UNIVERSITY OF CALIFORNIA SAN DIEGO

G-Protein-Coupled Receptor GPR65 Potentially Reduces Innate Lymphoid Cell-Related Type 2 Inflammation in Airway and Lung

A thesis submitted in partial satisfaction of the requirements
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by

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Professor Gen-Sheng Feng

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The Thesis of Yifei Zhou is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

I would like to dedicate this work to my parents and family. I am extremely grateful for your always support and unconditional love.

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This thesis is coauthored with Doherty, Taylor A. The thesis author was the primary author of this paper.

ABSTRACT OF THE THESIS

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by

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Group 2 innate lymphoid cells (ILC2s) promote asthma by releasing type 2 cytokines that promote allergic-type inflammation. As a subset of innate lymphoid cells (ILCs), ILC2s are activated by alarmin cytokines released by airway epithelial cells when exposed to an allergen. Previous studies examining the role of G-protein-coupled

receptor (GPR65) during an inflammatory response have identified its role as an activator of intracellular signaling pathways. However, the role of GPR65 on ILC2 function in ILC2-driven type 2 lung inflammation is unknown. To determine the role of GPR65 in an allergic asthma response, GPR65 knock-out (GPR65-/-) mice and Wild Type (WT) mice were challenged with *Alternaria* to observe the differences in lung inflammation and ILCs by flow cytometry and the differences in type 2 cytokines by enzyme-linked immunosorbent assay (ELISA). Our data shows that GPR65-/- mice have trending increases in airway and lung inflammation, proliferation of ILCs, and airway type 2 cytokines compared to WT mice. These preliminary findings suggest that GPR65 may suppress the activation of ILCs after exposure to *Alternaria*. Therefore, GPR65 may serve a protective role in type 2 lung inflammation and allergic airway disease.

INTRODUCTION

Asthma is a chronic respiratory disease with eosinophilic inflammatory obstruction in the airway, mainly caused by excess mucus production, airway narrowing, or airway wall remodeling (Lambrecht &Hammad, 2015). Asthma is a heterogeneous syndrome, but most patients have increased eosinophils in the airway that are induced by T helper 2 (Th2) cells and type 2 innate lymphoid (ILC2) cells.

Innate lymphoid cells (ILCs) are considered the innate counterparts of T lymphocytes that lack the antigen receptors for T cells and B cells (Artis & Spits, 2015). While T cells produce cytokines in response to a highly specific antigen, ILCs produce cytokines in response to alarmin molecules that are released from epithelial cells upon exposure to an allergen stimulus (Hammad & Lambrecht, 2021). Depending on the cytokines produced, ILCs can be categorized into three main subsets (Vivier et al., 2018). Interferon-gamma (IFN-γ) is produced by ILC1s; type 2 cytokines interleukin 4 (IL-4), IL-5, and IL-13 are produced by ILC2s; and Th17 cytokines IL-17 and IL-22 are produced by ILC3s (Vivier et al., 2018). These cytokines are small proteins that have individual targeted effects on inflammation (Vivier et al., 2018). ILC2s are the most common subset of ILC cells within the mouse lung, despite all subsets of ILCs being tissue-resident cells that can be found throughout the body (Mindt et al., 2018). Upon challenge of irritants and allergens, ILC2s rapidly and robustly respond to produce an inflammatory response and play key roles as a tissue-resident cell in the lung (Gasteiger et al., 2015).

Allergic asthma is hallmarked by airway hyperresponsiveness and mucus production and can be characterized by increased eosinophils, a common phenotype

referred to as type 2 inflammation (Lambrecht & Hammad, 2015). Upon exposure in the airway, allergens stimulate the epithelial cells to release alarmin molecules IL-33, IL-25, and thymic stromal lymphopoietin (TSLP), which activate ILC2s to produce the type 2 cytokines IL-4, IL-5, and IL-13 (Divekar & Kita, 2015; Vivier et al., 2018). Among them, IL-5 and IL-13 lead to the type 2 asthma phenotype by induction of eosinophils, mucus production, and airway hyperresponsiveness (Lambrecht et al., 2009; Kim et al., 2010; Klein Wolterink et al., 2012).

