PI3Kδ Inhibition Prevents IL33, ILC2s and Inflammatory Eosinophils in Persistent Airway Inflammation

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Abstract

Background: Phosphoinositide-3-kinase-delta (PI3Kδ) inhibition is a promising therapeutic approach for inflammatory conditions due to its role in leucocyte proliferation, migration and activation. However, the effect of PI3Kδ inhibition on group-2-innate lymphoid cells (ILC2s) and inflammatory eosinophils remains unknown. Using a murine model exhibiting persistent airway inflammation we sought to understand the effect of PI3Kδ inhibition, montelukast and anti-IL5 antibody treatment on IL33 expression, group-2-innate lymphoid cells, inflammatory eosinophils, and goblet cell metaplasia.

Results: Mice were sensitised to house dust mite and after allowing inflammation to resolve, were re-challenged with house dust mite to re-initiate airway inflammation. ILC2s were found to persist in the airways following house dust mite sensitisation and after re-challenge their numbers increased further along with accumulation of inflammatory eosinophils. In contrast to montelukast or anti-IL5 antibody treatment, PI3Kδ inhibition ablated IL33 expression and prevented group-2-innate lymphoid cell accumulation. Only PI3Kδ inhibition and IL5 neutralization reduced the infiltration of inflammatory eosinophils. Moreover, PI3Kδ inhibition reduced goblet cell metaplasia.

Conclusions: Hence, we show that PI3Kδ inhibition dampens allergic inflammatory responses by ablating key cell types and cytokines involved in T-helper-2-driven inflammatory responses.

Background

Inflammation is maintained due to presence of specific cells responsible for the rapid production of cytokines. For example, group 2 innate lymphoid (ILC2) cells have been identified as key promoters of eosinophilic persistence in allergic airway inflammation [1]. Inflammatory conditions observed in asthma can be recapitulated in murine models of house dust mite (HDM)-induced persistent pulmonary inflammation [2]. The complex composition of HDM enables interaction with structural and immune cells present in the lung [3, 4]. Due to these inherent properties and the fact that HDM is a clinically important allergen, it is believed the development of murine models utilising HDM as the allergic insult are clinically relevant [5].

Despite much research in the asthma field, inhaled corticosteroids (ICS) have remained a mainstay of anti-inflammatory treatment. However, the symptoms of some patients persist even though high doses of ICS are prescribed [6] and there is a need for non-steroidal or novel approaches in these patient groups. Montelukast, a cysteinyl leukotriene D4 receptor antagonist, is also used as an anti-inflammatory treatment for asthma [7, 8]. However, clinical evidence demonstrates that its effectiveness may be more asthma endotype dependent [9, 10] and indeed montelukast may be more appropriate for atopic paediatric and exercise-induced asthma sub-populations [11]. Phosphatidylinositol 3-kinase delta (PI3Kδ) inhibitors have been reported as potential novel non-steroidal anti-inflammatory agents for pulmonary inflammatory disorders [12-15]. However, to date clinical studies of PI3Kδ in asthma have not produced anticipated results. For example, the PI3Kδ inhibitor nemiralisib did not significantly reduce FEV1 after 28
days of treatment in adults with uncontrolled asthma. However, the levels of pro-inflammatory cytokines including IL5, and IL13 measured in the sputum of patients treated with nemiralisib were reduced when compared to the placebo group [16]. These observations support the potential PI3Kδ inhibitors as anti-inflammatories and further studies are required in order to define the right patient population and clinical setting. While the effect of PI3Kδ inhibitors is well described on TH2-driven airway inflammation [17], currently no data exists on the impact of PI3Kδ inhibition on group 2 innate lymphoid (ILC2) cells. In order to address this question, we evaluated the effect of PI3Kδ inhibition on IL33 induction, accumulation of ILC2 cells, migration of inflammatory (Siglec-F$^{hi}$) eosinophils and initiation of goblet cell metaplasia. These were compared to the effects of montelukast and anti-IL5 antibody treatment.

In the experiments presented here, mice were sensitised to HDM via the topical route to establish a persistent inflammatory profile in the airways. We found that ILC2 cells remained in the airways after a period of resolution. Re-exposure to HDM at a later time-point caused a rapid cytokine induction, reinitiated pulmonary inflammation and revealed infiltration of an inflammatory sub-population of eosinophil along with enhanced IL33 expression and goblet cell metaplasia. Using this model, we demonstrate that PI3Kδ inhibition but not leukotriene receptor antagonism reduces the expression of IL33 along with infiltration of ILC2 cells into the airways. In addition, we observed inhibition of inflammatory (Siglec-F$^{hi}$) eosinophil infiltration and goblet cell metaplasia.

