OT-II T cells 3 d after intra-peritoneal immunization with OVA in alum adjuvant occurred comparably with that of wild-type OT-II T cells (not depicted), indicating that this repressive pathway might only act in certain contexts. To test whether LysoPS could affect the induction of Foxp3−expressing cells in vitro, naive T cells were cultured under TGF-β–mediated T reg cell–skewing conditions. The addition of LysoPS reduced the number and frequency of Foxp3+ cells in wild-type T cell cultures, whereas these effects were not observed in Gpr174−/Y T cell cultures (Fig. 3 E). These influences of LysoPS signaling via GPR174 might contribute to the T reg cell accumulation observed in Gpr174−/Y mice.

Enhanced T reg cell activity and reduced
Th1 responses of Gpr174−/Y T cells

In addition to affecting T reg cell development and turnover, we considered that GPR174 might alter T reg cell functionality. In vitro CD4+ T cell suppression assays can reveal some aspects of T reg cell function (Thornton and Shevach, 1998). In this assay, GPR174-deficient T reg cells could more potently suppress the anti-CD3 and DC-induced proliferation of naive CD4+ responder T cells compared with wild-type control T reg cells (Fig. 3 F). When we separated T reg cells into CD103+ and CD103− subsets, we found that the CD103− subset of Gpr174−/Y T reg cells were better suppressors than their wild-type counterparts (Fig. 3 F). Furthermore, CD103+ T reg cells were more potent suppressors than CD103− T reg cells for both wild-type and Gpr174−/Y cells.
This phenotype was observed even in the absence of exogenous LysoPS. The addition of 200 nM or 2 µM LysoPS increased the frequency of Th1 cells, but unlike its activities in T reg cell–skewing cultures, it did not reveal GPR174-specific effects as the frequency of Th1 cells increased similarly in both wild-type and Gpr174−/− cell cultures (Fig. 3 G). In contrast, neither GPR174 deficiency nor exogenous LysoPS affected in vitro Th17 cell differentiation (Fig. 3 G). The reduced Th1 cell differentiation propensity of Gpr174−/− T cells could be a consequence of LysoPS influences during T cell development, as suggested by the CD25−CD4+ SP thymocyte Nur77-GFP expression profile (Fig. 3 A);
pools of LysoPS to be distinguished, it is notable that a concentration of 1 µg/g corresponds to ~2 µM LysoPS. Plasma contained much lower concentrations of all three LysoPS isoforms that were below the limit of detection for our assay (not depicted). We also analyzed LysoPS concentrations in two common sites of immunopathology. Spinal cord tissues exhibited slightly lower 18:0 and 16:0 LysoPS concentrations compared with lymphoid tissues but contained almost an order of magnitude more 18:1 LysoPS (Fig. 4 A). Colon tissues contained an order of magnitude more 18:0 LysoPS compared with any other tissue measured, in addition to high levels of 18:1 and 16:0 LysoPS (Fig. 4 A).

The network of enzymes that regulates the generation and degradation of LysoPS is likely to be complex. To assess the abundance of known enzymes with effects on PS → LysoPS reactions or the degradation of LysoPS in immune tissues, we performed quantitative PCR analyses. ABHD16a, an enzyme with established activity in generating LysoPS in vivo (Kamat et al., 2015), was broadly expressed in DCs, macrophages, lymphocytes, and lymphoid organs (Fig. 4 B). Transcripts for Pla1a mRNA, which encode an enzyme that can both generate and degrade LysoPS (Sato et al., 1997), were most abundant in myeloid cells. Levels of Pla2g2d and Pla2g5 mRNA transcripts were high in lymphocytes and DCs but low in peritoneal macrophages. Pla2g5 mRNA transcript levels were markedly lower in the thymus than in the spleen. Transcripts encoding ABHD6 and ABHD12, enzymes that have been shown in vitro and in vivo to degrade LysoPS (Blankman et al., 2013; Thomas et al., 2013), could also be detected in lymphoid cells and spleen but were notably underexpressed in the thymus (Fig. 4 B). The expression in DCs of several LysoPS synthetic enzymes (Abhd16a, Pla2g2d, and Pla2g5) raises the possibility that LysoPS was acting in a paracrine fashion in the in vitro suppressor experiments (Fig. 3 F). Although we were not able to measure changes in LysoPS in the culture supernatants with our current LC-MS/MS procedure (not depicted), it is notable that activated myeloid cells can secrete LysoPS (Kamat et al., 2015), which might allow for localized changes in ligand abundance. Future studies of mice lacking individual phospholipase enzymes will be needed to elucidate the key enzymes responsible for LysoPS production and metabolism in lymphoid cells and tissues.