During the allergic response, the airway becomes acidic in part due to the accumulation of eosinophils (Kottyan et al., 2009). The eosinophils can then induce the release of lactate and protons, promoting an acidic environment (Lardner, 2001), which can change the airway pH from 7.7 to 5.2 (Hunt et al., 2000). The G-protein-coupled receptor called GPR65 is a pH-sensitive, proton-sensing receptor that is known to stimulate intracellular signaling pathways and is expressed on immune and lymphoid cells (Okajima, 2013). When tissue pH drops below 7.2, GPR65 is internalized and activates the intracellular signaling pathway to form cAMP (Ishii et al., 2005). Curiously, in a GWAS study, SNPs in GPR65 were linked to a more severe asthma phenotype with Chronic Obstructive Pulmonary Disease (COPD) overlap (Hardin et al., 2014). However, despite several separate studies examining the acidic lung environment and the functions of GPR65, the role of GPR65 regulation in asthma is largely unexplored.

Our lab has found that GPR65 was highly expressed on mouse lung ILCs using RNA-seq studies. We also found that GPR65 is expressed higher on human lung ILC2s compared with ILC1s and ILC3s for both an asthmatic and non-asthmatic donor.

Furthermore, the GPR65 level decreased on mouse ILCs after the allergen challenge, indicating a potential role for GPR65 during an asthmatic response. Considering the function of GPRs as an activator of intracellular signaling pathways, our data showing a reduction in GPR65 expression upon allergen challenge suggests that GPR65 may play an inhibitory and protective role during an innate immune response.

In general, long-term steroid therapy can effectively relieve asthma symptoms by inhibiting airway inflammation and bronchoconstriction; however, steroids have no significant effect on severe asthmatics (Nabe, 2020). It has been found that ILC2s develop corticosteroid resistance with alarmin stimulation (Kabata et al., 2013). Around 5% to 10% of patients are resistant to steroids (Holgate & Polosa, 2006), but these asthma patients account for almost 50% of all asthma healthcare (Licari et al., 2018). Since severe asthma patients represent much of the morbidity and high cost of the disease and often cannot be treated effectively with steroids, research on ILC2 regulation could provide insight into novel alternative treatments for asthma (Ray et al., 2016).

In this study, we utilized the fungal allergen *Alternaria alternata (Alternaria)* to induce an allergic asthma response in mice. We challenged GPR65 knock-out (GPR65-/-) mice and Wild Type (WT) mice to determine the role of GPR65 in an allergic asthma model and examined the mice for differences in phenotype, inflammation, cytokine production, and cell proliferation. Here we show that GPR65 may play a protective role in innate type inflammation by suppressing eosinophilia, ILC2 proliferation, and production of type 2 cytokines IL5 and IL13.