**Results**

3 weeks of repeated topical HDM sensitisation resulted in persistent pulmonary allergic inflammation and cells involved in the allergic response remained in the lung following a period of resolution

The challenge and sensitisation protocol used in the current study (Figure 1) resulted in establishment of an eosinophilic, neutrophilic and lymphocytic infiltration into the airways of mice and a significant induction of pro-inflammatory cytokines (p≤0.001 in all cases compared to saline exposed mice, Figure 2A and B). Cessation of HDM dosing caused resolution of inflammation, except for macrophages, which persisted and increased over time. In addition, lymphocytes and eosinophils were present in significant numbers in the BAL of HDM sensitised mice at the end of the resolution period compared to mice exposed to saline (p<0.05 compared to saline controls in all cases (Figure 2C). Further investigation of lung tissue cells in HDM sensitised mice at the end of the resolution period revealed a sustained presence of ILC2, TH2, TH17, T-regs, B cells, dendritic cells and monocytes (p<0.05 compared to naïve mice, Figure 2D and E).

**Re-challenge to HDM induced rapid cytokine and chemokine production followed by infiltration of myeloid and lymphoid subsets into the lung tissue**

BAL and serum cytokine responses using 100µg of HDM re-challenge (Figure 3A) peaked between 2 and 4 hours post HDM re-challenge except for BAL IL5 which peaked at 24 hours (Figure 3B and C). HDM re-challenge (of HDM sensitised mice) evoked a rapid neutrophil response in the BAL, peaking at 6 hours.
The numbers in the BAL stabilised between 24 hours and 7 days post re-challenge but remained above saline challenged levels (Figure 3D). Macrophage numbers in BAL peaked at 3 days post HDM re-challenge and returned to saline levels by day 7 (Figure 3D). Eosinophil and lymphocyte numbers in BAL followed a similar time-course of infiltration into BAL post HDM re-challenge, both apparent in significant numbers at 2 days compared to saline controls (p<0.01, Figure 3D). Infiltrating lung inflammatory eosinophils (Siglec-F\(^{hi}\)) peaked at day 3 post re-challenge and were present in significant numbers from 24 hours after re-challenge. These cells were differentiated from resident populations based on Siglec-F expression (Figure 3E and F). Lymphocyte subsets in BAL (ILC2, B and CD4\(^+\)Treg cells) peaked at day 3 post HDM re-challenge except for TH2 cells which were present in significant numbers at day 7 (p<0.01 at day 7, Figure 4A and Figure E1 in the online method supplement). In lung, lymphocyte numbers peaked at day 3 post HDM re-challenge (ILC2 and TH2 cells) or persisted in elevated numbers (B and T regulatory cells) compared to saline challenged controls where numbers of these cells continued to resolve (Figure 4B and Figure E1 in the online method supplement). Sustained presence of CD19\(^+\) B cells in the lung was associated with a significant increase of HDM-specific IgE at day 7 (p<0.05, Figure 4C).

Inhibition of PI3K\(\delta\) but not antagonism of the leukotriene D4 receptor significantly reduced both cytokine response and infiltration into the airways of ILC2 and inflammatory eosinophils post HDM re-challenge

Inhibition of PI3K\(\delta\) using PI-3065 caused dose-dependent reductions in infiltration of CD4\(^+\) T helper, CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T regulatory and CD19\(^+\) B cells into the airways of mice re-challenged with HDM (p<0.001 at 100mg/kg PI-3065, Figure 5A). Moreover, treatment with PI-3065 dose-dependently inhibited a range of BAL cytokines that are important in TH2 cell and eosinophil recruitment and antibody class switching in B cells (p<0.01 in all cases at 100mg/kg PI-3065, Figure 5B). In line with the inhibition of cytokines, PI-3065 treatment resulted in dose-dependent reductions in recruitment of eosinophils into the lung in particular the Siglec-F\(^{hi}\) expressing inflammatory eosinophil sub-population, without significantly affecting numbers of lung resident eosinophils (Figure E2 in the online method supplement). TH2 and ILC2 cell recruitment was also diminished (p<0.001 at 100mg/kg PI-3065 in both cases, Figure 5C). Treatment with montelukast did not exhibit significant inhibition in recruitment of any of the inflammatory cell types nor induction of cytokines in the BAL (p>0.05 in all cases, Figure 5B). As expected, and previously published, neutralisation of IL5 resulted in significant inhibition of eosinophil migration, including inflammatory eosinophils (p<0.001 compared to IgG1 isotype control, Figure 5C). However, anti-IL5 antibody treatment did not inhibit either TH2 or ILC2 cells (p>0.05 when compared to IgG1 isotype control, Figure 5C).

