Cytokine 162 (2023) 156091



Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine





IL-4 and IL-13, not eosinophils, drive type 2 airway inflammation, remodeling and lung function decline

George Scott [1], Seblewongel Asrat [1], Jeanne Allinne, Wei Keat Lim, Kirsten Nagashima, Dylan Birchard, Subhashini Srivatsan, Dharani K. Ajithdoss, Adelekan Oyejide, Li-Hong Ben, Johnathon Walls, Audrey Le Floc'h, George D. Yancopoulos, Andrew J. Murphy, Matthew A. Sleeman, Jamie M. Orengo [*]

Regeneron Pharmaceuticals Inc., 777 Old Saw Mill River Road, Tarrytown, NY 10591, USA

ARTICLE INFO

Keywords:
IL-4
IL-13
Airway inflammation
Asthma
House dust mite
Lung remodeling
Anti–IL-5
Anti–IL-4R
Type 2 inflammation

ABSTRACT

Rationale: Type 2 (T2) asthma is characterized by airflow limitations and elevated levels of blood and sputum eosinophils, fractional exhaled nitric oxide, IgE, and periostin. While eosinophils are associated with exacerbations, the contribution of eosinophils to lung inflammation, remodeling and function remains largely hypothetical.

Objectives: To determine the effect of T2 cytokines IL-4, IL-13 and IL-5 on eosinophil biology and compare the impact of depleting just eosinophils versus inhibiting all aspects of T2 inflammation on airway inflammation.

Methods: Human eosinophils or endothelial cells stimulated with IL-4, IL-13 or IL-5 were assessed for gene changes or chemokine release. Mice exposed to house dust mite extract received anti-IL-4Rα (dupilumab), anti-IL-5 or control antibodies and were assessed for changes in lung histological and inflammatory endpoints.

Measurements and main results: IL-4 or IL-13 stimulation of human eosinophils and endothelial cells induced gene expression changes related to granulocyte migration; whereas, IL-5 induced changes reflecting granulocyte differentiation. In a mouse model, blocking IL-4Rα improved lung function by impacting multiple effectors of inflammation and remodeling, except peripheral eosinophil counts, thereby disconnecting blood eosinophils from airway inflammation, remodeling and function. Blocking IL-5 globally reduced eosinophil counts but did not impact inflammatory or functional measures of lung pathology. Whole lung transcriptome analysis revealed that IL-5 or IL-4Rα blockade impacted eosinophil associated genes, whereas IL-4Rα blockade also impacted genes associated with multiple cells, cytokines and chemokines, mucus production, cell:cell adhesion and vascular permeability.

Conclusions: Eosinophils are not the sole contributor to asthma pathophysiology or lung function decline and emphasizes the need to block additional mediators to modify lung inflammation and impact lung function.

1. Introduction

Asthma is a heterogenous disease characterized by chronic airway inflammation, remodeling and airway hyper-responsiveness resulting in variable expiratory airflow limitation and symptoms including wheezing, coughing and chest tightness. Clinical parameters associated with severe asthma include exacerbations and lung function decline. Most of the current biologics are approved for people with uncontrolled moderate-to-severe asthma with elevated biomarkers of type 2 (T2) inflammation, which is inflammation driven by the downstream effects of the T2 cytokines IL-4, IL-13, and IL-5. Since it is challenging to measure T2 cytokines in circulation, downstream biomarkers have been

Abbreviations trying to add T2 type 2: HDM, house dust mite; IL, Interleukin; AHR, airway hyperresponsiveness; GCM, goblet cell metaplasia; FEV, forced expiratory volume; DC, dendritic cell; TARC, thymus and activation regulated chemokine; GSEA, Gene Set Enrichment Analysis; GO, Gene Ontology; HMVEC-L, human lung microvascular endothelial cells; MCP, monocyte chemoattract protein; MDC, macrophage derived chemokine; VCAM-1, vascular cell adhesion protein 1; Mch, methacholine; NGS, next generation sequencing.

* Corresponding author.
E-mail address: jamie.orengo@regeneron.com (J.M. Orengo).

[1] Authors contributed equally to this work.

https://doi.org/10.1016/j.cyto.2022.156091
Received 14 August 2022; Received in revised form 2 November 2022; Accepted 14 November 2022
Available online 5 December 2022
1043-4666/© 2022 Regeneron Pharmaceuticals. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

valuable to aid in patient identification, including blood and sputum eosinophil counts, fractional exhaled nitric oxide (FeNO), IgE, and periostin [1].

Despite the approval of many biologics targeting T2 inflammation, there have been no placebo-controlled head-to-head clinical studies. With respect to approved biologics that impact T2 cytokines, all have been shown to reduce asthma exacerbations [1–11], however, there have been notable differences in other secondary endpoints, namely fractional exhaled nitric oxide (FeNO) and FEV_1 (Forced Expiratory Volume-1 s), accepted measures of T2 inflammation and lung function, respectively. Clinical studies with the IL-4Rα blocking antibody, dupilumab, demonstrated that inhibiting signaling of the T2 cytokines, IL-4 and IL-13, results in decreased exacerbations and consistent improvements in FEV_1 and in FeNO without decreasing circulating eosinophils [8,11,12]. Studies with the humanized IL-5Rα specific monoclonal antibody, benralizumab, which effectively depletes eosinophils, results in decreased exacerbations and an improved FEV_1 profile, but only decreases FeNO in subjects with higher levels of FeNO (greater than 75 ppb) [13,14]. In contrast, studies using humanized IL-5 antagonist monoclonal antibodies, mepolizumab and reslizumab, have shown inconsistent improvements in FEV_1 and have not improved FeNO despite marked sustained decreases in eosinophils and decreased exacerbations [15,16]. Of note, when mepolizumab was further studied in the long-term open-label extension studies, COLUMBA and COSMOS, initial improvements in lung function were observed, however, appeared to wane over time [17,18]. This gradual decline was not observed in the dupilumab and benralizumab extension trials, TRAVERSE [19] and BORA [13].

The mechanism of these drugs may play a role in shaping these distinctions. IL-5 is an essential maturation factor for eosinophils and the basis of mepolizumab's and reslizumab's eosinophil-driven mechanism of action. On the other hand, dupilumab a fully human monoclonal antibody binds to the shared co-receptor IL-4Rα and inhibits signaling of two key T2 cytokines, IL-4 and IL-13, that drive many T2 inflammatory events [20]. These differences raise valid questions concerning the role eosinophils play in asthma and their contribution to T2 inflammation. In contrast, benralizumab, an afucosylated IL-5Rα-specific antibody, depletes IL-5Rα–expressing cells via antibody-dependent cellular cytotoxicity (ADCC) and this mechanism extends benralizumab's effects beyond relatively deeper depletion of eosinophils and includes IL-5Rα expressing basophils. Basophils are T2 effector cells and known producers of IL-4 [21,22]; therefore, the broader impact of benralizumab is an additional factor that warrants consideration when comparing mechanisms of all the biologics targeting the IL-5 pathway. Thus, only the anti-IL-5 biologics allow for a specific interrogation of the role of eosinophils in asthma pathophysiology and will be used herein [23,24].

Mouse studies using eosinophil-deficient and eosinophil granule-specific proteins deficient mice have supported the notion that there are significant disease aspects such as remodeling, lung function and T2 inflammation that are not dependent on eosinophils [25,26]. Considering the observations in mouse models and clinical data with anti-IL-5 biologics, we sought to further investigate the contribution of the main T2 cytokines to airway inflammation. Using human eosinophils stimulated with IL-4, IL-13 or IL-5, we identified differential gene expression patterns. In addition, using a mouse model of airway inflammation, we performed a head-to-head comparison of dual IL-4/IL-13 blockade (anti-IL-4Rα) versus IL-5 blockade and dissected the relevance of circulating vs tissue eosinophils in driving airway inflammation, remodeling and lung function decline and have thereby decoupled peripheral blood eosinophil counts from impact on tissue inflammation, remodeling and function.

Reducing exacerbations have become the gold standard for treatment efficacy in asthma and by this yardstick, most biologics are able to achieve some level of efficacy in the asthmatic patient population. Consequently, this has led to very similar indication labels and treatment population overlap in clinical guidelines leading to a patient qualifying for more than one biologic [27]. Ultimately, the questions we attempt to address may explain the mechanistic differences that lead to the nuances in clinical observations between therapies and why these differences should have merit.

2. Results

2.1. (Fig. 1) unbiased identification of genes impacted by IL-4Rα vs IL-5 blockade in HDM-induced inflammation

To understand the contribution of the main T2 cytokines to lung inflammation, we used the relevant mouse model of house dust mite (HDM)-induced airway inflammation using $Il4ra^{hu/hu}Il4^{hu/hu}$ mice [20] (Fig. 1A) and took an unbiased approach to identify the impact of anti-IL-4Rα (dupilumab) vs anti-IL-5 on gene changes by whole lung transcriptome analysis. HDM exposure induced significant gene changes compared to saline (Fig. 1B) and a significant proportion of these gene changes were normalized by anti-IL-4Rα, while anti-IL-5 had a minor effect (Fig. 1B). Specifically, of the 737 genes perturbed by HDM exposure (Table E1), 208 were normalized uniquely by anti-IL-4Rα (Fig. 1B). These changes included T2 cytokines and chemokines (eg. *Ccl11 (Eotaxin-1), Ccl24 (Eotaxin-2), Ccl9 (Mip-1 g), Ccl8 (Mcp-2)*), genes involved in mucus production and secretion (eg. *Muc5ac, Tff1, ITLN-1*) and genes implicated in airway remodeling, bronchoconstriction and airway hyperresponsiveness (AHR) (eg. *Mmp12, Arg1, Mrgprg*) (Table E1). Unexpectedly, only 1 HDM-responsive gene was impacted by anti-IL-5 alone (*Tnfrsf17*), while 8 genes were impacted by both anti-IL-5 and anti-IL-4Rα (Fig. 1B and 1C). Interestingly, these 8 genes were primarily related to eosinophilia (*Cebpe, Prg2, Ccr3, Gapt, Il5Ra, Inpp5j, P2rx1* and *Cyp4f18*) (Fig. 1B and as a heatmap in 1C). The 217 genes impacted by HDM and reversed by anti-IL-4Rα and/or anti-IL-5 are shown as a heatmap in Fig. 1D. Aside from the illustrated genes that passed the defined significance threshold, anti-IL4Rα also reversed the overall gene changes induced by HDM exposure with Spearman's rank correlation coefficient, $\rho = -0.821$, a trend not observed with anti-IL-5 (Figure E1). Together, these data show that IL-5 blockade has highly specific effects on genes associated with eosinophil function, whereas IL-4Rα blockade impacted genes associated with T2 inflammation more broadly.

2.2. (Fig. 2) IL-4Rα blockade prevents tissue recruitment and activation of eosinophils while IL-5 blockade inhibits eosinophil maturation in the bone marrow.

To further explore the roles of IL-4/IL-13 and IL-5 on eosinophil infiltration and biology, we directly compared the impact of IL-4Rα blockade to IL-5 blockade on eosinophil levels in different tissue compartments. Given the highly vascularized nature of the lung, we used a double staining method to distinguish immune cells circulating in the lung vasculature from tissue infiltrating cells (Fig. 2A; Fig. E2A) [28]. This method demonstrated that in naïve mice, eosinophils were restricted to the vasculature and bone marrow (BM), but not in lung tissue. In contrast, eosinophils were present and increased in both lung vasculature and tissue following HDM exposure (Fig. 2A and 2B). No difference was observed in frequency of eosinophils in BM between naïve and HDM exposed animals. Increased vascular and lung tissue eosinophilia in patients with asthma has also been described [29]. We confirmed the observations that eosinophils can be detected in lung capillaries, but not in the tissue of naïve mice, whereas they can be detected in both the tissue and capillaries in HDM-exposed mice using electron microscopy (Fig. E2B).