RESULTS

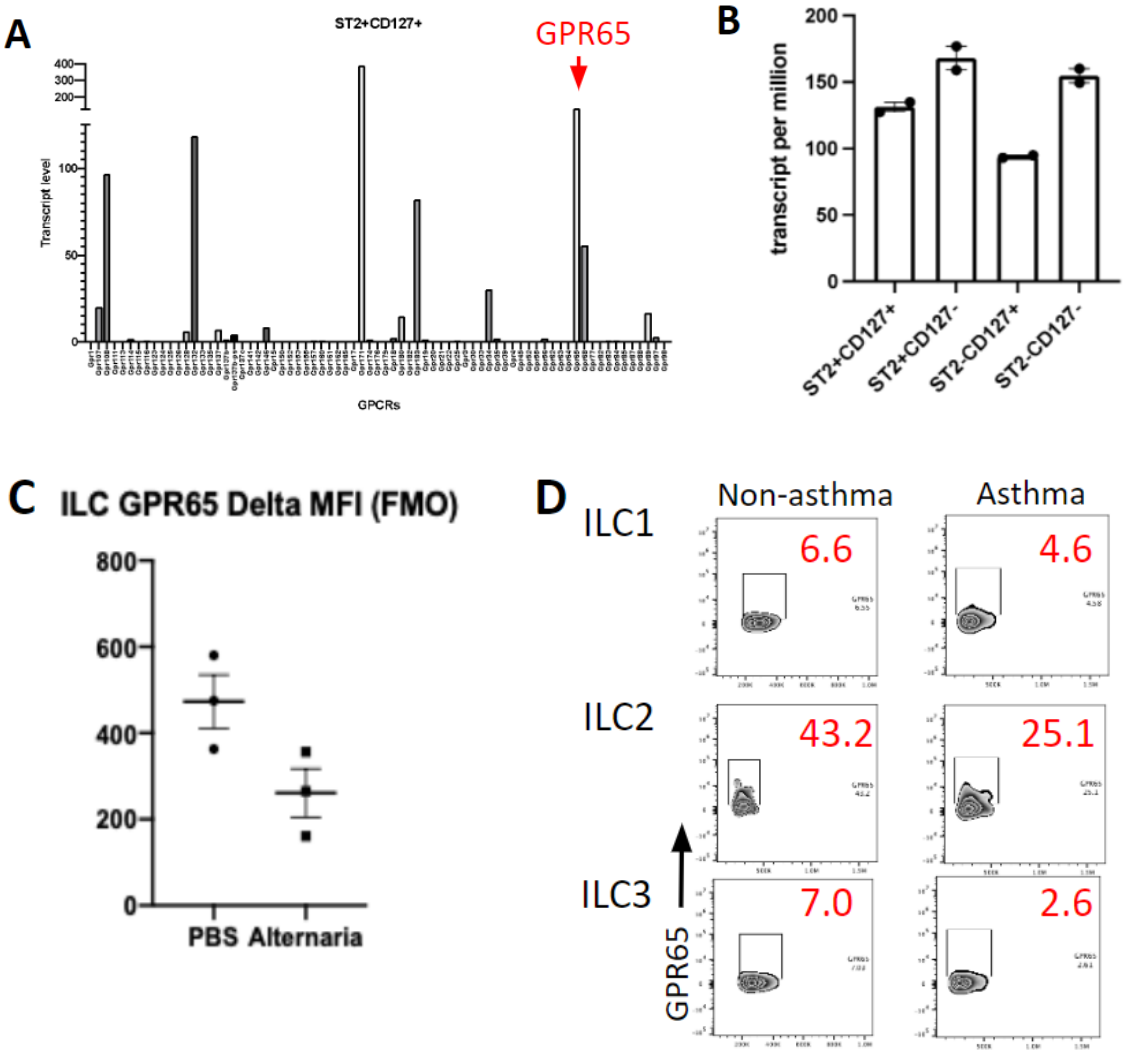

Figure 1. GPR65 is highly expressed in mouse and human lung ILCs. Transcript levels in mouse lung ILC2s (1a) and transcript per million (1b) were detected by RNA-sequencing of mouse ILC subsets based on CD127 and ST2. Mean fluorescence intensity of ILC2 GPR65 protein expression in mice after allergen challenge (1c). FACS plots of percent GPR65 on ILCs from human cadaveric non-asthmatic and asthmatic lungs (1d).

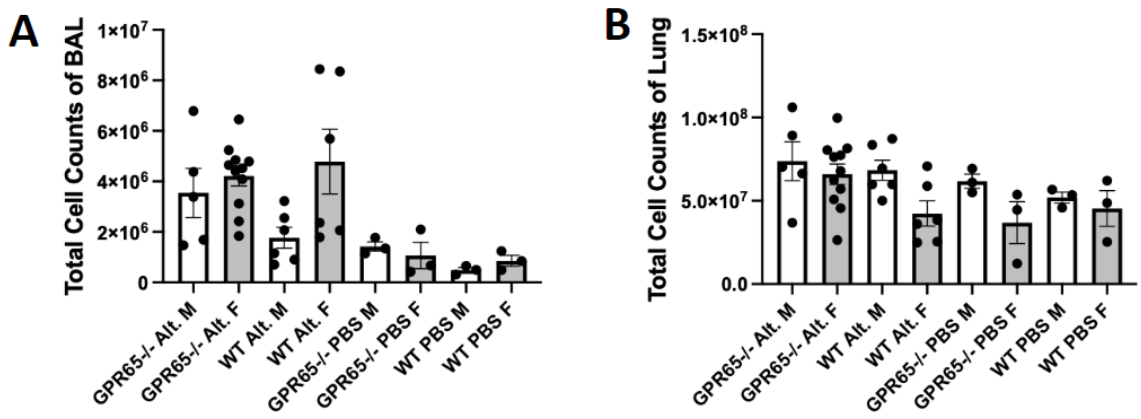

Figure 2. GPR65 potentially plays a protective role in innate lung inflammation in female mice. GPR65 knock-out (GPR65-/-) mice and Wild Type (WT) mice were challenged with 10µg *Alternaria* (Alt.) or PBS three times over 7 days, of which PBS-challenged mice were the control group. Data was collected by 4 independent experiments with n=40 in total. Total cell counts were calculated from bronchoalveolar lavage (BAL) fluid (a) and the whole lung (b). The error bars represent the standard error. *p<0.05, **p<0.01, ***p<0.001, Kruskal-Wallis test.

