Shh production and Gli signaling is activated *in vivo* in lung, enhancing the Th2 response during a murine model of allergic asthma

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Summary: Hh/Gli signals are received by multiple pulmonary and immune cell types in response to allergen inhalation *in vivo*; this autocrine/paracrine activation enhances Th2 immune responses.

Running title: Hh/Gli signaling in allergic airways disease

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Abbreviations

AAD: Allergic airways disease

APC: Antigen presenting cell

BAL: Broncho-alveolar lavage

Dhh: Desert hedgehog

GFP: green fluorescent protein

Gli: Glioma-associated oncogene

GliA: transcriptional activator Gli

GliR: transcriptional repressor Gli

HDM: House dust mite

Hh: Hedgehog

Hhip: Hedgehog interacting protein

Ihh: Indian hedgehog

KO: knockout

LN: Lymph node

Ptch: Patched

Shh: Sonic hedgehog

Smo: Smoothened

Th2: T-helper 2

Wk: week/s

WT: wild type

Abstract

The pathophysiology of allergic asthma is driven by T-helper 2 (Th2) immune responses following aeroallergen inhalation. The mechanisms that initiate, potentiate and regulate airways allergy are incompletely characterized. We have previously shown that Hedgehog (Hh) signaling to T-cells, via downstream Gli transcription factors, enhances T-cell conversion to a Th2 phenotype. Here, we show for the first time that Gli-dependent transcription is activated in T-cells *in vivo* during murine allergic airways disease (AAD) a model for the immunopathology of asthma; and that genetic repression of Gli signaling in T-cells decreases the differentiation and/or recruitment of Th2 cells to the lung. We report that T-cells are not the only cells capable of expressing activated Gli during AAD. A substantial proportion of eosinophils and lung epithelial cells, both central mediators of the immunopathology of asthma, are also able to undergo Hh/Gli signaling. Finally, we show that Shh increases *Il4* expression in eosinophils. We therefore propose that Hh signaling during AAD is complex, involving multiple cell types, signaling in an auto- or paracrine fashion. Improved understanding of the role of this major morphogenetic pathway in asthma may give rise to new drug targets for this chronic condition.

Introduction

Asthma is a complex, heterogeneous respiratory disease characterized by airway inflammation, hyperresponsiveness, obstruction and remodeling. For many, asthma is a chronic, life-long condition controlled by inhaled corticosteroids, leukotriene antagonists, and short or long-acting bronchodilators. Approximately three people a day die as a result of asthma in the UK alone (Asthma UK Data Portal & Office for National Statistics). Asthma can be sub-divided into a series of variable phenotypes, categorized by the prominent cellular infiltrates and pathophysiology observed in the airway [1]. The majority of asthma is thought to be triggered by allergens (allergic asthma) and is characterized by eosinophilia and T-helper type 2 (Th2) immune responses. Allergic conditions are linked to epithelial barrier dysfunction, which potentiates immune responses to allergens, although the mechanisms behind this are not clear [2].

Allergic asthma results from misguided immune reactivity to aeroallergen, the most common of which are derived from house dust mite species (HDM) [3]. Sensitization is complex and is dependent on a series of genetic and environmental factors including extent of aeroallergen exposure, predisposition to atopy, composition of personal microbiota, exposure to pollutants and time spent indoors in early life [4]. Aeroallergens are ingested by mucosal antigen presenting cells (APC) and presented to T-cells in local lymph nodes (LN). T-cell activation and conversion to a Th2 phenotype triggers the production of IgE, subsequent activation of mast cells, eosinophils and the production of inflammatory mediators. Persistent allergen exposure, sustained immune infiltration and chronic inflammation result in excessive mucus production, smooth muscle contraction, airway remodeling and hyperresponsiveness, leading to the symptoms of asthma.

The mechanisms driving Th2 conversion and persistence in response to inhaled allergens are incompletely characterized. The heterogeneity of asthma phenotypes, disease course and treatment response implies that there are many factors that synergize to determine phenotype and increase overall risk of disease and/or treatment failure. It is therefore important to dissect the molecular mechanisms that contribute to the induction and persistence of Th2 immune pathology.

We have previously shown that Hedgehog (Hh) signaling to T-cells potentiates differentiation of naïve T-cells to a Th2 phenotype, and that Sonic Hedgehog (Shh) ligand is upregulated in the airway of mice with allergic airways disease, a murine model of asthma pathophysiology [5]. This work indicates that Shh is one such factor that can contribute to Th2-mediated responses. The relationship between Shh signaling and allergic asthma has not been explored in detail to date.

Hh proteins are inter-cellular signaling molecules that are vital for patterning during embryogenesis but also play a number of roles postnatally in tissue homeostasis [6]. There are three mammalian Hh proteins: Sonic Hh (Shh), Desert Hh (Dhh) and Indian Hh (Ihh), all of which share a common signaling pathway. Upon ligand binding to the Hh receptor Ptch, signal transduction is initiated by de-repression of Smo, which triggers an intracellular signaling cascade culminating in the activation of Gli transcription factors. Gli-dependent transcription of target genes is driven by the abundance of activated Gli (GliA) relative to transcriptional repressor Gli (GliR) in the cell. Gli1 acts as an activator of transcription, Gli2 as an activator and repressor, whereas Gli3 is predominantly a transcriptional repressor [7]. Target genes of Gli are wide-ranging and cell-context dependent. We have shown that in mature CD4+ T-cells, Gli target genes include Hh signaling molecules: *Ptch1* [8], *Smo* [5], Th2-modulators: *Il4*, *Il1rl1* [5], and genes linked to allergic disease: *Tgfb3* [9], *Igf1r* [10] and *Csf2* [11].

Several genes related to the Hh signaling pathway have been linked to asthma. Large genome-wide association studies (GWAS) have implicated *HHIP* [12], *PTCHD1* [13] and *PTCHD3* [14] in poorer asthmatic lung function. Gene expression analyses comparing Th2-high and Th2-low asthma phenotypes found differences in expression of *Dhh* and *Disp1* between groups [15]. These findings have been predominantly discussed in the context of developmental biology, the authors suggesting that the Hh pathway may be linked to lung function as a consequence of its role in lung branching morphogenesis.

We have shown that Hh signaling via Gli-dependent transcription potentiates the conversion of naïve T-cells to Th2 effectors by upregulation of Th2-related genes including the key cytokine IL-4 [5]. We therefore propose that the linkage between Hh signaling and asthma may be due to the influence of Hh on Th2 immune responses in addition to any structure-function effects driven by differences in morphogen signaling during lung development and postnatally in lung tissue homeostasis. Here, we investigate the relationship between Hh/Gli signaling and allergic immune responses *in vivo* using murine models of asthma pathology.

Materials and methods

Mice, tissues and cells

Lck-Gli2ΔC2 (C2, Gli2R [8]), GFP-Gli reporter mice (Gli binding site-GFP transgenic, GBS-GFP Tg [16]) and littermate/age-matched controls, all C57BL/6 background, C57BL/6 and BALB/c WT mice were bred and maintained at UCL under UK Home Office regulations. Allergic airways disease (AAD) was induced as described [5]. HDM allergen was given three times per week for the number of weeks stated. BAL, lung lobes and draining LN were harvested. Lung tissue was cryopreserved for sectioning or homogenization or minced and digested with 1.5mg/ml Liberase (Roche) and 0.5mg/ml DNAse (Roche), subject to erythrocyte lysis and prepared for flow cytometry, or subject to lysis for RNA extraction. To obtain SiglecF+ cells (eosinophils), cells were purified from pooled BALB/c lung and spleen leukocytes by Streptavidin-based magnetic bead positive selection (eBioscience) using anti-SiglecF-biotin (Miltenyi). Cells were then cultured at $2.5x10^5$/ml in AIMV media (LifeTechnologies) for 24h in the presence or absence of 500ng/ml recombinant Shh (R&D Systems) prior to lysis for RNA extraction.