As previously described [6], HDM exposure induces a dramatic influx of eosinophils into lung tissue, which was completely prevented by anti-IL-4Rα (Fig. 2B). Of note, anti-IL-4Rα did not impact circulating or BM eosinophils, whereas blockade of IL-5 decreased eosinophil levels in all compartments: lung tissue, circulation and BM. This is consistent

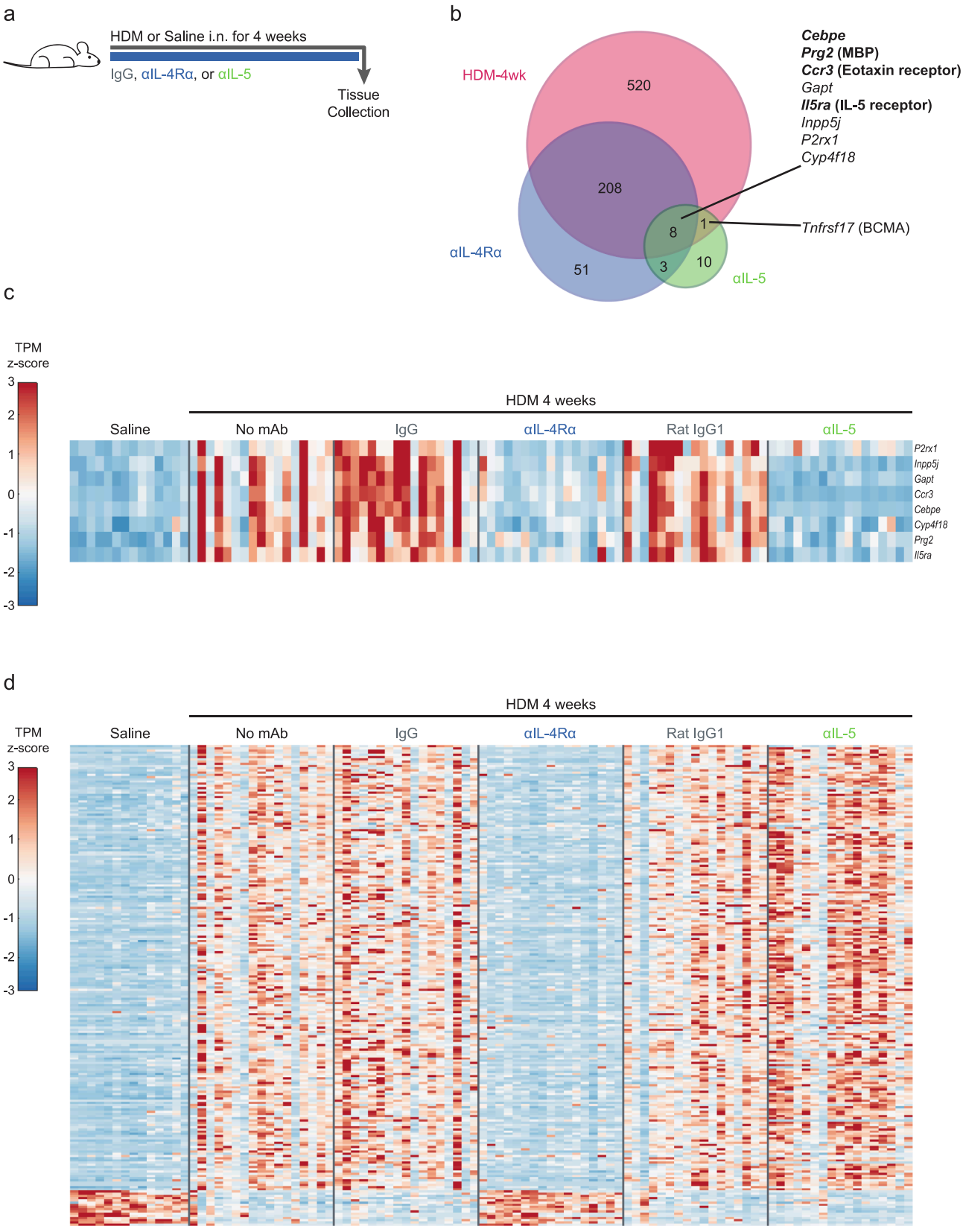


Fig. 1. Unbiased identification of genes impacted by IL-4Rα vs IL-5 blockade in HDM-induced lung inflammation. (A) *Il4ra*$^{hu/hu}$*Il4*$^{hu/hu}$ mice were exposed to HDM for 4 weeks and received either twice weekly αIL-4Rα, αIL-5 or an IgG or Rat IgG1 or no mAb. (B) Venn diagram of HDM signature vs anti-IL-4Rα vs anti-IL-5 signatures (adjusted p-value < 0.05 & absolute fold change ≥ 2). Treatment signatures correspond to statistically significant gene changes in treatment as compared to isotype control. (C-D) Heatmaps represent the z-score of gene expression (TPM) for the 8 HDM induced genes that were reversed by both αIL-4Rα and αIL-5 treatments (C) and the 217 genes reversed by αIL-4Rα and/or αIL-5 treatments (D).

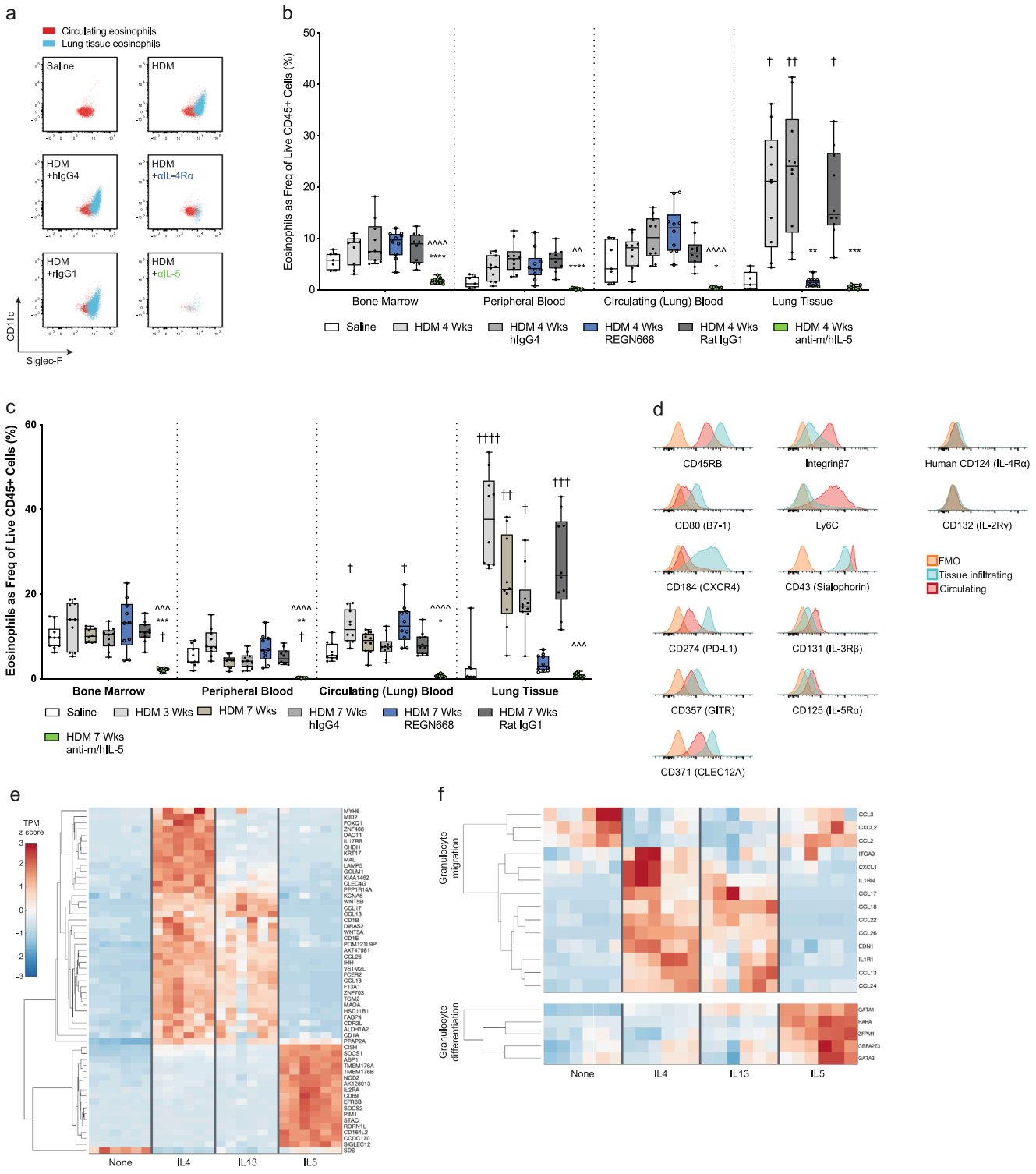


Fig. 2. IL-4Rα blockade prevents tissue recruitment of eosinophils while IL-5 blockade inhibits eosinophil maturation in the BM. *Il4rα*$^{hu/hu}$*Il4*$^{hu/hu}$ mice were exposed to HDM for 4 weeks with prophylactic treatment of αIL-4Rα, αIL-5 or isotype controls. (A) Representative flow cytometric plots from dissociated lungs. (B) Eosinophils reported as a frequency of live CD45^{+} cells in the indicated tissue. Each point reflects a single mouse. Symbols represent statistical significance compared to saline (†), relevant isotype control (*) and αIL-4Rα treated group (^). (C) Mice administered HDM for 7 weeks and treated with either αIL-4Rα, αIL-5 or isotype controls starting at week 3. Eosinophils in various tissue compartments are shown as a frequency of live CD45^{+} cells in the indicated tissue. (D) Mice administered HDM for 6 weeks. Overlaid flow cytometry histograms show the expression of gated eosinophils from the circulation and lung tissue with the FMO control for the respective marker. (E) Human eosinophils from 2 donors were stimulated with IL-4, IL-13 or IL-5 overnight. Heatmap shows the top genes regulated by each of these cytokines (adjusted p-value < 0.05 and absolute fold change ≥ 10). (F) Heatmap shows the genes from GO terms "granulocyte migration" and "granulocyte differentiation" that are significantly induced by IL-4, IL-13 or IL-5 in human eosinophils.

with the role of IL-5 in eosinophil maturation and survival [30]. Strikingly, similar results were observed when either antibody was administered therapeutically at a time of elevated tissue eosinophilia (Fig. 2C; Figure E3). These data suggest that each pathway modulates eosinophil biology in different and distinct ways: IL-4Rα blockade prevents HDM-induced infiltration of eosinophils into the inflamed lung tissue whereas IL-5 blockade globally reduces eosinophil levels.

Since anti-IL-4Rα effectively reduced eosinophils in the lung tissue without significantly decreasing eosinophil levels in circulation (Fig. 2B, C), we sought to investigate whether eosinophils in the periphery were phenotypically different from those that had infiltrated the lung. We found that lung tissue eosinophils of HDM-exposed mice showed differential expression of several immunomodulatory surface markers reported to be increased on tissue eosinophils in asthma: CD45Rb, CD80 (B7-1), CD184 (CXCR4), CD274 (PD-L1), CD357 (GITR) and CD371 (CLEC12A) (Fig. 2D) [31–33]. Notably, a decrease in IL-5Rα (CD125) was observed in eosinophils that infiltrated tissue, which is consistent with earlier reports of an activated eosinophil phenotype [34]. However, blood eosinophils from mice exposed to HDM showed no difference in surface marker expression compared to blood eosinophils from naïve mice. This remained consistent when also distinguishing tissue-infiltrating and circulating eosinophils. Together these data indicate that the activation and/or effector state of eosinophils is dependent on migration into inflamed lung tissue.