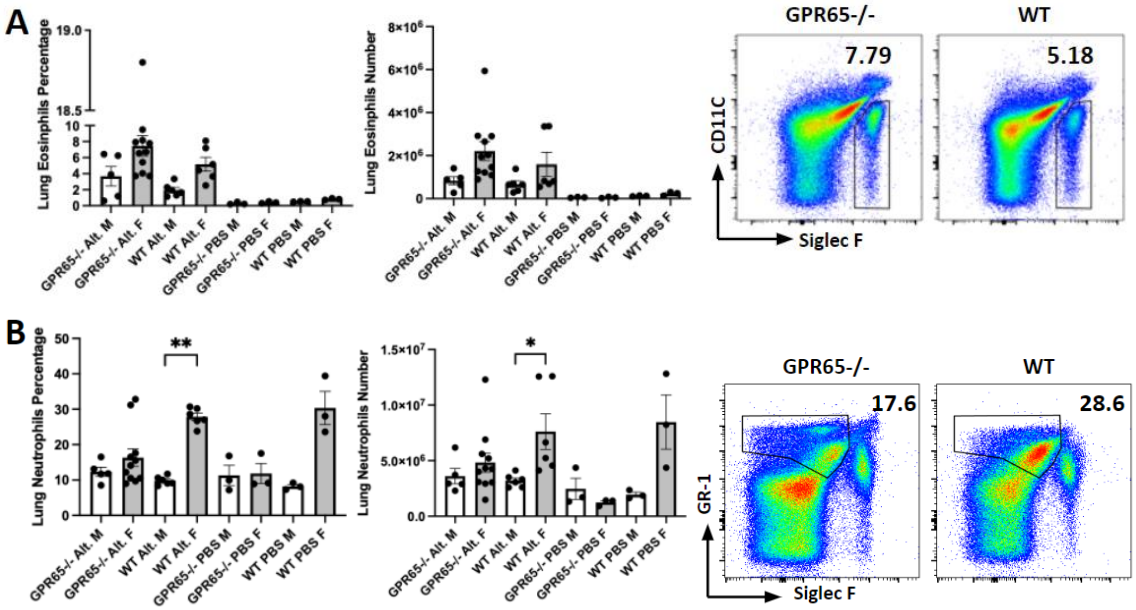

Figure 3. GPR65 may suppress airway and lung eosinophilic responses in *Alternaria*-challenged female mice. GPR65 knock-out (GPR65-/-) mice and Wild Type (WT) mice were challenged with 10µg *Alternaria* (Alt.) or PBS three times over 7 days, of which PBS-challenged mice were the control group. Data was collected by 4 independent experiments with n=40 in total. (a) Lung eosinophils percentage, number, and representative FACS plots. (b) Lung neutrophils percentage, number, and representative FACS plots. The error bars represent the standard error. *p<0.05, **p<0.01, ***p<0.001, Kruskal-Wallis test.