Flow cytometry and cell sorting

Samples were stained using antibodies from eBioscience (UK), acquired on a C6 Accuri flow cytometer (BD) and analyzed using FlowJo v10 (Tree Star). For cell sorting, samples were acquired on a MoFlo XDP or FACSDiva (Beckman Coulter) and defined as follows: Epithelium: CD45-Epcam+; Endothelium: CD45-Epcam-CD31+; Eosinophils: SiglecF+CD11b+; neutrophils: CD11b+Ly6G(IA8)^{hi}; Alveolar macrophages: CD11b^{lo}CD11c+; CD4+ T-cells: CD4+CD11c-.

qPCR

qPCR was performed in triplicate for at least two independent experiments using Quantitect primers (Qiagen) on a Rotor-Gene thermal cycler (Qiagen) or a Biorad iCycler. Data were normalized to *Hprt* expression and are represented as relative mean expression±SD of independent experiments, the number of data points indicating the number of mice/samples analyzed.

Immunofluorescence

Immunofluorescence was performed on fresh frozen acetone fixed 5µm sections of OCT-embedded lung tissue. All antibodies were from eBioscience unless otherwise stated. To detect Shh: goat anti-Shh clone N19 (Santa Cruz), followed by donkey anti-goat biotin (Alpha Diagnostic) and streptavidin-Alexa Fluor 555 or anti-biotin Alexa Fluor 488; E-Cadherin: anti-E-cadherin followed by anti-rat IgG1 PE; SiglecF: anti-mouse SiglecF and anti-rat IgG2a eFluor570; CD16: anti-mouse CD16/32 followed by anti-rat IgG2a eFluor570; CD45: anti-mouse CD45.2-FITC. CD31: anti-mouse CD31-APC. Data were captured on an Olympus BX63 epifluorescent microscope or Zeiss LSM 710 confocal microscope and analyzed using cellSens (Olympus) and Image J (NIH) software. Magnifications are given as power of microscope of objective, where multiple magnifications are used in one figure scale bars are also included.

Lung histology

Lung lobes were formalin-fixed paraffin embedded (FFPE) samples. 5µm sections were subjected to Periodic Acid Schiff staining with hematoxylin counterstaining. Sections were assessed by a blinded observer and scored for cellular infiltration and PAS+ mucus production. Scores denote infiltration/mucus production as 0-1: minimal; 1-2: moderate; 2-3: severe.

Data analysis

Statistical analyses were performed using Microsoft Excel or Prism 4 (Graph Pad). Two-tailed unpaired Student's *t*-tests were used to assess statistical significance, which was accepted at $p<0.05$. All data are represented as mean±SEM, with the exception of qPCR data, which are displayed as stated above.

Results and Discussion

Shh expression increases in lung during AAD induction by allergen administration

We have previously shown that Shh signals to T-cells to favor a Th2 immune response and that Shh protein is increased in the lung tissue of mice after three weeks of allergen dosing [5]. To further understand when and where Hh ligand is expressed during AAD induction, we examined expression of Shh in lung during a time course of allergen administration. BALB/c mice were given three doses of HDM allergen per week for 0, 1, 2, 3 or 5 weeks. A progressive induction of a classical Th2 immune response was observed including the appearance of CD4+T1ST2+ (Th2) cells in BAL (Fig. S1A), lung (Fig. S1B) and eosinophilia (Fig. S1C). Concurrently, we observed a progressive increase in Shh mRNA (Fig. S1D) and protein (Fig. S1E) in lung tissue. Shh co-localized with E-cadherin+ airway epithelia (Fig. S1F), supporting our published observations. Further, as early as one week into the AAD time course we also observed Shh expression around pulmonary vessels (v) and in areas of cellular infiltration (i) around bronchi/bronchioles (Fig. S1F). Interestingly, Shh co-localised with a subset of peribronchiolar CD45+ cells (Fig. S1G), indicating that in addition to epithelia, cells of hematopoietic origin express Shh during AAD. We therefore show that there are several potential cellular sources of Shh, some of which are recruited to the disease site during AAD.

Lymphocytes respond to Gli-activating signals in vivo during AAD

To directly test our hypothesis that lymphocytes receive Hh/Gli-activating signals *in vivo* during AAD [5], we performed a time course allergen dosing experiment on GFP-Gli reporter mice [16] and WT (non-GFP) littermates. GFP-Gli+ mice are engineered to express GFP in cells that have received an activating signal upstream of the Gli transcription factors. GFP expression inside cells reflects the presence of GliA and therefore activation of Gli-dependent

transcription. To ensure that the reporter did not have any background effect on our experiments, we compared AAD immune parameters in GFP-Gli+ and GFP-Gli- littermates. Across a five week AAD time course there were no differences in immune cell recruitment between the two genotypes, indicating that the presence of the reporter construct does not itself have any influence on AAD progression (Fig. 1A).

Using GFP-Gli+ mice, we then examined expression of GFP in lymphocytes and CD4+ cells during an AAD time-course. GFP+CD4+ cells were observed in draining LN after only one week of allergen dosing with a further increase at three weeks (Fig. 1B, C). GFP+ cells were also evident in BAL (Fig. 1D, E) and lung (Fig. 1F, G), indicating that lymphocytes undergo Gli-dependent transcription during AAD. Upon allergen inhalation, the number of GFP+CD4+ cells increased in LN and particularly in BAL, which represents a major site of immune reactivity at the interface between the epithelium and the airspaces. These data suggest that there is a subset of T-cells *in vivo* that are able to respond to Hh or other Gli-activating signals and that these cells increase in number during AAD. Our previous work suggested that such cells would potentiate a Th2 response [5]. We therefore hypothesized that repression of Gli activity in T-cells would limit a Th2 response *in vivo*, and dampen allergic pathology.

Repression of Gli activity in T-cells impairs the recruitment of CD4+ and Th2 cells during AAD

To test the effect of repressing Gli activity in T-cells on AAD, we induced disease in transgenic mice that overexpress the repressor form of Gli2 (Gli2R) under the control of the Lck promoter (C2, Lck-Gli2R). Gli-dependent transcription is therefore repressed in these mice in T-cells only: C2 T-cells do not efficiently upregulate Gli target genes compared to WT T-cells upon receipt of a Hh signal [8]. C2 and WT littermates were given PBS or HDM

allergen for three weeks and the T-cell phenotype was examined. As predicted, there were significantly fewer CD4+T1ST2+ Th2 cells recruited to the lungs (Fig. 2A) and LN (Fig. 2B) of C2 compared to WT mice during AAD. This indicates that there is a specific effect of Gli2 repression on the differentiation and/or recruitment of CD4+ Th2 cells during AAD. It is particularly noteworthy that C2 tissues contain fewer CD4+T1ST2+ cells during AAD because these transgenic mice show enhanced selection of CD4+ single positive cells in the thymus, and an increased proportion and enhanced signaling/activation/proliferation potential of CD4+ cells in peripheral lymphoid tissues [8, 17].