We next determined the direct effect of IL-4, IL-13 and IL-5 on human eosinophils and performed an unbiased global transcriptome analysis. Genes were ranked by fold change in each cytokine treatment and Gene Set Enrichment Analysis (GSEA) using gene sets derived from Gene Ontology (GO) biological process was implemented. While all three cytokines induced significant gene changes with minimal overlap (Fig. 2E), two distinct granulocyte-related GO terms were significantly enriched: granulocyte migration and granulocyte differentiation (Figure E4). Specifically, IL-4/IL-13 regulated genes associated with granulocyte migration (i.e. cytokines/chemokines, such as thymus and activation regulated chemokine (TARC, *Ccl17*), Eotaxin-2 (*Ccl24*), GROα (*Cxcl1*); integrin genes, such as *Itgα9*; and vasoconstriction genes, such as *Edn1)*. Whereas, IL-5 induced gene changes related to granulocyte differentiation (i.e. transcription factors and regulators involved in eosinophil development, such as *Gata1, Gata2* and FOG-1 (*Zfmp1*)) [35] (Fig. 2F).

2.3. (Fig. 3) IL-4Rα blockade prevents lung tissue entry of eosinophils by blunting chemotaxis and activation of the endothelium

Tissue transmigration of circulating immune cells requires a series of sequential events including chemotaxis, rolling, adhesion and diapedesis [36] with the endothelium being central to this process, as endothelial cells can produce chemokines and upregulate adhesion molecules in response to various stimuli. To investigate the effects of IL-4, IL-5 and IL-13 on these events, we assessed their effects on chemokine production and surface adhesion receptor expression using primary human lung microvascular endothelial cells (HMVEC-L). IL-4 and IL-13 each induced robust production of all three human eotaxins (CCL11, CCL24 and CCL26), the primary chemoattractants for eosinophils, as well as other chemokines such as monocyte chemoattract protein (MCP)-3 and MCP−4, chemoattractants for monocytes and lymphocytes; and T-cell chemoattractants, macrophage derived chemokine (MDC) and TARC (CCL17) (Fig. 3A). IL-5 had no effect on release of any chemokine measured from primary human endothelial cells since IL-5Rα expression is restricted to eosinophils and basophils [37]. Anti-IL-4Rα blocked chemokine release induced by IL-4 or IL-13 to baseline (Fig. 3B). The specific effects of IL-4 and IL-13 were confirmed *in vivo*, as IL-4Rα blockade completely prevented the HDM-induced increase in eotaxin expression in lung while IL-5 blockade had no effect (Fig. 3C), suggesting that IL-4 and IL-13 drive lung eotaxin expression.

Similarly, we found that IL-4 and IL-13 induced the cell adhesion molecule, vascular cell adhesion protein 1 (VCAM-1), on primary human lung endothelial cells (Fig. 3D) at comparable levels as the positive control TNF-α [38–40]. Altogether, these data suggest that IL-4 and IL-13 are potent activators of lung endothelium and that blocking these cytokines will inhibit tissue immune cell infiltration by blunting chemotaxis and adhesion of immune cells to the endothelium.

2.4. (Fig. 4) IL-4Rα blockade has a broader impact on T2 airway inflammation

Asthma is an inflammatory airway disease with complex pathophysiology [42,43], so we explored effects of anti-IL-4Rα and anti-IL-5 beyond eosinophil biology. In addition to lung eosinophilia (Fig. 2B&C), HDM induced infiltration of activated B cells, shown by increased CD23 expression. CD23 expression on B cells has been reported to be increased by IL-4 stimulation and correlates with circulating IgE levels in allergic inflammation [20,44–47]. Additionally, induction of ST2 + T cells, which have been described to be memory antigen-specific Th2 cells that produce IL-5 and IL-3 [48–50]; loss of alveolar macrophages, a sign of significant tissue inflammation; and skewing of the DC landscape toward one that aligns with a T2 immune response, a shift that is best captured by the cDC1:DC2 ratio, are observed. IL-4Rα blockade prevented all measured HDM-induced pathological changes, normalized the cDC1:DC2 ratio (Fig. 4A; Figure E5) and prevented the increase of lung cytokines and chemokines observed following HDM exposure (Fig. 4B). Similarly, IL-4Rα blockade inhibited the humoral response to HDM exposure, as measured by circulating IgE and HDM-specific IgG1 (Fig. 4C) as well as IgG1 and IgE class-switching in spleen (as described in [72]), which in turn prevented priming of FcεRI-expressing cells, such as basophils in spleen (Fig. 4D). Notably, IL-5 blockade did not significantly impact these pathological changes and suggests that reducing eosinophil numbers alone in all compartments by blocking IL-5 does not appear to reduce the levels of other inflammatory endpoints associated with T2 inflammation.

2.5. (Fig. 5) IL-4Rα blockade prevents HDM-induced epithelial barrier dysfunction and vascular pathology

Loss of epithelial barrier integrity in the lung is a hallmark of asthma, enhancing the risk of allergen sensitization, promoting production of epithelial-derived T2 cytokines and reducing mucociliary clearance [51]. Disruption of epithelial junction complexes can be caused by inhaled allergens, respiratory viruses or inflammatory cytokines, which creates a feedback loop that allows better penetration of allergens and further activation of lung immune responses [51]. Repeated HDM exposure induced significant gene changes associated with lung apical junction complexes (*Cldn1*, *Gjb1*), cell–cell adhesion (*Cdh5*, *Cdh13*, *Cdh22*, *Pcdh1*, *Pcdhac2*, *Pcdhgc3*) and basement membrane assembly (Dag1, Lamc2) (Fig. 5A and B). Only anti-IL-4Rα prevented these changes (Fig. 5A and 5B), demonstrating a role of IL-4 and IL-13 as drivers of airway barrier dysfunction during allergen challenge.

To understand the role of IL-4, IL-13, and IL-5 in HDM-induced lung pathology, histopathological analysis was performed. HDM-induced lung lesions were characterized by mixed cell inflammation (particularly perivascular and peribronchiolar) (Fig. 5C), and vascular changes (endothelial hypertrophy/hyperplasia, intimal proliferation/media thickening with or without luminal narrowing) (Fig. 5D). Only anti-IL-4Rα significantly reduced or prevented the development of lung lesions (Fig. 5C and 5D).

Histopathological analysis of lung sections also revealed an HDM-induced significant increase in mucus-producing cells (goblet cell metaplasia, (GCM)) which was prevented by anti-IL-4Rα (Fig. 5E) further demonstrating a distinct role of IL-4 and IL-13 in airway remodeling.

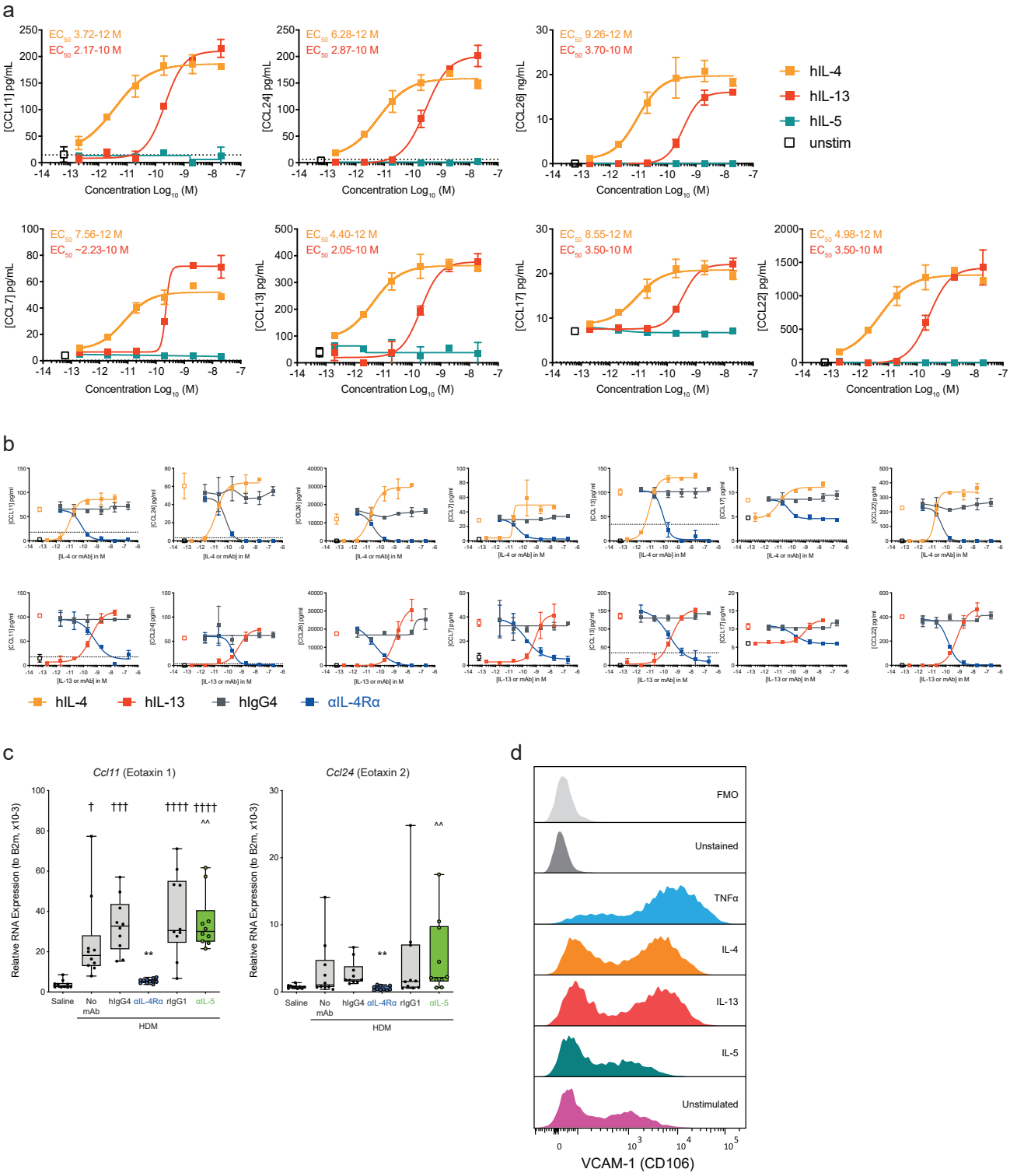


Fig. 3. IL-4Rα blockade prevents lung tissueeosinophil entry by blunting chemotaxis and activation of the endothelium. (A) HMVEC-L cells were incubated for 2 days with increasing concentrations of IL-4, IL-13 or IL-5, and chemokine release was assessed by MSD. (B) HMVEC-L cells were incubated for 2 days with increasing concentrations of serial dilutions of dupilumab (αIL-4Rα) or a control Ig(hIgG4) in the presence of 50 pM IL-4 (top panel) or 5 nM IL-13 (bottom panel) and chemokine release was assessed by MSD. [41] Error bars represent SD from samples run in duplicate. Dotted lines represent lower limit of detection. Data are representative of at least 3 independent experiments. (C) Lung chemokine gene expression as detected by using qRT-PCR (TaqMan) assays. For all graphs, boxes show medians and 25th-75th percentiles, and whiskers show minimum and maximum values. Each point reflects a single mouse. Symbols represent statistical significance compared to saline (†), compared to relevant isotype control (*) and compared to αIL-4Rα-treated group (^). (D) HMVEC-L were stimulated overnight with either media control, TNF-α, IL-4, IL-13, or IL-5 and then stained with anti-VCAM-1 for flow cytometry. Histograms represent changes in VCAM-1 surface expression compared to controls.

