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Lysophosphatidylserine suppression of T cell activation via GPR174 requires Gαs proteins

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Abstract

G protein-coupled receptors regulate diverse aspects of T cell activity and effector function. Recently, we showed that GPR174 mediates the suppression of T cell proliferation *in vitro* induced by the polar lipid lysophosphatidylserine (LysoPS). Here, we investigated the *in vivo* activity of this pathway and characterized the mechanisms involved. Using *in vivo* models of T cell proliferation induced by sublethal irradiation or regulatory T cell depletion, we show that GPR174 expression can constrain T cell proliferation. *In vitro* experiments established that Gαs G proteins are needed for LysoPS/GPR174-mediated suppression of T cell proliferation. Mechanistically, LysoPS acts via GPR174 and Gαs to suppress IL-2 production by activated T cells and limit upregulation of the activation markers CD25 and CD69. Together, our findings identify GPR174 as an abundantly expressed Gαs-dependent receptor that can negatively regulate naive T cell activation.

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Lysophosphatidylserine (LysoPS) can suppress T cell proliferation via GPR174. Here, we show that GPR174 negatively regulates T cell proliferation in vivo using models of homeostatic proliferation and regulatory T cell depletion. Mechanistically, LysoPS reduced early T cell activation and IL-2 production, and required G(alpha)s G-protein subunits to mediate these effects.

Keywords

Lysophosphatidylserine; G protein-coupled receptors; GPR174; T cell; proliferation; IL-2

INTRODUCTION

In addition to the T cell receptor (TCR) and co-stimulatory surface molecules, various signaling inputs can act to determine the magnitude of T cell activation and proliferation. Over 20 years ago, Bellini and Bruni found that a phosphatidylserine metabolite, lysophosphatidylserine (LysoPS), could act to inhibit human T cell proliferation *in vitro* by

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CONFLICTS OF INTEREST
The authors declare that they have no conflicts of interest related to this study.

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an undefined mechanism.[1] Recently, receptors for LysoPS were identified by Inoue and colleagues who developed cell-based reporter assays for detecting G protein-coupled receptor (GPCR) activity and coupling to most G protein subunits.[2] They showed that four mouse GPCRs could respond to LysoPS: GPR34, GPR174, P2RY10, and P2RY10-L (the latter is a pseudogene in humans). This cell-based reporter assay indicated that GPR34 can couple to Gαi-containing heterotrimeric G-proteins, whereas the latter three receptors are able to couple to Gα12 and/or Gα13.

The potential importance of these receptors, all of which are located on the X-chromosome, is highlighted by genetic association studies that identified linkages of *GPR174* to Grave's disease and Addison's disease,[3–5] and of *P2RY10* to rheumatoid arthritis.[6] We have studied the functions of these LysoPS receptors in mouse models, and recently reported that GPR174 can act in a receptor-selective manner to intrinsically limit the generation and activity of Treg cells.[7] Naive and some effector subsets of T cells also express high levels of GPR174. *In vitro*, we observed that LysoPS inhibition of T cell proliferation occurred in a GPR174-dependent manner.[7] However, the relevance and mechanisms involved in this interaction have remained unclear. In this study, we characterized the GPR174-dependent suppression of T cell proliferation using *in vivo* models of T cell proliferation and *in vitro* T cell activation assays in the presence of LysoPS.

RESULTS AND DISCUSSION

To test whether GPR174-deficiency affected T cell proliferation *in vivo*, we used models of homeostatic and antigen-induced proliferation. In mice rendered lymphopenic by a sublethal dose of irradiation, adoptively transferred T cells undergo lymphopenia-induced homeostatic proliferation. When equal numbers of CFSE-labeled wild-type or *Gpr174*$^{-/Y}$ (CD45.2$^+$) and congenically distinct wild-type (CD45.1/2$^+$) naive CD8$^+$ T cells were transferred into recipients at one day post-radiation, an increased extent of *Gpr174*$^{-/Y}$ cell division was observed seven days later (Fig. 1a). This corresponded with the recovery of a greater number of *Gpr174*$^{-/Y}$ cells compared to co-transferred wild-type (CD45.1/2$^+$) cells (Fig. 1a). In a second model, we bred cohorts of *Gpr174*$^{-/Y}$ and wild-type mice that carried a *Foxp3-DTR* transgene and ablated all Treg cells by the injection of diphtheria toxin (DT).[8] Treg cell ablation results in the self-antigen-driven T cell proliferation of conventional CD4$^+$ and CD8$^+$ T cells prior to the development of lethal autoimmunity ~2 weeks after DT treatment.[9] In *Gpr174*$^{-/Y}$ mice, Treg cell ablation resulted in greater splenomegaly (Fig. 1b) and a significantly increased accumulation of CD4$^+$ T cells one week after DT injection (Fig. 1c). These data establish a role for GPR174 in restraining T cell proliferation *in vivo*.