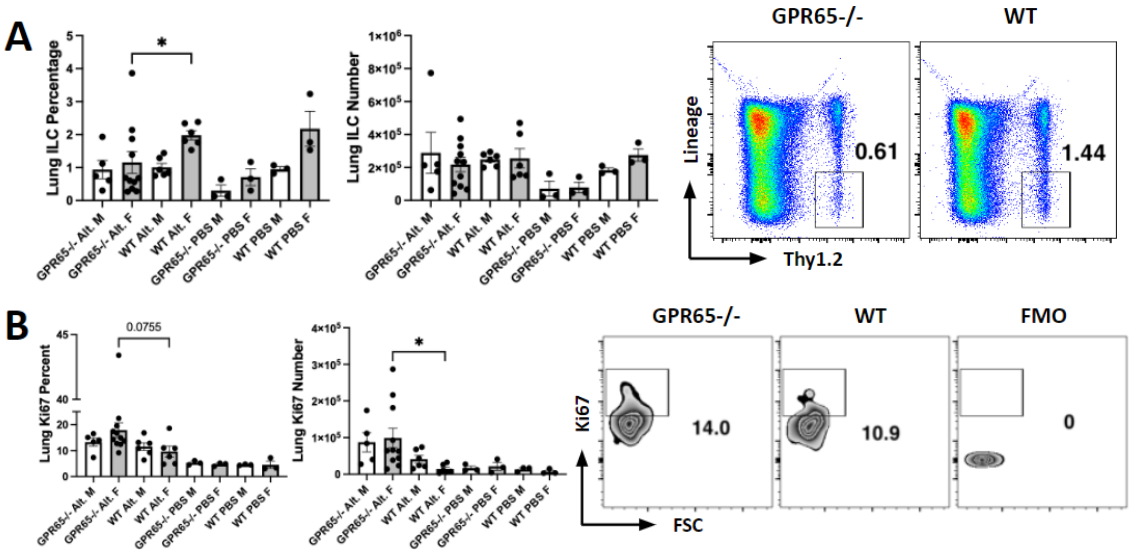

Figure 4. Female GPR65-/- mice have increased proliferating ILCs. GPR65 knock-out (GPR65-/-) mice and Wild Type (WT) mice were challenged with 10µg Alternaria (Alt.) or PBS three times over 7 days, of which PBS-challenged mice were the control group. Data was collected by 4 independent experiments with n=40 in total. (a) Lineage-Thy1.2+ ILC percentage, number, and representative FACS plots. (b) Ki67-producing ILCs percentage, number, and representative FACS plots. Ki67+ gating based on FMO control. The error bars represent the standard error. *p<0.05, **p<0.01, ***p<0.001, Kruskal-Wallis test.

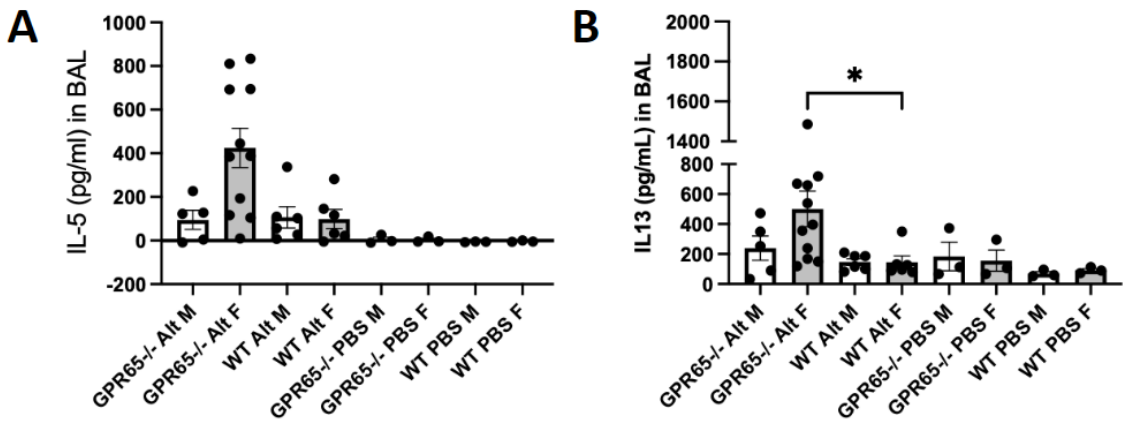

Figure 5. GPR65 may suppress the production of type 2 cytokines IL5 and IL13 from airway in female mice. GPR65 knock-out (GPR65-/-) mice and Wild Type (WT) mice were challenged with 10µg *Alternaria* (Alt.) or PBS three times over 7 days, of which PBS-challenged mice were the control group. Data was collected by 4 independent experiments with n=40 in total. Type 2 cytokines IL-5 (a) and IL-13 (b) were detected in bronchoalveolar lavage (BAL) fluid via enzyme-linked immunosorbent assay (ELISA). The error bars represent the standard error. *p<0.05, **p<0.01, ***p<0.001, Kruskal-Wallis test.