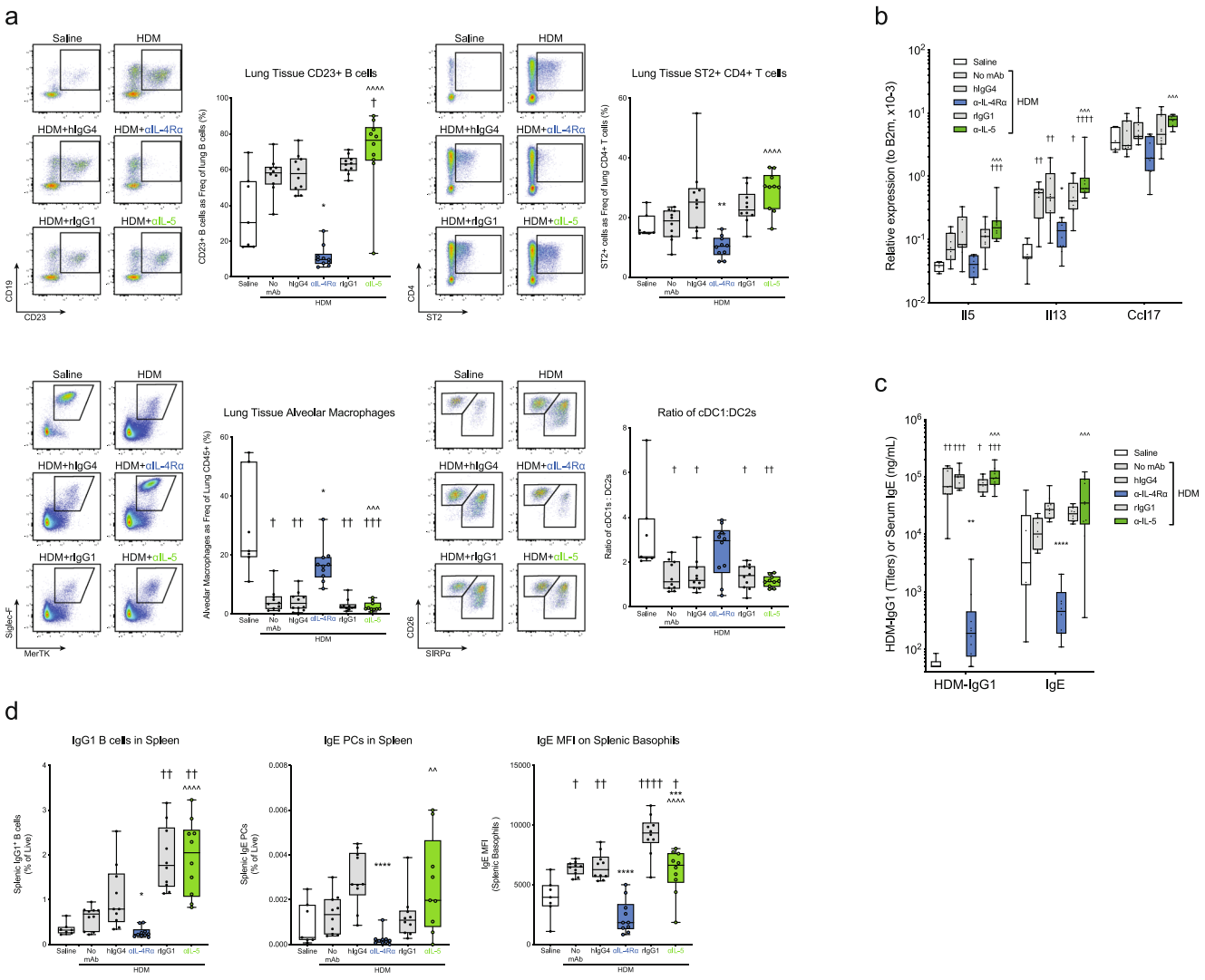


Fig. 4. IL-4Rα blockade has a broader impact on lung inflammation than IL-5 blockade. (A) Quantification of lung immune cell infiltrates, as determined by flow cytometry, in mice exposed to saline or HDM with the indicated treatments. Representative fluorescence-activated cell sorting plots and frequencies of $CD23^+$ B-cells, $ST2^+$ $CD4^+$ T-cells, alveolar macrophages and the ratio of cDC1:DC2s. (B) *Il5*, *Il13* and *Ccl17* expression as detected by qRT-PCR (TaqMan). (C) Serum concentrations of HDM-specific IgG1 or total IgE as determined by ELISA in mice exposed to saline or HDM as indicated. (D)Frequencies of splenic $IgG1^+$ B cells, splenic IgE plasma cells and quantification of IgE surface expression as measured by mean fluorescence intensity (MFI) of IgE on splenic basophils. Each point reflects a single mouse. For all graphs, boxes show medians and 25th-75th percentiles, and whiskers show minimum and maximum values. Symbols represent statistical significance compared to saline (†), compared to relevant isotype control (*) and compared to αIL-4Rα-treated group (^). For gating strategies, see Figure E5.

2.6. (Fig. 6) IL-4Rα blockade prevents HDM-induced lung function decline.

Given the wider impact of IL-4Rα blockade on many of the cardinal features of asthma, we determined how lung function is impacted by blocking these respective pathways. As anticipated HDM exposure caused significant lung function impairment with a decline in $FEV_{0.1}$ (forced expiratory volume, FEV, in 0.1 s), reduced airflow (peak expiratory flow, PEF) and reduced lung capacity (forced vital capacity, FVC). IL-4Rα blockade protected mice from lung function decline by preventing significant airflow obstruction while simultaneously improving endpoints indicative of airway restriction (Fig. 6A). Strikingly, these effects were sustained post-methacholine (Mch) challenge, demonstrating an effect of anti-IL4Rα in preventing airway hyperresponsiveness (Fig. 6B; Figure E6). Anti-IL-5 had no impact on either baseline or Mch-challenge measurements of lung function.

3. Discussion

This manuscript explores the impact of reducing eosinophils alone compared to blocking broader T2 inflammation in asthma pathophysiology by comparing neutralization of IL-5 to blockade of IL-4Rα. In head-to-head comparison studies, we used an IL-5 neutralizing antibody to deplete eosinophils and an IL-4Rα specific antibody to inhibit signaling of IL-4 and IL-13, which more globally inhibits T2 inflammation [20]. The results of all the endpoints provide evidence of the role T2 inflammation can play in remodeling and lung function in the absence of eosinophils. Additionally, these studies were bolstered with *in vitro* experiments that investigated the inflammatory effect of these pathways on endothelial cells and how these cytokines prime eosinophils for entry into the tissue. We ultimately show that these processes are dependent on IL-4 and IL-13 and reinforce clinical findings.

Eosinophils are a well-known biomarker of T2 airway disease. Clinical studies with IL-5 blocking antibodies have demonstrated a clear and significant reduction in exacerbations associated with a reduction in circulating eosinophils [3,4,6,23]. Interestingly, dupilumab also

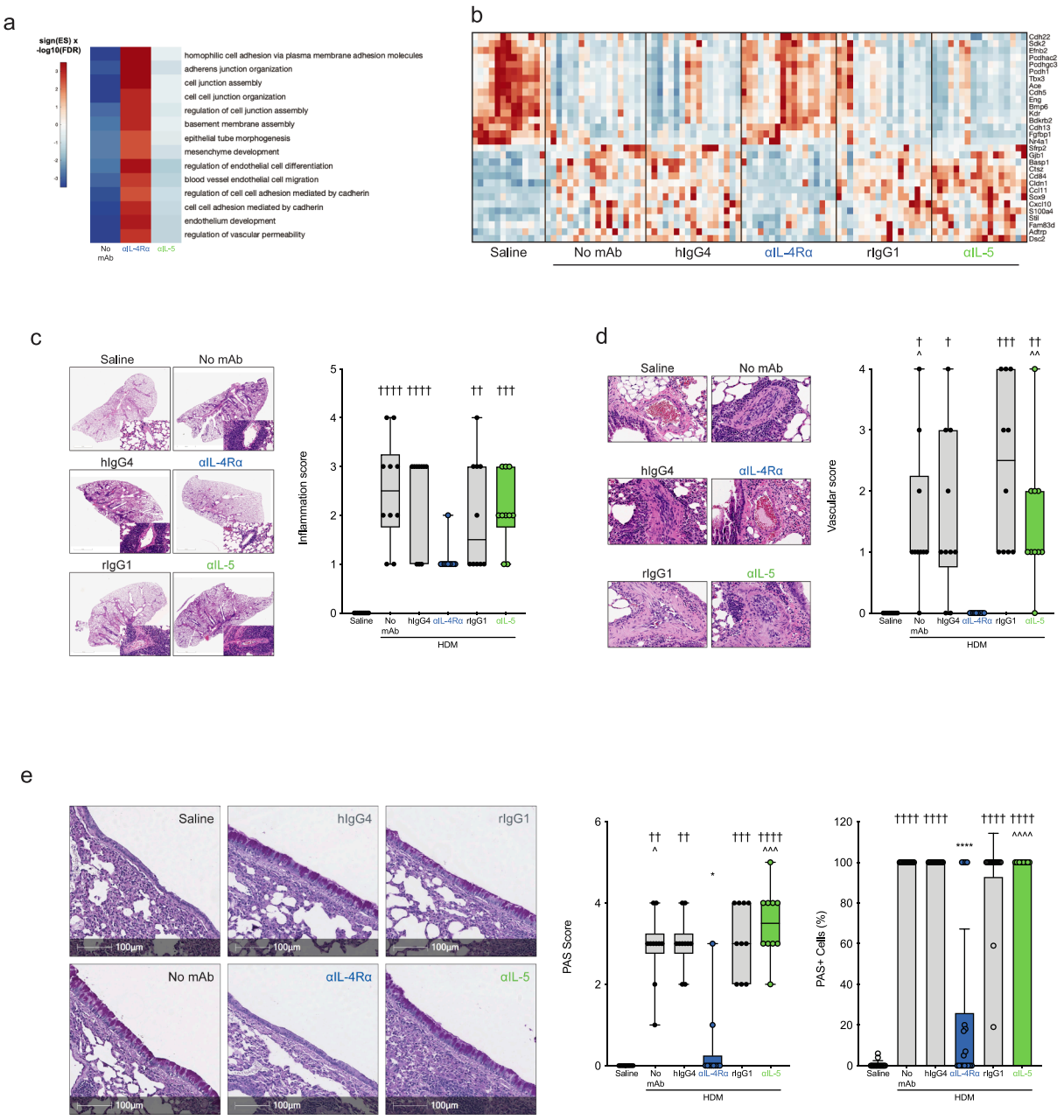


Fig. 5. IL-4Rα blockade, but not IL-5 blockade, prevents HDM-induced epithelial barrier dysfunction and vascular pathology (A) Heatmap shows the significant level of selected gene ontology (GO) biological processes that are impacted by HDM exposure and reversed by αIL-4Rα treatment (B) Summary of differentially expressed genes from each of the GO terms indicated (C) Representative H&E images showing normal lung or lung with inflammation (insert shows higher magnification of a vessel and surrounding parenchyma) and a graph showing pathology score for severity of inflammation.(D). Representative H&E lung images showing normal vessel or vessel with pathology and a graph showing vascular score for severity of vascular lesions. (E). Representative images of PAS stained lung sections showing normal airway or airway with increased goblet cell metaplasia (GCM). Graphs show the severity of GCM, measured as PAS score and % PAS positive cells.

