GPR174 signals via Gαs to control a CD86-containing gene expression program in B cells

by
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To my parents, Bert Wolf and Karen Welty-Wolf –

thank you for giving me your support, values, and love.

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CONTRIBUTIONS TO PRESENTED WORK

All work presented in this dissertation was performed under the direct supervision and guidance of Dr. Jason G. Cyster. Chapter 2 is adapted from a manuscript accepted for publication at Proceedings of the National Academy of Sciences, "GPR174 signals via Gαs to control a CD86-containing gene expression program in B cells". The thesis abstract was adapted from this work. The co-authors for this publication were Zachary P. Howard, Lihui Duan, Hanson Tam, Ying Xu, and Jason G. Cyster. Jason G. Cyster and I conceptualized the study and designed the experiments. Zachary Howard and Lihui Duan assisted with RNA seqeucing data analyses, Hanson Tam assisted with LC-MS/MS analyses, and Ying Xu assisted with RNA sequencing library preparation and vector cloning. I conducted all experiments described in the paper, analyzed the data with the help of those mentioned, and prepared the figures. Dr. Jason G. Cyster and I collaboratively wrote and revised the manuscript. Michael Barnes made this project possible with his prior work on GPR174 and his demonstration that GPR174 signals via Gαs to restrain T cell proliferation.

GPR174 signals via Gαs to control a CD86-containing gene expression program in B cells

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ABSTRACT

GPR174 is abundantly expressed in B and T lymphocytes and has a role in restraining T cell responses, but the function of GPR174 in B cells is less clear. Here we report that upon in vitro culture B cells undergo a spontaneous GPR174-dependent activation process that is associated with marked changes in gene expression, including upregulation of *Cd86*, *Nr4a1*, *Ccr7* and phosphodiesterases. B cells lacking Gαs show a block in induction of the GPR174-dependent program. Spontaneous upregulation of CD86 in cultured B cells is dependent on protein kinase A. Both GPR174- and Gαs-deficient B cells show enhanced survival in culture. In vivo, GPR174 contributes to NUR77 expression in follicular B cells and is needed for establishing a marginal zone compartment of normal size. Treatment of mice with lysophosphatidylserine (lysoPS), a GPR174 ligand, is sufficient to promote CD86 upregulation by follicular B cells. These findings demonstrate that GPR174 can signal via Gαs to modulate B cell gene expression and show this can occur in vivo in response to lysoPS. Additionally, the findings illuminate a pathway that might be targeted to improve systems for the in vitro study of B cell responses.

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CHAPTER ONE

Introduction

Gαs-coupled GPCRs in the immune system

G-protein coupled receptors (GPCRs) and their ligands play a ubiquitous role in the mammalian immune system, and are one of the most important classes of protein for drug discovery and treatment of human disease. GPCR agonists and antagonists are among the most widely used pharmaceutical agents.

Following ligand engagement, GPCRs couple to heterotrimeric G-proteins, which include combinations of alpha, beta, and gamma subunits. Coupling occurs via the alpha subunits, which include Gαi, Gα12/13, and Gαs, and initiate intracellular signaling pathways that promote (or inhibit) cell migration, proliferation, and differentiation. Gαi-coupled GPCRs generally promote cell migration, whereas GPCR coupling to Gα12/13 proteins causes migration inhibition. Gαs signaling leads to adenyl cyclase activation and generation of the small molecule cAMP, which activates the cAMP receptors protein kinase A (PKA) and exchange proteins directly activated by cAMP (EPAC1 and EPAC2), resulting in complex and often suppressive effects on immune cell activation and proliferation.

One of the most widely studied Gαs-coupled GPCRs, the β_2 adrenergic receptor (β_2AR), promotes smooth muscle relaxation and vasodilation, and has been shown to suppress immune cell function. In one study, engagement of the β_2AR on lymphocytes inhibited egress from lymph nodes and impaired T cell recruitment to peripheral tissues during inflammation (1). In other studies, β_2AR antagonism has been shown to suppress inflammatory response in dendritic cells (2,3). Given the wide usage of β_2AR-modulating drugs in lung and cardiovascular disease, it is important to understand the subsequent effects on the immune system.

Another widely studied Gαs-coupled receptor expressed on lymphocytes is the adenosine receptor (A2AR). A2AR is expressed on T cells and adenosine suppresses various aspects of T

cell function, including activation and anti-tumor activity (4–6), and this receptor had been studied as a potential point of tumor therapy (7–9).

GPR174 is broadly expressed in lymphocytes, including in conventional and regulatory T cells (10). Work from this laboratory revealed an intrinsic role for GPR174 in restraining T regulatory (Treg) cell development and function (10). Further work from the Cyster and Aoki laboratories showed that GPR174 engagement can suppress conventional T cell proliferation and IL-2 production, and these effects were suggested to be dependent on Gαs proteins (10–12).

In addition to demonstrated suppressive effects of cAMP downstream of β_2AR, A2AR, GPR174, and other Gαs-coupled GPCRs, cAMP can also be directly transferred from Treg cells to conventional T cells and dendritic cells to inhibit their function (13–15). cAMP may also promote adenosine production by Tregs, thus creating a feed-forward loop (16). Interestingly, the importance of Treg-generated cAMP in Treg functionality is in conflict with the cell-intrinsic role of GPR174, which likely leads to cAMP production via Gαs-dependent adenyl cyclase activation, suppressing Treg development. Despite these nuances, cAMP has a generally suppressive function in the immune response.

Gαs-dependent gene induction

Signaling via Gαs-coupled receptors and subsequent cAMP generation leads to protein kinase A (PKA) activation, which then phosphorylates and activates the cAMP-response element binding (CREB) family of transcription factors, including CREB **(Fig. 1)**, cAMP-response element modulator (CREM), and activating transcription factor 1 (ATF1) (17). Nearly one quarter of all genes may be regulated by CREB family proteins via cAMP-response element (CRE) sites, although a much smaller fraction is thought to be dynamically regulated in a given

cell type (18). Further adding to the complexity of CREB family-dependent gene expression is a group of induced cAMP early repressor (ICER) proteins, generated from alternate isoforms of CREM (19,20). ICER proteins contain the DNA binding domain (DBD) of CREM but lack the transactivator (TA) domain, and thus occupy CRE sites and block cAMP-responsive transcription **(Fig. 1)**. ICER proteins have been shown to play a role in promoting Th17 T cell differentiation and may play a role in the pathogenesis of systemic lupus erythematosus (SLE) (21).

A number of cAMP- and CREB-responsive genes have been demonstrated to play a role in lymphocytes. One of the most classic families of cAMP-responsive genes are *Nr4a* family members, including *Nr4a1,* encoding NUR77, and *Nr4a3* (22,23). NUR77 is an orphan transcription factor which is strongly induced by B-cell receptor (BCR) and T-cell receptor (TCR) signaling (24) and plays a role in peripheral B cell tolerance (25) and promoting clonal diversity in T-independent responses (26). BCR is not thought to engage Gαs, so it is unknown how cAMP and BCR-derived signals differentially regulate NUR77 and its family members.

CD86 is an important costimulatory molecule which is expressed on antigen presenting cells, including B cells (27,28). Interestingly, although CD86 is most often considered in its role downstream of BCR or cytokine signaling (29–31), it was originally identified as a cAMP-responsive gene in both humans and mice (32–35). There is some evidence for direct regulation of the *CD86* locus by CREB in human cells, but this site is not conserved between humans and mice (18,36).

Other cAMP-responsive genes include multiple cAMP-specific phosphodiesterases, including Pde3b, Pde4b, and Pde4d (37). The induction and activation of these phosphodiesterases by cAMP serves as a negative feedback loop on cAMP generation **(Fig. 1)**.

Importantly, Pde4b inhibitors are of current interest in the treatment of B-cell malignancies (38). Increased expression of *PDE4* is associated with poor prognosis in diffuse large B-cell lymphomas (DLBCLs), and elevated cAMP following inhibition of Pde4b in DLBCL is proposed to reduce tumor viability and proliferation (39).

Despite growing interest in Gαs-coupled GPCRs in development of T cell therapies, comparatively less is known about how these GPCRs might regulate the B cell response.

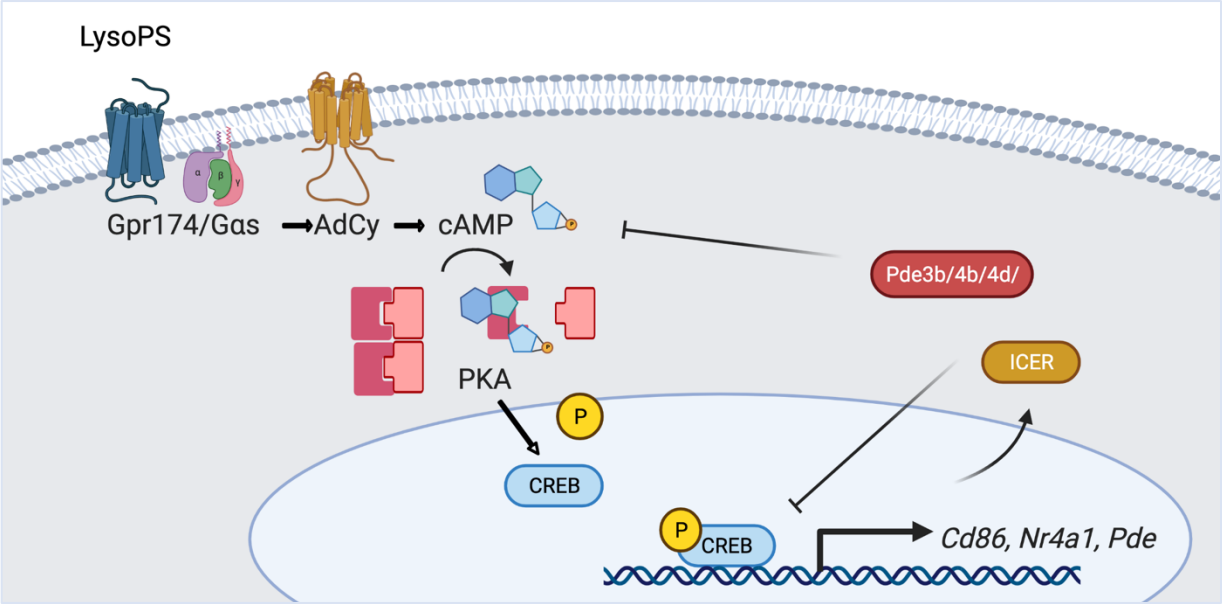


Figure 1.1 Mechanism and pathway intermediates of ligand-mediated induction of cAMP and subsequent gene induction for a Gαs-coupled GPCR, with proposed LysoPS-GPR174 interaction.

LysoPS receptors in leukocytes

GPR174 is one of three X-linked lysophosphatidylserine (lysoPS) receptors present in both humans and mice, along with GPR34 and P2RY10, with an additional mouse receptor P2RY10L which is not present in humans (40,41). These receptors have been shown to have various roles in regulating cell migration, proliferation, and other aspects of the immune response.

GPR34 was initially described as the lysoPS receptor responsible for mast cell degranulation (40), although this activity has since been challenged (42). Recently, GPR34 expression on ILC3s was shown to promote tissue repair via recognition of lysoPS derived from apoptotic neutrophils (43). GPR34 is suggested to couple to Gαi proteins based on lysoPS-mediated suppression of cAMP generation in GPR34-expressing cells (40) and by TGF-α shedding assay (44) **(Fig. 2)**. In this same shedding assay, P2RY10 was shown to couple predominantly to Gα12/13, with additional coupling to Gαi (41,44). In vivo, P2RY10 was shown to act in CD4 T cells to promote experimental autoimmune encephalomyelitis (EAE) in mice. This was attributed to activity of both ATP and lysoPS on the receptor augmenting the response to several chemokines. The G-protein involved was not determined though the strong stimulation of RhoA by P2RY10 is consistent with signaling via Gα12/13, although a pro-migratory effect of P2RY10 may suggest additional coupling to Gαi (45) **(Fig. 2)**. The authors of this study noted that in vitro CD4 T cell migration may be reduced by lysoPS (45) but did not comment on the receptor dependence of this phenotype, and further inquiry may provide evidence of coupling to Gα12/13.

Although the initial study characterizing GPR174 as a lysoPS receptor demonstrated coupling to Gα12/13 (41), additional studies by this group showed stronger coupling to Gαs proteins (44). The work by our group that revealed a role for GPR174 in restraining T cell proliferation in vitro and in vivo suggested this effect was dependent on Gαs proteins (12). Further support for Gαs coupling comes from studies of receptor overexpression on Chinese hamster ovary (CHO) cells, in which GPR174 leads to decreased proliferation and increased cAMP (46). However, a recent study on murine B lymphocytes reported GPR174-dependent migration toward the CCR7 ligands CCL19 and CCL21 (47). This study suggested GPR174

coupling to Gα12/13 in activated B cells, with a shift to Gαi association following stimulation with CCL21 in a sex-dependent manner (47). These data suggest GPR174 may couple to multiple ligands and G proteins in a context and cell-type dependent manner, with important consequences for the outcome of ligand engagement **(Fig. 2)**.

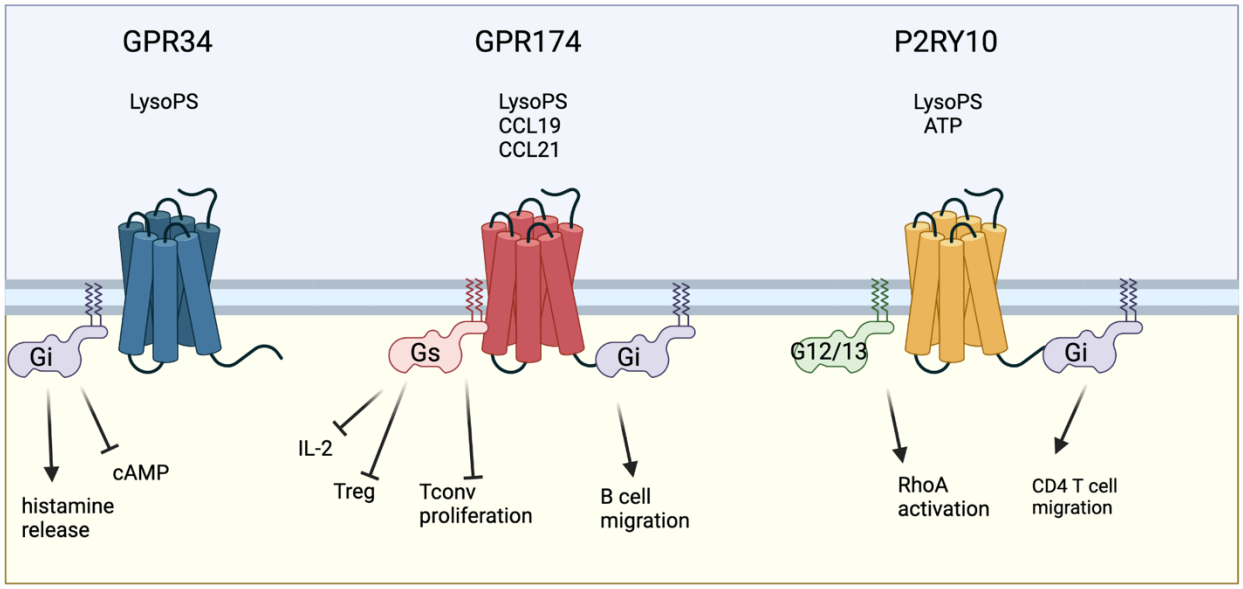


Figure 1.2. Schematic of suggested ligands and G-protein couplings for lysoPS receptors, with corresponding outcomes.

GPR174 is associated with human autoimmune disease

SNPs at the Gpr174 locus have been associated in multiple studies with Graves' (48–52) and Addison's (53) disease, and *GPR174* mRNA was shown in one study to be negatively correlated with sepsis in ICU patients (54). GPR174 expression in Tregs in a murine model of autoimmunity, EAE, was shown to contribute to disease severity (10), whereas GPR174 expression on B cells in another study was protective against EAE severity (47). Further investigation will be important to understand how GPR174 expression on different cell types contributes to autoimmune disease and the implications this has for understanding the role of SNPs in humans.