Interestingly, we noted a significant decrease in the presence of neutrophils in the lung and BAL of C2 mice after allergen administration (Fig 2C). This implies that the repression of Gli activity in T-cells has a secondary effect on the recruitment, activation or differentiation of neutrophils in response to HDM inhalation. There are comparable neutrophil numbers in lung and BAL in the PBS treated C2 and WT groups (Fig 2C), suggesting that neutrophil development is not impaired in C2 mice. It therefore seems plausible that the presence of GliR in T-cells alters the production of a factor that controls neutrophil proliferation, mobilization or chemotaxis to the lung in response to inflammation/danger. Of interest, C2 CD4+ T-cells express lower levels of *Csf2* transcript than WT [5] – this gene encodes GM-CSF, a key cytokine for neutrophil recruitment and activation [18].

However, when we measured AAD pathology in the lungs of C2 and WT mice we noted no significant differences between the two genotypes. Despite the decrease in the number of Th2 cells, eosinophilia was not significantly different (Fig. 2D), Periodic Acid Schiff staining revealed comparable mucus production, and histological examination of lung infiltration suggested that overall, AAD pathology was not diminished in the lungs (Fig. 2E, F). Th2-related cytokines in whole lung homogenates were equivalently induced by HDM treatment in WT and C2 groups (Fig. 2G), despite the fact that T1ST2+ population was reduced in C2

mice. This suggests that other cells are responsible for the majority of IL4, IL5 and IL13 in lung. Indeed, Th2 cells are not the only source of these cytokines: among other cells, eosinophils, recruited in large numbers during AAD, also produce IL-4, IL-5 and IL-13 [19].

We therefore considered the possibility that there are other, as yet uncharacterized cells in the lung that are able to respond to Shh during AAD and influence Th2 responses.

Innate immune cells and lung epithelium respond to Gli-activating signals in vivo

To assess whether other cells involved in AAD are able to respond to Hh/Gli signaling, we screened for GFP expression and thus GliA during an AAD time course in GFP-Gli+ reporter mice. We examined sections of lung lobes to determine the location of GFP+ cells. We found no GFP expression in GFP-Gli- littermate lung samples (Fig. 3A), but GFP was highly expressed around lung structures including bronchial/bronchiolar and alveolar epithelium and diffusely throughout other areas of the lung during AAD in GFP-Gli+ lungs (Fig. 3B). We noted variable and graded expression of GFP throughout the tissue, implying that some cells expressed higher levels of Gli than others. This is consistent with graded signaling as would be expected with a morphogen and is detectable using this reporter, which has been previously used to model the dynamics of morphogen signaling in neural development [16]. It is currently unknown to what extent morphogen gradients function in adult tissue. Hh signaling in adult lung has promoted pro-mitogenic [20, 21] and anti-proliferative [22] effects in models of acute lung injury. These conflicting results could be due to differences in experimental models or may reflect functional differences in morphogen gradients in tissues.

Co-immunofluorescence analyses showed that GFP signal was strongest in E-cadherin+ bronchial cells and in peri-bronchial cells following 3wk allergen inhalation, particularly at the air-interface (Fig. 3C). Weaker GFP signal (seen on merged images as pink-yellow, depending on the strength of the GFP signal) also co-localized with a subset of peri-bronchial

SiglecF^{hi} cells, possibly those eosinophils about to egress into the airspaces (and therefore BAL), after HDM dosing (Fig. 3D). Co-localization of GFP was also observed with some CD16+ cells, thus we also detect Gli activity in a subset of phagocytic cells (Fig. 3E). We did not detect GFP expression in CD31+ cells at 3wk, indicating that pulmonary endothelium does not express active Gli in AAD at this time-point (Fig. 3F). These data indicate that lung epithelial cells, some eosinophils and phagocytes are all subject to Hh/Gli signaling during AAD.

We also quantified cells expressing GliA by flow cytometry in whole lung homogenates and in bronchial alveolar lavage (BAL). Interestingly, at 3 weeks the majority of eosinophils in BAL expressed GFP (Fig. 4A) as did a lower but still substantial proportion of neutrophils (Fig. 4B). GFP expression was sustained in these cells into the 5th week of allergen dosing (Fig. 4C). GFP expression was also observed in a proportion of lung eosinophils (Fig. 4D, E), in a minority of lung neutrophils and a subset of lung macrophages (Fig. 4F). These data indicate that innate immune cells, in particular a significant subset of eosinophils, are capable of responding to Hh or other Gli-activating signals during allergen inhalation. To our knowledge, this has not been previously reported. We noted a much higher frequency of GFP+ eosinophils in BAL and lung digests when we analyzed samples from our AAD experiments by flow cytometry (Fig. 4) compared to immunofluorescence (Fig. 3). This may be due to enhanced sensitivity of cytometry or to cytometric gating strategies and lung cryosections, which are extensively washed during staining, may simply not mimic the cellular content of intact whole lung digests. Alternatively, this may represent a real difference in Gli activation; BAL contains eosinophils that have egressed from pulmonary tissue, which may well have received Hh signals when passing through the epithelial barrier. Lung/BAL eosinophils are also functionally distinct during AAD, with inflammatory eosinophils located in BAL and peri-bronchial areas and regulatory eosinophils found largely

in lung parenchyma [23]. The function of eosinophils can thus be heavily influenced by their location in tissue presumably via responses to local microenvironmental signals; we propose that Shh/Gli signaling is one such local signal, active during allergic responses.

Lung epithelium, endothelium and innate immune cells, particularly eosinophils, express key molecules of the Hh signaling pathway

To confirm that lung tissue and innate immune cells express the machinery required for Hh/Gli signaling, we sorted epithelial and endothelial cells, eosinophils, neutrophils, macrophages and CD4+ T-cells from lung digests of WT mice and performed qPCR to assess gene expression. In control mice, epithelial cells expressed *Shh* (as in [5]), *Ihh*, *Hhip, Ptch, Smo* and *Gli1* indicating potential to both produce Hh ligand and receive Hh signals (Fig. 5A). Endothelial cells expressed negligible levels of *Shh* but did express Hh signaling machinery (Fig. 5A). Upon HDM inhalation, epithelial cells expressed all of the genes as above. Interestingly, compared to epithelial cells, endothelial cells expressed slightly higher levels of *Hhip, Ptch, Smo* and *Gli1* (Fig. 5B). This indicates that the Hh signaling capacity in the endothelial cells of the pulmonary vasculature may change from their baseline state following allergen exposure.

The respiratory epithelium is an important physical barrier and 'immune' organ, increasingly linked to the initiation and maintenance of unwanted allergic immune responses. House dust mite allergens including Derp1 protease disrupt epithelial tight junctions [24], permitting transepithelial passage of allergen and immune priming. Derp1 also stimulates the release of pro-inflammatory cytokines from respiratory epithelium [25], which are important in subsequent immune skewing. We show for the first time that lung epithelia also expresses Hh signaling machinery during AAD, suggesting that auto/paracrine Hh signaling is possible in this compartment following allergen inhalation. Embryogenic morphogens such as Shh are

re-expressed in adult lung to maintain epithelial and mesenchymal cell quiescence [22]. Dysregulation of this signaling is strongly implicated in pulmonary fibrosis in mouse models [20] and man [26]. It is plausible that Shh signaling in epithelial cells may contribute to the long-term fibrotic airway remodeling seen in chronic asthma. Hh signaling regulates the epithelial to mesenchymal transition (EMT) during lung development and branching morphogenesis. Asthma has been proposed as a disorder of dysregulated EMT, although this is controversial. Nevertheless, morphogenetic pathways appear to be good candidates for driving EMT during lung disease, including asthma (reviewed in [27]).