drastically impacted exacerbations; however, the levels of blood eosinophils remained the same, and in some patients, a transient but slight elevation was observed [8,11,52]. In our mouse model, we showed that IL-4Rα blockade prevented eosinophil infiltration into lung tissue without affecting circulating eosinophil levels. These findings were upheld even when anti-IL-4Rα was administered therapeutically at the peak of eosinophilia. Of note, the impact of IL-4Rα blockade extended beyond the eosinophils and also prevented immune cell infiltration. While anti-IL-5 profoundly reduced eosinophil levels in all compartments studied, levels of other immune infiltrates were not reduced. Sequencing of lung tissue from our mouse model indicated that the absence of eosinophils only significantly affected a handful of genes largely related to eosinophil development. Moreover, these genes were also a subset of genes that were normalized by anti-IL-4Rα treatment. Our *in vitro* studies support this observation by showing that IL-4 and IL-13 stimulation of human eosinophils increases gene changes related to migration, while the effects of IL-5 are constrained to expression of differentiation genes. Despite reports demonstrating that endothelial cells can respond to IL-5 stimulation [53–55], IL-5 stimulated human lung microvessel endothelial cells (HMVEC-L) did not exhibit the release of eotaxins and cytokines that was observed with IL-4 and IL-13 stimulations. This suggests that IL-5 does not directly or indirectly drive the recruitment of other cells or the production of key T2 cytokines and chemokines [56]. Overall, these data indicate a direct role of IL-4 and IL-

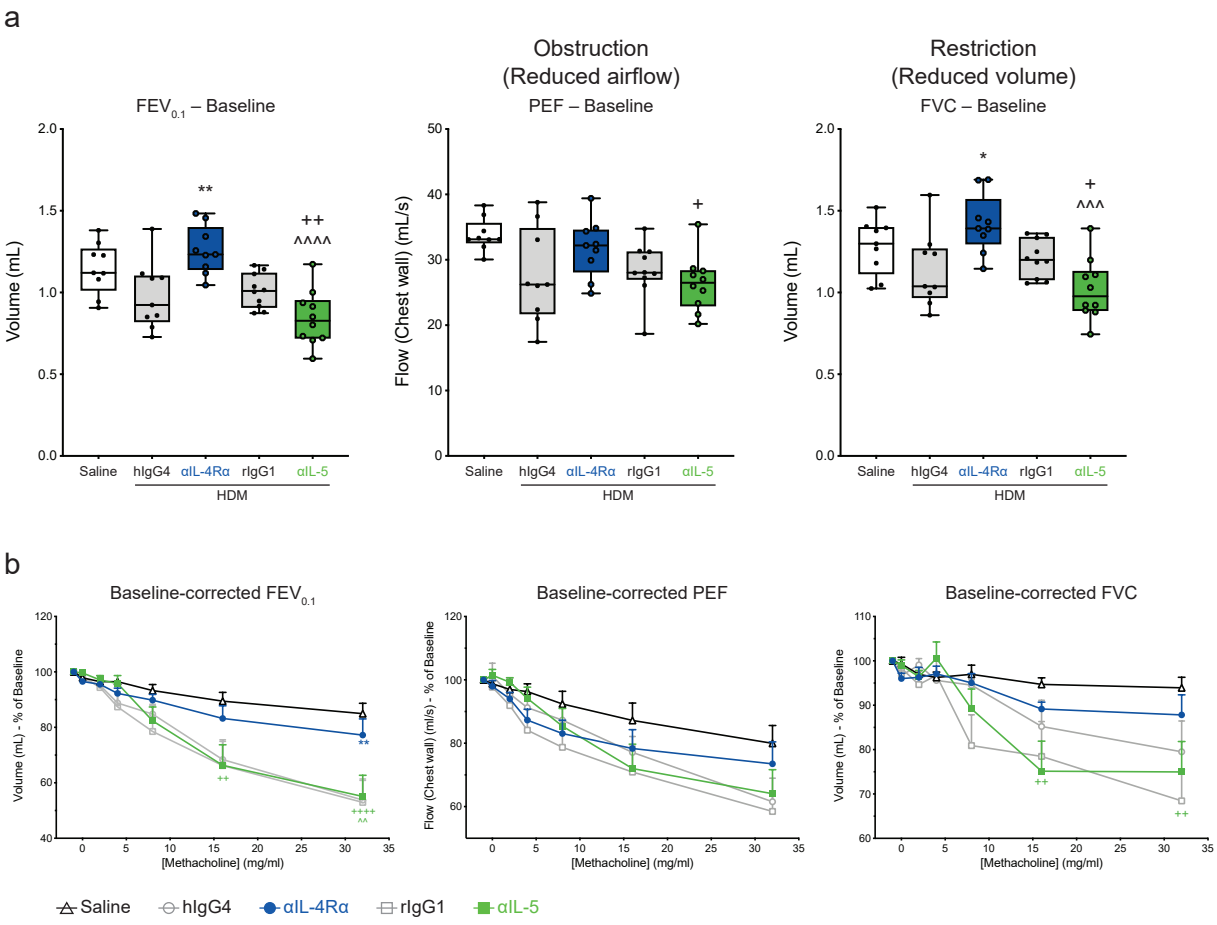


Fig. 6. IL-4Rα blockade, but not IL-5 blockade, prevents HDM-induced tissue remodeling and lung function decline. (A) Respiratory mechanics at baseline measured by using negative pressure forced expiration (NPFE) maneuvers for $FEV_{0.1}$, PEF and FVC. (B) $FEV_{0.1}$, PEF and FVC measured at the indicated methacholine dose and plotted as percent of baseline.

13 on eosinophils and endothelial cells that is different from the role of IL-5 and provides insight into the mechanisms that drive different pathological outcomes.

Despite an association between eosinophilic inflammation and lung function decline, there is no concrete evidence directly implicating IL-5 as a driver of T2 inflammation and overall lung function decline in asthmatics. Earlier findings in other mouse models of eosinophil depletion determined that eosinophils were not required for allergen-induced changes in airway pathology such as mucus production but could contribute to some of the fibrotic changes that occur with asthma [57,58]. In accordance, we found no attenuation of inflammation, but also no significant improvement in lung function or AHR as well as structural changes such as epithelial barrier disruption and vascular remodeling.

The epithelial barrier is structurally and functionally compromised in patients with asthma, atopic dermatitis, CRSwNP, allergic rhinitis and eosinophilic esophagitis [59,60]. In asthma, loss of barrier integrity is associated with defects in tight junction and reductions in adherens junction proteins such as cadherins and claudins, which leads to an increase in epithelial permeability [61]. This permeability is conducive to allergen sensitization, secretion of pathogenic cytokines and chemokines and disruption of mucus clearance, which in turn creates an environment for chronic inflammation [59]. IL-4 and IL-13 can also directly interfere with tight junctions in human bronchial epithelial cells [61]. Our studies show that repeated HDM exposure in mice significantly impacted genes associated with barrier integrity, including different cadherins (*Cdh22*, *Cdh5*, *Cdh13*) and claudins (*Cldn1*), which may be expressed in the airway epithelium and are associated with tight junction formation. Genes involved in cell-to-cell attachment were also significantly impacted, such as *Dsc2*, *Pcdh1*, *Sdk2*. Of note, cadherins have been shown to play a role in asthma pathogenesis [62]. Interestingly, changes in these genes were only impacted by IL-4Rα blockade, and not by depletion of eosinophils, demonstrating a distinct role for IL-4 and IL-13 in driving airway structural damage.

Although genes were significantly impacted, without protein expression data, protein localization data, and single cell analysis, it is challenging to make conclusions around the specific impact these gene changes have on barrier function. Nevertheless, the significant gene changes we observed in pathways associated with barrier function, paired with reports in the literature [63–65] gives credence that there is an impact on barrier-associated genes.

Vascular remodeling has been recognized as one of the components of poorly controlled, severe persistent asthma [66]. It has been reported that inflammation of pulmonary and bronchial blood vessels can significantly impact asthma pathophysiology, leading to decreased lung capacity and airflow [66]. Interestingly, genes implicated in vascular remodeling and angiogenesis in asthmatics were impacted in the HDM model, such as genes involved in endothelium development and migration (eg. *Kdr*, *Efnb2* and *Fgfbp1* [67–70]). Vascular changes in the lung were also observed by histology, including endothelial hyperplasia, intimal proliferation and media thickening and these changes were only normalized by IL-4Rα blockade highlighting a specific role for IL-4 and IL-13 in driving important structural changes associated with airway remodeling.

Overall, these studies propose processes by which dual IL-4 and IL-13 blockade can prevent lung function decline and airway remodeling and

give important insight into the contributors of long-term lung function decline in asthmatics. Nevertheless, mouse models may not capture all features of asthma pathophysiology and direct translation to human disease maybe challenging. Exacerbations, for example, are not evaluated in this model. And while eosinophils and IL-5 have strong association with exacerbations, the role they play in lung remodeling is less clear. Our findings support the notion of a separate processes driving lung function impairment and inflammation. Furthermore, this study disconnects peripheral eosinophil counts from impacts on tissue inflammation, remodeling and function that are dependent on IL-4 and IL-13 and collectively demonstrates that multiple factors of T2 inflammation are required to protect lung function and truly modify disease. Biologics targeting T2 cytokines have revolutionized the treatment of severe asthma but, due to the complex and heterogenous nature of this disease, how each of these different treatments improve disease remains poorly defined. *In vivo* models can help understand the different pathways and mechanisms of severe asthma and lead us to consider additional clinical studies that can improve and enhance patient care.

4. Methods

4.1. Mouse models of HDM-induced asthma

All procedures were conducted in compliance with protocols approved by the Institutional Animal Care and Use Committee of Regeneron Pharmaceuticals. Female, 8–12 week old mice generated in a hybrid 129S6/C57BL/6 background were genetically modified to replace both endogenous IL-4 and the IL-4Rα ectodomain with their corresponding human sequences [20] were used for all experiments. The humanized mice were exposed to house dust mite (HDM) three times per week for 4 weeks (for prophylactic treatment studies) or for 7 weeks (for therapeutic treatment studies) and received twice-weekly subcutaneous injections of 25 mg/kg dupilumab, anti-IL-5 (TRFK5), their respective isotype control antibody, or no antibody. At the end of the study, mice were either subjected to lung-function testing (FlexiVent®) or sacrificed for analysis of cellular infiltrates in lung blood and tissue, cytokine and chemokine levels, and gene expression and circulating immunoglobulin levels. All animal experiments were performed in accordance with the Guidelines of the Institutional Animal Care and Use Committee at Regeneron Pharmaceuticals, Inc.

4.2. Flow cytometry

Spleen, BM (femur), and lung were collected at end of HDM study - and processed to single cell suspension for flow cytometric analysis. For BM preparation, collected femurs were cut at both ends and centrifuged, while spleens were mechanically dissociated. Lung samples underwent enzymatic digestion and mechanical dissociation. Additionally, to enable flow cytometric analysis of circulating versus tissue-infiltrating immune cells in the lung, mice were injected intravenously with an anti-CD45 BV650 antibody 5 min prior to sacrifice to selectively label immune cells still in the vasculature while leaving cells that had infiltrated the lung parenchyma and alveolar space unlabeled. Cells - from all tissue compartments were then stained for the identification of different immune cell types and subsets. Samples were acquired on an LSR Fortessa X-20 or a FACSymphony cell analyzer using the HTS attachment (BD). Data analysis was performed using FlowJo v10 Software (BD).

4.3. Histopathology

At end of study, and after exsanguination, the whole left lung of each mouse was collected and kept in 4 % (w/v) paraformaldehyde at room temperature for 3 days. Lung samples were subsequently blotted dry and transferred to 70 % ethanol prior to being sent to Histowiz (Brooklyn, NY) for paraffin embedding, sectioning, and staining (periodic acid Schiff [PAS], and H&E). Inflammation, vascular lesions, and goblet cell metaplasia (GCM) were assessed and scored by board-certified veterinary pathologists. GCM was additionally quantified as the percentage of PAS-positive goblet cells in the epithelium as determined by visual counting along a roughly millimeter length (100 epithelial cells) of primary bronchus.