LysoPS as an immunomodulatory lipid

LysoPS is a minor lysophospholipid present in cell membranes and can be detected in many tissues and cell types, with relatively higher levels in the brain, spinal cord, colon, and immune tissues (10,11). LysoPS is produced via deacetylation of the abundant lipid phosphatidylserine (PS) by the secreted phospholipase PS-PLA_1 (55–57), which acts largely on extracellular PS, or ABHD16a (BAT5) (58,59), which acts on intracellular PS. Early studies on the effects of phospholipids on the immune system identified PS as a stimulator of mast cell degranulation and histamine release (60,61), activity which was largely attributable to contaminating lysoPS in PS preparations (62). Subsequent studies showed a repressive effect of PS and lysoPS on T cell proliferation, and activity of PS required a phospholipase (63–65). Furthermore, lysoPS production by leukocytes was able to stimulate histamine production by mast cells (66).

PS is preferentially located on the inner leaflet of the plasma membrane in healthy cells due to the activity of phospholipid flippases (67), and is thus inaccessible to PS-PLA enzymes for conversion to lysoPS (57,68). However, PS is flipped to the outer membrane in multiple important physiological contexts including apoptosis (69,70), during platelet activation and blood clot formation (71,72), and by some tumor cells (73–75). High PS-PLA_1 expression is associated with multiple autoimmune diseases, including systemic lupus erythematosus and Graves' disease (51,76).

ABHD12 and ABHD6 are serine hydrolases that have been identified as lysoPS degrading enzymes (59,77,78). Both are integral membrane proteins. While the active site of ABHD6 is predicted to face the cytoplasm, the active site of ABHD12 faces either lumenally or

extracellularly, allowing these enzymes to access and degrade distinct pools of lysoPS (79). Indeed, mouse studies show elevated lysoPS species when either of these enzymes are knocked out (77,78). ABHD12 is expressed broadly at low levels in immune cell types, with high expression in macrophages (Immgen.org). ABHD16a expression is similarly ubiquitous. In contrast, PS-PLA_1 and ABHD6 have low expression in the immune system (Immgen.org).

Taken together, the varied and complex effects of PS and lysoPS in mediating cross-talk between immune cells and with other tissues raise interesting possibilities for studying and treating human disease.

CHAPTER TWO

GPR174 signals via Gαs to control a CD86-containing gene expression program in B cells

SIGNIFICANCE STATEMENT

Modeling immune responses in vitro is critical for studying many facets of the B cell response. We show that during culture without stimulation, mouse B cells undergo massive changes in gene expression. Many of these changes are promoted by GPR174 signaling via Gαs. GPR174 and Gαs also contribute to reduced B cell viability during culture. We suggest that GPR174 antagonists may be useful to reduce the shift in gene expression and to augment B cell survival during culture. We also provide evidence that ligand engagement of GPR174 can activate this pathway in vivo. Variants in the GPR174 locus have been associated with autoimmune diseases. Our findings provide knowledge for understanding how alterations in GPR174 expression may contribute to disease.

ABSTRACT

GPR174 is abundantly expressed in B and T lymphocytes and has a role in restraining T cell responses, but the function of GPR174 in B cells is less clear. Here we report that upon in vitro culture B cells undergo a spontaneous GPR174-dependent activation process that is associated with marked changes in gene expression, including upregulation of *Cd86*, *Nr4a1*, *Ccr7* and phosphodiesterases. B cells lacking Gαs show a block in induction of the GPR174-dependent program. Spontaneous upregulation of CD86 in cultured B cells is dependent on protein kinase A. Both GPR174- and Gαs-deficient B cells show enhanced survival in culture. In vivo, GPR174 contributes to NUR77 expression in follicular B cells and is needed for establishing a marginal zone compartment of normal size. Treatment of mice with lysophosphatidylserine (lysoPS), a GPR174 ligand, is sufficient to promote CD86 upregulation by follicular B cells. These findings demonstrate that GPR174 can signal via Gαs to modulate B cell gene expression and show this can occur in vivo in response to lysoPS. Additionally, the findings illuminate a pathway that might be targeted to improve systems for the in vitro study of B cell responses.

INTRODUCTION

G-protein coupled receptors (GPCRs) comprise a large family of seven-transmembrane signaling proteins, many of which have roles in the development and function of the mammalian immune system. The outcome of GPCR signaling is determined via coupling to various G proteins. Signaling via Gαi-coupled GPCRs promotes cell migration, whereas GPCR coupling to Gα12/13 proteins often leads to migration inhibition. In contrast Gαs signaling exerts its effect through cAMP production and leads to complex and often suppressive effects on immune cell activation and proliferation. GPCR GPR174 is one of several related X-linked receptors that are highly expressed on lymphocytes (10,41,47). Several GWAS studies have linked *Gpr174*-associated single nucleotide polymorphisms (SNPs) to autoimmune disorders including Graves and Addison's disease (48,49,52).

GPR174 was found to be a receptor for lysophosphatidylserine (lysoPS) and was suggested to signal via Gα12/13-containing heterotrimeric G proteins (41). Studies on GPR174-deficient mice revealed a role for the receptor in restraining T regulatory (Treg) cell development and function (10) and conventional T cell proliferation and IL-2 production (11,12). In these reports, signaling was suggested to occur via Gαs-containing heterotrimeric G proteins (11,12). In another study, GPR174 was reported to support B cell migration to spleen stromal cell culture supernatants and biochemical fractionation led to the suggestion that CCL19 and CCL21, well defined CCR7 ligands, were ligands for GPR174 (47). In that study, GPR174 was suggested to signal via Gαi and Gα13 (47). Taken together, there is currently a lack of clear understanding regarding the signaling pathway(s) engaged downstream of GPR174.

CD86 is a critical costimulatory molecule in antigen presenting cells including B cells (27,28). Induction of CD86 by B cell receptor (BCR) signaling is an important feature of the

BCR-induced activation program, though CD86 can also be induced in B cells by CD40 and cytokine signaling (29,80,81). Interestingly, *Cd86* was originally characterized as a cAMP inducible gene in B cells (31–35). However, since the BCR, CD40 and cytokines are not thought to induce CD86 via cAMP, the receptors leading to cAMP-mediated induction of CD86 in B cells have been unclear.

The study of B cells in vitro has been critical to numerous advances in the understanding of adaptive immunity and it remains a crucial method for dissecting the B cell response. Although not widely reported on, it is generally understood by B cell biologists that B cells undergo some amount of gene expression change during in vitro culture. Indeed, this aspect of cultured B cells presented a major challenge to efforts by the Alliance for Cell Signaling (82,83) to dissect the signaling circuits in mouse B cells. The pathways involved in causing these gene expression changes are not understood.

Here we report that cultured follicular B cells rapidly upregulate CD86 independently of stimulation in a GPR174- and Gαs-dependent manner. The GPR174 mediated response was not dependent on exogenous ligand or BCR signaling. RNA-sequencing anlaysis revealed that B cells underwent changes in expression of ~1000 genes after 4 hours of unstimulated culture and many of these changes were GPR174-dependent. This included induction of *Cd86, Nr4a1, Ccr7* and phosphodiesterases and downregulation of immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing receptors. There was a strong overlap in the alterations in gene expression between GPR174- and Gαs-deficient B cells, indicating that GPR174 signals via Gαs in follicular B cells. When B cells were maintained in vitro for 1-2 days in the absence of stimulation, GPR174- and Gαs-deficiency were associated with augmented survival. In vivo GPR174-deficiency led to reduced NUR77-GFP reporter expression, and GPR174- and Gαs-

deficient mice had a reduced marginal zone (MZ) B cell compartment. Treatment of mice with lysoPS was sufficient to cause increased CD86 expression by follicular B cells. These findings establish GPR174 as a receptor capable of exerting a large influence on B cell gene expression and they provide insight into the B cell properties that can be influenced by the receptor.

RESULTS

GPR174-deficient B cells have a defect in CD86 upregulation in vitro

To examine the role of Gpr174 in B cell activation, we utilized mice in which the single coding exon of *Gpr174* was replaced with an in-frame tdTomato allele. These mice show high reporter expression in naive T and B cells (10). In accordance with Immgen RNAseq data of *Gpr174* expression, there was high reporter expression in spleen follicular and MZ B cells, while minimal expression was observed in $CD23^{-}CD93^{+}$ transitional B cells **(Fig. 2.1A).** B cell reporter expression in $Gpr174^{+/-}$ female mice, in which random X-inactivation is predicted to yield 50% reporter positive (and thus GPR174-deficient) cells, displayed a bimodal population in follicular and MZ B cells, with minimal expression in early transitional B cells **(Fig. 2.1A).**

In order to determine whether GPR174 plays a role in the initial response to activating signals we performed in vitro stimulations of WT and GPR174-deficient B cells and analyzed the expression of surface markers by flow cytometry after 6 hours of culture. As anticipated, the activation markers CD86, CD69, and CD83 were strongly induced within 6 hours by BCR ligation with anti-IgM compared to cells kept on ice **(Fig. 2.1B and Fig. S2.1A).** However, in contrast to CD69 and CD83, which were upregulated to a similar extent in WT and GPR174-deficient B cells, CD86 (B7-2) upregulation was significantly reduced in GPR174-deficient cells **(Fig. 2.1B)**. Stimulation with anti-CD180 revealed a comparable defect in CD86 upregulation in GPR174-deficient B cells **(Fig. S2.1B).** In the course of these studies, we noted that substantial CD86 upregulation occurred in B cells cultured in the absence of added stimulation, and that this upregulation was strongly GPR174 dependent **(Fig. 2.1B and 2.1C)**. In contrast, CD69 was not spontaneously upregulated **(Fig. 2.1B and 2.1C)** and CD83 was minimally induced **(Fig. S2.1A)**.

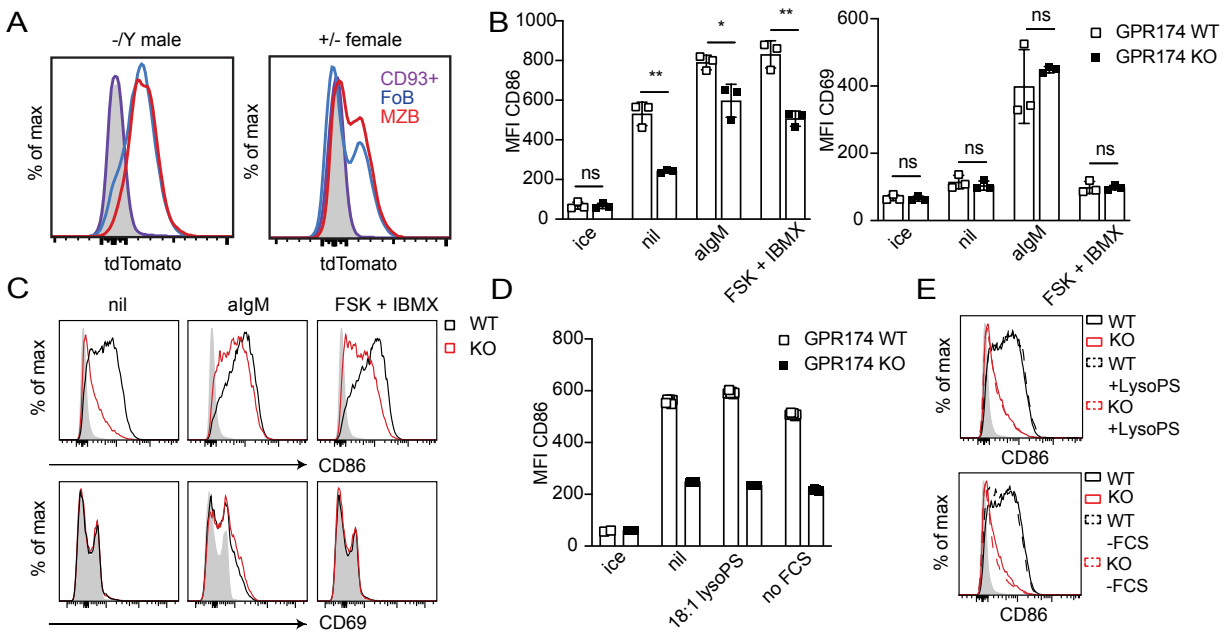


Figure 2.1. GPR174 promotes CD86 induction. (A) Representative tdTomato reporter expression in *Gpr174*$^{-/Y}$ and *Gpr174*$^{+/-}$ mice for transitional B (CD93+), splenic follicular B (FoB), and marginal zone B cells (MZB) versus reporter negative (gray). (B) Summary MFI data of CD86 (left) and CD69 (right) on B cells cultured for 6 hours with or without the indicated stimuli or maintained on ice (N=3 mice per genotype from one experiment representative of more than 10). (C) Representative histogram plots of the data presented in (B) with the gray histograms corresponding to WT cells kept on ice. (D) MFI of CD86 on B cells of the indicated genotypes (triplicate wells) incubated for 6 hours in normal media (nil), media with 18:1 lysoPS (10 μM), media without FCS, or maintained on ice. (E) Representative histograms of the data presented in (D) versus WT cells maintained on ice (gray). Statistical significance for (B) determined by unpaired T-test. ns = not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Similar findings were made using cultures of total splenocytes or lymph node cells **(Fig S2.1C)**, and with sorted B cells **(Fig. S2.1D)** indicating that the CD86 induction was not dependent on factors from non-B cells. The effects of GPR174 expression were cell-intrinsic, as gating on reporter-positive and -negative B cells from *Gpr174*$^{+/-}$ female mice showed less CD86 induction in the reporter+ cells (that lack GPR174), with similar levels of CD69 **(Fig. S2.1E and S2.1F)**. TdTomato reporter expression, was comparable between cultured cells and cells kept on ice and was unaffected by the various stimulations **(Fig S2.1G)**.

Engagement of Gαs-coupled GPCRs drives cAMP generation, and CD86 is cAMP-inducible (31–35). Forskolin induces cAMP production by adenyl cyclase and 3-isobutyl-1-methylxanthine (IBMX) prevents cAMP degradation by phosphodiesterases (37,84). As anticipated, combined treatment of B cells with forskolin and IBMX led to induction of CD86, but not of CD69 or CD83 **(Fig. 2.1B and Fig. S2.1A)**. This treatment did not fully rescue the defect in CD86 induction in GPR174-deficient B cells. We suspect that this reflects a reduced ability of forskolin to engage adenylate cyclase in cells that lack a dominant Gαs-coupled receptor, as we discuss further below.

Lysophosphatidylserine (lysoPS) is a ligand for GPR174 (10–12,41,85,86). Addition of lysoPS to the B cell cultures did not alter CD86 induction **(Fig. 2.1D and 2.1E)**. In order to test whether this might be due to the presence of lysoPS in serum, we cultured B cells in the absence of fetal calf serum (FCS). Cells cultured under these conditions spontaneously induced CD86 to a comparable level as cells cultured in FCS-containing media **(Fig. 2.1D and 2.1E)**.