GPR65 is highly expressed in mouse and human lung ILCs

WT mice were challenged intranasally with *Alternaria* 3 times over 7 days. Lungs were harvested into single-cell suspensions and cells were sorted to obtain a highly pure population of ILC subsets, separated based on the expression of ST2 and CD127. The ILC subsets then underwent RNA sequencing (RNA seq). Based on the RNA seq, GPR65 showed high RNA expression levels on ST2+CD127+ ILC2s (**Figure 1a**). Further examination of all of the ILC subsets showed high mRNA GPR65 transcript levels (**Figure 1b**). PBS-challenged (control) mice showed higher protein expression of GPR65 on ILCs compared to *Alternaria*-challenged mice (**Figure 1c**). Furthermore, we examined the expression of GPR65 on human lung ILCs. Non-asthmatic human lung ILCs showed increased GPR65 expression compared to asthmatic lung (**Figure 1d**). ILC2s in particular have higher GPR65 expression compared to ILC1 and ILC3 (**Figure 1d**). This suggests that GPR65 is highly expressed on human and mouse ILC2s at both RNA and protein levels.

GPR65 potentially plays a protective role in innate lung inflammation in female mice

To determine the influence of GPR65 on total cell number during inflammation, GPR65-/- mice and WT mice were challenged with 10μg *Alternaria* challenge 3 times over 7 days to model an asthma environment. Bronchoalveolar lavage (BAL) fluid total cells were modestly decreased in female GPR65-/- mice versus female WT mice (**Figure 2a**). However, total lung cells were increased in female GPR65-/- mice compared to female WT mice (**Figure 2b**). This suggests that GPR65 potentially plays

a protective role in innate lung inflammation by preventing an increase in the overall number of cells that enter the lung, though the potential differences between genders are currently not resolved.

GPR65 may suppress airway and lung eosinophilic responses in *Alternaria*-challenged female mice

To determine the influence of GPR65 on airway and lung inflammation, GPR65-/- mice and WT mice were challenged with 10μg of *Alternaria*, 3 times over 7 days. Eosinophils and neutrophils were analyzed as endpoints for airway and lung inflammation by collecting the whole lung. Total BAL (in males) and lung cells (in females) showed a modest increase in GPR65-/- mice (**Figure 2**). There was no significant difference in lung eosinophils percentage and number between *Alternaria* challenged GPR65-/- mice and WT mice (**Figure 3a**). However, there was a trend that GPR65-/- female mice had increased lung eosinophils compared to WT mice (**Figure 3a**). Conversely, GPR65-/- mice had decreased lung neutrophil percentage and number compared to WT mice in trends, and WT female mice had significantly increased lung neutrophil percentage and number compared to male mice (**Figure 3b**). This suggests that the population of eosinophils during type 2 inflammation could be suppressed by GPR65 upon the *Alternaria* challenge. Paired with the increasing number and percent of lung neutrophils, this may be indicative that GPR65 shifts inflammation from an eosinophilic inflammatory response toward a neutrophilic inflammatory response.

Female GPR65-/- mice have increased proliferating ILCs

To determine the influence of GPR65 on the ILCs population, GPR65-/- mice and WT mice were challenged with 10μg *Alternaria* challenge 3 times over 7 days and the lungs were harvested and analyzed for changes in the ILC population. GPR65-/- female mice had significantly decreased ILCs percentage compared to the WT female mice upon *Alternaria* challenge (**Figure 4a**). GPR65-/- female mice had marginally increased Ki67-producing ILCs in percentage and significantly increased Ki67-producing ILCs in number compared to the WT female mice upon the *Alternaria* challenge (**Figure 4b**). As a proliferation marker for ILCs, we expected that the total ILC population would be increased but we found no difference in total ILC numbers between groups. It is possible that another unidentified mechanism such as increased apoptosis (which we did not analyze) or activation-induced death in GPR65-/- mice may account for this.

GPR65 may suppress the production of type 2 cytokines IL5 and IL13 from airway in female mice

To determine the function of GPR65 on ILCs, GPR65-/- mice and WT mice were challenged with 10μg *Alternaria* 3 times over 7 days to model an asthma environment. We assessed ILC function by analyzing the production of ILC2s effector cytokines, IL5 and IL13. We collected the BAL fluid and performed an enzyme-linked immunosorbent assay (ELISA) to assess IL5 and IL13 levels in the airway. GPR65 suppressed the production of type 2 cytokine IL5 in clear trends in female *Alternaria*-

challenged mice (**Figure 5a**). GPR65 also significantly suppressed the production of type 2 cytokine IL13 in female *Alternaria*-challenged mice (**Figure 5b**). This suggests that the GPR65 may play a role in suppressing the production of the type 2 cytokines IL5 and IL13.