PI3K\(\delta\) inhibition but not montelukast or anti-IL5 antibody treatment resulted in reduced IL33 expression and improvement of goblet cell metaplasia

IL33 immunoreactivity was confined to Type II pneumocytes. HDM re-challenge resulted in significant increase in both the number of positive cells and the intensity of their immunoreactivity (p<0.01 compared to saline controls, Figure 6A and B). PI3K\(\delta\) inhibition, but not montelukast or anti-IL5 antibody treatment caused a statistically significant decrease in IL33 immunoreactivity (p<0.001, Figure 6A and B).
Alcian Blue/ Periodic Acid Schiff (AB/PAS) staining showed a statistically significant increase in the number of goblet cells in the proximal bronchioles of HDM re-challenged mice compared to saline controls (p<0.01, Figure 6C and D). PI3Kδ inhibition, but not Montelukast or anti-IL5 antibody treatment resulted in a significant reduction in the number of goblet cells present in the airways (p<0.05, Figure 6C and D).

**Discussion**

We demonstrate that PI3Kδ inhibition, but not leukotriene D4 receptor antagonism or IL5 neutralisation, inhibits the expression of IL33 and accumulation of ILC2 cells in the airways (Figure 7). Migration of inflammatory eosinophils was attenuated by both PI3Kδ inhibition and IL5 neutralisation but not by montelukast. Moreover, airway goblet cell metaplasia was ablated by PI3Kδ inhibition but not by montelukast or anti-IL5 antibody treatment.

Modelling the pulmonary allergic inflammatory process in animals can be challenging and appropriate animal models reflecting all aspects of human disease do not exist [5, 18]. To assess the impact of novel therapeutic approaches and to benchmark established therapies, we sought to develop a murine system which modelled the processes of airway sensitisation, resolution and re-exposure to allergen as observed in asthma patients using a clinically relevant allergen.

We found that sensitisation by repeated airway exposure using HDM, results in persistent pulmonary allergic inflammation without the need for systemic adjuvants, as has been reported previously by Johnson and colleagues [2]. In addition, the mouse model reported in this paper employed a period where there was no HDM exposure to mimic resolution of the inflammatory portion of the airway sensitisation response. The profile of inflammatory cell types, which included ILC2 cells, in the airways of mice at the end of the resolution period was found to be similar to that observed in eosinophilic asthma patients [19, 20].

ILC2 cells are critical in rapidly mounting and maintaining TH2 type cellular responses to the airways and promote migration of dendritic cells to local draining lymph nodes [21]. They are found in increasing numbers in the airways of severe asthmatics [22] and upon activation they rapidly produce a range of TH2 cytokines such as IL2, IL4, IL5 and IL13 [23], even when high doses of oral corticosteroid are used [1]. In addition, ILC2 cells have been identified as key biomarkers of eosinophilic airway inflammation in asthma patients [24] and as cells that are responsible for exacerbations [25]. ILC2 cells are activated in the presence of epithelial damage-induced cytokines such as IL33 [23, 26-28], the expression of which is increased in asthmatics [28].

In the current mouse model, a single allergen re-challenge at the end of the resolution period resulted in a re-induction of TH2 cytokines, enhanced expression of IL33 in the tissue, amplified goblet cell metaplasia and increased accumulation of myeloid and lymphoid cells, including ILC2 cells, in the airways. Eosinophils in the tissue were sub-divided into two populations based on the expression of Sialic acid-binding immunoglobulin-type lectin-F (Siglec-F). A stable resident population of eosinophil that expressed
intermediate levels of Siglec-F (Siglec-F^int) and an infiltrating population, apparent only after allergen re-challenge expressing high levels of Siglec-F (Siglec-F^{hi}). Mesnil and colleagues in a murine model also using HDM [29, 30] showed that Siglec-F^{int} eosinophils were important for immune regulation and homeostasis and were differentiated from inflammatory eosinophils [29].