Naïve (Fig. 5C) and allergen exposed (Fig. 5D) eosinophils expressed *Ptch* and *Smo*, Hh receptor and signal transduction molecules respectively, and *Gli1* at higher levels than any of the other innate immune cells, and indeed higher than CD4+ T-cells. Interestingly, naïve eosinophils also expressed Hh ligands, *Shh* and *Dhh,* and *Hhip* (Fig. 5C). These cells are recruited to BAL (hundreds of thousands) and lung (millions) in abundance during the induction of AAD (Fig. S1C) and thus are likely to be a major source of Hh ligand. Surprisingly, following allergen treatment, *Ihh* expression was induced in eosinophils (Fig. 5D). There is little literature on this, although Ihh has a role in thymocyte development [28] and macrophage polarization [29]. It will be important to fully characterize this and the role of Hh signaling in other innate immune cells before Hh inhibitors are investigated as a potential therapy for Th2 mediated disease. Neutrophils and macrophages from HDM-exposed mice expressed Hh signal transduction molecules *Ptch* and *Smo*, but at lower levels than eosinophils (Fig. 5D). As in baseline conditions (Fig. 5C), neutrophils retained the ability to express Hh ligands. As neutrophil recruitment is impacted by repression of Gli-dependent transcription in T-cells (Fig. 2C) and that neutrophils are Gli responsive (Fig. 4), it will be very interesting to examine the role of Hh/Gli signaling in neutrophilic/Th17 asthma. Macrophages expressed *Shh* and *Ihh* but not *Dhh* after HDM inhalation (Fig. 5D). Shh/Gli

ligand expression and/or signaling has been previously reported in macrophages/monocytes [29-31]. There is evidence that macrophages upregulate both Shh and Ihh following exposure to schistosome antigens and that this can lead to M2 polarisation and a subsequent pro-fibrotic environment in the liver [29]. It remains to be seen whether Hh signaling in lung during AAD favors M2 differentiation and pulmonary fibrosis or airway remodeling.

HHIP is expressed in adult lung tissue including the lung epithelium (Fig. 5), pulmonary endothelium (Fig. 5) and eosinophils (Fig. 5). Several genome-wide association studies (GWAS) have repeatedly identified SNPs at the HHIP locus, the endogenous inhibitor of Hedgehog ligands, to affect lung function [32-34] and to predispose to increased susceptibility to asthma [12]. Patients with COPD have decreased HHIP mRNA and protein expression [35]. Functionally, two of the associated SNPs have been shown to be within a HHIP enhancer region which interacts with the HHIP promotor, and both SNPs reduce promotor activity [35]. Altered HHIP levels may well lead to enhanced Shh signaling, and we speculate perhaps consequently similar corollaries as we have reported here.

Shh activates Gli-dependent transcription via the canonical pathway - we have established that the components of the canonical hedgehog pathway are present in the lung tissue and immune cells. However we also acknowledge that Hh ligand can signal non–canonically and that Gli-dependent transcription can be triggered by non-Hh ligands, such as TGF beta [36]. The crosstalk of these signaling pathways in AAD and asthma therefore warrants further investigation.

Eosinophils, recruited to lung and BAL in high numbers in AAD, can respond to Hh signals by upregulating transcription of Il4

Our data suggest that eosinophils, key cells in the Th2 response, are able to produce Hh ligands and respond to Hh signals. To directly test the effect of Shh signaling to eosinophils,

we purified SiglecF+ cells from murine lung and spleen and cultured the cells in the presence or absence of recombinant mouse Shh. As in CD4+ T-cells [5], Shh treatment triggered upregulation of *Il4* in eosinophils (Fig. 5E). This suggests that Hh ligands, produced by various lung cell types during AAD can further potentiate Th2 cytokine production. This could explain why repression of Gli activity in T-cells only (Lck-Gli2R transgenic) did not significantly alter total IL-4 concentration in AAD lung tissue (Fig. 2C). However, the Smo inhibitor cyclopamine promotes eosinophilic differentiation of leukemic myeloid cells [37], thus it is currently unclear what role Hh signaling plays in eosinophil differentiation and/or function.

Our initial model proposing that Shh is released from lung epithelial cells following allergen inhalation therefore requires modification to include CD45+ innate leukocytes such as eosinophils and neutrophils (Fig. 6, white arrows). The model should also be extended to reflect the Hh/Gli signaling potential of eosinophil, CD16+ and epithelial cell subsets (Fig. 6, black arrows), and the influence of Shh on *Il4* transcription in eosinophils, potentially exacerbating the local Th2 response (Fig. 6, dashed arrow). The full consequences of Gli activation in each of the cell types examined here are currently unknown. In addition to its roles in maintaining quiescence and tissue homeostasis, postnatal re-expression of Shh in lung may therefore also have evolved as a beneficial Th2-potentiating mechanism to protect against lung parasites. Of note, Shh production is increased in astrocytes of mice infected with *Angiostrongylus cantonensis,* the rat lungworm [38]. However, in allergy, excess Shh in lung tissue is potentially pathological as it may be exacerbating Th2-driven disease. This requires further investigation in order to fully characterize the role of Hh signaling in the allergic lung, which is clearly complex. Of further interest are Group 2 innate lymphoid cells (ILC2), which lack antigen receptors but are early producers of Th2 cytokines in immune responses. These cells are particularly important in driving Th2 responses in the papain-

induced AAD model [39]. Interestingly, ILC2 development is regulated by morphogeneic pathways NOTCH and Wnt/β-catenin (reviewed in [40]), thus it is important to establish whether there is a role for Hh/Gli signaling in the differentiation/function of this cell type.

In summary, this study has uncovered a new role for Hh signaling in the cross-talk between pulmonary cells and infiltrating leukocytes during the induction of an allergic immune response. Future work should determine the role of canonical and non-canonical Hh/Gli signaling in eosinophils, neutrophils and airway epithelia in the presence and absence of allergens. The Hh/Gli signaling pathway is complex and subject to several regulatory and feedback mechanisms. It will be very interesting to dissect the kinetics of Hh/Gli signaling in these cell types during AAD. It is not currently clear in which cell type, if any, Hh signaling is absolutely required for AAD induction. It may well be the case that Hh signaling is another pathway that contributes to, rather than controls, disease pathogenesis. Together this information should uncover novel biological mechanisms and allow the rational design of potential modulators of Th2-driven lung disease.

Authorship

ASIS designed, performed and analyzed experiments and drafted the paper; DYM and RR performed experiments; TC contributed to the conception of the study, provided valuable insight and critically revised the paper; ALF conceived the study, designed, performed, analyzed and interpreted experiments and wrote the paper.

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Conflicts of interest statement

We declare no conflicts of interest.

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Figure legends

Figure 1: Lymphocytes respond to Hh signaling in vivo during AAD

GFP-Gli reporter mice (GFP-Gli+, GFP+) and WT (GFP-Gli-, GFP-) littermates (n=3 per group) underwent repeated intranasal challenge with PBS or 25µg HDM allergen in PBS. (A) Mean±SEM cell number in BAL, leukocytes in LN and lung digests (row across, all p-values n/s), in GFP-Gli+ groups (B, C) GFP status of CD4+ cells in LN, numbers on plots indicates %cells positive. Mean±SEM number of (D) GFP+ lymphocytes and (E) CD4+ cells in BAL and in (F, G) lung respectively. *p≤0.05; **p≤0.005, compared to the control group at t=0 wks.