Immunization-induced T cell responses were tested using GPR174-deficient OT-II TCR transgenic T cells. We co-transferred CFSE-labeled *Gpr174*$^{-/Y}$ (CD45.2$^+$) and wild-type (CD45.1/2$^+$) OT-II T cells into congenically distinct (CD45.1$^+$) recipient mice. The next day, mice were immunized with sheep red blood cells conjugated to ovalbumin[10] or ovalbumin in alum. In these settings, *Gpr174*$^{-/Y}$ and wild-type OT-II T cells were found to have proliferated similarly three days post-immunization ([7] and data not shown) suggesting that either the inflammatory setting or the relatively strong ovalbumin–OT-II TCR signal may overcome the influence of GPR174. Additionally, the availability of endogenous GPR174

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ligands, which may include LysoPS and yet to be identified molecules, may be affected by the inflammatory context.

We next sought to characterize the mechanism downstream of GPR174 that constrained T cell proliferation. As GPR174 was first reported to be a Gα13-coupled GPCR,[2] we generated $CD4^{cre}Gna12^{-/-}Gna13^{fl/fl}$ mice that lacked expression of both of these G-protein subunits. However, wild-type and $CD4^{cre}Gna12^{-/-}Gna13^{fl/fl}$ T cells proliferated equivalently *in vitro* with stimulation by anti-CD3 and anti-CD28 (data not shown), which suggested that GPR174 coupling to another G-protein subunit might mediate the suppression of T cell proliferation. Another study that used transfected CHO cells suggested that GPR174 may be unique among LysoPS receptors in its ability to couple to Gαs-containing heterotrimeric G-proteins.[11] The suppression of wild-type, but not $Gpr174^{-/Y}$ naive $CD4^+$ T cell proliferation can be observed *in vitro* when micromolar concentrations of LysoPS are present (Fig. 2a). When naive $CD4^+$ T cells lacking expression of *Gnas*, which encodes Gαs, were cultured similarly, they showed a loss of proliferation inhibition in the presence of LysoPS (Fig. 2b). These observations suggest that LysoPS suppression of T cell proliferation requires GPR174 signaling through Gαs. We note that the increased baseline T cell proliferation of Gαs-deficient cells may indicate the presence of other Gαs-dependent proliferation inhibitory factors in the cultures.

In co-cultures of wild-type and $Gpr174^{-/Y}$ T cells, LysoPS could intrinsically suppress the proliferation of wild-type, but not $Gpr174^{-/Y}$ cells (Fig. 3a). The inhibition of proliferation was greatest when LysoPS was added to cultures of T cells prior to activation compared to 24 h post-activation, suggesting that LysoPS affected very early events involved in T cell activation (Fig. 3b). Additionally, we noted that the suppression of wild-type T cell proliferation by LysoPS was weaker in co-cultures with $Gpr174^{-/Y}$ T cells (Fig. 3a) than in cultures of wild-type T cells alone (Fig. 2a), indicating that the generation of *trans*-acting factors was inhibited by LysoPS. Indeed, we found that LysoPS dose-dependently reduced IL-2 production by T cells in a GPR174- and Gαs-dependent manner (Fig. 3c). Therefore, the suppression of IL-2 production accounts for some, but not all of the T cell proliferation-inhibitory activity of LysoPS.

Analysis of T cell activation marker expression 24 h post-activation showed that LysoPS repressed the anti-CD3- and anti-CD28-induced upregulation of both CD25 and CD69 in wild-type, but not $Gpr174^{-/Y}$ T cells (Fig. 4a). Although CD25 levels can be affected by the presence of IL-2, wild-type cells cultured in the presence of LysoPS and saturating amounts of IL-2 still showed reduced CD25 levels (Fig. 4b). These effects of LysoPS also required Gαs (Fig. 4c) and we noted elevated baseline levels of CD25 and CD69 in Gαs-deficient cells, which was analogous to the elevated baseline proliferation and IL-2 production. Overall, these observations are consistent with LysoPS suppressing T cell proliferation via GPR174 and Gαs by inhibiting IL-2 production and affecting additional pathways involved in T cell activation.