GPR174 deficiency in T reg cells protects from neuroinflammation

Finally, given the constraining influence of GPR174 on T reg cell development and functionality, we examined whether GPR174 deficiency might protect mice from autoimmune pathology. To induce EAE, we administered myelin oligodendrocyte glycoprotein (MOG) peptide in CFA along with pertussis toxin. In this disease model, Th1 and Th17 cells drive CNS inflammation, whereas T reg cells can limit disease severity and promote the remission of symptoms (Stromnes and Goverman, 2006). Although the day of disease onset was unaffected, Gpr174<sup>−/−</sup> male mice showed significantly reduced
brief definitive report

considerable range in $Gpr174^{+/−}$ females (Fig. 2 A). To make the T reg cell compartment in $Gpr174^{+/−}$ females nearly completely GPR174 deficient, we crossed $Gpr174^{+/−}$ females to Foxp3DTR/Y males to yield offspring with a $Gpr174^{−/−}$ allele on one X chromosome and a Foxp3DTR allele on the other. Administering diphtheria toxin to these mice selectively ablated the $Gpr174^{Foxp3DTR}/Y$ T reg cells, in accord with past work (Fig. 5 E; Pierson et al., 2013), and allowed GPR174-deficient T reg cells to fill the niche (not depicted). At day 10 after diphtheria toxin treatment, we induced EAE as before and found that T reg cell–specific GPR174 deficiency significantly protected mice from EAE (Fig. 5 F). Therefore, we identify T reg cell expression of GPR174 as a nonredundant factor that can contribute to immunopathology.

Concluding remarks

Our in vitro and in vivo findings show that GPR174 acts in Foxp3+ T cells to constrain their development and immune regulatory function. Thus, under inflammatory conditions where LysoPS production can increase (Kamat et al., 2015), GPR174 could contribute to down-modulating T reg cell activity to favor effector responses. Although this may be beneficial during responses to infections, it might allow more severe tissue damage during autoimmune responses. The complete mechanism whereby GPR174 signaling restrains T reg cell

Figure 5. GPR174 deficiency in T reg cells limits the severity of EAE. (A) EAE was induced in 10-wk-old male wild-type or $Gpr174^{+/−}$ littermate mice by injecting a MOG35–55 + CFA emulsion (s.c.) followed by pertussis toxin (i.v.). Mice were scored daily for disease symptoms; mean disease scores are shown and error bars indicate SEM. (B) The frequency of Foxp3+ T reg cells among total CD4+ T cells was determined in the indicated tissues 12 d after EAE induction in 10-wk-old male wild-type or $Gpr174^{+/−}$ littermate mice by flow cytometry. (C) EAE was induced in a cohort of wild-type mice, and levels of LysoPS were measured by LC-MS/MS in the inguinal LN that drained the MOG + CFA emulsion (dotted lines) and in the spinal cord (solid lines) throughout the course of disease; $n = 3–5$. (B and C) Error bars show SEM. (D) EAE was induced in 10-wk-old female $Gpr174^{+/−}$ or wild-type littermate mice as in A; error bars show SEM. (E and F) In $Gpr174^{+/−}$ or wild-type female mice heterozygous for an X-linked Foxp3-DTR allele, diphertheria toxin (DTx) was injected on days −10, −7, and −4. EAE was induced as in A on day 0. The ablation efficiency was assessed by analyzing the frequency of DTR-GFP+ cells among blood CD4+CD25+ T reg cells at the indicated time points (E); error bars show SD. Disease was scored daily after MOG immunization (F); $n = 11$; error bars show SEM. Data are representative of two (E and F) or three or more (A–D) independent experiments. Differences between groups were compared using unpaired Student’s t test for each time point: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