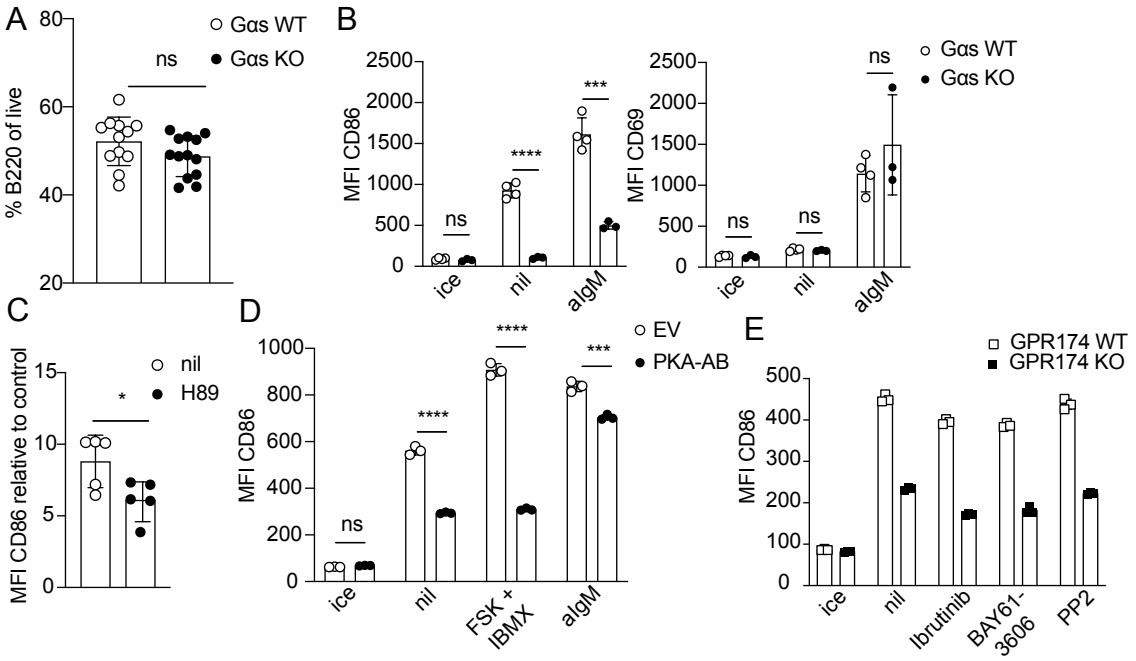


Figure 2.2. GPR174-dependent CD86 induction is Gαs and PKA-dependent and BCR-independent. (A) Percentage B220+ cells in the spleens of *Gnas*$^{+/+}$ *CD19 Cre*$^{+/-}$ (Gαs WT, N=12) and *Gnas*$^{fl/fl}$ *CD19 Cre*$^{+/-}$ (Gαs KO, N=13) mice. (B) MFI of CD86 (left) and CD69 (right) on Gαs WT (N=4 mice) or Gαs KO (N=3 mice) splenic follicular B cells cultured with or without anti-IgM. Representative of more than 3 individual experiments. (C) MFI of CD86 relative to ice on WT B cells cultured with or without H89 (10 μM). (D) MFI of CD86 on vector+ (gating shown in Suppl. Fig. 2A) B cells from chimeras transduced with EV or PKA-AB constructs and cultured with or without anti-IgM or forskolin IBMX or maintained on ice (triplicate wells, from one experiment representative of three). (E) MFI of CD86 on B cells of the indicated genotype treated with or without Ibrutinib (1 μM), BAY61-3606 (1 μM), or PP2 (20 μM) (triplicate wells, representative of at least 3 experiments). Statistical significance for (B-D) determined by unpaired T-test. ns = not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

We also examined the effect of GPR174-deficiency in a lipopolysaccharide (LPS)-induced B cell proliferation assay. WT and GPR174-deficient B cells proliferated equivalently after 4 days and addition of lysoPS to the culture medium had no effect on the response **(Fig. S2.1H)**. This contrasted with CD4 T cells stimulated with anti-CD3 and anti-CD28, where lysoPS repressed CD4 T cell proliferation in a GPR174-dependent manner **(Fig. S2.1H)** as previously observed (10). These discrepant effects on B and T cells may in part be explained by differences in GPR174 expression. LPS stimulated B cells downregulated the tdTomato reporter after 4 days **(Fig. S2.1I)** in accord with plasma cells and germinal center (GC) B cells having minimal expression of the *Gpr174* locus (Immgen.org), whereas CD4 T cells activated with anti-CD3 and anti-CD28 maintained tdTomato reporter expression at similar levels to unstimulated cells **(Fig. S2.1I)**.

We considered the possibility that exogenous lysoPS failed to augment CD86 in unstimulated B cells because endogenous lysoPS increased in B cells during culture. Indeed, when lysoPS was quantitated in cultured B cells using liquid chromatography and mass spectrometry (LC-MS/MS), the amounts of 18:0 lysoPS were increased within 10 minutes of incubation and remained elevated for at least 60 minutes (**Fig. S2.1J**).

GPR174 induction of CD86 is dependent on Gαs and PKA and is BCR-independent

Given prior studies indicating that CD86 is a cAMP-inducible gene (31–35) and work in T cells suggesting GPR174 function was Gαs dependent (12) we asked whether *Gnas*, the gene encoding Gαs, was required for GPR174 signaling in B cells. For these studies, we crossed *Gnas* floxed mice (87) with *Cd19-Cre* mice (88), yielding mice that conditionally lacked *Gnas* in B

cells beginning in early development. Gαs-deficient (*Gnas*$^{fl/fl}$ *Cd19-Cre*$^{+/-}$) mice had a comparable percentage of B220^{+} cells in the spleen to Gαs WT *(Gnas*$^{+/+}$ *Cd19-Cre*$^{+/-}$) mice **(Fig. 2.2A)**. As above, WT B cells upregulated CD86 spontaneously over 6 hours of culture. Importantly, Gαs-deficient B cells did not upregulate CD86 above cells kept on ice, representing an even stronger phenotype than observed in GPR174-deficient mice **(Fig. 2.2B)**. Gαs-deficient B cells also displayed a defect in CD86 induction in response to anti-IgM treatment while CD69 induction occurred normally **(Fig. 2.2B)**. Forskolin binding to and activation of adenyl cyclase and subsequent cAMP generation is dependent on Gαs proteins (84). Accordingly, forskolin and IBMX treatment of Gαs-deficient B cells did not induce CD86 **(Fig. S2.2A)**.

Gαs activation leads to elevations of cAMP and subsequent activation of the cAMP-dependent kinase, PKA (89). To test for involvement of PKA in spontaneous CD86 upregulation we cultured B cells in the presence of H89, a PKA inhibitor. H89 treatment partially blocked spontaneous CD86 upregulation **(Fig. 2.2C)**. Pharmacological inhibitors of PKA are known to have off-target effects (90). The regulatory type I subunit of PKA has two cAMP binding sites, A and B, and mutation of these sites yields a dominant negative PKA (91). In order to confirm that the effect of H89 on spontaneous CD86 induction was via PKA, we generated retroviral bone marrow (BM) chimeras expressing the dominant negative PKA-AB construct or empty vector (EV), and a GFP reporter, in hematopoietic cells. Following 8 weeks of reconstitution, we cultured splenocytes from these chimeras for 6 hours in either media alone or with forskolin and IBMX. Compared to EV GFP^{+} B cells, PKA-AB GFP^{+} B cells induced less CD86 **(Fig. 2.2D),** suggesting that CD86 upregulation during unstimulated culture is PKA-dependent. This defect was most dramatic in the highest GFP-expressing PKA-AB cells, which showed a near-total loss of CD86 induction **(Fig. S2.2B)**. Expression of PKA-AB GFP also blocked all CD86 induction

in response to forskolin and IBMX treatment, and partially reduced CD86 induction following anti-IgM treatment **(Fig. 2.2D and Fig. S2.2B)**. These data suggest that GPR174 and Gαs signal at least in part via PKA to induce CD86.

Since BCR signaling is a strong inducer of CD86 we wanted to determine whether a BCR-derived signal was required for spontaneous CD86 induction. Inhibition of Btk, Syk, or Src-family kinases, critical kinases downstream of the BCR (92), had a minimal effect on spontaneous CD86 induction **(Fig. 2.2E)**. These inhibitors strongly inhibited anti-IgM induced CD86 upregulation, confirming their efficacy **(Fig. S2.2C)**.

We tested if a similar pathway operated in humans B cells by culturing peripheral blood mononuclear cells (PBMC) for 6 hours. Human B cells showed increased CD86 expression in the absence of stimulation and this induction was significantly inhibited by PKA antagonism (**Fig. S2.2D**).

RNAseq of cultured WT and GPR174-deficient B cells reveals numerous expression changes

To investigate the full extent of the gene expression program regulated by GPR174 in cultured B cells we performed RNA sequencing on mouse follicular B cells immediately post-sort (0 hr) or after 4 hours of culture without stimulation. Immediately ex vivo there were very few differences between WT and GPR174-deficient follicular B cells **(Fig. 2.3A and 2.3B)**, whereas gene expression diverged considerably after 4 hours of culture as observed by principle component analysis **(Fig. 2.3A and 2.3C)**. Importantly, cultured WT B cells underwent a massive change in gene expression compared to uncultured WT cells, with over 1000 genes up- or downregulated **(Fig. S2.3A and Dataset S1).** Cultured GPR174-deficient B cells underwent

many fewer changes compared to their uncultured counterparts **(Fig. S3B and Dataset S1)**. The top 100 differentially expressed genes (DEGs) that either increased or decreased expression from 0 to 4 hours in WT B cells were highly enriched for genes that were increased in expression in WT compared to KO B cells at 4 hours **(Fig. S2.3C)**. The top 100 DEGs that either increased or decreased from 0 to 4 hours in GPR174-deficient B cells were less enriched for differential gene expression between WT and KO B cells at 4 hours **(Fig. S2.3D)**. Together, these data indicated that the majority of differential gene expression came from increased or decreased expression in WT cells, whereas GPR174-deficient cells were comparatively static.

After 4 hours of culture, WT B cells more strongly expressed genes indicative of cAMP signaling than GPR174-deficient B cells, including the CREB family member cAMP response element modulator *(Crem)* and the cAMP-specific phosphodiesterases *Pde3b*, *Pde4b*, and *Pde4d* **(Fig. 2.3D)**. As expected, *Cd86* was also highly differential **(Fig. 2.3D)**. WT B cells showed elevated expression of the chemokine receptor *Ccr7* and the additional G-protein coupled receptors *Gpr171*, *Gpr132* and *P2ry10* **(Fig. 2.3D)**. Other DEGs that showed higher expression in WT than GPR174-deficient B cells at the 4-hour timepoint included immediate early response gene 2 (*Ier2*) and the *Nr4a* family of orphan transcription factors *Nr4a1* (encoding NUR77), *Nr4a2*, and *Nr4a3*, as well as the AP-1 transcription factors *Junb* and *Fosl2* **(Dataset S1)**. In contrast, GPR174-deficient B cells had higher expression of inhibitory signaling molecules like *Btla*, *Cd22*, and *Cd200*, as well as class II MHC transactivator, *Ciita*, and *Fcer2a* (CD23) than WT B cells after 4 hours of culture **(Fig. 2.3C and 2.3D)**. Notably these genes were reduced in expression in WT cells and unchanged or less reduced in expression in GPR174-deficient cells compared to uncultured cells **(Dataset S1)**.

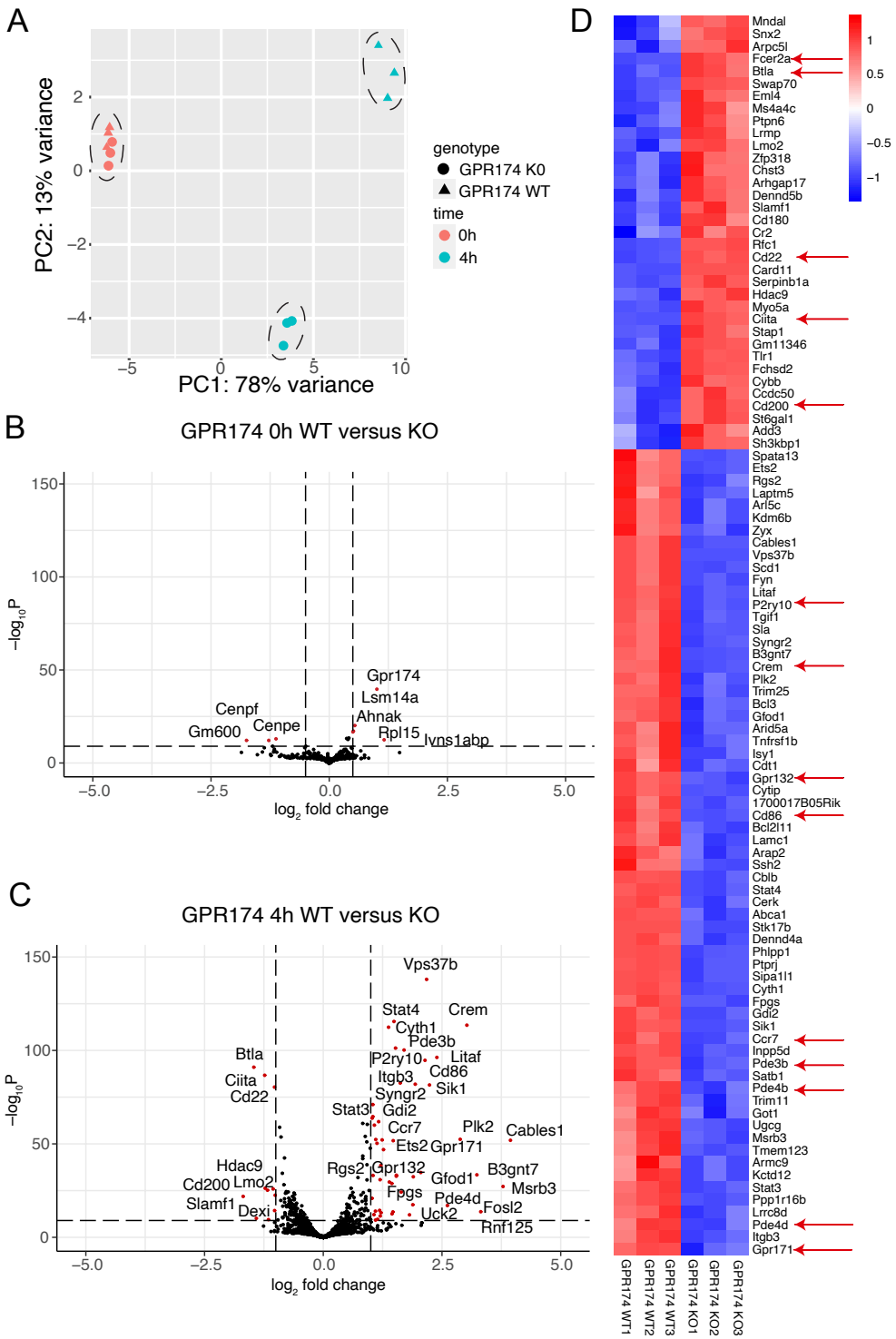


Figure 2.3. RNAseq of cultured WT and GPR174-deficient B cells reveals numerous expression changes. (A). PCA plot of RNAseq data from sorted GPR174 WT and KO B cells cultured for the indicated times. (N=3 mice per genotype). (B and C) Volcano plots of DEGs between WT and KO B cells at 0 hours (B) and 4 hours (C). Dashed lines indicate fold-change and p-value cutoffs for highlighted DEGs in red ($P_{adj}<1x10^{-10}$ and $|log_2FC|>1$). (D) Heatmap of top 100 DEGs in GPR174 WT versus KO follicular B cells after 4 hours of culture. Red arrows indicate genes referenced in the text.

We wondered whether expression of early response genes, such as *Ier2* and *Nr4a1*, suggested multiple waves of gene expression, with some expression changes less proximal to a GPR174-derived signal. In order to determine this, we performed RNA sequencing on sorted follicular B cells that had been cultured for 1 hour. By 1 hour of culture, many genes were already differential, although fewer than at the 4-hour time point **(Dataset S1 and S2)**. WT B cells displayed higher expression of *Pde4b*, *Nr4a* family members, AP-1 family members, *Crem*, *Cd86, and Ccr7,* whereas few genes had significantly higher expression in GPR174-deficient B cells **(Fig. S2.3E and S2.3F)**. These data suggest that very rapid signaling occurs via GPR174 in cultured B cells to drive early gene expression changes.

Gαs-deficient B cells phenocopy gene expression changes in GPR174-deficient cells

To further explore whether GPR174 signaling in B cells was Gαs-dependent, we performed RNA sequencing on sorted follicular B cells from Gαs WT and Gαs-deficient mice after 0 or 4 hours of culture without stimulation. As for GPR174-deficiency, very few genes were differentially expressed between WT and Gαs-deficient B cells immediately ex vivo **(Fig. 2.4A and 2.4B)**, whereas large changes occurred after 4 hours **(Fig. 2.4A and 2.4C).** Following 4 hours of culture, WT and Gαs-deficient B cells showed a strikingly similar pattern of divergent gene expression to WT and GPR174-deficient B cells. Gαs WT B cells had increased expression of *Cd86*, *Crem*, phosphodiesterases, *Nr4a* family members, *Ccr7*, *Gpr132* and *Gpr171* compared to Gαs-deficient B cells **(Fig. 2.4D).** *P2ry10*, which is adjacent to *Gpr174* on the X chromosome, was increased in WT compared to Gαs-deficient B cells, indicating that the *P2ry10* increase observed in WT versus GPR174-deficient B cells was not due to disruption of the locus in generating the *Gpr174 tdTomato* allele. Similarly to GPR174-deficient B cells, Gαs-

deficient B cells had higher expression of *Cd22*, *Btla*, and *Cd200* than WT cells **(Fig. 2.4D)**. As expected, Gαs WT B cells showed similar gene induction to GPR174 WT B cells during culture **(Fig. S2.4A)**. Similarly to GPR174-deficient B cells, Gαs-deficient B cells displayed fewer changes in gene expression, and the changes that did occur overlapped with those in WT cells **(Fig. S2.4B)**.