Recently, Shinjo and colleagues also reported that LysoPS suppressed IL-2 production by T cells via GPR174 using knockout mice and LysoPS receptor-selective forms of LysoPS.[12] Interestingly, they observed increased generation of LysoPS after T cell activation and

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speculated that it may account for higher baseline IL-2 production by GPR174-deficient cells. Considered in the context of our findings using Gαs-deficient cells, LysoPS represents one of at least several Gαs-dependent ligands produced in T cell cultures that can affect T cell activation. To better understand the roles of GPR174 and other LysoPS receptors in homeostasis and an immune response, it will be important to identify the most potent endogenous GPR174 ligands and enzymes responsible for their generation in future studies.

In accord with our findings for LysoPS and GPR174, prostaglandin E2 signals via EP2 and EP4 to Gαs in T cells to suppress T cell IL-2 production and proliferation.[13,14] However, prostaglandin E2 can promote Th1 and Th17 effector responses,[15,16] whereas LysoPS signaling via GPR174 has shown more pronounced effects on baseline Treg cell activity and numbers.[7] Whether the ability of GPR174 to also couple with Gα13 contributes to the differing activities of these receptors should be tested in future work.

Our findings and those of others[11,12] suggest that GPR174–Gαs signaling triggers elevated cyclic AMP levels and protein kinase A (PKA) activity, which mediate suppression of T cell proliferation and IL-2 production. The mechanism whereby increased cyclic AMP levels cause these effects are complex, but may involve effects on cell cycle regulators of the G1→S transition[17] and transcription factors bound to the IL-2 locus, such as CREB and CREM/ICER,[18,19] respectively. Moreover, signaling studies have shown that cyclic AMP and PKA can act to antagonize proximal TCR signaling, for example by increasing Csk and decreasing Lck activity, by antagonizing NFAT, and by antagonizing Raf1.[20] Future experiments will be needed to determine the extent to which these pathways are engaged during GPR174-Gαs signaling. Cyclic AMP and PKA can also antagonize STAT5 signaling, which could potentially contribute to GPR174-mediated repression of IL-2 responsiveness.[21] Finally, as antagonists of Gαs-coupled receptors for adenosine and prostaglandin E2 are under consideration as immuno-oncology agents,[22,23] studies of GPR174 and LysoPS bioavailability in the context of tumors are warranted. In conclusion, our study establishes GPR174 as an abundantly expressed receptor that can intrinsically modulate conventional T cell activation and proliferation by coupling to Gαs-containing heterotrimeric G-proteins.

METHODS

Mice and reagents

C57BL/6J wild-type ($CD45.2^+$) and congenically distinct wild-type (BoyJ; $CD45.1^+$) mice were from Jackson Labs. *Gpr174*$^{-/Y}$ mice were generated at UCSF as described previously.[7] *Cd4*^{cre}*Gnas*$^{fl/fl}$ and control *Cd4*^{cre}*Gnas*$^{fl/+}$ bone marrow (both $CD45.2^+$) was obtained from Eyal Raz at UCSD and used to reconstitute irradiated recipients ($CD45.1^+$).[24] *Foxp3-DTR* mice were generated in the Rudensky lab[8] and bred with *Gpr174*$^{-/Y}$ mice at UCSF; we note that both the *Foxp3-DTR* allele and *Gpr174* are on the X-chromosome, and recombination events were identified among male progeny that were *Foxp3*$^{DTR/Y}$ *Gpr174*$^{-/Y}$.

The 18:1 LysoPS was obtained from Avanti Polar Lipids. Recombinant IL-2 was a gift from the Lanier lab (UCSF). Flow cytometry antibodies were purchased from Biolegend or Tonbo. Anti-CD3 and anti-CD28 antibodies used for T cell stimulation were from eBioscience.