Figure 2: Repression of Gli activation in T-cells lowers Th2 cell recruitment and neutrophilia in AAD

C2 (Gli2R) and WT littermates (n=5 or 6 per group) underwent intranasal challenge with PBS or 25µg HDM allergen in PBS, receiving nine doses over three weeks. Flow cytometric analysis of mean±SEM number of (A) CD4+T1ST2+ Th2 cells in lung digests, (B) in LN, (C) percentage of CD11b^{hi}Ly6G(IA8)+CD11c-SiglecF- neutrophils in live lung (grey bars) and BAL (white bars) leukocyte gates, and (D) number of eosinophils in BAL (top panel) and lung (lower panel). (E) Periodic Acid Schiff (PAS) and hematoxylin staining of FFPE lung sections, *indicates highly cellular immune infiltrates, magnification x20, representative examples are shown (F) operator-blinded scoring of cellular infiltration and mucus production in PAS/hematoxylin sections: 0-1 = minimal, 1-2 = moderate, 2-3 = severe. (G) ELISA analysis of cytokine concentration in whole lung homogenates. *p≤0.05; **p≤0.005, ng = negligible.

Figure 3: GFP-Gli is expressed in epithelial cells and subsets of innate leukocytes

(A) GFP expression in GFP-Gli- control and (B) in GFP-Gli+ lung cryosections, magnification x10. Immunofluorescence of GFP-Gli+ lung cryosections after 3wk allergen inhalation following co-staining with anti- (C) E-cadherin, (D) SiglecF, (E) CD16 and (F) CD31. (C-F) top panels, magnification x10; lower panels, magnification x100, scale bars are shown on last panels in each row. Co-localization in red/green merged images can be seen as yellow or paler pink depending on the intensity of green fluorescence.

Figure 4: Innate immune cells express can respond to Hh/Gli signals during AAD in vivo

GFP-Gli reporter mice (n=3 in each group) underwent repeated intranasal challenge with 25μg HDM allergen in PBS as described. Flow cytometry was used to analyze (A) eosinophils (B) neutrophils in BAL. Representative flow profiles are shown at 3 weeks, numbers indicate % of eosinophils, bold numbers indicate % of live gate. (C) Percentage and number per ml BAL of GFP+ eosinophils and neutrophils, (D) %GFP+ in lung CD4+ T-cell populations and number of GFP+ lung (E) eosinophils, (F) neutrophils and macrophages. *$p \leq 0.05$; **$p \leq 0.005$, compared to the control group at t=0 wks.

Figure 5: Innate immune cells, particularly eosinophils, express key molecules of the Hh signaling pathway

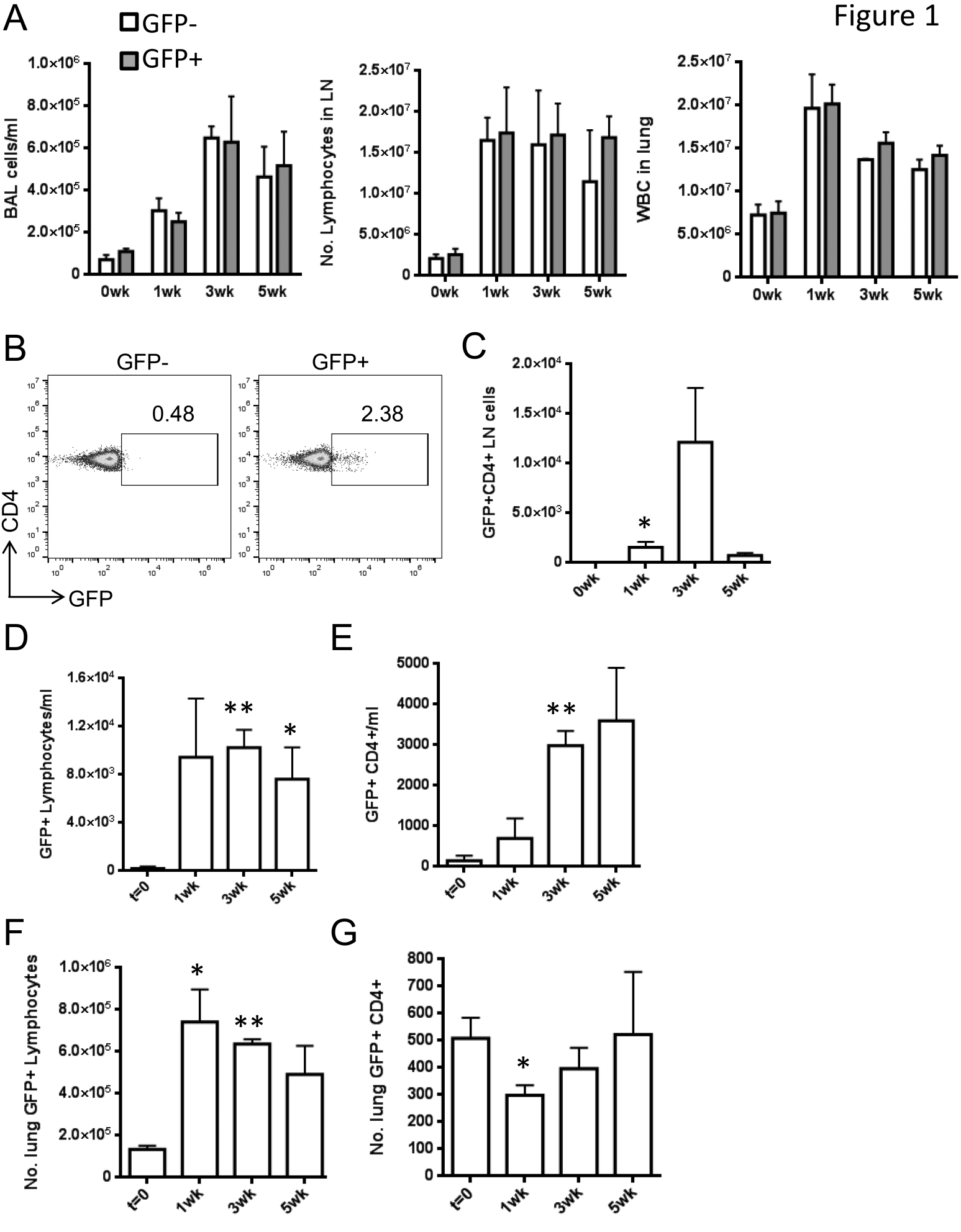
Lungs were harvested from (A, B) C57BL/6 (n=3 per group) or (C, D) Balb/c WT (n=2 per group) allergen naïve (A, C) or HDM-treated (B, D) mice and (A, B) lung epithelial and endothelial, or (C, D) resident lung eosinophils, neutrophils, macrophages and CD4+ T-cells were sorted by FACS as in Methods and subjected to gene expression analysis by qPCR. Expression of *Shh, Hhip, Ptch, Smo, Gli1, Ihh* and *Dhh* are expressed relative to *Hprt* in each