Using the current mouse model, we chose to study the effect of therapeutics on a pre-inflamed background prior to allergen re-challenge as would occur in allergic asthma patients. Given the position of ILC2 cells as initiators of the allergic inflammatory cascade, we sought to determine if our model could be used to determine the effect of current and future therapies on their function. Of the standard non-steroidal therapies used for asthma management, we did not find any reports documenting the direct inhibitory effect of anti-IL5 antibody treatment on the activation or accumulation of ILC2 cells in the airways. Our data clearly show that this treatment had no effect on the increase in ILC2 or TH2 cell numbers in the airways, hence we conclude that migration of both these cell types into inflamed airways was not dependent on IL5. However, anti-IL5 antibody treatment inhibited the migration of the Siglec-F^{hi} inflammatory eosinophils, a finding that is consistent with their reported reliance on IL5 for migration to the airways [29].

Montelukast, a cysteinyl leukotriene D4 receptor antagonist has been shown to inhibit IL4 production in isolated human ILC2 cells [31]. However, this is in contrast to our own findings, where in a complex in vivo setting, montelukast failed to exhibit significant inhibition of not only IL4 but also of other cytokines and chemokines involved in the allergic response. Moreover, montelukast did not inhibit ILC2 infiltration into inflamed airways, hence we conclude that migration of ILC2 cells to the airways in the current mouse model was not dependent on eicosanoids such as leukotriene D4. In addition, we did not observe any effect of montelukast dosing on the accumulation of Siglec-F^{hi} inflammatory eosinophils in the airways.

Our data demonstrates that the PI3Kδ inhibitor PI-3065 reduces the expression of IL2 and IL33, key mediators of ILC2 cell proliferation and activation [32, 33]. As a consequence, we observed a profound inhibition of ILC2 accumulation into inflamed airways with PI-3065 treatment. Overall, our data suggest a critical function of PI3Kδ which to our knowledge has not been reported. This function is in addition to the known roles of PI3Kδ in allergic asthma such as; leucocyte migration into inflamed tissues [34-36], release of asthma relevant cytokines (IL4 and IL5) [37] and chemokines (RANTES and eotaxin) [38]. Moreover, PI-3065 treatment reduced airway goblet cell metaplasia which was concomitant with reduction in IL13 levels, a cytokine that induces goblet cell metaplasia in the airways [39, 40]. We also report for the first time that PI3Kδ inhibition attenuated the migration of Siglec-F^{hi} inflammatory eosinophils into the airways post allergen re-challenge without effecting the lung resident and regulatory Siglec-F^{int} eosinophil population. Furthermore, PI-3065 reduced levels of RANTES (CCL5), another important mediator for the haematopoiesis, survival and chemotaxis of eosinophils to asthmatic airways [41].

In these studies, we have developed a murine model incorporating sensitisation, resolution and allergen re-challenge which allowed us to uncover the persistence of ILC2 cells in the airways of mice. Airway
accumulation of this cell type was found to be PI3Kδ dependent. Re-challenge to allergen after a period of inflammation resolution, uncovered an inflammatory population of eosinophils, the migration of which we found to be dependent on PI3Kδ. Key cytokines that activate ILC2 cells (IL33) or induce goblet metaplasia (IL13), were ablated by PI3Kδ inhibition and cytokines resulting from ILC2 activation were also dampened. To date PI3Kδ inhibitors tested in asthmatics have not met the primary endpoint of improving lung function in their clinical trials. This indicates a lack of translatability of our model with asthma, which is a heterogeneous disease resulting from a combination of multiple factors beyond allergens. Of notes, the levels of the type 2 pro-inflammatory cytokines including IL5, and IL13 measured in the sputum of patients treated with nemiralisib were reduced when compared to those treated with placebo [16]. This agrees with a critical role for PI3Kδ in type 2 inflammatory cells including ILC2s. Our data combined underscores the need to define the right allergic patient population and clinical setting in which ILC2s play a critical pathological role [42].

Conclusions

In this study, we develop a murine system which models the processes of airway sensitisation, resolution and re-exposure to allergen. In the model, we uncover the persistence of ILC2 cells in the airways of mice, which we show to be sensitive to PI3Kδ inhibition. Our results point towards a patient stratification approach based on elevated numbers of ILC2 cells in the targeted tissue. Such an innovative approach may lead to improved clinical outcomes for PI3Kδ inhibitors in refractory allergic and inflammatory conditions.