Interestingly, expression of *Ier2* and *Gpr171*, which had increased expression in WT versus GPR174-deficient B cells at 4 hours and both of which are predicted to contain conserved TATA-associated CRE sites (18), were higher in Gαs WT than Gαs-deficient B cells immediately ex vivo **(Dataset S3)**.

GSEA analysis of DEGs in WT versus Gαs-deficient B cells at 4 hours revealed a strong enrichment for differential expression in the WT versus GPR174-deficient 4-hour dataset **(Fig. S2.4C)**. Furthermore, analysis of de novo transcription factor binding motifs in DEGs that increased in GPR174 or Gαs WT B cells during culture showed significant enrichment for CREB binding motifs, whereas this was not the case for de novo motif analyses of GPR174- or Gαs-deficient B cells **(Fig. S2.4D)**. The level of similarity between GPR174- and Gαs-dependent transcript induction indicated that GPR174 was the primary Gαs-coupled receptor in this context, despite the presence of additional Gαs-coupled GPCRs in B cells, including ADRB2 and PTGER4 (93,94) (Immgen.org). *Pde3b* was one gene that was more strongly affected by Gαs than GPR174 deficiency, as it was moderately upregulated in GPR174-deficient but not in Gαs-deficient B cells **(Fig. S2.3B and Fig. S2.4B)**. PDE3b is activated by PKA phosphorylation (95), and Gαs-mediated induction of this gene may be part of a negative feedback loop when cAMP is present.

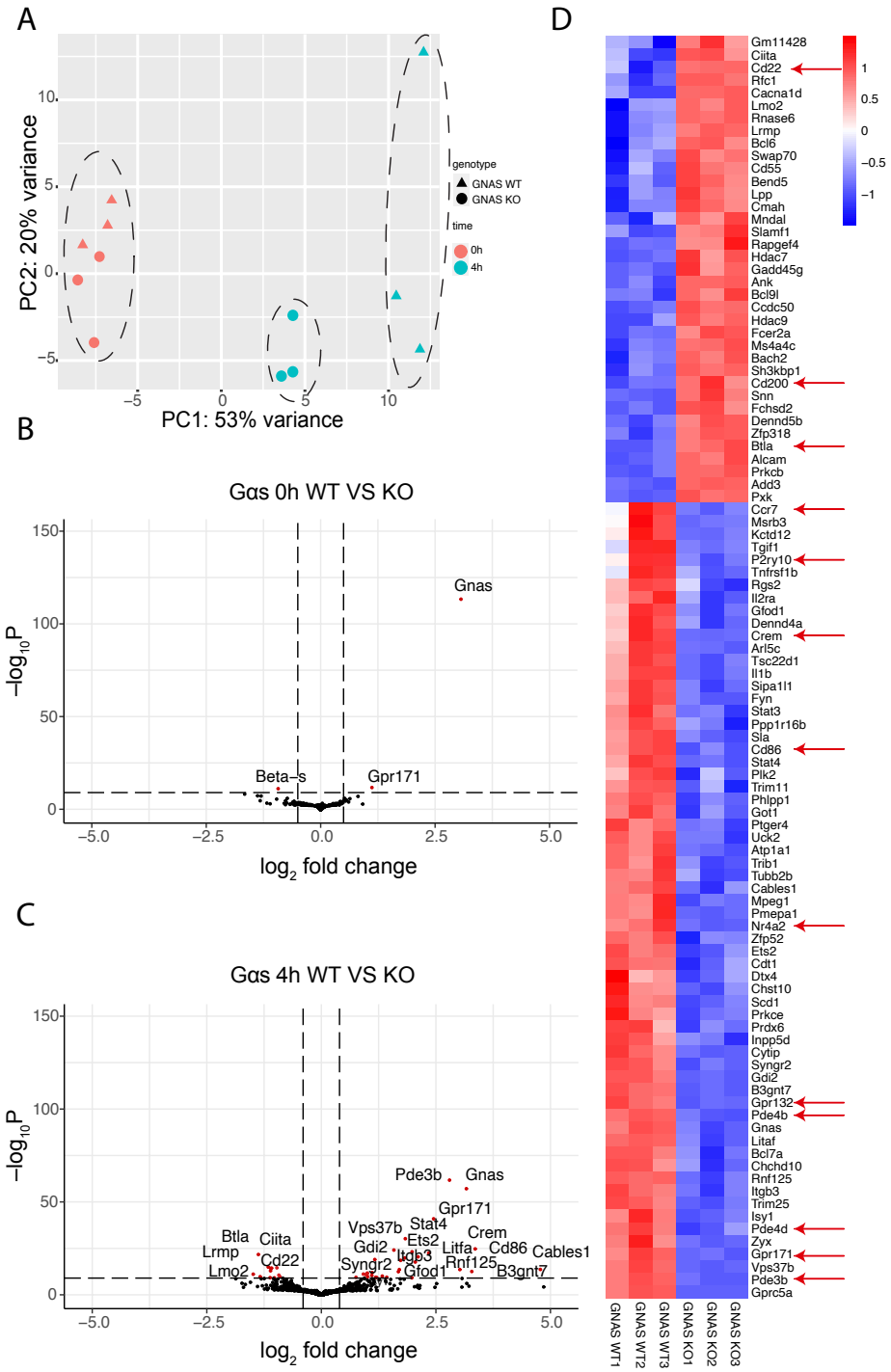


Figure 2.4. Gαs-deficient B cells phenocopy gene expression changes in GPR174-deficient cells. (A) PCA plot of RNAseq data from sorted Gαs WT and KO B cells cultured for the indicated times (N=3 mice per genotype). (B and C) Volcano plots of DEGs between WT and KO B cells at 0 hours (B) and 4 hours (C). Dashed lines indicate fold-change and p-value cutoffs for highlighted DEGs. (D) Heatmap of top 100 DEGs in Gαs WT versus KO B cells after 4 hours of culture. Red arrows indicate genes referenced in the text. (E) GSEA plots of the top 100 DEGs increased (top) or decreased (bottom) in Gαs WT versus KO B cells compared to GPR174 WT and KO B cells that were cultured for 4 hours.

Amongst the genes that increased in expression over time in GPR174- and Gαs-deficient B cells, most increased to a lesser extent than in WT cells. However, there was a small set of genes that increased in expression more in GPR174- and Gαs-deficient than WT cells during culture. Comparing overlap of these DEGs revealed multiple genes involved in cholesterol uptake and biosynthesis, including *Ldlr*, *Sqle*, and *Hmgcs1* **(Fig. S2.4E)**. This may reflect a Gαs-dependent repression of a latent cholesterol synthesis drive, and could have relevance for understanding the repressive effect of some Gαs-coupled receptors on lymphocyte proliferation (5,10,12,81).

Increased survival of GPR174- and Gαs-deficient B cells in culture

When murine B cell cultures were extended to one day, CD86 expression on WT B cells was reduced compared to 6 hours of culture and the difference between WT and GPR174-deficient B cells was no longer evident, whereas Gαs-deficient B cells continued to show decreased CD86 expression **(Fig. 2.5A**). In the one day cultures there was considerable B cell death **(Fig. 2.5B)**. Notably, GPR174-deficient B cells underwent significantly less cell death than WT B cells, and this phenotype was more prominent with Gαs-deficient B cells **(Fig. 2.5B)**. Addition of forskolin and IBMX to the cultures led to a substantial further reduction in B cell viability and this effect was partially dependent on GPR174 and fully dependent on Gαs **(Fig. 2.5B)**. Although WT B cell cultures had few viable cells after 48 hours of unstimulated incubation, GPR174 deficiency still conferred a protection against cell death **(Fig. 2.5C).** This viability effect was not dependent on the presence of other cells in the culture, as FACS purified B cells showed a similar viability difference to B cells from total splenocytes **(Fig. 2.5D)**.

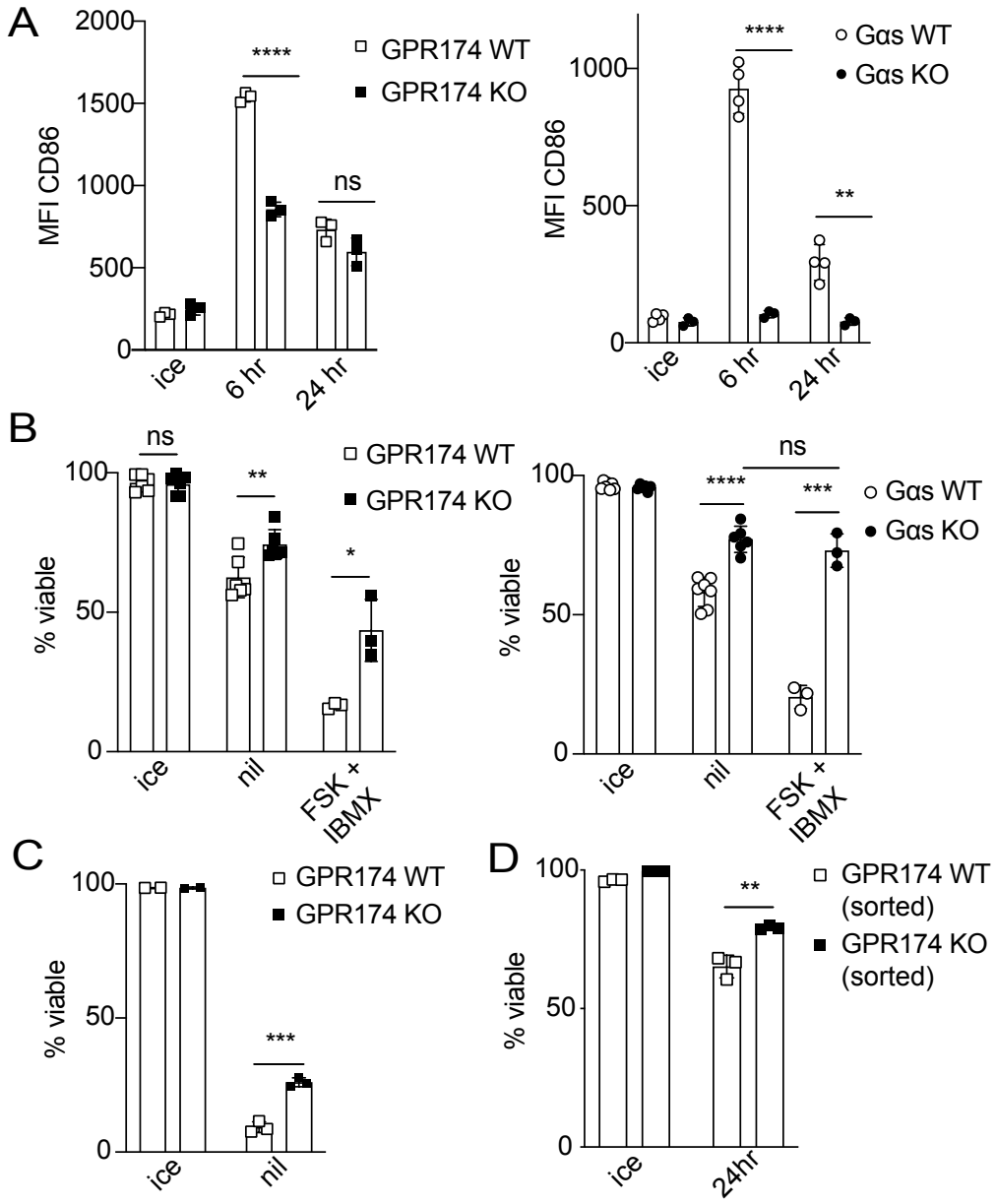


Figure 2.5. GPR174- and Gαs-deficient B cells show increased viability in vitro. (A) MFI of CD86 on GPR174 (left, N=3 mice per genotype) or Gαs (right, N=4 WT, N=3 KO mice) B cells after 6 or 24 hours of unstimulated culture. Representative of at least 3 experiments. (B) Percent viable of GPR174 (left, N=6 mice per genotype for ice/nil, N=3 mice per genotype for FSK+IBMX) or Gαs (right, N=7 WT, N=6 KO for ice/nil, N=3 mice per genotype for FSK+IBMX) B cells after 24 hours of unstimulated culture with or without forskolin + IBMX. (C) Percent viable of GPR174 WT and KO B cells after 48 hours of unstimulated culture (triplicate wells). (D) Percent viable of sorted GPR174 WT and KO B cells after 24 hours of unstimulated culture. Statistical significance for all panels determined by unpaired T-test. ns = not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

GPR174-Gαs promotes NUR77 expression in vitro and in vivo

One of the differentially expressed genes in cultured WT B cells at both 1 and 4 hours was *Nr4a1*, encoding NUR77, an early response gene that is well studied in lymphocytes for its induction via antigen receptor signaling (96,97). In order to validate GPR174- and Gαs-dependent induction of *Nr4a1* we performed intracellular staining of NUR77 in WT and Gαs-deficient mice. NUR77 protein levels were spontaneously induced in WT but not Gαs-deficient B cells within one hour of culture in the absence of stimulation **(Fig. 2.6A)**. While treatment with anti-IgM increased NUR77 expression in both WT and Gαs-deficient B cells, expression in Gαs-deficient B cells was not fully rescued. WT and Gαs-deficient B cells showed comparable MFIs of NUR77 protein immediately ex-vivo. However, B cells from WT and NUR77 KO mice also showed similar levels of NUR77 staining **(Fig S2.5A)**, indicating that the antibody staining was not sufficient to detect the low levels of NUR77 that exist at the steady state in mature B cells.

NUR77-GFP mice show a high level of reporter in mature naive B cells due to the stability of GFP, despite rapid turnover of NUR77 in vivo (24,98,99). Thus, we reasoned that the NUR77 reporter on a GPR174-deficient background might reveal subtle differences in NUR77 production or turnover. NUR77-GFP conferred a high level of GFP in follicular B cells compared to reporter negative mice, and B cells from WT NUR77-GFP mice had elevated GFP levels across multiple tissues compared to GPR174-deficient NUR77-GFP B cells **(Fig. 2.6B and Fig. S2.5B)**. Furthermore, WT NUR77-GFP B cells displayed more spontaneous GFP induction than GPR174-deficient NUR77-GFP B cells upon in vitro culture **(Fig. 2.6B and Fig. S2.5B)**. These observations provide evidence that GPR174 signaling influences gene expression in vivo under homeostatic conditions.