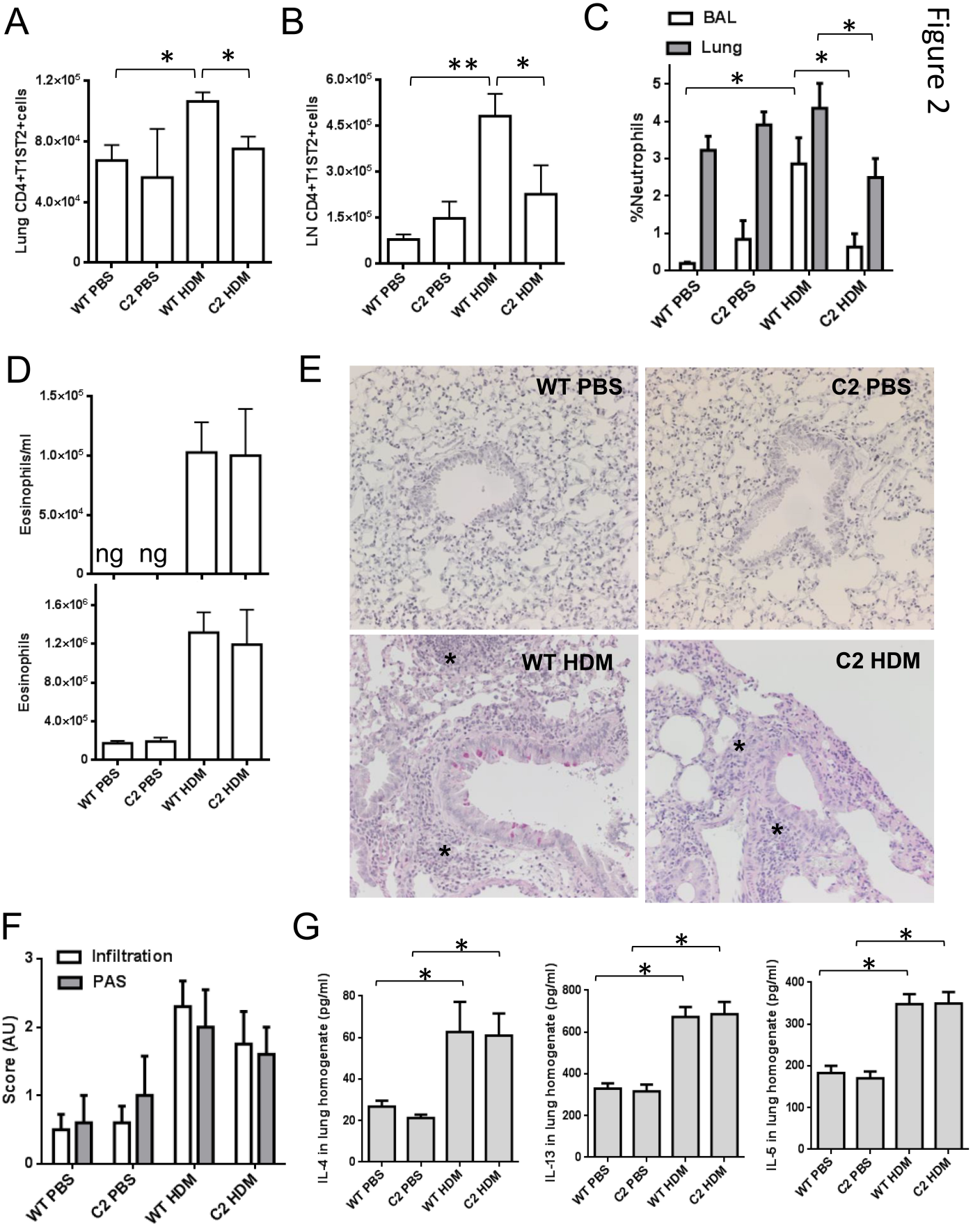
cell subset, data points represent individual mice. (E) SiglecF+ cells were separated from WT mice (n=2 independent experiments) and cultured for 24h in the presence (+Shh) or absence (control) of 500ng/ml recombinant mouse Shh protein. Expression of *Il4* in the treated group was assessed by qPCR in triplicate, relative to the control samples.

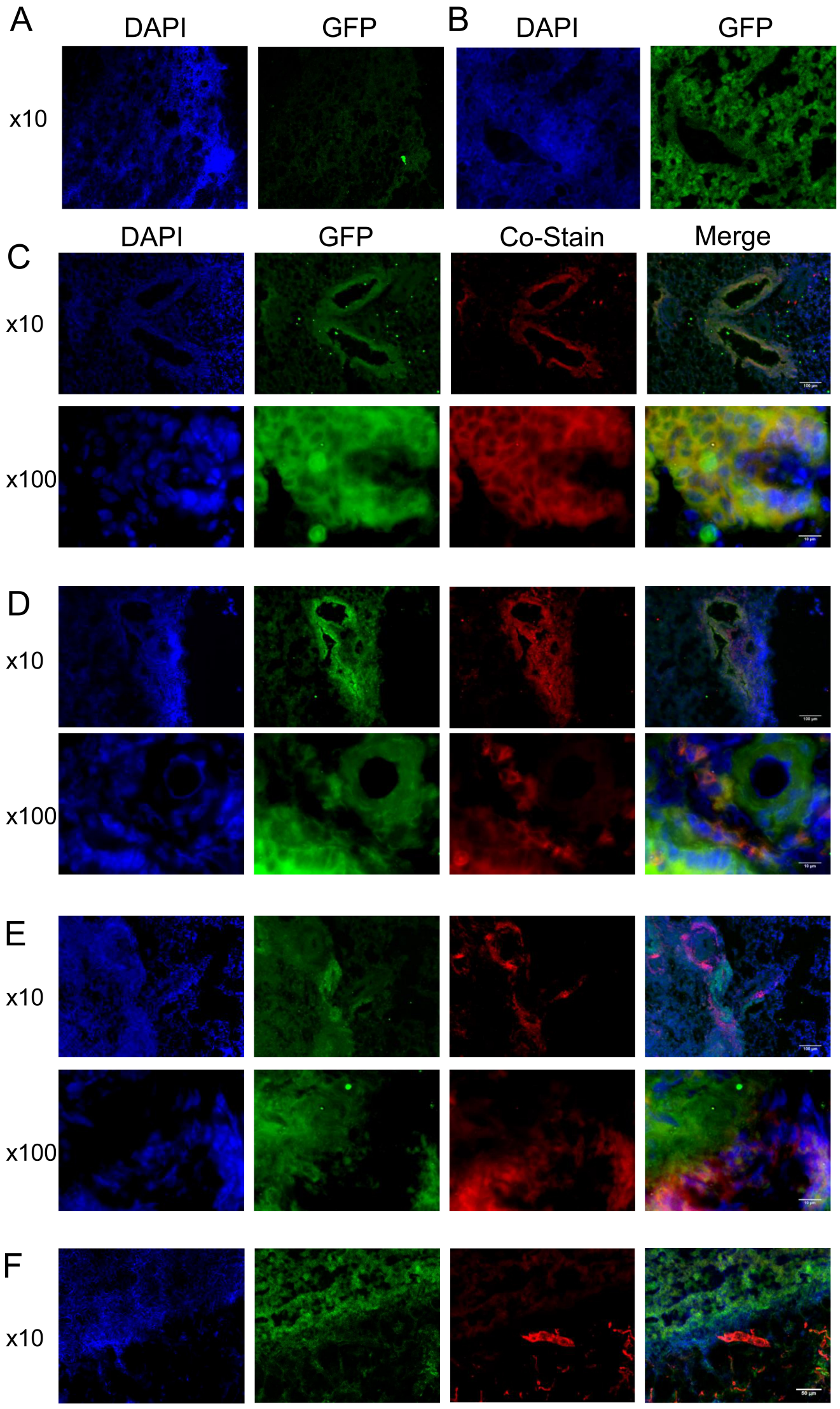
Figure 6: Multiple cell types in lung produce and respond to Shh, potentiating Th2 responses as a consequence of allergen inhalation in AAD

Our working model [5] has been updated to incorporate our new findings. We propose that in addition to triggering allergic immune responses via CD4+ Th2 activation, HDM allergen also induces the release of Shh in lung (HDM action: grey arrows). Shh is the primary Hh ligand secreted in the lung during AAD (Shh ligand, black circles) and that the source cells include epithelia, eosinophils and other CD45+ leukocytes (Shh release: white arrows). T-cells are able to respond to Shh, leading to Gli activation and upregulation of genes involved in Th2 skewing, leading to the potentiation of Th2 pathology and further inflammation/injury, potentially further increasing Shh expression. Epithelial cells and some CD16+ cells express active Gli (black arrows), likely in response to Shh, but the outcome of the signal to these cell types is currently unknown. Eosinophils also express activated Gli, and one consequence of this via Shh signaling is the upregulation of IL4, which may further drive Th2 activation and pathology.