4.4. Electron microscopy

At end of study, and after exsanguination, a paraformaldehyde and glutaraldehyde mixture (Electron Microscopy Sciences, cat. #15720, Mixture 2) was instilled into the lungs through the trachea. Lungs were then harvested into the fixative, and samples targeting the area of the first bifurcation of the main bronchus were taken using a 1 or 2 mm biopsy punch. Punches were then shipped in fixative to Charles River Laboratories, Pathology Associates (Durham, NC) for processing and imaging via transmission electron microscopy.

4.5. Gene expression analysis

Differential gene expressions were analyzed using DESeq2 package [73]. Genes with absolute fold change ≥ 2 and FDR-adjusted p-value < 0.05 were selected as the significantly perturbed gene signatures. Functional analysis was performed using Gene Set Enrichment Analysis (GSEA) [71] with GO biological process gene sets from the Molecular Signatures Database (MSigDB, c5.bp.v7.0).

4.6. Statistical analysis

All statistical analyses were performed using GraphPad Prism™. Normality of the data was evaluated using the Shapiro-Wilk test. If data passed the normality test and standard deviations of the different groups were not statistically different from each other as assessed by the Brown-Forsythe test, results were interpreted by one-way analysis of variance (ANOVA) followed by the Tukey *post hoc* test for multiple comparisons. If data failed to pass the normality test, or if standard deviations were significantly different, results were interpreted using the Kruskal-Wallis test followed by the Dunn's *post hoc* test for multiple comparisons. Differences were considered to be statistically significant when $p < 0.05$ (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$).

Funding sources: This study was sponsored by Regeneron Pharmaceuticals, Inc, and Sanofi.

5. Data and materials availability

The authors declare that the data supporting the findings of this study are available within the article and its supplementary information files or are available upon reasonable requests to the authors.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: All authors reports financial support was provided by Regeneron Pharmaceuticals, Inc. and Sanofi-Aventis France SA. All authors reports a relationship with Regeneron Pharmaceuticals Inc that includes: current or former employment and equity or stock or stock options. Andrew Murphy has patent #HUMANIZED IL-4 AND IL-4Ra ANIMALS issued to Regeneron Pharmaceuticals.

Data availability

Data will be made available on request.

Acknowledgments

The authors thank the following contributors for providing support: Wen-Chi Chen, Paulina Pedraza, Xia Liu, Amit Agrawal, Viola Woo, Navneet Kaur, Nikhil Amin, Sivan Harel, Andre Limnander, and Amanda Atanasio for assistance in data acquisition and helpful discussion; the VelociGene team for generating all the mice used in this study; members of the departments of Therapeutic Proteins, Protein Expression Sciences, and Preclinical Manufacturing and Process Development, for immunizations and production of antibodies, stable cell lines, and large scale production of proteins; the dupilumab team for helpful discussions and thoughtful input.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2022.156091.

References

[1] W. Busse, W. Neaville, Anti-immunoglobulin E for the treatment of allergic disease, Curr. Opin. Allergy Clin. Immunol. 1 (1) (2001) 105–108.
[2] M. Solèr, J. Matz, R. Townley, R. Buhl, J. O'Brien, H. Fox, J. Thirlwell, N. Gupta, G. Della Cioppa, The anti-IgE antibody omalizumab reduces exacerbations and steroid requirement in allergic asthmatics, Eur. Respir. J. 18 (2) (2001) 254–261.
[3] J.M. FitzGerald, E.R. Bleecker, P. Nair, S. Korn, K. Ohta, M. Lommatzsch, G.T. Ferguson, W.W. Busse, P. Barker, S. Sproule, G. Gilmartin, V. Werkstrom, M. Aurivillius, M. Goldman, investigators Cs. Benralizumab, an anti-interleukin-5 receptor alpha monoclonal antibody, as add-on treatment for patients with severe, uncontrolled, eosinophilic asthma (CALIMA): a randomised, double-blind, placebo-controlled phase 3 trial. *Lancet* 2016; 388: 2128-2141.
[4] E.R. Bleecker, J.M. FitzGerald, P. Chanez, A. Papi, S.F. Weinstein, P. Barker, S. Sproule, G. Gilmartin, M. Aurivillius, V. Werkstrom, M. Goldman, investigators Ss. Efficacy and safety of benralizumab for patients with severe asthma uncontrolled with high-dosage inhaled corticosteroids and long-acting beta2-agonists (SIROCCO): a randomised, multicentre, placebo-controlled phase 3 trial. *Lancet* 2016; 388: 2115-2127.
[5] E.H. Bel, S.E. Wenzel, P.J. Thompson, C.M. Prazma, O.N. Keene, S.W. Yancey, H. G. Ortega, I.D. Pavord, S. Investigators, Oral glucocorticoid-sparing effect of mepolizumab in eosinophilic asthma, N. Engl. J. Med. 371 (2014) 1189–1197.
[6] H.G. Ortega, M.C. Liu, I.D. Pavord, G.G. Brusselle, J.M. FitzGerald, A. Chetta, M. Humbert, L.E. Katz, O.N. Keene, S.W. Yancey, P. Chanez, M. Investigators, Mepolizumab treatment in patients with severe eosinophilic asthma, N. Engl. J. Med. 371 (2014) 1198–1207.
[7] L. Bjermer, C. Lemiere, J. Maspero, S. Weiss, J. Zangrilli, M. Germinaro, Reslizumab for Inadequately Controlled Asthma With Elevated Blood Eosinophil Levels: A Randomized Phase 3 Study, Chest 150 (4) (2016) 789–798.
[8] M. Castro, J. Corren, I.D. Pavord, J. Maspero, S. Wenzel, K.F. Rabe, W.W. Busse, L. Ford, L. Sher, J.M. FitzGerald, C. Katelaris, Y. Tohda, B. Zhang, H. Staudinger, G. Pirozzi, N. Amin, M. Ruddy, B. Akinlade, A. Khan, J. Chao, R. Martincova, N.M. H. Graham, J.D. Hamilton, B.N. Swanson, N. Stahl, G.D. Yancopoulos, A. Teper, Dupilumab Efficacy and Safety in Moderate-to-Severe Uncontrolled Asthma, N. Engl. J. Med. 378 (26) (2018) 2486–2496.
[9] J. Corren, J.R. Parnes, L. Wang, M. Mo, S.L. Roseti, J.M. Griffiths, R. van der Merwe, Tezepelumab in Adults with Uncontrolled Asthma, N. Engl. J. Med. 377 (10) (2017) 936–946.
[10] A. Menzies-Gow, J. Corren, A. Bourdin, G. Chupp, E. Israel, M.E. Wechsler, C. E. Brightling, J.M. Griffiths, Å. Hellqvist, K. Bowen, P. Kaur, G. Almqvist, S. Ponnarambil, G. Colice, Tezepelumab in Adults and Adolescents with Severe Uncontrolled Asthma, *N. Engl. J. Med.* 384 (19) (2021) 1800–1809.
[11] K.F. Rabe, P. Nair, G. Brusselle, J.F. Maspero, M. Castro, L. Sher, H. Zhu, J. D. Hamilton, B.N. Swanson, A. Khan, J. Chao, H. Staudinger, G. Pirozzi, C. Antoni, N. Amin, M. Ruddy, B. Akinlade, N.M.H. Graham, N. Stahl, G.D. Yancopoulos, A. Teper, Efficacy and Safety of Dupilumab in Glucocorticoid-Dependent Severe Asthma, N. Engl. J. Med. 378 (26) (2018) 2475–2485.
[12] S. Wenzel, L. Ford, D. Pearlman, S. Spector, L. Sher, F. Skobieranda, L. Wang, S. Kirkesseli, R. Rocklin, B. Bock, J. Hamilton, J.E. Ming, A. Radin, N. Stahl, G. D. Yancopoulos, N. Graham, G. Pirozzi, Dupilumab in persistent asthma with elevated eosinophil levels, N. Engl. J. Med. 368 (26) (2013) 2455–2466.
[13] W.W. Busse, E.R. Bleecker, J.M. FitzGerald, G.T. Ferguson, P. Barker, S. Sproule, R. F. Olsson, U.J. Martin, M. Goldman, A. Yañez, M. Fernández, A. Tolcachier, J. Belloni, J. Taborda, M. De Salvo, J. Maspero, C. Victorio, M.C. Navarta, M. Grilli, P. Rodríguez, M. Otaola, V. Cambursano, P. Malamud, A. Stok, G. Arce, O. Roza, F. Scherbovsky, P. Elias, M.S. Saez, M. Peters, M. Phillips, J. Upham, P. Gibson, F. Thien, J.o. Douglass, P. Thomas, P. Bardin, D. Sajkov, M. Hew, D. Langton, A. Pez, C. Fritscher, J. Hetzel, W. Mattos, R. Stelmach, M. Antila, A.L. Fernandes, H. Metev, Y. Ivanov, A. Bogdanova, A.D. Markova, M. Peneva, R. Veselinova, T. Petkova, V. Petrova, E. Shopova, Y. Ivanova, R. Yotsova, G. Kirkova, G. Petrova, S. Dachev, K. Sotirova, M. Stoyanova, O. Yakov, V. Ilieva-Fartunova, C. Lemiere, M. Laviolette, W. Yang, J.M. FitzGerald, B. Pek, W.P. Killorn, C. Poirier, G. Chouinard, L. Melenka, R. Leigh, P. Nair, D. Dorscheid, J. Martin, J. Pavie, A. Rosenblut, C. Cartagena, C. Quilodran, S. Muñoz, I. Malkusova, J. Veverka, S. Holub, J. Mares, R. Kozel, D. Kopecka, V. Zindr, P. Cerva, P. Chanez, A. Bourdin, G. Devouassoux, C. Taille, F. De Blay, F. Goupil, C. Leroyer, D. Gourcerol, T. Didi, A. Didier, G. Garcia, P. Bonniaud, R.R. Vasram, M. Lommatzsch, U. Harnest, A. Linnhoff, C. Keller, G. Hoheisel, K. Förster, T. Schultz, I. Schenkenberger, J. Kirschner, T. Schmoller, S. Zielen, M. Kornmann, E. Beck, L. Volgmann, R. Dichmann, A. Overlack, J. Winkler, P. Mikloweit, S. Ballenberger, M. Rolke, S. Korn, H. Pabel, M. Hoffmann, J. Feimer, K. Rabe, R. Deckelmann, T. Welte, H. Ohbayashi, S. Hozawa, T. Shimoda, M. Yoshida, T. Nagakura, T. Kamei, Y. Sano, S. Kagami, H. Takahashi, M. Haida, G. Kimura, H. Nakamura, Y. To, M. Shinkai, T. Shimokawaji, M. Kudo, N. Shijyubo, T. Tochigi, M. Kato, A. Tanaka, Y. Gon, N. Sasaki, T. Ohdaira, C. Omori, T. Takahashi, S. Takeuchi, K. Nakajima, H. Makita, M. Kaneko, K. Furuta, M. Taniguchi, Y. Inobe, H. Miki, Y. Nakamura, N. Miyazawa, S. Yano, M. Iijima, T. Yamato, J. Diaz, A. Matsuno, E. Felix, A. Guerreros, A. Daly, F. Rodriguez Chiarse, D. Salazar, S. Castro, W. Chavez, R. Estrella, T. Aquino, T. Atienza, R. Samoro, M.G.D. Isidro, P. Kuna, A. Antczak, M. Bednarek, D. Mądra-Rogacka, T. Hofman, M. Jutel, E. Pisarczyk-Bogacka, A. Kot, R. Mróz, M. Czupryńska-Borkowska, M. Żurowska-Gębala, A. Dyczek, G. Jasieniak-Pinis, P. Wojnowski, E. Trębas-Pietraś, M. Dobryniewska, M. Stobiecki, M. Tarnowska-Matusiak, A. Magner, A. Kolczyński, T. Kachel, Z. Bartuzi, B. Majorek-Olechowska, P. Śliwiński, V. Balicka, E. Hajoł, J. Stachera, M. Rachel, M. Michnar, J. Tałałaj, E. Springer, P. Napora, T. Grzelewski, R. Bijata-Bronisz, A. Świderska, G. Mincewicz, Z. Sankowski, T. Fijołek, A. Kwaśniewski, J. Jerzyńska, J. Cygler, R. Olesiejuk, I. Von Steiner, W. Machowiak, K. Filipek, P. Piskorz, E. Uhryn, M. Nittner-Marszalska, E. Rybicka-Liszewska, A. Bodzenta-Łukaszyk, M. Chełmińska, I. Krupa-Borek, W. Skucha, A. Płoszczuk, T. Mihaescu, I.D. Petrui, A. Savu, G. Apti, C. Toma, A. Trailescu, S. Sokurenko, V. Trofimov, I. Leshchenko, N. Shaporova, I. Ilyashevich, I. Stitsenko, A. Vizel, Y. Ilkovich, D. Tikhanov, S. Goncharova, A. Ezhov, G. Ignatova, N. Izmozherova, N. Berdnikova, L. Goryachkina, V. Popova, L. Bolieva, A. Rebrov, V. Nosov, O. Magnitskaya, S. Nedogoda, V. Abrosimov, A. Molotkov, G. Reshedko, M. Smirnov, M. Osipenko, E. Pavlysh, S. Myasoedova, L. Lenskaya, V. Chistyakov, E. Vishneva, L. Uon, A. Zateyschikova, S. Laleko, T. Pak, A. Bruning, V. Naidoo, J. Reddy, R. Van Zyl Smit, H.-S. Park, Y.J. Cho, S.-H. Cho, S.H. Lee, B.J. Lee, J.S. Kim, Y.S. Cho, C.-S. Park, M.-K. Kim, Y.-I. Koh, J.-H. Kim, S.Y. Lee, S.P. Lee, J.W. Park, K.H. Yoo, H. Yoon, J. Lee, G.-Y. Hur, S.K. Lee, HeeKyoo Kim, D. Ramos Barbón, E. Laffond Yges, E. Martínez Moragón, M. Arias Guillen, A. Saura Vinuesa, F. Sánchez-Toril López, J.L. Velasco Garrido, B. García-Cosio Piqueras, L. Pérez de Llano, A. L. Valero Santiago, M.C. Rubio, L. Bjermer, B.o. Lundbäck, B. Gemicioglu, S. Bavbek, D. Ediger, M. Erdinc, I. Hanta, S. Nayci, S. Arinc, L. Iashyna, Y. Mostovoy, M. Ostrovskyy, L. Kuryk, I. Lemko, T. Ospanova, L. Moskalenko, O. Korzh, V. Blazhko, O. Krakhmalova, L. Romaniuk, Y. Dytyatkovs'ka, O. Yakovenko, V. Gavrysyuk, H. Burhan, D. Saralaya, S. Scott, A. Morice, E. Jackson-Voyzey, T. Win, S. Nasser, R. Harrison, R. Chaudhuri, M. Nordstrom, P. De, M. Masoli, A. Mansur, A. Gunstone, R. Niven, T. Harrison, S. Babu, S. Bhuchar, S. Kureishy, J. Zwetchkenbaum, L. Kaplan, E. Bleecker, S. Reyes, F. Fakih, M. Heuer, R. Klein, M. Marcus, I. Hussain, T. Tanus, H. Raissy, S. Alpizar, H. Rodriguez, G. Gleich, T. Bridges, J. Pouzar, G. Steven, J. Matz, S. Krstevska, D. Ziworitin, A. Kaplan, J. Sublett, J. DeAngelo, A. Darter, J. Agaiby, J. Hagan, E. Davis, D. Siri, C. Mcevoy, C. Dharma, N. Karimjee, S. Kelsen, M. Frade, A. Silverthorn, J. Kline, F. Averill, J. Ruiz, E. Robinette, Z. Shaikh, C. Aguilar, W. Nish, G. Alvarez, Y. Ismail, P. Parikh, M. Leong, D. Murphy, F. Garcia, A. Goldsobel, A. Blanco, E. Campbell, G. Perez, G. Chupp, T. Bates, V. Perez, S. Wenzel, K. Shenoy, R. Katial, D. Bernstein, N. Parikh, W. Sims, G. Ferguson, D. Pham, A. George, R. Beasley, G. Perez, R. Garver, M. Garcia-Portela, S. Wong, W. Soong, R. Guillot, G. Hudes, A. Arif, J. Binker, M. Villareal, U. Ziegner, J. Ojile, S. Goswami, C. Piniella, I. Segal, H. Zafra, M. Lee, M. Tarpay, J. Panuto, R. Merchant, P. Springsteen, T. Scialla, P. Shapero, K. Allen, M. Moss, H. Cruz, J. Rumbyrt, A. Boyd, R. Nathan, J. Bernstein, J. Jacobs, E. Ginchansky, J. Johnston, B. Baxter, T. Johnson II, S. Apaliski, C. Corder, D. Fuentes, J. Diaz, C. Ngo, L. Le, Long-term safety and efficacy of benralizumab in patients with severe, uncontrolled asthma: 1-year results from the BORA phase 3 extension trial, Lancet Respir. Med. 7 (1) (2019) 46–59.
[14] A.P. Hearn, J. Kavanagh, G. d'Ancona, C. Roxas, L. Green, L. Thomson, M. Fernandes, B.D. Kent, J. Dhariwal, A.M. Nanzer, D.J. Jackson, The relationship between Feno and effectiveness of mepolizumab and benralizumab in severe eosinophilic asthma. *J. Allergy Clin. Immunol. Pract.* 9 (2021) 2093-2096 e2091.
[15] I.D. Pavord, S. Korn, P. Howarth, E.R. Bleecker, R. Buhl, O.N. Keene, H. Ortega, P. Chanez, Mepolizumab for severe eosinophilic asthma (DREAM): a multicentre, double-blind, placebo-controlled trial, Lancet 380 (9842) (2012) 651–659.
[16] P. Haldar, C.E. Brightling, B. Hargadon, S. Gupta, W. Monteiro, A. Sousa, R. P. Marshall, P. Bradding, R.H. Green, A.J. Wardlaw, I.D. Pavord, Mepolizumab and exacerbations of refractory eosinophilic asthma, N. Engl. J. Med. 360 (10) (2009) 973–984.
[17] S. Khatri, W. Moore, P.G. Gibson, R. Leigh, A. Bourdin, J. Maspero, M. Barros, R. Buhl, P. Howarth, F.C. Albers, E.S. Bradford, M. Gilson, R.G. Price, S.W. Yancey, H. Ortega, Assessment of the long-term safety of mepolizumab and durability of clinical response in patients with severe eosinophilic asthma. *J. Allergy Clin. Immunol.* 143 (2019) 1742-1751 e1747.
[18] N. Lugogo, C. Domingo, P. Chanez, R. Leigh, M.J. Gilson, R.G. Price, S.W. Yancey, H.G. Ortega, Long-term Efficacy and Safety of Mepolizumab in Patients With Severe Eosinophilic Asthma: A Multi-center, Open-label, Phase IIIb Study, Clin. Ther. 38 (2016) 2058–2070, e2051.
[19] M.E. Wechsler, L.B. Ford, J.F. Maspero, I.D. Pavord, A. Papi, A. Bourdin, H. Watz, M. Castro, N.M. Nenasheva, Y. Tohda, D. Langton, G. Cardona, C. Domingo, H.