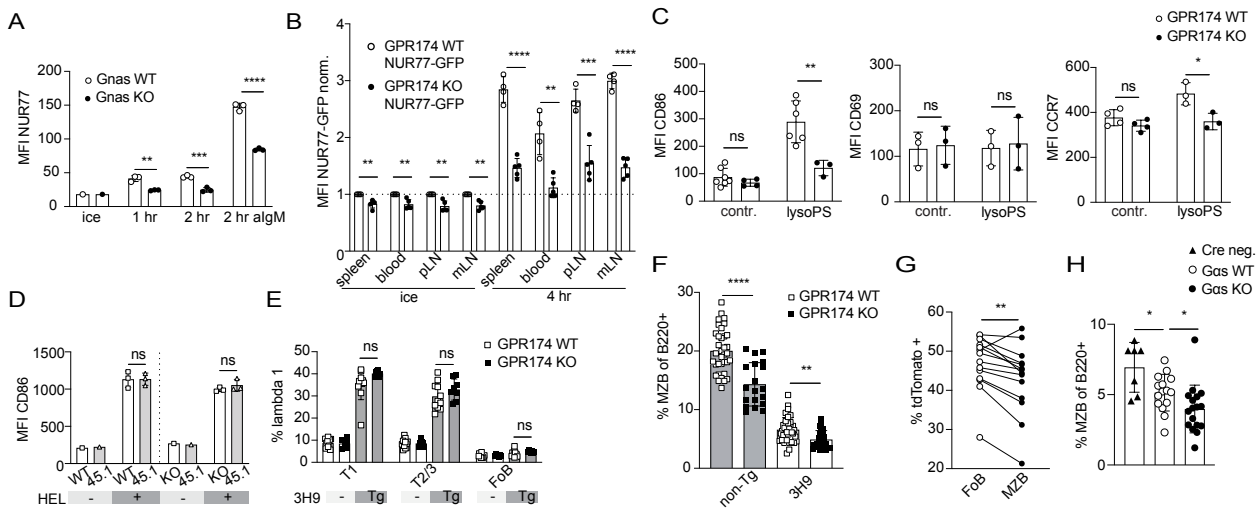


Figure 2.6. GPR174-Gαs promotes NUR77 expression in vivo and influences MZ size. (A) MFI of intracellular NUR77 on splenic B cells from Gαs WT or KO mice cultured for the indicated times with or without anti-IgM or maintained on ice (triplicate wells). (B) MFI of NUR77-GFP reporter on GPR174 WT (N=4 mice) or KO (N=5 mice) follicular B cells from indicated tissues either immediately after harvesting (left) or 4 hours of unstimulated culture (right). All values are normalized within each tissue to the WT sample that was maintained on ice. (C) MFI of CD86 (left), CD69 (middle) and CCR7 (right) on follicular B cells from draining popliteal lymph nodes of GPR174 WT or KO mice 4-6 hours after foot pad injection of 25 ug 18:1 lysoPS. Contr., contralateral (non-draining) lymph node. (D) MFI of CD86 on adoptively transferred GPR174 WT or KO MD4 (or CD45.1 congenic WT MD4) B cells 4-6 hours after I.V. injection of 1 mg HEL or saline. (E) Percent λ1 positive splenic T1 transitional (T1, CD93+ CD23-), T2/3 or follicular (FoB) B cells from GPR174 3H9 transgenic or non-transgenic mice (N≥7 mice per genotype). (F) Percent MZ B cells (MZB, CD21/35+ CD23-) of total splenic B220+ cells in GPR174 3H9 transgenic or non-transgenic mice (N≥20 mice per genotype). (G) Percent tdTomato reporter positive of follicular or MZ B cells from *Gpr174*$^{+/-}$ female mice (N=14 mice). Lines connect percent between compartments within the same mouse (H) Percent MZB of splenic B220+ cells from *Gnas*$^{+/+}$ *CD19 Cre*$^{+/-}$ (WT/ N=15), *Gnas*$^{fl/fl}$ *CD19 Cre*$^{+/-}$ (KO, N=17), or Cre^{-} control mice (N=7). Statistical significance for (A-F, H) determined by unpaired T-test. Statistical significance for (G) determined by paired T-test. ns = not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Chemokines do not alter GPR174-dependent CD86 induction

GPR174 has been reported to bind and respond to CCR7 ligands CCL19 and CCL21 (47). At concentrations that promote the migration of CCR7-expressing cells, recombinant CCL19 and CCL21 had no effect on the CD86 upregulation by WT B cells in vitro **(Fig. S2.5C)**. The prior work had found reduced migration of *Gpr174*$^{-/Y}$ (male) B cells pre-activated for 48 hours with anti-IgM and anti-CD40 to CCL19 and CCL21. In the mice studied here we found no effect of GPR174-deficiency on pre-activated male B cell migration to CCL19 or CCL21 **(Fig. S2.5D)**. The prior work also reported that CCR7-transfected 293T cells bound to a histidine (His)-tagged form of CCL21 (47). CCL21 has a highly positively charged C-terminus that can lead to binding to negatively charged proteoglycans (100). In contrast, CCL19 lacks this positively charged C-terminus, so we used recombinant CCL19-Fc, a reagent previously used to study CCR7 expression (101), to test for binding to GPR174. Using transfected 293T cells that had high surface expression of the GPR174 epitope tag (OX56) we were unable to detect CCL19-Fc binding **(Fig. 52.SE)**. Expression of GRP174 fused at the C-terminus with GFP also did not confer binding to CCL19-Fc **(Fig. S2.5E)**. In comparison, cells transfected with CCR7 showed a high level of CCL19-Fc binding as expected **(Fig. S2.5E)**. Similarly, CCL19-His bound to CCR7 but not GPR174 expressing 293T cells (**Fig. S2.5F**).

Our RNA sequencing result showed that cultured B cells undergo a GPR174- and Gαs-dependent induction of *Ccr7* **(Fig. 2.3C and 2.4C)**. In agreement with these data, surface levels of CCR7 increased to a greater extent in cultured WT versus GPR174-deficient B cells **(Fig. S2.5G)**. Unstimulated T cells increased CCR7 expression to a lesser extent after 6 hours of culture and the level was not different in WT versus GPR174-deficient cells **(Fig. S2.5G)**. CCL19-Fc binding to B cells increased in accord with CCR7 levels **(Fig. S2.5H)** and there was

no binding of CCL19-Fc to freshly isolated or cultured CCR7-deficient B cells **(Fig. S2.5H)**. In support of the ability of Gαs-cAMP to induce CCR7, stimulation with forskolin and IBMX increased CCR7 expression and CCL19-Fc binding in B cells **(Fig. S2.5G and S2.5H)**. Gαs signaling has previously been shown to induce CCR7 expression and function in monocytes (102).

LysoPS promotes B cell CD86 expression in vivo

In vitro studies have established that several forms of lysoPS (16:1, 18:0, and 18:1 in particular) can act as ligands for GPR174 (10–12,41,85,86). Although we had not detected an effect of exogenous lysoPS on B cell GPR174 signaling in vitro, the rapid increase in lysoPS that occurred upon B cell culture (**Fig. S2.1J**) led us to ask whether we could observe an effect of elevating lysoPS abundance in vivo. Mice were injected with lysoPS in the footpad and the draining popliteal lymph node monitored four hours later. LysoPS treatment led to upregulation of CD86 but not CD69 in draining lymph node B cells, and not in contralateral lymph node B cells, in a GPR174-dependent manner **(Fig. 2.6C)**. LysoPS also led to a detectable increase in B cell CCR7 expression **(Fig. 2.6C)** and NUR77-GFP reporter expression **(Fig. S2.5I)**. A similar experiment in conditional Gαs-deficient mice showed that Gαs was required for lysoPS-mediated induction of CD86 and CCR7 **(Fig. S2.5J)**.

To test for altered antigen-induced CD86 upregulation in GPR174-deficient cells in vivo, B cells from GPR174-deficient mice carrying hen egg lysozyme (HEL) specific transgenic IgM and IgD (MD4) were transferred into WT hosts. Four to six hours following HEL immunization there was comparable induction of CD86 on WT and GPR174-deficient MD4 B cells **(Fig. 2.6D)**. These data indicate that under these in vivo conditions, BCR-signaling induced CD86

does not depend on GPR174 signals. Consistent with these findings, Gαs-deficient mice mounted a normal GC response to a T-dependent antigen **(Fig. S2.5K)**.

GPR174 influences marginal zone B cell compartment size

SNPs in *GPR174* are associated in several GWAS studies with autoimmune disease (48,49,52), and NUR77 plays a role in the pruning of peripheral autoreactive B cells in a mouse model of lupus (25). V_H3H9 mice express a transgenic Ig heavy chain which, when paired with endogenous light chains, leads to dsDNA reactive B cells which are deleted during development (103–105). The λ1 light chain is the most autoreactive and deletion in 3H9 mice preserves tolerance. Given the coincidence of GPR174 expression in B cell development with the pruning of dsDNA autoreactive B cells and a potential role of this receptor in human autoimmune disease, we asked whether loss of GPR174 influenced tolerance in this model. WT 3H9 and GPR174-deficient 3H9 mice showed similar levels of λ1 light chain deletion from T1 transitional to mature follicular B cells **(Fig. 2.6E)**. Thus, tolerance appears to be intact. However, in the course of this work we noted that the percent of splenic MZ B cells, which is expanded in 3H9 transgenic mice, was decreased in GPR174-deficient 3H9 compared to WT 3H9 mice **(Fig. 2.6F)**. An assessment of a large number of WT and GPR174-deficient mice showed that GPR174 deficiency led to a reduction in the MZ B cell compartment of polyclonal mice **(Fig. 2.6F)**. This GPR174-dependent defect was cell intrinsic, as *Gpr174* heterozygous females, in which random X-inactivation would yield approximately 50% reporter positive cells, had lower percentages of tdTomato positive (GPR174-deficient) cells in the MZ versus the follicular B cell compartment **(Fig. 2.6G)**. Testing the effect of Gαs-deficiency on the MZ compartment was complicated by the *Cd19-Cre* allele being a KO allele of CD19, since CD19 loss is associated with a reduction in

the MZ (106). *Cd19-Cre* heterozygosity did lead to reduced MZ size but comparing *Gnas*$^{+/+}$ *Cd19-Cre*$^{+/-}$ and *Gnas*$^{fl/fl}$ *Cd19-Cre*$^{+/-}$ mice suggested that Gαs was also required for maintenance of a MZ compartment of normal size **(Fig 2.6H).**

Discussion

In this work we reveal that a GPR174-dependent gene expression program is induced in cultured B cells. This program is dependent on Gαs, providing evidence that GPR174 in B cells signals via Gαs-containing heterotrimeric G proteins. Notable GPR174-Gαs induced genes are *Nr4a1*, *Cd86, Ccr7* and phosphodiesterases while GPR174-Gαs repressed genes include ITIM-containing receptors *Cd22* and *Btla*. Importantly, GPR174-Gαs signaling reduces B cell viability in culture. We provide in vivo evidence that GPR174 can signal via this pathway through the finding of reduced NUR77-GFP reporter expression in mice lacking the receptor and the demonstration that lysoPS treatment increases CD86, NUR77 and CCR7 in lymph node B cells. GPR174 and Gαs also contribute to the induction or maintenance of the MZ B cell compartment. These findings complement earlier observations on GPR174 function in T cells (10–12) in advancing our understanding of how variants in the *GPR174* locus could contribute to human autoimmune disease (48,49,52). They also provide insights that may enable the development of improved approaches for studying B cells in culture.

The cAMP inducibility of CD86 was observed in multiple early studies with mouse and human cells (31–35,81,107,108). However, most subsequent work highlighting the ability of the BCR and other B cell activating receptors to induce CD86 has been consistent with the NFkB inducibility of this costimulatory molecule (29,109,110). The BCR is not thought to engage Gαs signaling, and we speculate that the weaker upregulation of CD86 after in vitro BCR stimulation

in GPR174- and Gαs-deficient B cells is reflective of the CD86 induction in WT B cells representing the additive effect of the spontaneous GPR174-Gαs (cAMP) signal and the BCR-induced (NFkB) signal. The physiological context in which cAMP contributes to CD86 induction in B cells remains to be defined though the β2-adrenergic receptor and prostacyclin receptor PTGIR have also been implicated in this process (108)(111).

LysoPS can be generated from phosphatidylserine (PS) by intracellular and extracellular enzymes (59,112), and lysoPS abundance in tissue increases under inflammatory conditions (43,59). A surprising observation in our work was that GPR174-dependent CD86 induction was unaffected by the addition of lysoPS to the cultures. However, lymphocytes can generate lysoPS (11,45,113) and our findings indicate that lysoPS abundance in B cells increases within minutes of in vitro incubation. We speculate that the lack of activity of exogenously added lysoPS reflects efficient occupancy of the receptor by lysoPS in an autocrine manner. However, our mouse lysoPS treatment data suggest that under homeostatic conditions in vivo the receptor is not fully occupied and exogenous lysoPS can engage the receptor and induce a CD86-containing gene expression program.

It is important to consider how our findings relate to evidence that GPR174 is a receptor for CCL19 and CCL21 (47). Our inability to detect an influence of CCL19 or CCL21 on GPR174-dependent CD86 induction is consistent with our inability to detect CCL19 binding to GPR174. Further work will be needed to resolve the discrepancy between Zhao et al., (47) detecting CCL21-His binding, and our inability to detect CCL19-His binding to GPR174-transfected HEK293T cells. Given our in vitro and in vivo findings that CCR7 can be upregulated in naïve B cells in a GPR174- and Gαs-dependent manner, we speculate that under some conditions GPR174 promotes CCL19 and CCL21 responses by increasing CCR7

expression. Zhao et al., provided biochemical evidence for GPR174 coupling to Gαi and Gα13 in anti-IgM plus anti-CD40 activated B cells (47). Although we have not performed biochemical studies, we believe the very close phenocopy observed in our in vitro and in vivo experiments between GPR174-deficient and Gαs-deficient B cells provides strong evidence that GPR174 signals via Gαs-containing heterotrimeric G-proteins in naïve B cells. A profiling study of GPCR-coupling capabilities reported that GPR174 strongly engaged Gαs but could stimulate Gα13 and Gαi more weakly (44). Zhao et al., noted as an unpublished observation that when CCR7 is ablated in activated B cells, GPR174-Gαi coupling and GPR174-mediated migration are markedly reduced (47). Further studies will be needed to discern whether GPR174 undergoes a change in G-protein coupling during B cell activation and whether this involves GPR174 interaction with CCR7.

cAMP signaling in activated B cells has previously been associated with reduced viability (114). The basis for GPR174 and Gαs signaling reducing B cell viability in vitro may reflect induction of several genes. For example, NUR77 restrains B cell survival (115). *Cables1* (Cdk5 and Abl enzyme substrate 1), one of the most strongly GPR174 dependent genes, can promote apoptosis (116). A growth repressive influence of Gαs on B cells is suggested by the finding of *GNAS* mutations in human diffuse large B cell lymphomas (117). Based on studies on T cells and innate immune cells, cAMP signaling is generally considered to be immune-suppressive (118). Indeed, Gαs-coupled adenosine receptors are targets of therapeutic antagonists that are being developed to increase tumor-specific T cell responses. However, studies in Gαs-deficient T cells showed a positive role for Gαs in promoting Th17 and Th1 cell differentiation and inflammatory function (119). When the activity of the GPR174-Gαs pathway in promoting costimulatory and

reducing ITIM molecule expression in B cells is taken together with its activity in reducing their viability, we suggest it may have both stimulatory and suppressive influences on B cells, helping fine-tune certain responses.