Figure 1


A
GFP-
GFP+
BAL cells/ml
1.0x10^6
8.0x10^5
6.0x10^5
4.0x10^5
2.0x10^5
0
0wk
1wk
3wk
5wk
No. Lymphocytes in LN
2.5x10^7
2.0x10^7
1.5x10^7
1.0x10^7
5.0x10^6
0
WBC in lung
B
GFP-
GFP+
0.48
2.38
CD4
GFP
C
GFP+CD4+ LN cells
2.0x10^4
1.5x10^4
1.0x10^4
5.0x10^3
*
D
GFP+ Lymphocytes/ml
1.6x10^4
1.2x10^4
8.0x10^3
4.0x10^3
**
*
t=0
E
GFP+ CD4+/ml
5000
4000
3000
2000
1000
F
No. lung GFP+ Lymphocytes
G
No. lung GFP+ CD4+
800
700
600
500
400
300
200
100


A
Lung CD4+T1ST2+cells
1.2×10^5
8.0×10^4
4.0×10^4
0
WT PBS
C2 PBS
WT HDM
C2 HDM
B
LN CD4+T1ST2+cells
6.0×10^5
4.5×10^5
3.0×10^5
1.5×10^5
C
BAL
Lung
%Neutrophils
D
Eosinophils/ml
1.5×10^5
1.0×10^5
5.0×10^4
ng
ng
Eosinophils
1.6×10^6
1.2×10^6
8.0×10^5
4.0×10^5
E
WT PBS
C2 PBS
WT HDM
C2 HDM
F
Infiltration
PAS
Score (AU)
G
IL-4 in lung homogenate (pg/ml)
IL-13 in lung homogenate (pg/ml)
IL-5 in lung homogenate (pg/ml)
Figure 2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DAPI
GFP
B
DAPI
GFP
x10
C
DAPI
GFP
Co-Stain
Merge
x10
x100
D
x10
x100
E
x10
x100
F
x10

Figure 4

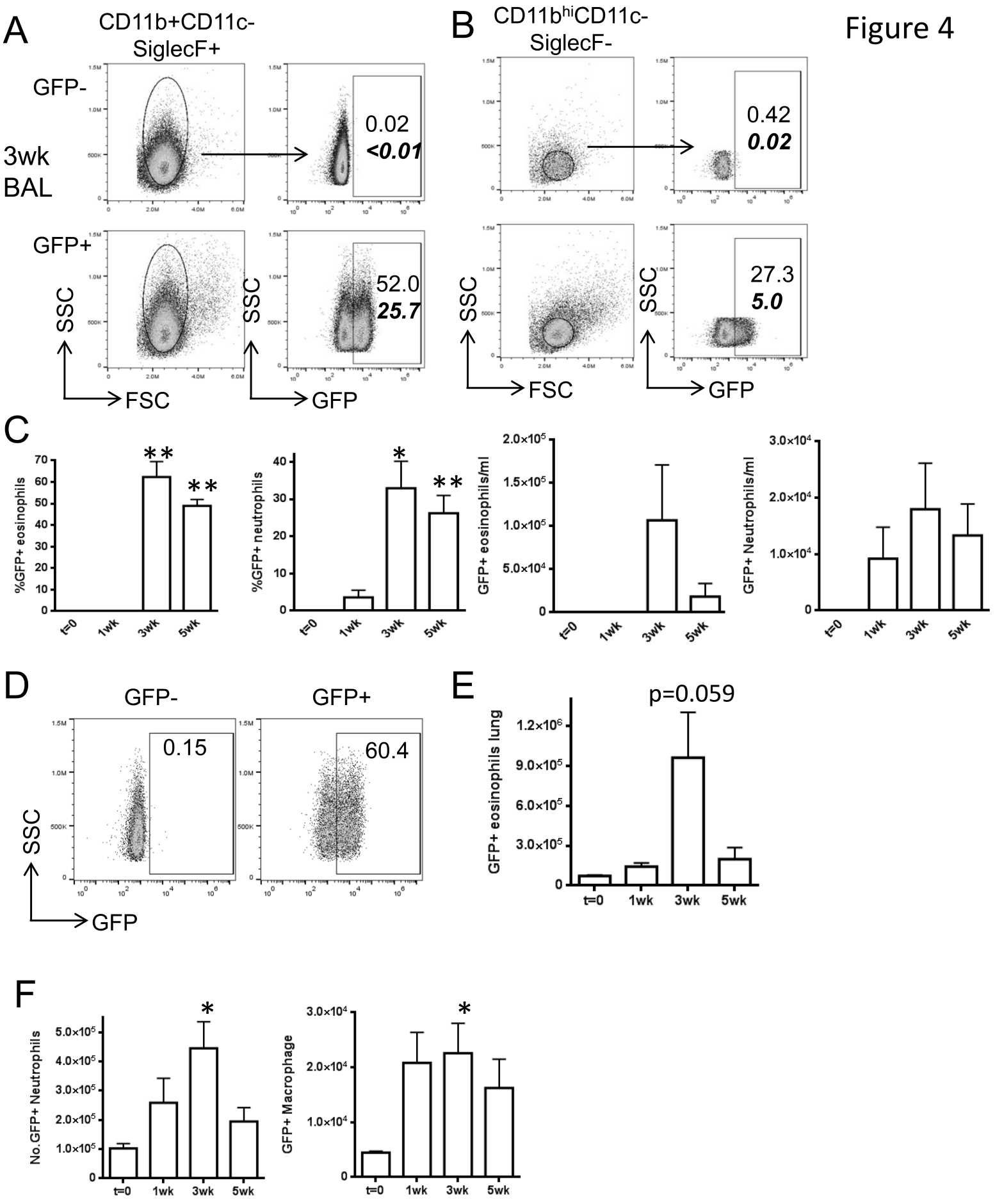
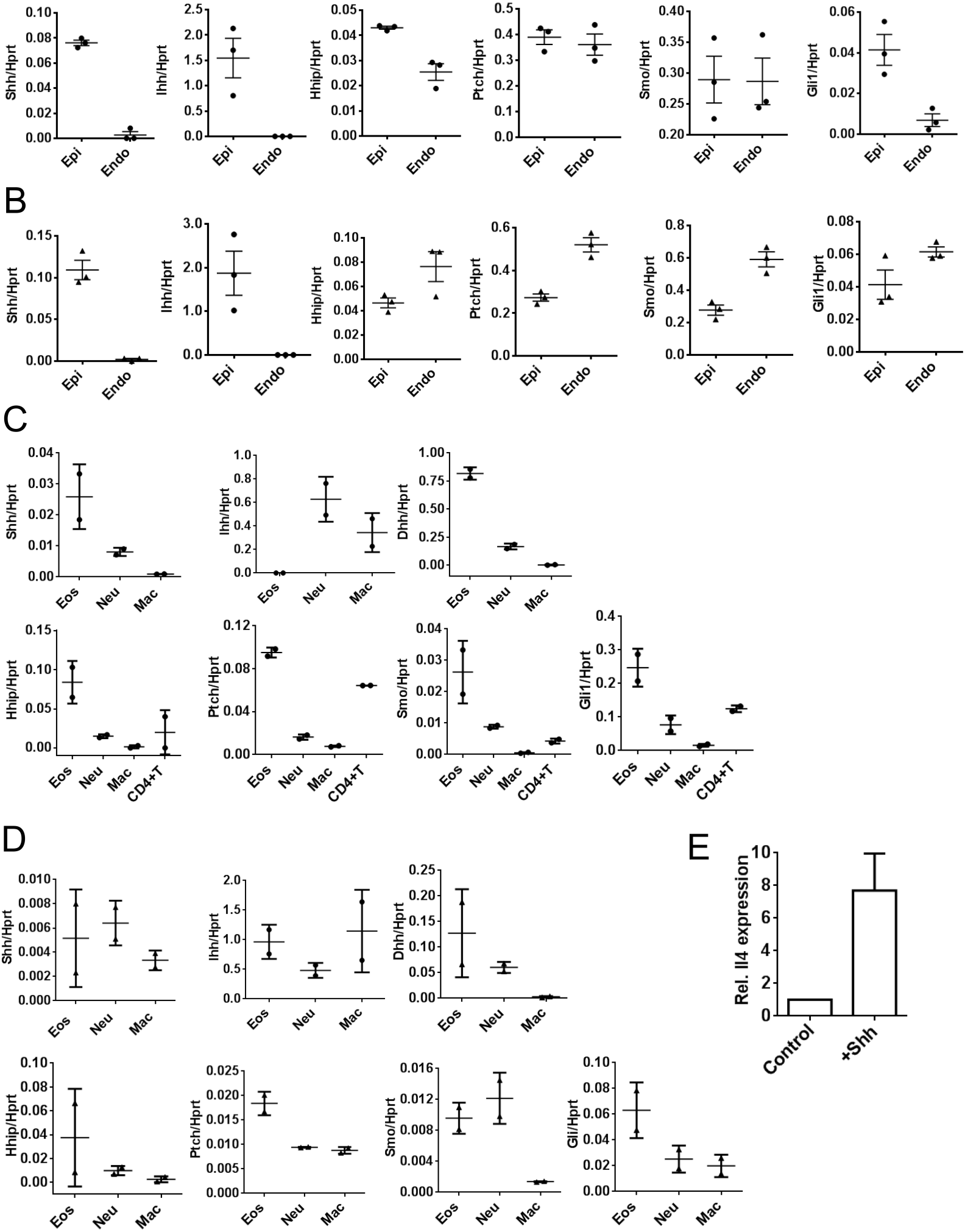