S. Park, K.R. Chapman, X. Mao, Y.i. Zhang, A.H. Khan, Y. Deniz, P.J. Rowe, U. Kapoor, F.A. Khokhar, L.P. Mannent, M. Ruddy, E. Laws, N. Amin, M. Hardin, Long-term safety and efficacy of dupilumab in patients with moderate-to-severe asthma (TRAVERSE): an open-label extension study, Lancet Respir. Med. 10 (1) (2022) 11–25.

[20] A. Le Floc'h, J. Allinne, K. Nagashima, G. Scott, D. Birchard, S. Asrat, Y. Bai, W. K. Lim, J. Martin, T. Huang, T.B. Potocky, J.H. Kim, A. Rafique, N.J. Papadopoulos, N. Stahl, G.D. Yancopoulos, A.J. Murphy, M.A. Sleeman, J.M. Orengo, Dual blockade of IL-4 and IL-13 with dupilumab, an IL-4Ralpha antibody, is required to broadly inhibit type 2 inflammation, Allergy 75 (2020) 1188–1204.

[21] B.M. Salter, G. Nusca, D. Tworek, J.P. Oliveria, S.G. Smith, R.M. Watson, T. Scime, C. Obminski, R. Sehmi, G.M. Gauvreau, Expression of activation markers in circulating basophils and the relationship to allergen-induced bronchoconstriction in subjects with mild allergic asthma, J. Allergy Clin. Immunol. 137 (2016) 936–938, e937.

[22] E. Ono, M. Taniguchi, N. Higashi, H. Mita, K. Kajiwara, H. Yamaguchi, S. Tatsuno, Y. Fukutomi, H. Tanimoto, K. Sekiya, C. Oshikata, T. Tsuburai, N. Tsurikisawa, M. Otomo, Y. Maeda, M. Hasegawa, E. Miyazaki, T. Kumamoto, K. Akiyama, CD203c expression on human basophils is associated with asthma exacerbation, J. Allergy Clin. Immunol. 125 (2) (2010) 483–489.

[23] M. Koike, K. Nakamura, A. Furuya, A. Iida, H. Anazawa, K. Takatsu, N. Hanai, Establishment of humanized anti-interleukin-5 receptor alpha chain monoclonal antibodies having a potent neutralizing activity, Hum. Antibodies 18 (1-2) (2009) 17–27.

[24] W.W. Busse, R. Katial, D. Gossage, S. Sari, B. Wang, R. Kolbeck, A.J. Coyle, M. Koike, G.L. Spitalny, P.A. Kiener, G.P. Geba, N.A. Molfino, Safety profile, pharmacokinetics, and biologic activity of MEDI-563, an anti-IL-5 receptor alpha antibody, in a phase I study of subjects with mild asthma, J. Allergy Clin. Immunol. 125 (2010) 1237–1244, e1232.

[25] K.L. Denzler, M.T. Borchers, J.R. Crosby, G. Cieslewicz, E.M. Hines, J.P. Justice, S. A. Cormier, K.A. Lindenberger, W. Song, W. Wu, S.L. Hazen, G.J. Gleich, J.J. Lee, N.A. Lee, Extensive eosinophil degranulation and peroxidase-mediated oxidation of airway proteins do not occur in a mouse ovalbumin-challenge model of pulmonary inflammation, J. Immunol. 167 (3) (2001) 1672–1682.

[26] K.L. Denzler, S.C. Farmer, J.R. Crosby, M. Borchers, G. Cieslewicz, K.A. Larson, S. Cormier-Regard, N.A. Lee, J.J. Lee, Eosinophil major basic protein-1 does not contribute to allergen-induced airway pathologies in mouse models of asthma, J. Immunol. 165 (10) (2000) 5509–5517.

[27] "Global strategy for asthma management and prevention: GINA executive summary." E.D. Bateman, S.S. Hurd, P.J. Barnes, J. Bousquet, J.M. Drazen, J.M. FitzGerald, P. Gibson, K. Ohta, P. O'Byrne, S.E. Pedersen, E. Pizzichini, S.D. Sullivan, S.E. Wenzel and H.J. Zar. Eur Respir J 2008; 31: 143-178. *Eur Respir J* 2018; 51.

[28] K.G. Anderson, K. Mayer-Barber, H. Sung, L. Beura, B.R. James, J.J. Taylor, L. Qunaj, T.S. Griffith, V. Vezys, D.L. Barber, D. Masopust, Intravascular staining for discrimination of vascular and tissue leukocytes, Nat. Protoc. 9 (1) (2014) 209–222.