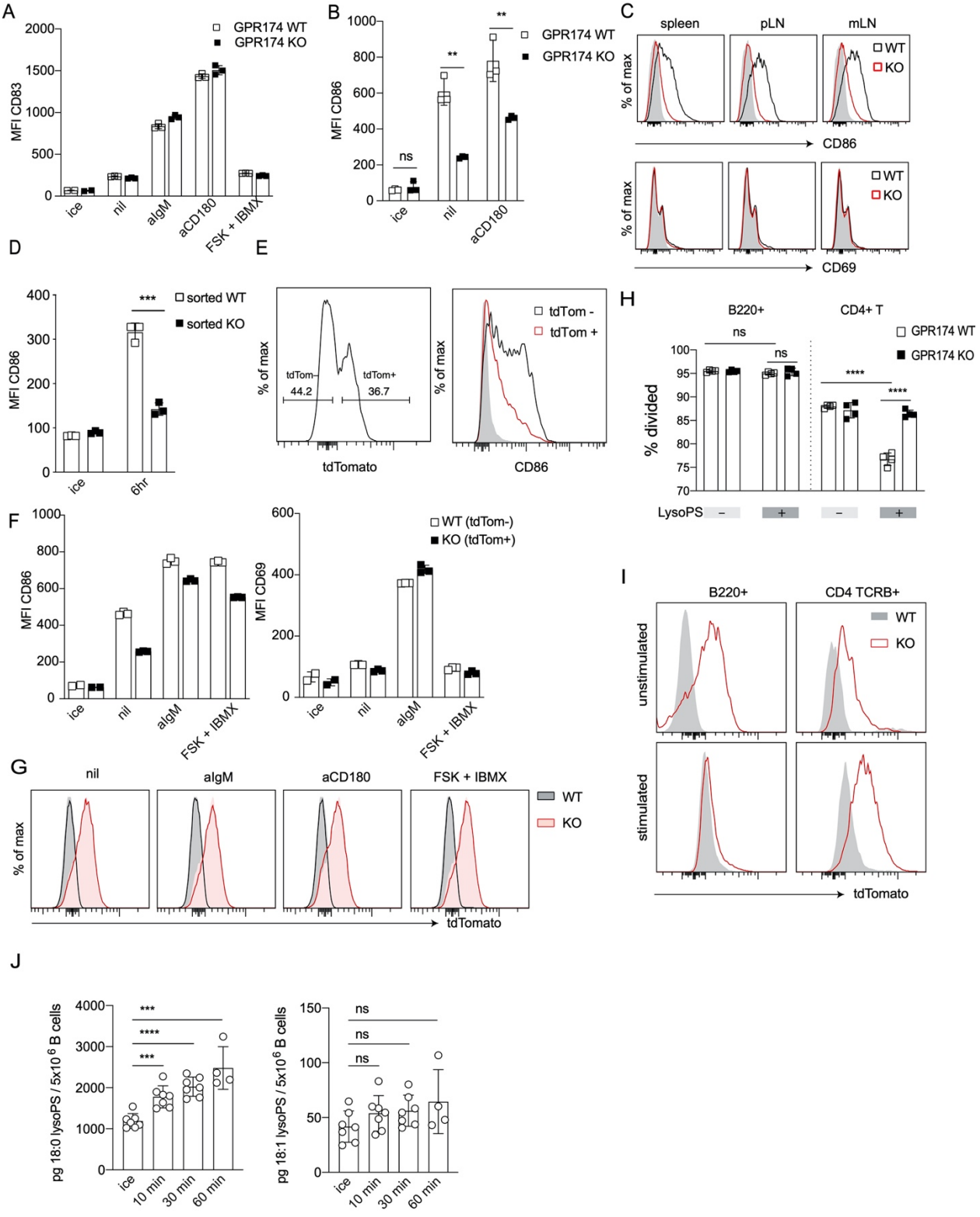
Our findings demonstrate that *Nr4a1* is a Gαs-inducible gene in B cells. Consistent with these data, *Nr4a1* is a CREB target gene in adrenal medulla-derived PC12 cells (22) and hepatocytes (23). NUR77 has a role in restricting the survival of self-reactive peripheral B cells and restraining B cell responses to antigen in the absence of signal 2 (25,115). The latter activity involved partially redundant roles of *Nr4a* family members. Reduced expression of *Nr4a1* and other *Nr4a* family members in GPR174-deficient B cells may lead to less effective control of self-reactive B cells. Whether the *Cd86* gene is directly induced by CREB is less clear though the GPR174-dependent upregulation of transcripts within 1 hour of incubation suggests direct induction by a cAMP responsive factor. A study of human cells provided evidence for functional CREB binding sites in the *CD86* promoter (36) but these sites are not well conserved in the mouse *Cd86* promoter (18). However, CREB can bind to many non-canonical sites (120,121). Future pCREB ChIPseq studies in B cells will help identify the full set of genes that are directly targeted by Gαs signaling.

The MZ B cell compartment has unique signaling requirements compared to follicular B cells including a dependence on BCR, CD19 and Notch signaling (106,122). MZ B cells are also dependent on Gαi- and Gα12/13-coupled GPCRs for their positioning and homeostasis (123,124). How GPR174 and Gαs signals integrate with these other inputs to promote the normal accumulation of MZ B cells is not yet clear. A previous study examining a different GPR174-deficient mouse line, also on the C57BL/6 background, suggested that GPR174-deficiency was associated with a marked increase in the MZ compartment (125). The basis for

this discrepancy in findings is unclear but there is some evidence that the microbiome, which is likely different between facilities, can influence the MZ compartment (126,127). Although our GPR174-deficient mice were generated by gene targeting in 129-background ES cells followed by extensive backcrossing to C57BL/6J, our findings are unlikely to be a consequence of linked gene variants because we obtained similar results in Gαs-deficient mice.

In summary, we provide evidence that GPR174 is the dominant Gαs-coupled GPCR in naïve B cells. We find that B cells undergo substantial gene expression change during the early period of in vitro culture, and that a considerable amount of this change is GPR174- and Gαs-dependent. In vivo, GPR174 appears capable of engaging a similar pathway and we propose that the receptor helps tune B cell responses based on the tissue microenvironment and state of inflammation. Altered activity of the GPR174 signaling pathway in B cells may contribute to development of Graves, Addison's and possibly other autoimmune diseases.

Supplementary Figures


A
MFI CD83
GPR174 WT
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ice
nil
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C
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tdTom +
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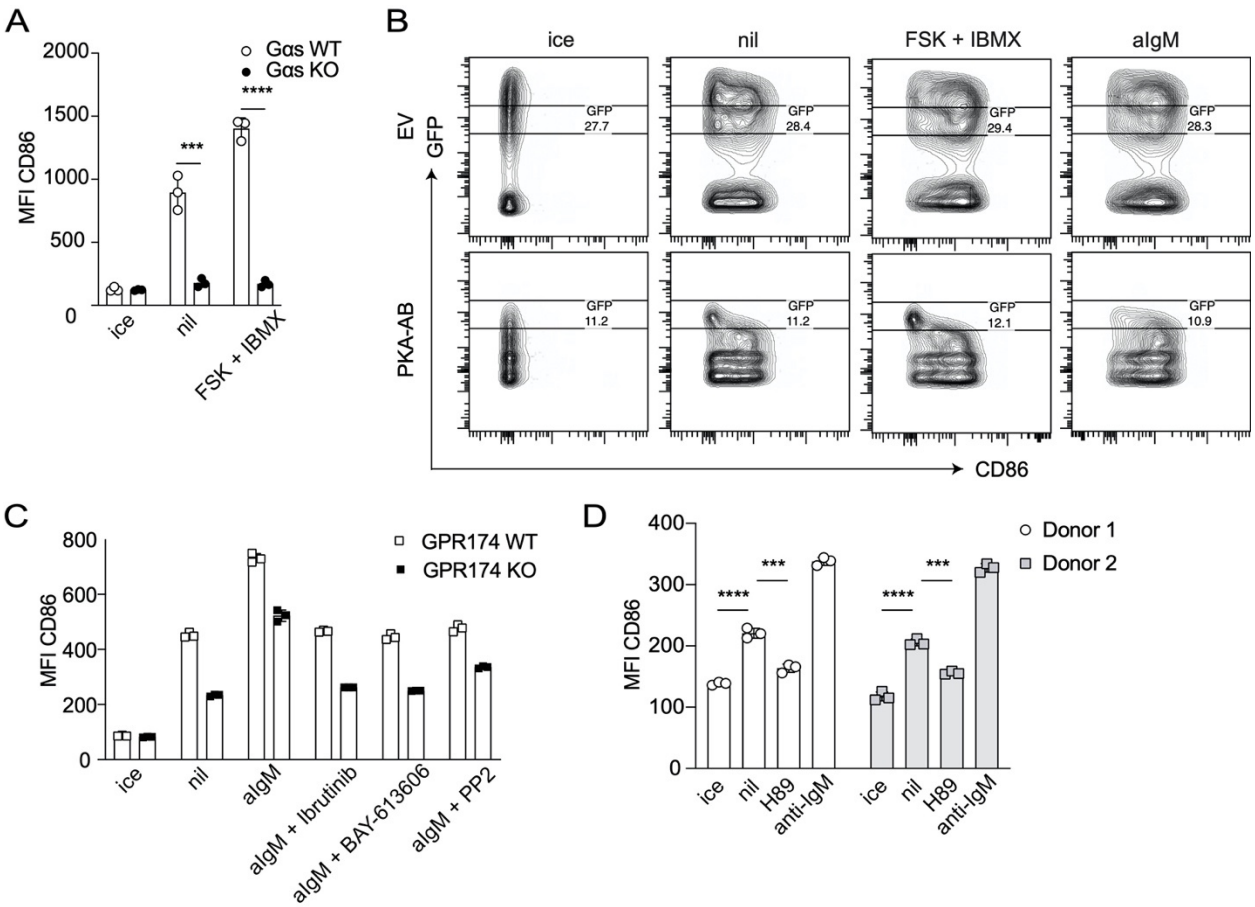
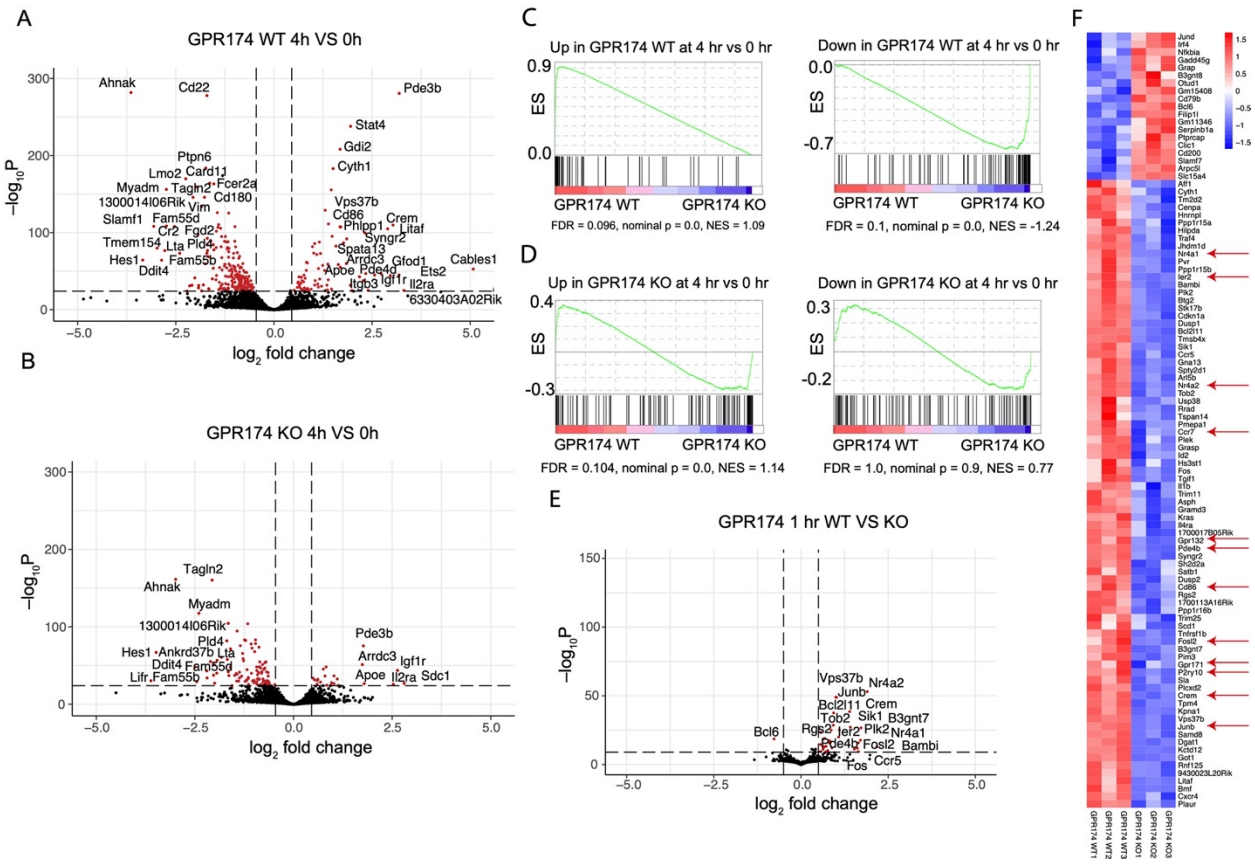
MFI CD86
MFI CD69
WT (tdTom-)
KO (tdTom+)
G
nil
aIgM
aCD180
FSK + IBMX
tdTomato
H
B220+
CD4+ T
% divided
LysoPS
I
B220+
CD4 TCRB+
unstimulated
stimulated
J
pg 18:0 lysoPS / 5x10^6 B cells
pg 18:1 lysoPS / 5x10^6 B cells
10 min
30 min
60 min

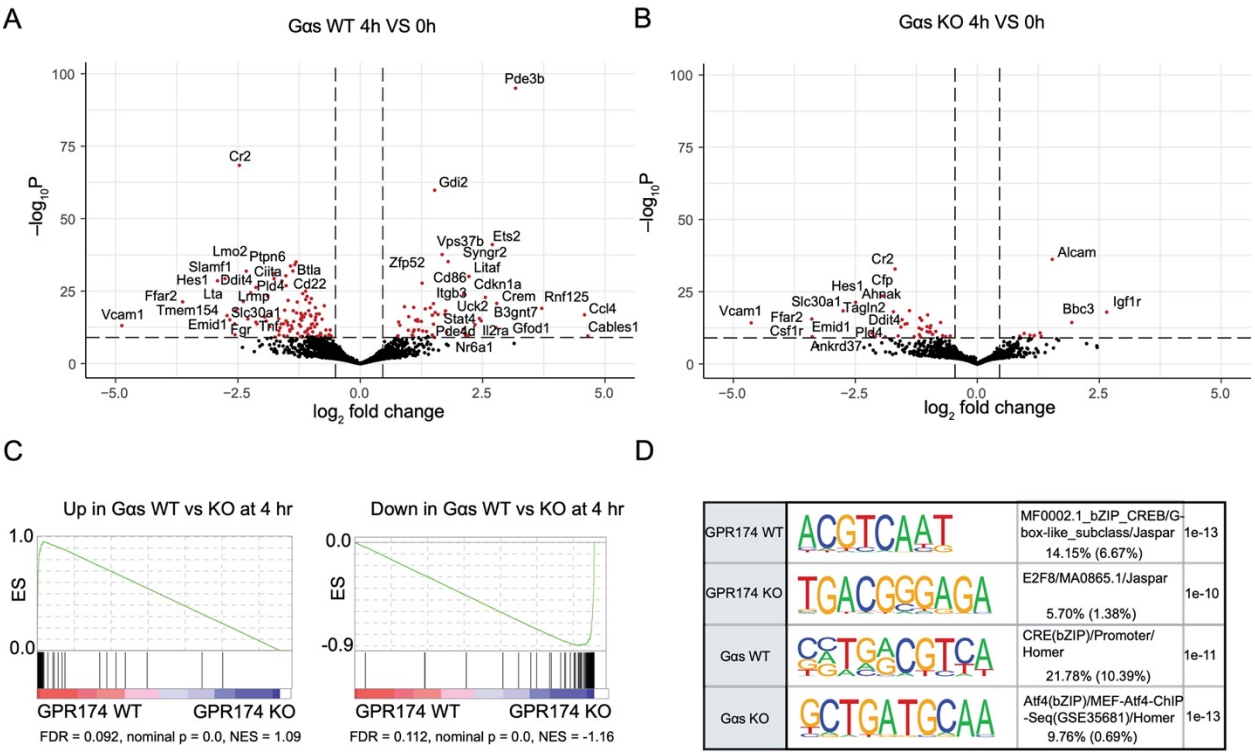
Supplementary Figure 2.1. GPR174 promotes CD86 induction. (A) MFI of CD83 on B cells from GPR174 WT or KO mice cultured for 6 hours with the indicated treatment or maintained on ice (triplicate wells). (B) MFI of CD86 on splenic follicular B cells from indicated genotypes cultured for 6 hours with or without anti-CD180 (0.5 μg ml^{-1}) or maintained on ice (N=3 mice per genotype). (C) Representative histograms of CD86 and CD69 expression on GPR174 WT or KO B cells from the indicated tissues after 6 hours of unstimulated culture, relative to cells maintained on ice (gray). (D) MFI of CD86 on sorted B cells immediately post-sort (ice) or after 6 hours of unstimulated culture (N=3 mice per genotype). (E) Representative gating of tdTomato reporter (left) and histogram of CD86 expression (right) of reporter negative or positive B cells from spleens of *Gpr174*$^{+/-}$ female mice after 6 hours of unstimulated culture, relative to cells maintained on ice (gray). (F) MFI of CD86 (left) or CD69 (right) on tdTomato reporter negative or positive B cells from *Gpr174*$^{+/-}$ female mice after 6 hours of culture with or without anti-IgM or forskolin + IBMX or maintained on ice (N=3 mice per genotype). (G) Representative histograms of tdTomato reporter expression on GPR174 WT or KO B cells immediately after isolation (solid) versus 6 hours of culture (lines) with the indicated stimulation. (H) Percent of viable B220+ or CD4+ T cells divided after 4 days in culture with or without 18:1 lysoPS (10 μM). Cells from GPR174 WT or KO spleens were enriched by depletion to >95% purity, CTV labeled, and stimulated with lipopolysaccharide (10 μg ml^{-1}) from *E. coli* (B cells) or plate-bound anti-CD3 (2 μg ml^{-1}) and anti-CD28 (2 μg ml^{-1}) (T cells). (I) Representative histograms of tdTomato reporter expression on cells from (H). Statistical significance for (B, D, H) determined by unpaired T-test. ns = not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.