A
CD11b+CD11c-
SiglecF+
GFP-
3wk
BAL
0.02
<0.01
GFP+
52.0
25.7
SSC
FSC
SSC
GFP
B
CD11b^hiCD11c-
SiglecF-
0.42
0.02
27.3
5.0
SSC
FSC
SSC
GFP
C
%GFP+ eosinophils
%GFP+ neutrophils
GFP+ eosinophils/ml
GFP+ Neutrophils/ml
t=0
1wk
3wk
5wk
D
GFP-
GFP+
0.15
60.4
SSC
GFP
E
p=0.059
GFP+ eosinophils lung
F
No.GFP+ Neutrophils
GFP+ Macrophage

Figure 5


A
Shh/Hprt
Ihh/Hprt
Hhip/Hprt
Ptch/Hprt
Smo/Hprt
Gli1/Hprt
Epi
Endo
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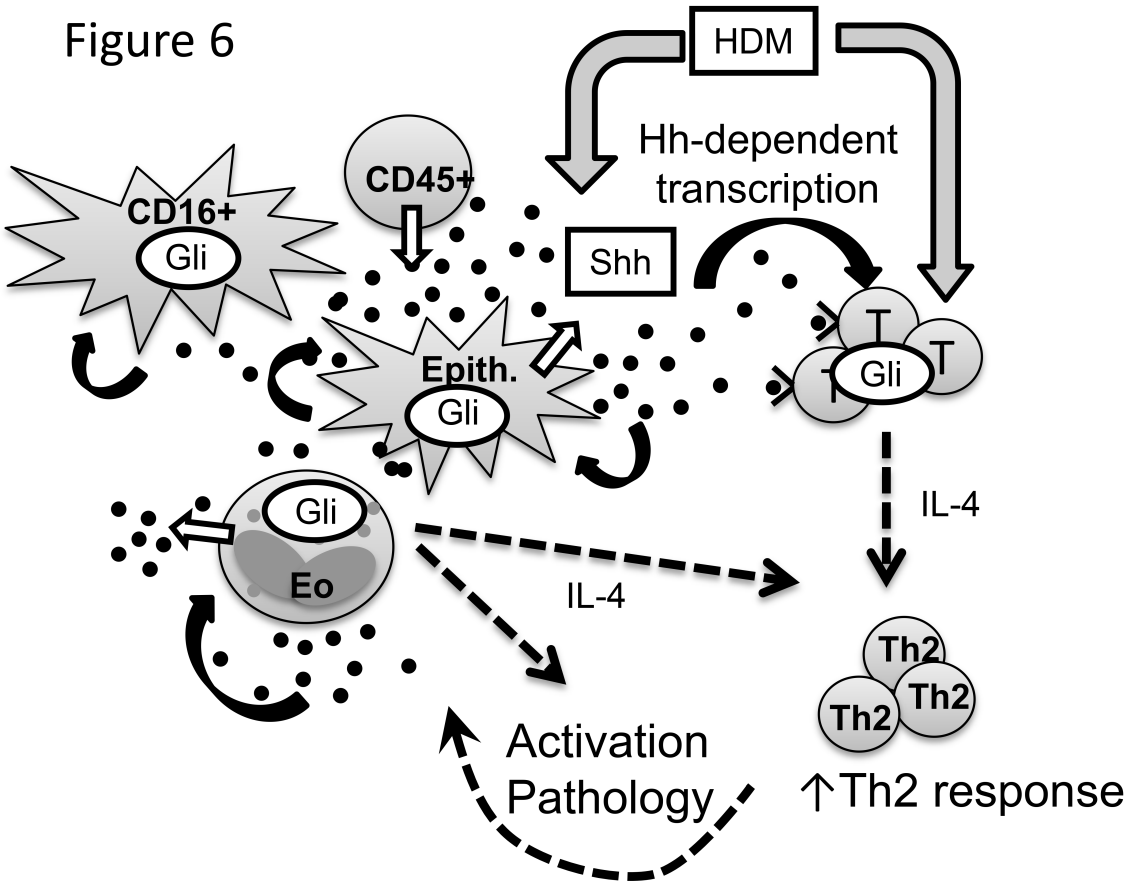
Shh/Hprt
Ihh/Hprt
Hhip/Hprt
Ptch/Hprt
Smo/Hprt
Gli1/Hprt
Epi
Endo
C
Shh/Hprt
Ihh/Hprt
Dhh/Hprt
Hhip/Hprt
Ptch/Hprt
Smo/Hprt
Gli1/Hprt
Eos
Neu
Mac
CD4+T
D
Shh/Hprt
Ihh/Hprt
Dhh/Hprt
Hhip/Hprt
Ptch/Hprt
Smo/Hprt
Gli/Hprt
Eos
Neu
Mac
E
Rel. Il4 expression
Control
+Shh

Figure 6


HDM
Hh-dependent
transcription
CD45+
CD16+
Gli
Shh
Epith.
Gli
Gli
Gli
IL-4
Gli
Eo
IL-4
Th2
Th2
Th2
Activation
Pathology
↑Th2 response