DISCUSSION

Here, our results demonstrate that GPR65 potentially plays a protective role during an *Alternaria* challenge. In response to the *Alternaria* challenge, GPR65 had decreased expression on mouse ILCs which parallels the lower GPR65 expression observed on asthmatic human ILCs. In challenged GPR65-/- mice, there was an increase in the eosinophil and the inflammatory response. Furthermore, considering their role in eosinophil recruitment, the increases in the type 2 cytokines IL5 and IL13 in GPR65-/- mice coupled with observed increases in eosinophils support the claim of a potentially protective role for GPR65. Since IL5 and IL13 are produced by ILC2s (Vivier et al., 2018), ILC2s may be suppressed by GPR65. Together, our data suggest that GPR65 may help prevent lung inflammation during *Alternaria* challenge.

While these experiments examined both male and female WT and GPR65-/- mice, it is known that the immune response is more robust in females compared to males (Klein & Flanagan, 2016). Moreover, there was some variability within each group making it difficult to completely conclude that GPR65 had a sex-dependent response. The GPR65-/- mice were somewhat difficult to breed and we used all available mice for these experiments. The difficulty of having enough mice of the same age and going through the experiment protocol simultaneously was considered a possible cause of the variability. Overall, the trends were most noticeable in female mice, which is interesting and may be related to overall increased ILC2 activation in female mice that can be suppressed by testosterone in males (Cephus et al., 2017).

Airway and lung inflammatory responses in *Alternaria*-challenged mice are potentially suppressed by GPR65 (**Figure 3a & 3b**). One possible mechanism by which

GPR65 can regulate an inflammatory response is through eosinophils. However, a previous report showed that eosinophils express GPR65 as an acid-sensing receptor and GPR65 helped increase the survivability of eosinophils (Kottyan et al., 2009). Thus, we would not expect that the increase in eosinophils in GPR65-/- female mice is an intrinsic effect of GPR65 in eosinophils but could be due to upstream ILC2 activation which we have to fully characterize. Another study found that in a mouse colitis model, the GPR65-/- mice had aggravated intestinal inflammation (Marie et al., 2022). This study supports our results as when we challenged GPR65-/- mice, inflammation appeared slightly suppressed in the lung.

When examining the proliferation of ILCs in Figure 4, we were surprised to find that despite increased Ki67 expression in ILCs in GPR65-/-, the overall total ILC population was not increased compared to WT mice. In a paper that examined T-cell activation, they found that apoptotic signals were induced when there was over-activation and proliferation of T cells due to antigenic stimulation (Zheng et al., 2017). The apoptosis balanced T cell proliferation and contraction during the immune response to prevent excessive accumulation of immune cells (Zheng et al., 2017). Therefore, we attribute the decreased total numbers of ILCs (**Figure 4a**) but increased proliferation of ILCs (**Figure 4b**) in GPR65-/- mice compared to WT mice to activation-induced apoptosis. In future studies, we will assess the apoptosis of ILC2s in these mice.

The production of type 2 cytokines IL-5 and IL-13 were shown to be suppressed by GPR65 (**Figure 5a & 5b**) by comparing with GPR65-/- mice and WT mice. Considering it is widely accepted that ILC2s produce IL-5 and IL-13, future

experiments will examine differences in cytokine production from ILC2s directly to help reinforce our findings of GPR65 here.

Overall, we identified the phenotype that GPR65 may suppress type 2 cytokine production from airway and may specifically regulate eosinophilic inflammation in lung. The protective role of GPR65 may prevent tissue damage during immune inflammation activated by allergen assault.

This thesis is coauthored with Doherty, Taylor A. The thesis author was the primary author of this paper.

MATERIALS AND METHODS

Mice

Male and female mice were C57BL/6J mice aged 9 to 14 weeks old. WT mice were obtained from Jackson Laboratory (Bar Harbor, ME). GPR65-/- mice were obtained via material transfer agreement from Li Yang (East Carolina University, Greenville, NC) and bred in-house. All mice experiments were approved by the University of California, San Diego Institutional Animal Care and Use Committee.