peak disease severity compared with wild-type littermates (Fig. 5 A). At day 12, a time point when $Gpr174^{+/−}$ and wild-type mice showed a similar disease score, the frequency of Foxp3+ T reg cells was not significantly affected by GPR174 deficiency in the secondary lymphoid organs or spinal cord (Fig. 5 B); however, the frequency of CD103+ cells among T reg cells remained elevated in the draining LN at days 8–12 (not depicted). Moreover, levels of 16:0, 18:0, and 18:1 LysoPS did not appear to markedly change in the draining LN or spinal cord throughout the experiment (Fig. 5 C). These whole tissue analyses do not exclude the possibility that there are changes in other LysoPS isoforms (Blankman et al., 2013; Kamat et al., 2015) or local changes in interstitial LysoPS concentrations that affect cell behavior.

To test whether intrinsic effects on T reg cells contributed to the reduced disease severity, we examined female $Gpr174^{+/−}$ mice because they have a mixture of wild-type and GPR174-deficient T reg and naive T cells as a consequence of X chromosome inactivation (Fig. 2 A). We predicted that if GPR174-deficient T reg cells in these mice had more potent activity, they might act in trans to suppress EAE. Indeed, disease was modestly, but significantly, reduced in $Gpr174^{+/−}$ female mice compared with wild-type littermates (Fig. 5 D). The frequency of inactivation of the wild-type versus the GPR174-deficient X chromosome showed a considerable range in $Gpr174^{+/−}$ females (Fig. 2 A). To make the T reg cell compartment in $Gpr174^{+/−}$ females nearly completely GPR174 deficient, we crossed $Gpr174^{+/−}$ females to Foxp3DTR/Y males to yield offspring with a $Gpr174^{−/−}$ allele on one X chromosome and a Foxp3DTR allele on the other. Administering diphtheria toxin to these mice selectively ablated the $Gpr174^{Foxp3DTR}/Y$ T reg cells, in accord with past work (Fig. 5 E; Pierson et al., 2013), and allowed GPR174-deficient T reg cells to fill the niche (not depicted). At day 10 after diphtheria toxin treatment, we induced EAE as before and found that T reg cell–specific GPR174 deficiency significantly protected mice from EAE (Fig. 5 F). Therefore, we identify T reg cell expression of GPR174 as a nonredundant factor that can contribute to immunopathology.
activities, and whether this involves Gα12, Gα13, or Gαs, is not yet clear and will require further investigation. Our in vitro experiments also suggest that there could be conditions in vivo whereby LysoPS exerts a direct T cell proliferation–inhibitory or Th1-skewing effect via GPR174 independently of its actions on T reg cells. Finally, although our findings are consistent with GPR174 functioning as a LysoPS receptor, we do not exclude the possibility that other physiologically relevant ligands exist for this receptor. Overall, our findings inform recent genome-wide association studies that linked human SNPs in or near the GPR174 locus to autoimmune conditions Graves’ disease and rheumatoid arthritis (Chu et al., 2013; Zhao et al., 2013; Okada et al., 2014). Given its function in T reg cells, studies of GPR174 antagonists to treat such conditions might be warranted.

MATERIALS AND METHODS

Mice. To generate Gpr174−/− mice, the Gpr174 locus was targeted using a standard homologous recombination approach in 129-background embryonic stem cells, as shown in Fig. 1 A. The entire Gpr174 open reading frame is depicted by a black box, and gray boxes represent untranslated regions. Dashed arrows indicate the homologous regions of the arms of the targeting construct. EcoKI sites that were used for the Southern blot confirmation of homologous recombination and the size of the predicted DNA fragments are indicated. In the Gpr174 open reading frame, we inserted a dTomato allele to track fluorescence as a surrogate for Gpr174 expression levels. Gpr174−/− mice were backcrossed to the C57BL/6 background (000664; The Jackson Laboratory) for 12 generations. Nur77-GFP (018974; The Jackson Laboratory) and Foxp3-diphtheria toxin receptor (DTR; 016958; The Jackson Laboratory and Foxp3–diphtheria toxin receptor (DTR; 016958; The Jackson Laboratory) and Foxp3-GFP (eBioscience) and Rudensky (Memorial Sloan-Kettering Cancer Center, New York, NY) laboratories, respectively. Gpr174−/− mice (Liebsher et al., 2011) were provided by T. Schoeberg and A. Schulz (Institut für Biochemie, Universität Leipzig, Leipzig, Germany).