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Homeostatic proliferation

To monitor homeostatic proliferation using a well-established model,[25] CD45.1$^+$ male BoyJ mice were treated with 600 cGy irradiation on day −1. The next day (day 0), naive CD8$^+$ T cells were isolated from donor male mouse splenocytes and lymph nodes by FACS. Naive CD8$^+$ T cells were CD8$^+$CD25$^-$CD44^{low}CD62L^{high} and sorted to greater than 99% purity using a FACS Aria sorter. Sorted T cells were labeled with 5 μM CFSE in PBS with 1% FBS at room temperature for 10 min. A total of 1.0×10^6 naive CD8$^+$ T cells from CD45.1/2$^+$ wild-type donors (5.0×10^5) and either CD45.2$^+$ wild-type or *Gpr174*$^{-/Y}$ donors (5.0×10^5 cells) were injected intravenously into irradiated recipients. On day 7, splenocytes of recipient mice were isolated and analyzed by flow cytometry to determine the extent of proliferation of the transferred cells based on CFSE dilution and the ratios of CD45.1/2$^+$-to-CD45.2$^+$ transferred cells.

In vitro T cell cultures

For all *in vitro* T cell assays, naive CD4$^+$CD25$^-$CD44^{low}CD62L^{high} T cells were used. T cells were enriched from total splenocytes and lymph node cells using a FACS Aria sorter to greater than 99% purity. After sorting, cells were labeled with CFSE as described above. For all assays, a total of 4.0×10^5 naive CD4$^+$ T cells were plated in 24-well plates coated with anti-CD3 (2 μg mL^{-1}) and anti-CD28 (2 μg mL^{-1}). Cells were cultured in standard RPMI media supplemented with 10% FBS, penicillin–streptomycin, 2-mercaptoethanol, and L-glutamine. Cell division was assessed based on CFSE dilution 72 h later. IL-2 production and cell surface marker levels were measured 24 after T cell activation.

ELISA

Levels of IL-2 in cell culture supernatants were measured 24 h after T cell activation using a Ready-Set-Go ELISA kit (eBioscience) according to the manufacturer's instructions. Cells were cultured and activated *in vitro* as described above.

Statistical analyses

Flow cytometry data were analyzed using FlowJo software. The CFSE analysis module was used to calculate the cell division index. For statistical calculations, GraphPad Prism was used to run Student *t*-tests to detect between-group differences; $P < 0.05$ was used as a threshold for significance. All assays were run multiple times and error bars show the standard deviation of triplicate measurements.

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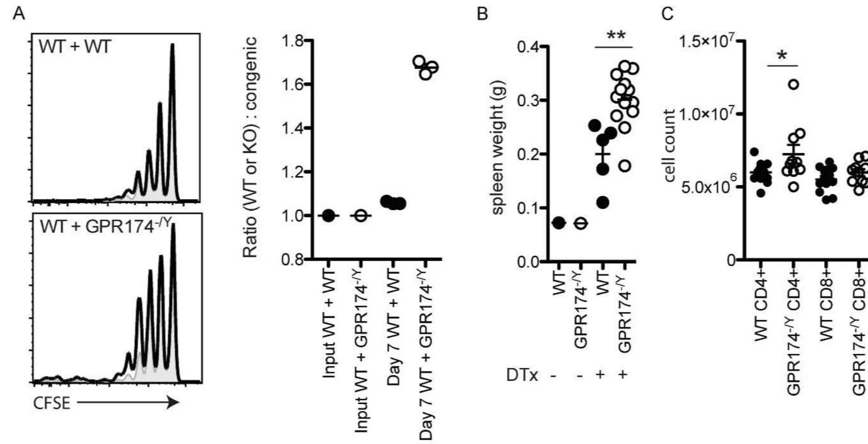

Figure 1.

GPR174 constrains T cell proliferation *in vivo*. (**a**) To assess homeostatic proliferation, a 1:1 mixture of wild-type (CD45.1/2$^+$; shaded gray plots) and either wild-type (CD45.2$^+$; upper left panel; open solid line) or *Gpr174*$^{-/Y}$ (CD45.2$^+$; lower left panel; open solid line) naive CD8$^+$ T cells were labeled with CFSE. A total of 1×10^6 T cells were transferred into CD45.1$^+$ recipient mice that had been irradiated the day before with 600 cGy irradiation. Homeostatic proliferation was assessed based on the CFSE dilution profile (left) and ratio of wild-type to wild-type or *Gpr174*$^{-/Y}$ cells recovered in the spleen of recipient mice seven days after transfer was determined (right). (**b–c**) To assess endogenous T cell proliferation in a Treg cell ablation model, cohorts of wild-type and *Gpr174*$^{-/Y}$ mice that expressed a *Foxp3-DTR* transgene were treated with diphtheria toxin intrapetironeally with an initial dose of 20 μg kg^{-1} on day 0, and then with 5 μg kg^{-1} on days 2 and 4. Spleen weights (**b**) and numbers of CD4$^+$ and CD8$^+$ T cells in the spleen (**c**) were quantified on day 7; * $P <$ 0.05; ** $P < 0.01$. Data are representative of two independent experiments; each dot represents a mouse.