Methods

Animals

Female BALB/c mice, aged 6-8 weeks, weighing approximately 20g were purchased from Charles River UK Ltd. All animal related protocols were reviewed and approved by the Animal Welfare Ethical Review Body (AWERB) of GSK. All animal studies were ethically carried out in accordance with relevant guidelines and regulations including the Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Animals and in compliance with the ARRIVE guidelines. Mice were given food and water ad libitum. For all investigations, mice were sacrificed using an intra-peritoneal overdose of sodium pentobarbitone (Dolethal, 200mg/ml, Vetoquinol, UK).

HDM sensitisation, inflammation resolution and re-challenge protocols

For all intranasal (i.n.) administrations, mice were anaesthetised using 2% isofluorane in oxygen. HDM sensitisation was carried out by i.n. dosing once a day for 5 days a week over a 3 week period (Days 0-18) with either sterile saline or 25µg HDM extract (Greer Laboratories, Lenoir, NC, USA) in 50µl sterile saline. Pulmonary inflammation was then allowed to resolve until day 33. Inflammation was then re-initiated by an i.n. re-challenge of HDM using 100µg of HDM extract (100µg) in 50µl saline. Respective
groups of mice were sacrificed at the end of the sensitisation period (day 33) and following re-challenge at pre-determined time-points (0.5 hours – 7 days) to ascertain the cellular and cytokine composition in both BAL and lung tissue.

**Compound and antibody dosing protocols**

Mice were dosed with the PI3Kδ inhibitor PI-3065, montelukast or vehicle (sterile distilled water containing 0.5% Hydroxypropyl methylcellulose (Sigma-Aldrich, UK), 0.2% Tween-80 (Sigma-Aldrich, UK)) via oral gavage in a total volume of 0.2ml. This occurred once daily for 7 days starting on day 26 (7 days prior to re-challenge) and ending on the day of HDM re-challenge. Anti-IL5 neutralizing antibody (100µg/mouse, Clone number: TRFK-5, BD Biosciences, UK) or Rat IgG1 (Clone number: R3-34, BD Biosciences, UK) were dosed intra-peritoneally in 0.2ml sterile saline, 1 hour prior to HDM re-challenge. Please refer to the online method supplement Figure E3 for the PK profiles of both PI-3065 and montelukast.

**Sample collection, processing procedures and analysis/quantification of cell composition**

Please refer to supplementary methods section for full details of sample/tissue (blood, BAL and lung) collection and preparation for cytokine and cell composition analysis by flow cytometry.

**Analysis of cytokines and IgE**

Analysis of cytokines in serum and BAL supernatants was carried out as per manufacturer’s instructions using mouse Magnetic Luminex® assay kits (R&D Systems, UK) and mouse V-Plex, Pro-inflammatory Panel 1 kits (Meso Scale Discovery®, US). Serum IgE was analysed using ELISA (please refer to online supplement for more details).

**Histological analysis**

Please refer to online supplementary methods section for full details of tinctorial (AB/PAS) and immunohistochemistry methods for detection and quantification of goblet cell metaplasia and IL33 expression in lung tissue.

**Statistical analysis:**

Except where stated, data in all figures is presented as arithmetic means ± standard error of the mean (SEM). Numbers of mice in are indicated in the figure legends. Except where stated, statistical analysis was performed using GraphPad Prism 5 and where necessary data were log transformed and a two-tailed students t-test or one-way analysis of variance (ANOVA) with Dunnets P value adjustment was used to analyse the data for significance. P values of less than or equal to 0.05 were considered significant.

**Abbreviations**

PI3Kδ: Phosphoinositide-3-kinase-delta
IL2: Interleukin-2
IL4: Interleukin-4
IL5: Interleukin-5
IL10: Interleukin-10
IL13: Interleukin-13
IL33: Interleukin-33
KC: Keratinocyte chemoattractant protein
TNFα: Tumour Necrosis Factor alpha
ILC2: Group 2 Innate lymphoid cell
IgE: Immunoglobulin E
TH2: T helper type 2 cells
HDM: House Dust Mite
BAL: Broncho-Alveolar Lavage
RANTES: Regulated on Activation, Normal T Cell Expressed and Secreted

Declarations

Ethics approval and consent to participate

All animal studies were ethically reviewed and carried out in accordance with Animals (Scientific Procedures) Act 1986 and the GSK policy on the Care, Welfare and Treatment of Animals.

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and/or analysed during the current study are not publicly available due to privacy restrictions but are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests
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Author contributions


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