To produce bone marrow chimeric mice, B6-LY5.2/Cr mice (01B96; The National Cancer Institute) were lethally irradiated with a split dose of 1,100 rad γ-radiation and then were i.v. injected with a mixture of bone marrow cells from F1 C57BL/6/J × C57BL/6-BoyJ (002014; The Jackson Laboratory) and F153, 522→153, 508→153, and 524→153, 522 mice that express GFP in all CD4+ naive T cells that were routinely at least 99% pure.

Flow cytometry. For cell surface staining, empirically determined dilutions of primary mAbs were used to stain single cell suspensions on ice for 20 min in FACS buffer (PBS with 2% FBS, 0.1% NaN3, and 1 µM EDTA). All mAbs were purchased from BioLegend unless otherwise indicated. The following mAb clones were used: B238-PE-Cy7, CD4–PECy5, CD8α–PerCP-Cy5.5, CD11b–PE-Cy7, CD11c–FITC, CD25–Alexa Fluor 647, CD44–FITC, CD45.1–FITC, CD45.2–BV605, CD62L–PE-Cy7, CD103–biotin (followed by streptavidin-BV711; BD), Foxp3–PE (eBioscience), Helios–APC, I-A(b)–PE; Ki–67–Alexa Fluor 647, and TCR-β–Pacific Blue. Dead cells were excluded using Fixable Viability Dye eFluor780 (eBioscience). Intracellular staining to detect Foxp3 expression was performed according to the manufacturer’s instructions, using the manufacturer’s protocol for Foxp3 staining in 96-well plates (eBioscience). Cells were analyzed using an LSR-II flow cytometer (BD) equipped with 405-, 488-, 552-, and 640-nm lasers. Flow cytometry data were processed using FlowJo version 9.7.5 software (Tree Star).

Sample preparation for LC-MS/MS. Mouse tissues were homogenized using a Precellys 24 homogenizer with a Cryolys cooling unit (Bertin Technologies). Individual mouse tissues were placed in 2-mL sample vials with 10 µL water (10-fold dilution) and seven homogenization beads. Homogenization was conducted by three cycles for 20 s at 5,000 rpm (with 30-s breaks) at a temperature lower than 10°C.

After sample homogenization, 20 µl homogenized samples were each pipetted into 13 × 100 mm tubes. Next, 20 µl water and 20 µl internal standard solution (0.1 µg/ml) were added, and the solution was vortexed for 10 s.

Precipitate proteins, 100 µl acetonitrile (ACN; CH3CN) was added, and the solution was centrifuged at 3,000 rpm for 10 min. The supernatant was injected into an LC-MS/MS system.

LC-MS/MS. Samples (10-µl volume) were injected into a Shimadzu Prominence UFLC system equipped with a binary pump and a SIL-20AC autosampler. Separation was achieved on a ZIC-HILIC column (4.6 × 50 mm, 3.5 µm; SaQuant). A gradient separation was used consisting of mobile phase A (ACN/H2O [95%/5%, vol/vol]) containing 1 mM ammonium formate [NH4HCO3] and mobile phase B (ACN/H2O [10%/90%, vol/vol]) containing 1 mM NH4HCO3 delivered at a flow rate of 1 ml/min. Mobile phase B was used at 0% from 0 to 2.0 min, and then was increased linearly to 20% B within 3.4 min, followed by a quick ramp within 0.1 min to 100% B, which was maintained at 100% B for 1.5 min. After a quick ramp back to 0% mobile phase B, it was maintained at 0% for another 2.9 min until the end of the analysis.