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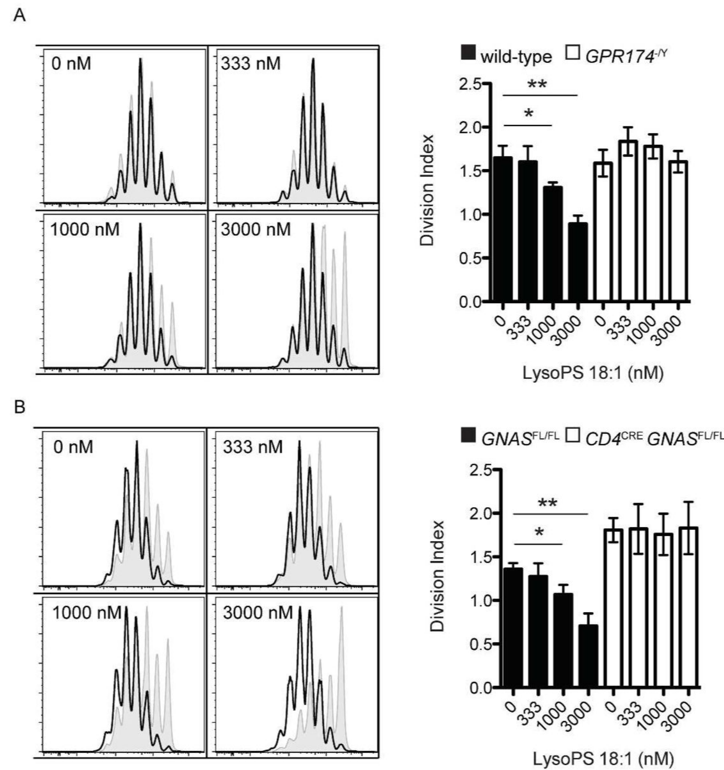

Figure 2.

Suppression of T cell proliferation by LysoPS requires GPR174 and Gαs. (**a**) Sorted naive CD4+ T cells were labeled with CFSE and incubated with plate-bound anti-CD3 and anti-CD28 for three days. On the left, histograms show the proliferation of wild-type (shaded, gray) and *Gpr174*$^{-/Y}$ (solid line, open) T cells cultured in the presence of the indicated concentration of 18:1 LysoPS. On the right, the division index of the cells is shown. (**b**) Experiments were carried out as in (A), except with *Cd4*^{cre}*Gnas*$^{fl/fl}$ naive CD4+ T cells; * $P < 0.05$; ** $P < 0.01$. Data are representative of three independent experiments. Error bars indicate standard deviation for triplicate measurements.

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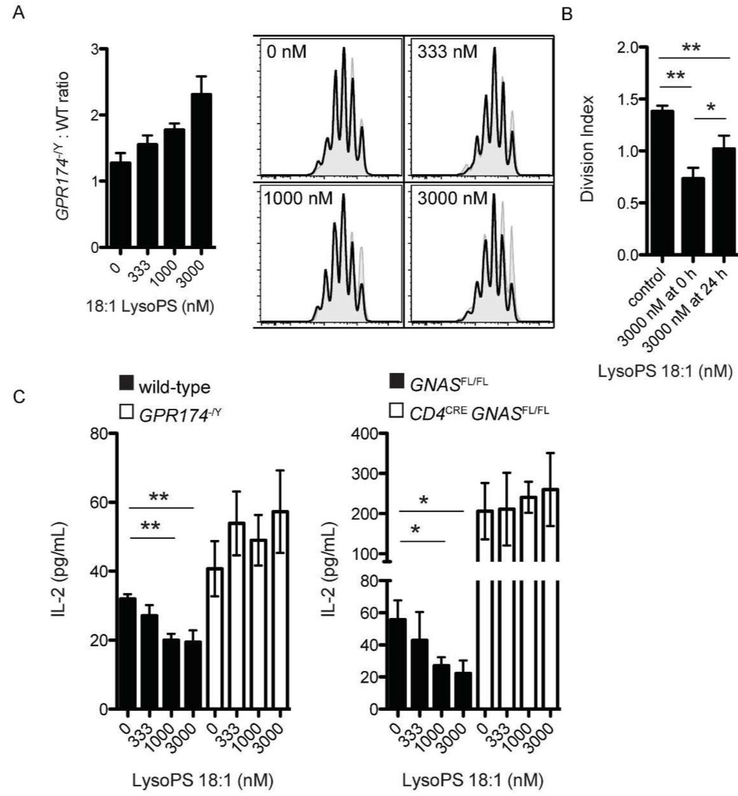