In Vivo Lung Inflammation Models

Alternaria alternata extract (Greer, Lenoir, NC) was used to challenge mice by intranasal injection three times every 72 hours over 7 days. 10µg *Alternaria* (lot number 387094) diluted in PBS was used to challenge mice to model the allergic asthma environment in mice; PBS was used to challenge mice as a control group to model non-allergic environment in mice.

BAL and Lung Processing

2% bovine serum albumin (BSA) (Sigma, St Louis, MO) was used to collect Bronchoalveolar lavage (BAL) fluid. Supernatant from the first 500µL BAL draw was stored for ELISA assay at -20°C. The whole lung was collected and the Miltenyi Lung Digest Kit and Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) were used to digest the lungs per the company's protocol. Novocyte flow cytometer (ACEA, San Diego, CA) was used to count cells based on size and granularity in preparation for further analysis.

ELISA

All BAL ELISA samples were stored at -20°C. IL-5 and IL-13 ELISA kits and R&D protocols (R&D Systems, Minneapolis, MN) were used to perform ELISA assays. Microplate reader Bio-Rad model 680 (Bio-Rad Laboratories, Hercules, CA) was used to read ELISA plates.

RNA Extraction

ILC RNA was extracted using RNA Easy Plus Micro kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. Before RNA-seq library preparation, samples were quantified as described previously (Seumois et al., 2014; Seumois et al., 2012) and quality of RNA was assessed by Fragment Analyzer (Advance Analytical, Santa Clara, CA). All samples had an RNA integrity number greater than 7.5.

RNA Sequencing

Purified total RNA (≈5 ng) was amplified following the Smart-seq2 protocol. In short, mRNA was isolated by using poly-dT oligos and was then reverse-transcribed into full-length cDNA via the template-switching oligo (Picelli et al., 2014; Rosales et al., 2018). cDNA was amplified by PCR for 15 cycles and purified by AMPure XP magnetic bead (0.9:1 (vol:vol)) ratio, Beckman Coulter, Brea, CA). Then, a standard NextEra XT sequencing library (NextEra XT DNA library prep kit and index kits; Illumina, San Diego, CA) was prepared with 1 ng of cDNA from each sample. Barcoded Illumina sequencing libraries (NextEra; Illumina) were generated using an

automated platform (Biomek FXP, Beckman Coulter). Whole-transcriptome amplification and sequencing library preparations in a 96-well format were used to reduce the variability caused by assay-to-assay. The optimal number of PCR preamplification cycles, fragment library size, and quality control steps were implemented to determine the quality and quantity of RNA. mm10 (mouse genome) was used to as the reference genome. After passing quality control, all samples were pooled at equimolar concentration and then loaded and sequenced on the Illumina Sequencing platforms (HiSeq2500, NovaSeq6000, Illumina). Sequenced libraries were used to obtain more than 10 million 50-bp reads mapping uniquely to mRNA mm10 reference.

Flow Cytometry

One-million lung cells were used to detect eosinophils, neutrophils, and ILCs via surface staining. Two-million lung cells were used to detect Ki67 via nuclear staining. CD16/32 (Biolegend, San Diego, CA) were used to block Fc receptors for 5 minutes. CD45.2 (BV650), Siglec-F (PE), GR-1 (APC), and CD11c (BV785) were used to stain eosinophils and neutrophils. Eosinophils were gated by CD45.2+CD11c-Siglec F+ and neutrophils were gated by CD45.2+Siglec F-GR1+. CD45.2 (BV650), Thy1.2 (eFluor 450), lineage cocktail (Biotin), and Streptavidin (BV785) were used to stain ILCs. ILCs were gate by Lineage-Thy1.2+ lymphocytes. The lineage cocktail included markers from Biolegend (Lin, CD11c, NK1.1, CD5, FceR1a, TcRβ, TcR$\gamma\delta$). Nuclear staining cells first went through cell surface staining as described above, and were then permeabilized using the FoxP3 kit (ThermoFisher, Waltham, MA). Finally,

the cells were then stained with Ki-67 (PE). All cells were run through the Novocyte flow cytometer (ACEA) and FlowJo (FlowJo, La Jolla, CA) was used to analyze the data.

Statistical Analysis

GraphPad PRISM Software (GraphPad Software, La Jolla, CA) was used to perform statistical analysis on flow cytometry and ELISA data. P-values were acquired by Kruskal-Wallis unpaired t test, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

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