Mass spectrometric detection was performed using an Applied Biosystems/SCIEX API 5000 triple quadrupole mass spectrometer operated in multiple reaction monitoring (MRM) mode via the negative electrospray ionization interface using the transitions (m/z) 496→153, 524→153, 522→153, and 508→153 for 16.0 LysoPS, 18:0 LysoPS, 18:1 LysoPS, and 17:1 LysoPS (as an internal standard), respectively. The ion source temperature was maintained at 400°C. The spraying needle voltage was set at −4.5 kV in positive mode. Curtain gas, collision gas (CAD), gas 1, and gas 2 were set at 20, 4, 50, and 50, respectively. The entrance potential (EP) was...
set at −10 V. The declustering potential and collision cell exit potential (CCE) were −150 mV and −15 V, respectively. Collinear energy (CE) was −35 eV for all compounds. Quantitation was performed based on the peak area ratio. Data acquisition and quantitative processing were accomplished using the Applied Biosystems Analyst version 1.5.1 software.

Measurements of gene expression. Total RNA was isolated using an RNeasy kit and in-column DNA digestion according to the manufacturer’s instructions (QIAGEN). For measurements of GPCR gene expression levels, RNA was isolated from 10⁶ MACS-enriched B cells, T cells, and DCs, day 4 thoglycinate–elicted peritoneal macrophages, and total spleen and thymus tissues. Gene expression levels were measured by real-time PCR using SYBR green PCR mix (Roche) on an ABI Prism 7300 sequence detection system (Applied Biosystems). Values were normalized to those of a housekeeping gene, Hprt. The following primers were used: Abhd6, (F) 5’-AAAGCCAG-GTGTGGTATGGTC-3’ and (R) 5’-TCTCATCCTCCGAGGCAAC-3’; Abhd12, (F) 5’-TCCAGTTTACCCCTTCCTAC-3’ and (R) 5’-CGTGGTGGCCTTCCCTCTA-3’; Abhd16a, (F) 5’-ATGTGTTG-CCGCCACGGG-3’ and (R) 5’-CTTCTAGGGGTGGTGAACAC-3’; Cpx34, (F) 5’-TGTATTTTCGTAGTCTCAGAT-3’ and (R) 5’-GCT- TTTACCTCCCTTGC-3’; Cpr174, (F) 5’-GGCTTATGTTTACCG- TACGG-3’ and (R) 5’-AGTCCAGACGTGAGTAGGT-3’; Hprt, (F) 5’-AGGTTGGAAGCTCTGGTGGT-3’ and (R) 5’-TAAGATCTCAT-TATAGTCAAGGGCA-3’; P2rly20, (F) 5’-AGGTCTCTGTATATTGCT- TCATT-3’ and (R) 5’-GATGGAATTACAGGCTATTTT-3’; Hprt, (F) 5’-AGGTTGGAAGCTCTGGTGGT-3’ and (R) 5’-TAAGATCTCAT-TATAGTCAAGGGCA-3’; P2rly20, (F) 5’-AGGTCTCTGTATATTGCT- TCATT-3’ and (R) 5’-GATGGAATTACAGGCTATTTT-3’; P2rly10, (F) 5’-TTTCCTGGCTGCTCCACTG-3’ and (R) 5’-CCCATCTGA-GTGTATATGTC-3’; Pta1a, (F) 5’-TGGATTATTATGGAGGA GGAGA-3’ and (R) 5’-GTGGGTTAGATGAGCCACCAT-3’; Pla2g2d, (F) 5’-GCTCTGCTGCTGCTGACATGTA-3’ and (R) 5’-CCCAGTGGTTG- GTTAACCCG-3’; and Pla2q5, (F) 5’-CTTACACTGCGCTTGGT-TCC-3’ and (R) 5’-CATTACCTGTTCTGTCAGAGC-3’.

Ex vivo T cell differentiation and proliferation. For T cell proliferation assays using total LNs, cellular LNs were meshed through 100-µm filters. Cells were resuspended in PBS + 0.1% BSA and labeled with 5 µM CFSE at 37°C in the dark for 10 min. Then, 4 × 10⁶ cells were added to round-bottom 96-well plates along with 0.25 µg/ml anti-CD3 and 2 µg/ml anti-CD28 mAbs as above. For the in vitro T cell suppressor assays, populations of CD103⁺ or CD103⁻, CD4⁺CD25⁺CD45RB⁻ T reg T cells were sorted along with concomitantly different CD4⁺CD25⁻CD45RB⁻ naïve T cells. Then, 4 × 10⁵ cells were added to wells at the indicated concentrations. Then, 4 d later, cells were harvested and Foxp3 expression was analyzed by flow cytometry.