Figure 3.

LysoPS limits IL-2 production and intrinsically suppresses $CD4^+$ T cell proliferation. (**a**) Co-cultures of wild-type ($CD45.1^+$; shaded, gray histograms) and *Gpr174*$^{-/Y}$ ($CD45.2^+$; solid line, open) naive $CD4^+$ T cells were labeled with CFSE and activated with plate-bound anti-CD3 and anti-CD28 for three days. Cells were initially mixed at a 1:1 ratio, and the ratio of *Gpr174*$^{-/Y}$ -to-wild-type cells on day three is shown for cultures with the indicated concentration of LysoPS (left) along with representative histograms (right). (**b**) Wild-type naive $CD4^+$ T cells were cultured for three days as in (A); LysoPS was added at the start of the cultures or 24 h after activation. (**c**) IL-2 production by naive $CD4^+$ T cells of the indicated genotype was measured by ELISA 24 h after activation in the presence of the indicated concentration of 18:1 LysoPS; * $P < 0.05$; ** $P < 0.01$. Data are representative of three independent experiments. Error bars indicate standard deviation for triplicate measurements.

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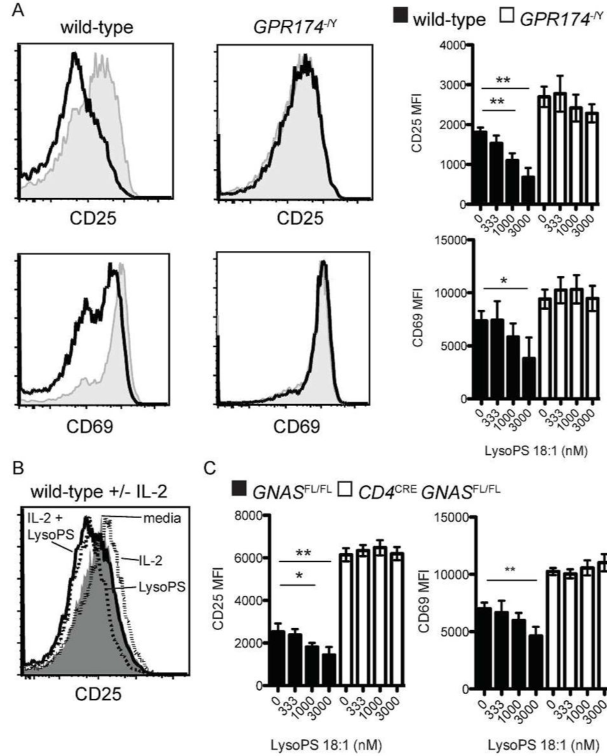

Figure 4.

LysoPS suppresses early T cell activation via GPR174 and Gαs. (**a**) Levels of CD25 and CD69 were determined by flow cytometry 24 after activation of naive CD4+ T cells incubated with plate-bound anti-CD3 and anti-CD28 for three days.. On the left, histograms of cells activated without LysoPS (gray, shaded) or in the presence of 3 μM 18:1 LysoPS are shown; MFI values for cells cultured with the indicated LysoPS concentrations are shown on the right. (**b**) Levels of CD25 expression are shown for cells activated as in (a) for 24 h in the presence of media alone (gray, shaded), media with 1000 U IL-2 (dotted line, open), media with 3 μM 18:1 LysoPS (dashed line, open), or media with 3 μM 18:1 LysoPS and 1000 U IL-2 (solid line, open). (**c**) Levels of CD25 and CD69 expression were measured as in (a) for *Cd4^cre^Gnas^fl/fl^* naive CD4+ T cells; * $P < 0.05$; ** $P < 0.01$. Data are representative of three independent experiments. Error bars indicate standard deviation for triplicate measurements.