Supplementary Figure 2.2. Induction of CD86 is Gαs and PKA dependent and BCR independent. (A) MFI of CD86 on B cells from Gαs WT or KO mice after 6 hours of culture with or without forskolin + IBMX or maintained on ice (N=3 mice per genotype). (B) Representative plots of vector GFP and CD86 expression on B cells from chimeras transduced with EV GFP or PKA-AB GFP and cultured with or without anti-IgM or forskolin + IBMX or maintained on ice. (C) MFI of CD86 on B cells treated with or without anti-IgM, Ibrutinib (1 μM), BAY61-3606 (1 μM), or PP2 (20 μM) or maintained on ice (triplicate wells). (D) MFI of CD86 on $CD19^+$ cells from PBMCs of two human donors after 6 hours of culture under the indicated conditions or maintained on ice. Statistical significance for (A, D) determined by unpaired T-test. ns = not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.



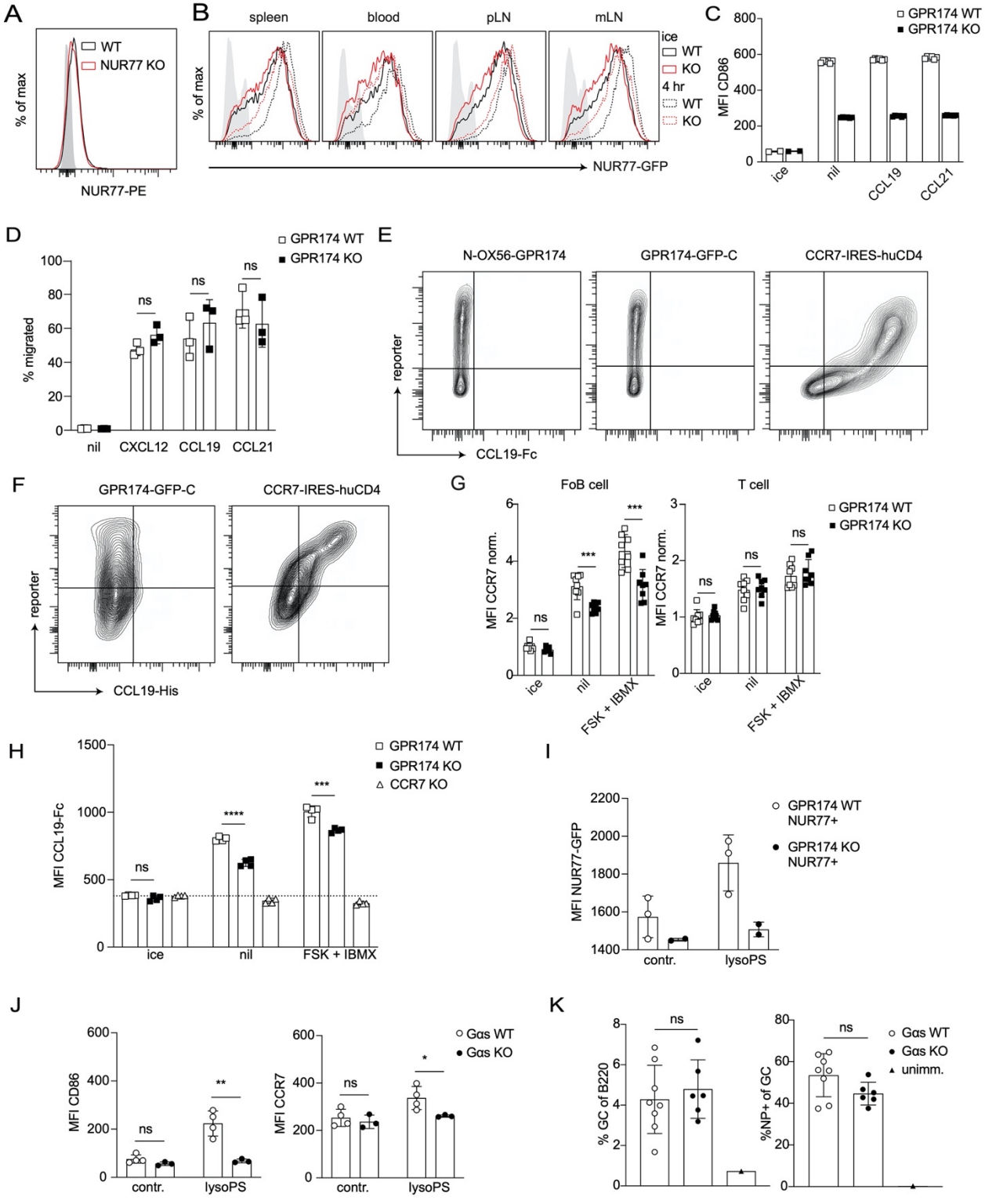
Supplementary Figure 2.3. RNAseq analysis of GPR174 B cells. (A, B) Volcano plots showing differential gene expression at 4 versus 0 hours in GPR174 WT (A) or KO (B) B cells. (C) GSEA plots of the top 100 DEGs that increased (left) or decreased (right) in GPR174 WT B cells during culture compared to GPR174 WT versus KO after 4 hr of culture. (D) GSEA plots of the top 100 DEGs that increased (left) or decreased (right) in GPR174-deficient B cells during culture compared to GPR174 WT versus KO after 4 hr of culture. (E) Volcano plot showing differential gene expression in GPR174 WT versus KO B cells after 1 hour of culture. (F) Heatmap of top 100 DEGs between GPR174 WT and KO B cells after 1 hour of culture. Red arrows indicate genes referenced in the text. For volcano plots, dashed lines indicate fold-change and p-value cutoffs for highlighted DEGs.



E

Gene		chol. synth
Alcam	Activated leukocyte cell adhesion molecule	no
Cyp51	Sterol 14-demethylase	yes
Fdft1	Farnesyl-diphosphate farnesyltransferase 1	yes
Fdps	Farnesyl diphosphate synthase	yes
Hmgcs1	Hydroxymethylglutaryl-CoA synthase	yes
Igf1r	Insulin-like growth factor 1 receptor	no
Ldlr	Low density lipoprotein receptor	yes
Lilrb4	Leukocyte immunoglobulin like receptor B4)	no
Mcoln2	Mucolipin-2	no
Pnpla7	Patatin-like phospholipase domain-containing protein 7	no
Sc4mol	Methylsterol monooxygenase 1	yes
Sqle	Squalene epoxidase	yes

Supplementary Figure 2.4. RNAseq analysis of Gαs and GPR174 B cells. (A, B) Volcano plots showing differential gene expression at 4 versus 0 hours in Gαs WT (A) or KO (B) B cells. Dashed lines indicate fold-change and p-value cutoffs for highlighted DEGs. (C) Top de novo motif logos (Homer) for DEGs upregulated during culture in B cells of the indicated genotype, with percent of DEGs containing the motif and, in parentheses, percent background. (D) Overlap of genes that increase in GPR174-deficient and Gαs-deficient B cells during culture more than in WT cells and their involvement in cholesterol biosynthesis.


A
WT
NUR77 KO
% of max
NUR77-PE
B
spleen
blood
pLN
mLN
ice
WT
KO
4 hr
WT
KO
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NUR77-GFP
C
GPR174 WT
GPR174 KO
MFI CD86
800
600
400
200
0
ice
nil
CCL19
CCL21
D
GPR174 WT
GPR174 KO
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100
80
60
40
20
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ns
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ns
nil
CXCL12
CCL19
CCL21
E
N-OX56-GPR174
GPR174-GFP-C
CCR7-IRES-huCD4
reporter
CCL19-Fc
F
GPR174-GFP-C
CCR7-IRES-huCD4
reporter
CCL19-His
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FoB cell
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GPR174 WT
GPR174 KO
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FSK + IBMX
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GPR174 WT
GPR174 KO
CCR7 KO
MFI CCL19-Fc
1500
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GPR174 WT
NUR77+
GPR174 KO
NUR77+
MFI NUR77-GFP
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Gαs KO
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contr.
lysoPS
MFI CCR7
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contr.
lysoPS
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% GC of B220
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%NP+ of GC
80
60
40
20
0
ns
Gαs WT
Gαs KO
unimm.

Supplementary Figure 2.5. GPR174 and Gαs influence expression in vitro and in vivo. (A) Representative staining of intracellular NUR77 on WT versus NUR77 KO in freshly isolated B cells.(B) Histogram of NUR77-GFP on GPR174 WT (black) or KO (red) B cells from the indicated tissue either immediately after isolation (solid line) or after 4 hours of unstimulated culture (dashed line), compared to reporter negative (solid gray). (C) MFI of CD86 on B cells treated with or without CCL19 (500 ng ml^{-1}) or CCL21 (500 ng ml^{-1}) (triplicate wells of N=2 mice per genotype). (D) Percent of GPR174 WT and KO B cells migrated to CXCL12 (100 ng ml^{-1}), CCL19 (500 ng ml^{-1}), or CCL21 (500 ng ml^{-1}) during 3 hour assay following 48 hours of pre-activation with anti-IgM and anti-CD40 (10 μg ml^{-1}) (N=3 wells, representative of at least 3 individual experiments). (E) MFI of CCL19-Fc binding on HEK 293T cells transfected with N-terminally OX56 epitope tagged GPR174, C-terminally GFP tagged GPR174, or CCR7-ires-huCD4. Representative of 3 experiments. (F) MFI of CCL19-His binding on HEK 293T cells transfected with C-terminally GFP tagged GPR174, or CCR7-ires-huCD4. (G) MFI of CCR7 (normalized to WT B cells maintained on ice) on B cells (left) or T cells (right) after 6 hours of culture with or without forskolin + IBMX or maintained on ice (combined wells from two experiments of N=1 mice per genotype, representative of at least 3 additional experiments). (H) MFI of CCL19-Fc binding to follicular B cells from GPR174 WT, GPR174 KO, and CCR7 KO splenocytes cultured for 6 hours under the indicated conditions or maintained on ice. Dashed line indicates MFI of CCL19-Fc on CCR7 KO cells maintained on ice (N=4 wells per condition, representative of two experiments). (I) Summary data of percent germinal center (GC, FAS^+ $GL7^+$) of total B cells (left) and % NP^+ of GC (right) from spleens of Gαs WT or KO mice immunized with 50 μg $NP_{(32)}$-KLH + 1 μg LPS in alum or an unimmunized control (unimm.). Data is representative of normal mice (N=2 WT and 4 KO) and chimeras (N=4 WT and 4 KO). Statistical significance for (D, F, G) determined by unpaired T-test. ns = not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Materials and Methods

Mice

All mice were bred internally. 3H9 and NUR77 reporter mice were obtained from J. Zikherman at UCSF. CD45.1 B6 (B6.SJL-PtprcaPepcb/BoyCrCrl) mice used for chimera recipients and cell transfer experiments were bred internally from founders ordered from JAX. All mice were analyzed between 8-20 weeks of age except for marginal zone B cell analyses, in which mice were analyzed at 10-20 weeks of age. Mice were co-caged littermates for all experiments. All data are representative of male and female mice unless otherwise noted. Animals were housed in a specific pathogen free environment in the Laboratory Animal Research Center at UCSF and all experiments conformed to ethical principles and guidelines approved by the UCSF Institutional Animal Care and Use Committee.

Bone marrow chimeras

CD45.1 B6 mice were lethally irradiated with 1,100 rads gamma-irradiation (split dose separated by 3 h) and then i.v. injected with relevant BM cells under isoflurane anesthesia. Chimeras were analyzed after 10-12 weeks of reconstitution.

Retroviral Constructs and Transductions

Murine PKA-AB sequence was obtained from S. McKnight and cloned into the MSCV2.2 retroviral vector followed by an internal ribosome entry site (IRES) and GFP as an expression marker. Retrovirus was generated by transfecting PLAT-E packaging cell line with 10 μg plasmid DNA and 25 μl Lipofectamine 2000 (Fischer). Bone marrow donors were injected

with 3 mg of 5-fluorouracil (Sigma) and bone marrow was collected 4 days later and cultured in DMEM containing 15% (vol/vol) FBS, antibiotics (penicillin (50 IU/mL) and streptomycin (50 μg/mL; Cellgro) and 10 mM HEPES, pH 7.2 (Cellgro), supplemented with IL-3, IL-6 and stem cell factor (at concentrations of 20, 50 and 100 ng/mL, respectively; PeproTech). Cells were 'spin-infected' twice at days 1 and 2 of culture at 2400 RPM and 37°C with viral supernatants and were transferred into irradiated recipients on day 3.

In vitro B cell culture

Spleens were isolated and sterilely mashed through a 100um filter on ice in cold MACS (PBS with 2% FBS and 1 mM EDTA). Spleens were washed and diluted in complete RPMI 1640 (containing 10% FBS, 10 mM HEPES, 55 μM 2-mercaptoethanol, 2 mM glutamine and 50 IU penicillin/streptomycin). Cells were plated ($5x10^5$) in 96-well flat-bottom plates. Plates were incubated at 37°C with 5% CO_2 for the indicated times and then collected, washed and stained in 96-well round-bottom plates. Chemicals and stimulations were added to cultures at the following concentrations; 10 μM forskolin (Cayman Chemicals) and 100 μM IBMX (Sigma); 10 μg ml^{-1} anti-IgM (Jackson ImmunoResearch); 10 μM 18:1 LysoPS (Avanti Polar Lipids); 100 ng ml^{-1} CXCL12 (Peprotech); 500 ng ml^{-1} CCL19 (R&D Systems); 500 ng ml^{-1} CCL21 (R&D Systems); 0.5 μg ml^{-1} LEAF-purified anti-CD180 (Fisher); 10 μM H89 (Fisher); 1 μM Ibrutinib (Selleck Chemicals); 1 μM BAY61-3606; 20 μM PP2

Flow cytometry

Cells were stained for 20 minutes on ice in MACS buffer (2% FCS in PBS with 1 mM EDTA) at 0.5-$1x10^6$ cells per well in 96 round bottom plates unless otherwise specified. All

mAbs were purchased from BioLegend unless otherwise indicated. The following monoclonal antibodies were used: TCRβ–BV421, TCRβ–PerCP-Cy5.5 (Tonbo), CD23–PE-Cy7, CD23–Alexa Fluor 488, CD86–Alexa Fluor 647, CD86–PerCP-Cy5.5, CD21/35–Pacific Blue, B220–BV785, CD69–FITC (BD), CD93–APC (eBioscience), CD4–BV605, CD45.2–PE, CD45.1–BV605, lambda-1 light chain–biotin (BD) (followed by streptavidin–BV605), CD19–PE, CCR7–biotin (followed by streptavidin–BV421). CCR7-biotin was stained for one hour at room temperature followed by streptavidin and other surface markers on ice. Dead cells were excluded using Fixable Viability Dye eFluor780 (eBioscience Cat# 65-0865-18). For time course experiments all samples were plated simultaneously, collected and kept on ice, and stained in parallel. Viability for 24-48 hour cultures was determined by pre-gating on B220+ TCRβ–. All samples were run on a BD LSRii at 5,000-10,000 events per second. Flow cytometry data was analyzed using FlowJo (v10.8.0).