To some cultures, 18:0 LysoPS (Avanti Polar Lipids) that was maintained as a 5 mM stock solution in cell culture–grade water with 0.5% DMSO was added to wells at the indicated concentrations. Then, 4 d later, cells were harvested and Foxp3 expression was analyzed by flow cytometry.

For TH1 and TH17 cultures, cells were sorted and cultured on plates coated with 2 µg/ml anti-CD3 and 2 µg/ml anti-CD28 mAbs as above. For TH1 cultures, 10 ng/ml IL-12 (PeproTech) and 10 µg/ml anti–IL-4 mAb were added. For TH17 cultures, 5 ng/ml TGF-β1, 10 ng/ml IL-1β, 20 ng/ml IL-6, and 10 ng/ml IL-23 (PeproTech), along with anti–IFN-γ, anti–IL-2, anti–IL-4, and anti–IL-12 mAbs (all 10 µg/ml) were added.

For the in vitro T reg cell suppressor assays, populations of CD103⁺ or CD103⁻, CD4⁺CD25⁺CD45RB⁻ T reg T cells were sorted along with concomitantly different CD4⁺CD25⁻CD45RB⁻ naïve T cells. After CFSE labeling, 10⁵ naïve T cells were cultured in round-bottom 96-well plates along with T reg cells at the indicated ratios in the presence of 2 × 10⁵ DCs (positively selected from splenocytes using anti-CD11c microbeads; Miltenyi Biotech) and 0.5 µg/ml anti-CD3e (LEAF grade; BioLegend). After 3.5 d of culture, effector T cell proliferation was assessed by CFSE dilution using flow cytometry and gating on live CD4⁺CD45R1⁻ T cells.

Induction of EAE. CNS inflammation was induced using a well-established model of EAE (Stromnes and Goverman, 2006). Briefly, groups of 10-wk-old age- and sex-matched mice were s.c. injected in the flank with 100 µg MOG₃₃₋₅₅ peptide (Genemed) in a 1:1 (vol/vol) water and CFA (Chondrex) emulsion using a 26 1/2-G needle. The same day and 2 d later, 200 µg pertussis toxin (List Biological Labs) in 200 µl physiological saline was injected i.v. Mice were monitored daily for signs of disease and/or weight loss. Disease severity was scored as follows: 0, normal; 1, limp tail; 2, tail paralysis and hind-limb weakness; 3, hind-limb paralysis; 4, paralysis of all limbs; and 5, moribund. If a mouse partially met the criteria for a disease score, an intermediate value was assigned to that mouse; e.g., a mouse with tail paralysis and the complete paralysis of only one hind-limb was scored as 2.5.

For Foxp3⁺ mice, 8-wk-old females were injected i.p. on days −10, −7, and −4 with 500 ng diphertheria toxin (List Biological Labs). Then EAE was induced as described above.

Statistical analyses. Data were analyzed using paired or unpaired Student’s t test as appropriate. Instances for which a statistically significant difference was detected are indicated by asterisks: *, P < 0.05; **, P < 0.01; ††, P < 0.001. Prism version 5 (GraphPad Software) was used for all statistical analyses and to generate plots. Each experiment was repeated at least three times, unless otherwise indicated in the figure legends.

We would like to thank members of the Cyster laboratory for helpful discussions, Andrea Reboldi and Hayakazu Sumida for critically reading this manuscript, and Scott Zamvil for advice regarding the EAE experiments.

J.G. Cyster is an investigator of the Howard Hughes Medical Institute. M.J. Barnes was supported by National Institutes of Health (NIH) grant T32 AI 7334-23. This work was supported in part by NIH grant AI45073. The authors declare no competing financial interests.

Submitted: 20 September 2014
Accepted: 19 May 2015

REFERENCES

Downloaded from on June 24, 2017