Cell sorting and RNA sequencing

Follicular B cells were sorted from spleens of male mice into cold MACS buffer using a BD FACSAria II. Sort purity was greater than 99% for *Gpr174*$^{+/Y}$ and *Gpr174*$^{-/Y}$ B cells and 99-95% for *Gnas*$^{+/+}$ *Cd19 Cre*$^{+/-}$ and *Gnas*$^{fl/fl}$ *Cd19 Cre*$^{+/-}$ B cells. 5x10^{6} cells were either frozen immediately post-sort or following 1 or 4 hours of culture in complete RPMI. The GPR174 4 hour experiment was prepared for sequencing using Ovation RNA-seq System V2 from Nugen, KAPA Hyper prep labeling kit, NEXTflex DNA barcodes Adapter kit from Bioo Scientific, and 50 bp single end was run on HiSeq2500 at the UCSF Institute for Human Genetics. The GPR174 1 hour and Gαs 4 hour experiments were prepared for sequencing using QuantSeq 3' kit from Lexogen and 50 bp single end was run on HiSeq4000 at the UCSF Center for Advanced

Technology. Sequences were aligned to the mm10 genome with STAR and mapped reads of each gene were counted with HTseq. DESeq2 was used for the gene differential expression analysis and with GSEA software. Heatmap was generated with pheatmap. Volcano plots were generated with EnhancedVolcano.

In vitro B and T cell proliferation

Follicular B cells were purified from GPR174 WT and KO spleens by depletion using biotinylated antibodies (anti-CD43, anti-TER119, anti-CD11c, anti-TCRβ, anti-CD4, and anti-CD8) and streptavidin-conjugated beads (EasySep Streptavidin RapidSpheres) to greater than 95% purity. CD4 T cells were purified using anti-TER119, anti-B220, anti-CD11c, and anti-CD8 and streptavidin-conjugated beads to greater than 95% purity. B/T cells were labeled with CellTrace Violet (Life Tech) according to the manufacturers protocol. 24-well plates were coated with anti-CD3 (2 μg ml^{-1}) and anti-CD28 (2 μg ml^{-1}) (both LEAF purified from BioLegend) in PBS for 3 hours at 37°C and T cells were plated ($4x10^5$ cells per well) in complete RPMI 1640 with or without 10 μM 18:1 LysoPS (Avanti Polar Lipids). B cells ($4x10^5$ cells per well) were plated in 24-well plates with LPS (10 μg ml^{-1} 0111:B4 from *E. coli*, Sigma) and with or without 18:1 LysoPS. Proliferation of viable cells was determined after 4 days.

Intracellular Nur77 staining

Splenocytes were isolated cultured as indicated in the figure legends except for ice samples, which were kept on ice until all samples were collected and then stained concurrently. Cells were collected and washed with cold MACS buffer (2% FCS in PBS with 1 mM EDTA) in 96-well round-bottom plates ($1x10^6$ cells/well). Cells were stained with Fixable Viability Dye

eFluor780 for 20 minutes on ice, washed twice with MACS, then resuspended in 100ul 2% PFA and incubated for 10 minutes at room temperature. Cells were washed twice with MACS and the pellet dislodged by vigorously tapping the plate, then 150ul of ice cold methanol was added dropwise to the wells. The plates were then kept at -20°C for 45 minutes and then washed and rehydrated for 5 minutes with MACS. Following another wash, cells were stained with Nur77-PE (eBiosciences Cat# 12-5965-80), B220–BV785, TCRβ–BV421, CD23–PE-Cy7and the relevant surface markers for 1 hour at room temperature and then washed twice, resuspended in MACS and collected on a BD LSRii.

Migration assays

B cells were enriched from spleens of co-caged littermate males by depletion using biotinylated antibodies (anti-CD43, anti-TER119, anti-CD11c, anti-TCRβ, anti-CD4, and anti-CD8) and streptavidin-conjugated beads (EasySep Streptavidin RapidSpheres). B cells were activated with 10 μg ml^{-1} F(ab′)2 goat anti-mouse IgM (Jackson Immunoresearch) and 10 μg ml^{-1} anti-CD40 (clone FGK4.5, Bio X Cell) in complete RPMI for 60 hours. B cells were collected, washed in pre-warmed migration medium (RPMI containing 0.5% fatty acid-free BSA, 10 mM HEPES and 50 IU penicillin/streptomycin) and rested in migration medium at 37 °C for 1 h. B cells (200 μl, $1x10^6$ cells) were then added to transwells (5 μm-pore, Corning Costar #93421) with the indicated chemokines in migration media (600 μl) in the bottom chamber and allowed to migrate for 3 hours at 37 °C and 5% CO_2. Migrated cells were then collected from the bottom chamber, stained for viable $B220^+$ cells, and collected on an LSRii cytometer for a fixed time under constant flow rate and normalized to an “input” well.

CCL19 binding assay

HEK 293T cells were seeded in 6-well plates with DMEM containing 10% FBS, 10 mM HEPES, 2 mM glutamine and 50 IU penicillin/streptomycin and grown to 75% confluency, then transfected with the plasmids indicated in the figure legends in Lipofectamine2000 and Optimem (Life Technologies). One day following transfection, cells were dislodged and $5x10^5$ cells were washed in 96-well round-bottom plates. Cells were then resuspended and incubated with 1% Fc block (Bio X Cell) in MACS buffer (2% FCS in PBS with 1 mM EDTA) for 15 minutes on ice, then CCL19-huFc (101) or CCL19-His (Cell Sciences CRM520A) was added without washing and incubated for 30 minutes on ice. Cells were washed twice with MACS and then stained with anti-huFc-PE (1:50, Jackson Immunoresearch 109-116-098) or anti-His-biotin (1:10, Miltenyi 130-099-423) for 20 minutes on ice. The secondary was pre-adsorbed with 2% each of normal rat and normal mouse serum. The His-biotin reagent was detected with streptavidin-BV421 (1:200 Biolegend 405226). Cells were washed twice and stained with Live/Dead and antibodies to identify vector positive cells (with 1% normal rat serum and 1% normal mouse serum), then washed twice and analyzed on a BD LSRii. Staining for detection of CCL19 binding on freshly isolated or 6 hour cultured splenocytes was performed as for 293T cells using anti-huFc-AF488 (1:50, Jackson Immunoresearch 709-546-098) to detect the CCL19-huFc reagent.

Immunization

Mice were immunized intraperitoneally with 50 μg NP_{32}-KLH and 1 μg LPS (0111:B4 from *E. coli*, Sigma) in 2% Alyhydrogel alum adjuvant (Invivogen) and spleens analyzed 14 days later.

LysoPS injections

Mice were anesthetized using isofluorane and injected in the right footpad with 25 ug 18:1 LysoPS (Avanti Polar Lipids) or an equivalent volume of diluted solvent (methanol) in the contralateral foot pad. Mice were euthanized four to six hours later and popliteal lymph nodes harvested for flow cytometric analysis.

Statistical analyses

Data were analyzed using paired or unpaired Student's t test as appropriate. Prism version 8 (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.

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Data availability

Raw and processed data files for RNA sequencing analysis have been deposited in the NCBI Gene Expression Omnibus under accession number GSE200800.

Competing Interests

JGC is a on the scientific advisory board of BeBio Pharma and MiroBio Ltd.

CHAPTER THREE

Conclusions

Conclusions

In this study we identify GPR174 as a Gαs-coupled GPCR which controls a large gene expression program in naïve B cells. This program includes *Cd86, Nr4a* family members, *Ccr7*, and cAMP-specific phosphodiesterases. The GPR174-Gαs signaling axis is induced in cultured B cells in the absence of added stimulation, and we show that this signaling explains most of the spontaneous activation in vitro. We propose that this spontaneous activation leads to loss of viability over time. In the absence of GPR174 or Gαs, B cells remain comparatively quiescent, with greater viability seen after 24 or 48 hours of culture, compared to control cells. Inhibition of this signaling may be a novel strategy for achieving B cell culture conditions that more closely mimic in vivo conditions.

Exogenous lysoPS does not augment GPR174-dependent gene expression in vitro, nor does the presence of serum factors. Purified follicular B cells maintain the GPR174- and Gαs-dependent activation and viability phenotypes, demonstrating that additional cell types are not required. T cells have been shown to produce lysoPS in response to stimulation with cytokines (45). We found that purified B cells spontaneously produce lysoPS within 10 minutes of in vitro incubation, and maintain this production for at least one hour. We propose that B cells produce a GPR174 ligand in sufficient amounts to generate the phenotypes studied **(Fig. 3.1)**. B cells express ABHD16a (Immgen.org), which deacetylates intracellular PS to form lysoPS (58,59), and this enzyme may account for some or all of the in vitro lysoPS production. Further studies using ABHD16a knockout mice (59) or a small molecule ABHD16a inhibitor (59) will address this question. We also found that B cells downregulate GPR174 during 4 days of in vitro proliferation, in contrast to CD4 T cells, which maintain receptor expression. These findings may

in part explain differences in B and T cell sensitivity to lysoPS in culture, and more work is needed to understand the dynamics of lymphocyte lysoPS production and receptor expression during immune responses.

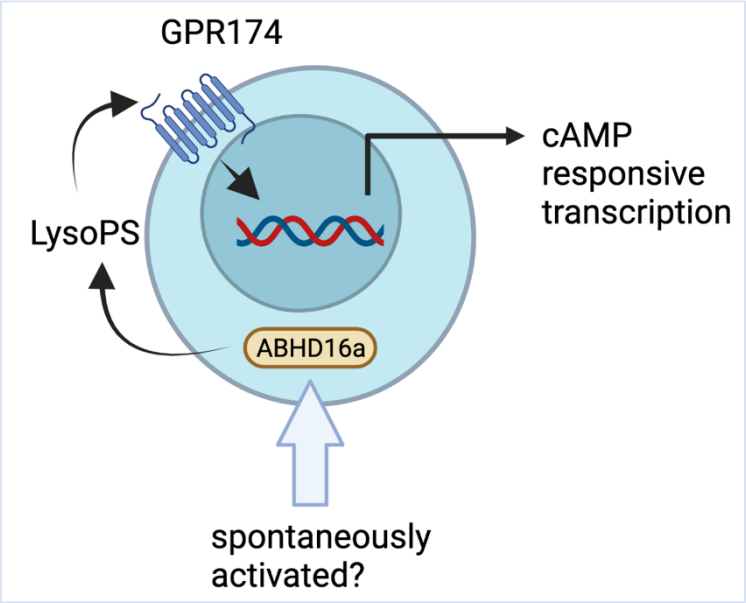

Figure 3.1. Model diagram of in vitro lysoPS production and GPR174 activation, suggesting Abhd16a-dependent lysoPS production and GPR174-responsive gene transcription.

GPR174- and Gαs-dependent gene induction occurs in vivo in response to injected lysoPS, and leads to increase surface expression of CD86, CCR7, and NUR77 within 4 hours. LC/MS-MS analyses have demonstrated that lysoPS is present across tissues, but it is not known whether this lysoPS is intracellular or extracellular. We found that freshly isolated B cells had a low level of lysoPS, and it may be that this amount is not sufficient to activate GPR174. It will be interesting in future work to determine conditions under which B cells produce elevated lysoPS in vivo.

PS is preferentially located on the inner leaflet of the plasma membrane in healthy cells (67). The secreted enzyme PS-PLA$_1$ can generate lysoPS from PS (56,57). Further work

elucidating the ability of PS-PLA$_1$ to regulate extracellular lysoPS in lymphoid organs is needed. Increased expression of PS-PLA$_1$ is associated with autoimmune disease (51,76), as are SNPs in GPR174 (48,49,51,52), and it will be important to understand lysoPS production under both homeostatic and disease contexts.

RNAseq analysis of GPR174- and Gαs-deficient follicular B cells revealed few expression differences compared to control B cells immediately ex vivo. However, following 4 hours of in vitro culture, control B cells upregulated more than 1000 genes. GPR174- and Gαs-deficient B cells upregulated far fewer genes, and many of these were not unique compared to control cells. This study supports GPR174 as the predominant GPCR expressed on B cells which drives spontaneous in vitro B cell activation. The majority of these gene expression changes seemed likely to be directly induced by GPR174 signaling as RNAseq analysis of B cells cultured for one hour showed many of the same expression changes. Top DEGs which increased in control B cells compared to GPR174- and Gαs-deficient cells included *Cd86*, *Ccr7*, and *Nr4a1*, all of which increased in B cells by in vivo surface expression (or reporter expression, in the case of *Nr4a1*/NUR77) following lysoPS injection. It remains to be determined whether the rest of the in vitro gene expression program is also regulated by GPR174 in vivo.

CCR7 is an important receptor in dictating lymphocyte migration, and binds the ligands CCL19 and CCL21 (128–130). A recent study described a GPR174-dependent response to CCL19 and CCL21 in B cells (47). In this study, B cell activation promoted association of GPR174 with Gαi proteins in a sex-dependent manner in vitro (47). The phenotypes described in our study were sex-independent and Gαs-dependent. Moreover, we were unable to detect

binding of CCL19 to GPR174. Further investigation will be necessary in understanding these discrepancies. We provide evidence that, under certain conditions in vitro and in vivo, GPR174 can signal via Gαs to augment CCR7 expression **(Fig. 3.2)**.

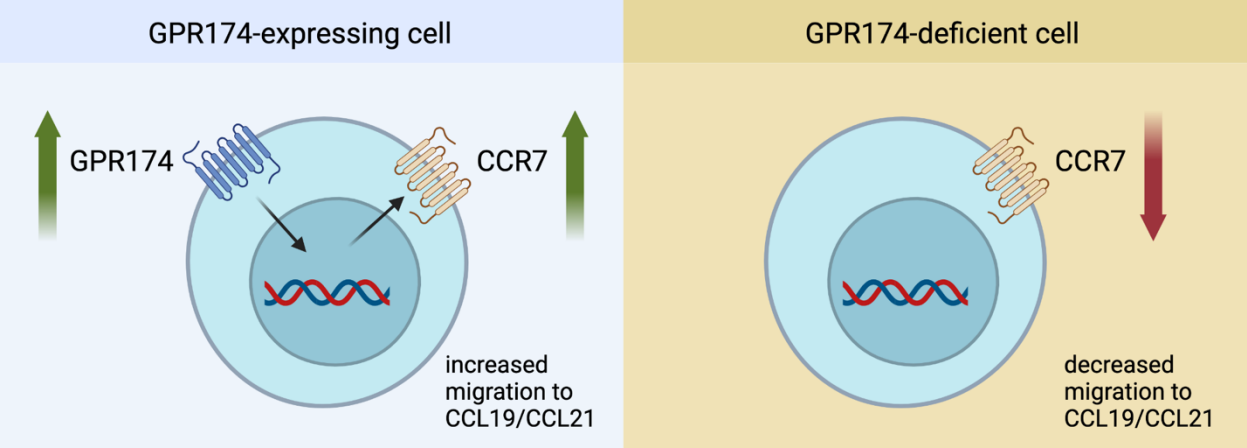

Figure 3.2. Model of GPR174-mediated augmentation of CCR7 function in B cells showing increased CCR7 expression downstream of GPR174.

NUR77 is described in multiple cell types as a Gαs- and cAMP-responsive gene (18,22,23). NUR77 is also induced in response to BCR and TCR engagement (24). Using intracellular staining, we found that NUR77 is spontaneously upregulated in a Gαs-dependent manner. NUR77 levels are low in follicular B cells due to rapid turnover, but NUR77-GFP transgenic mice show high levels of GFP due to reporter stability (24,98,99). We found that GPR174-deficient mice display lower levels of the NUR77-GFP reporter than control mice ex vivo, and the NUR77-GFP reporter is induced in vitro in a GPR174-dependent manner. While we found no GPR174-dependent effect on tolerance in V_H3H9 model, in which NUR77 has been shown to play a role in peripheral tolerance (25), we did observe a GPR174-dependent effect on the marginal zone size, which is expanded in V_H3H9 mice. Upon further investigation in GPR174-deficient non-transgenic mice, we observed a slight but significant reduction in marginal zone B cells, which was reproducible in Gαs-deficient mice. Interestingly, another

group found that GPR174-deficiency led to an increased marginal zone size (125). The marginal zone compartment can be influenced by the microbiome (126,127), which varies between mouse facilities. Microbial influences on GPR174 function may prove important in further understanding the complexities of this receptor.

In conclusion, we demonstrate that GPR174 can couple to Gαs to influence B cell responses in short term culture. In vitro B cell cultures are widely used to study aspects of the immune response, and we provide evidence that manipulating this axis may augment the study of B cell responses. In vivo, ligand engagement of GPR174 appears capable of inducing a similar pathway and we propose that the receptor helps tune B cell responses based on the tissue microenvironment and state of inflammation. SNPs in GPR174 are associated with Graves and Addison's disease, and altered activity of the receptor in B cells may contribute to development of autoimmune disease.

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