[29] P.L. Bruijnzeel, Eosinophil tissue mobilization in allergic disorders, Ann. N. Y. Acad. Sci. 725 (1994) 259–267.

[30] Y. Yamaguchi, Y. Hayashi, Y. Sugama, Y. Miura, T. Kasahara, S. Kitamura, M. Torisu, S. Mita, A. Tominaga, K. Takatsu, Highly purified murine interleukin 5 (IL-5) stimulates eosinophil function and prolongs in vitro survival. IL-5 as an eosinophil chemotactic factor, J. Exp. Med. 167 (1988) 1737–1742.

[31] C. Duez, A. Dakhama, A. Tomkinson, P. Marquillies, A. Balhorn, A.-B. Tonnel, D. L. Bratton, E.W. Gelfand, Migration and accumulation of eosinophils toward regional lymph nodes after airway allergen challenge, J. Allergy Clin. Immunol. 114 (4) (2004) 820–825.

[32] T. Tak, B. Hilvering, K. Tesselaar, L. Koenderman, Similar activation state of neutrophils in sputum of asthma patients irrespective of sputum eosinophilia, Clin. Exp. Immunol. 182 (2015) 204–212.

[33] H. Nagase, K. Kudo, S. Izumi, K. Ohta, N. Kobayashi, M. Yamaguchi, K. Matsushima, Y. Morita, K. Yamamoto, K. Hirai, Chemokine receptor expression profile of eosinophils at inflamed tissue sites: Decreased CCR3 and increased CXCR4 expression by lung eosinophils, J. Allergy Clin. Immunol. 108 (4) (2001) 563–569.

[34] L.Y. Liu, J.B. Sedgwick, M.E. Bates, R.F. Vrtis, J.E. Gern, H. Kita, N.N. Jarjour, W. W. Busse, E.A. Kelly, Decreased expression of membrane IL-5 receptor alpha on human eosinophils: II. IL-5 down-modulates its receptor via a proteinase-mediated process, J. Immunol. 169 (2002) 6459–6466.

[35] P.C. Fulkerson, Transcription Factors in Eosinophil Development and As Therapeutic Targets, Front. Med. (Lausanne) 4 (2017) 115.

[36] P.F. Weller, L.A. Spencer, Functions of tissue-resident eosinophils, Nat. Rev. Immunol. 17 (12) (2017) 746–760.

[37] N. Geijsen, L. Koenderman, P. Coffer, Specificity in cytokine signal transduction: lessons learned from the IL-3/IL-5/GM-CSF receptor family, Cytokine Growth Factor Rev. 12 (1) (2001) 19–25.

[38] C.C. Lin, C.S. Pan, C.Y. Wang, S.W. Liu, L.D. Hsiao, C.M. Yang, Tumor necrosis factor-alpha induces VCAM-1-mediated inflammation via c-Src-dependent transactivation of EGF receptors in human cardiac fibroblasts, J. Biomed. Sci. 22 (2015) 53.

[39] T.K. Kim, C.S. Park, H.J. Na, K. Lee, A. Yoon, J. Chung, S. Lee, Ig-like domain 6 of VCAM-1 is a potential therapeutic target in TNFalpha-induced angiogenesis, Exp. Mol. Med. 49 (2017) e294.

[40] R.P. Schleimer, S.A. Sterbinsky, J. Kaiser, C.A. Bickel, D.A. Klunk, K. Tomioka, W. Newman, F.W. Luscinskas, M.A. Gimbrone Jr., B.W. McIntyre, B.S. Bochner, IL-4 induces adherence of human eosinophils and basophils but not neutrophils to endothelium. Association with expression of VCAM-1, J. Immunol. 148 (1992) 1086–1092.

[41] D.F. Gudbjartsson, U.S. Bjornsdottir, E. Halapi, A. Helgadottir, P. Sulem, G. M. Jonsdottir, G. Thorleifsson, H. Helgadottir, V. Steinthorsdottir, H. Stefansson, C. Williams, J. Hui, J. Beilby, N.M. Warrington, A. James, L.J. Palmer, G. H. Koppelman, A. Heinzmann, M. Krueger, H.M. Boezen, A. Wheatley, J. Altmuller, H.D. Shin, S.-T. Uh, H.S. Cheong, B. Jonsdottir, D. Gislason, C.-S. Park, L. M. Rasmussen, C. Porsbjerg, J.W. Hansen, V. Backer, T. Werge, C. Janson, U.-B. Jönsson, M.C.Y. Ng, J. Chan, W.Y. So, R. Ma, S.H. Shah, C.B. Granger, A. A. Quyyumi, A.I. Levey, V. Vaccarino, M.P. Reilly, D.J. Rader, M.J.A. Williams, A. M. van Rij, G.T. Jones, E. Trabetti, G. Malerba, P.F. Pignatti, A. Boner, L. Pescollderungg, D. Girelli, O. Olivieri, N. Martinelli, B.R. Ludviksson, D. Ludviksdottir, G.I. Eyjolfsson, D. Arnar, G. Thorgeirsson, K. Deichmann, P. J. Thompson, M. Wjst, I.P. Hall, D.S. Postma, T. Gislason, J. Gulcher, A. Kong, I. Jonsdottir, U. Thorsteinsdottir, K. Stefansson, Sequence variants affecting eosinophil numbers associate with asthma and myocardial infarction, Nat. Publ. Group 41 (3) (2009) 342–347.

[42] S. Saglani, C.M. Lloyd, Novel concepts in airway inflammation and remodelling in asthma, Eur. Respir. J. 46 (6) (2015) 1796–1804.

[43] S.E. Wenzel, Asthma phenotypes: the evolution from clinical to molecular approaches, Nat. Med. 18 (5) (2012) 716–725.

[44] J.Y. Bonnefoy, S. Lecoanet-Henchoz, J.P. Aubry, J.F. Gauchat, P. Graber, CD23 and B-cell activation, Curr. Opin. Immunol. 7 (1995) 355–359.

[45] R. Selb, J. Eckl-Dorna, A. Neunkirchner, K. Schmetterer, K. Marth, J. Gamper, B. Jahn-Schmid, W.F. Pickl, R. Valenta, V. Niederberger, CD23 surface density on B cells is associated with IgE levels and determines IgE-facilitated allergen uptake, as well as activation of allergen-specific T cells, J. Allergy Clin. Immunol. 139 (290–299) (2017) e294.

[46] J. Punnonen, G. Aversa, B.G. Cocks, A.N. McKenzie, S. Menon, G. Zurawski, R. de Waal Malefyt, J.E. de Vries, Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells, PNAS 90 (8) (1993) 3730–3734.

[47] F.L. van der Heijden, R.J. Joost van Neerven, M. van Katwijk, J.D. Bos, M. L. Kapsenberg, Serum-IgE-facilitated allergen presentation in atopic disease, J. Immunol. 150 (1993) 3643–3650.

[48] Y. Endo, K. Hirahara, R. Yagi, D.J. Tumes, T. Nakayama, Pathogenic memory type Th2 cells in allergic inflammation, Trends Immunol. 35 (2) (2014) 69–78.

[49] M. Löhning, A. Stroehmann, A.J. Coyle, J.L. Grogan, S. Lin, J.-C. Gutierrez-Ramos, D. Levinson, A. Radbruch, T. Kamradt, T1/ST2 is preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function, PNAS 95 (12) (1998) 6930–6935.

[50] Calise J, Garabatos N, Bajzik V, Farrington M, Robinson D, Jeong D, Londei M, Wambre E. Optimal human pathogenic TH2 cell effector function requires local epithelial cytokine signaling. *J Allergy Clin Immunol* 2021; 148: 867-875 e864.

[51] S.N. Georas, F. Rezaee, Epithelial barrier function: at the front line of asthma immunology and allergic airway inflammation, J. Allergy Clin. Immunol. 134 (3) (2014) 509–520.

[52] J.D. Hamilton, S. Harel, B.N. Swanson, W. Brian, Z. Chen, M.S. Rice, N. Amin, M. Ardeleanu, A. Radin, B. Shumel, M. Ruddy, N. Patel, G. Pirozzi, L. Mannent, N. M.H. Graham, Dupilumab suppresses type 2 inflammatory biomarkers across multiple atopic, allergic diseases, Clin. Exp. Allergy 51 (7) (2021) 915–931.

[53] F. Bucher, J. Lee, S. Shin, M.S. Kim, Y.S. Oh, S. Ha, H. Zhang, K. Yea, Interleukin-5 suppresses Vascular Endothelial Growth Factor-induced angiogenesis through STAT5 signaling, Cytokine 110 (2018) 397–403.

[54] S.L. Park, T.W. Chung, S. Kim, B. Hwang, J.M. Kim, H.M. Lee, H.J. Cha, Y. Seo, S. Y. Choe, K.T. Ha, G. Kim, S.J. Yun, S.S. Park, Y.H. Choi, B.K. Kim, W.T. Kim, E. J. Cha, C. Patterson, W.J. Kim, S.K. Moon, HSP70-1 is required for interleukin-5-induced angiogenic responses through eNOS pathway, Sci. Rep. 7 (2017) 44687.

[55] S. Shahabuddin, P. Ponath, R.P. Schleimer, Migration of eosinophils across endothelial cell monolayers: interactions among IL-5, endothelial-activating cytokines, and C-C chemokines, J. Immunol. 164 (7) (2000) 3847–3854.

[56] M. Godar, K. Deswarte, K. Vergote, M. Saunders, H. de Haard, H. Hammad, C. Blanchetot, B.N. Lambrecht, A bispecific antibody strategy to target multiple type 2 cytokines in asthma, J. Allergy Clin. Immunol. 142 (1185–1193) (2018) e1184.

[57] A.A. Humbles, C.M. Lloyd, S.J. McMillan, D.S. Friend, G. Xanthou, E.E. McKenna, S. Ghiran, N.P. Gerard, C. Yu, S.H. Orkin, C. Gerard, A critical role for eosinophils in allergic airways remodeling, Science 305 (2004) 1776–1779.

[58] D.I. Blyth, T.F. Wharton, M.S. Pedrick, T.J. Savage, S. Sanjar, Airway subepithelial fibrosis in a murine model of atopic asthma: suppression by dexamethasone or anti-interleukin-5 antibody, Am. J. Respir. Cell Mol. Biol. 23 (2000) 241–246.

[59] P.W. Hellings, B. Steelant, Epithelial barriers in allergy and asthma, J. Allergy Clin. Immunol. 145 (2020) 1499–1509.

[60] J. Lyles, M. Rothenberg, Role of genetics, environment, and their interactions in the pathogenesis of eosinophilic esophagitis, Curr. Opin. Immunol. 60 (2019) 46–53.

[61] L. Petecchia, F. Sabatini, C. Usai, E. Caci, L. Varesio, G.A. Rossi, Cytokines induce tight junction disassembly in airway cells via an EGFR-dependent MAPK/ERK1/2-pathway, Lab. Invest. 92 (2012) 1140–1148.

[62] H. Yuksel, M. Ocalan, O. Yilmaz, E-Cadherin: An Important Functional Molecule at Respiratory Barrier Between Defence and Dysfunction, Front. Physiol. 12 (2021), 720227.

[63] E.J. Swindle, J.E. Collins, D.E. Davies, Breakdown in epithelial barrier function in patients with asthma: identification of novel therapeutic approaches, J. Allergy Clin. Immunol. 124 (2009) 23–34, quiz 35-26.

[64] S.M. Warner, D.A. Knight, Airway modeling and remodeling in the pathogenesis of asthma, Curr. Opin. Allergy Clin. Immunol. 8 (2008) 44–48.
[65] K. Sweerus, M. Lachowicz-Scroggins, E. Gordon, M. LaFemina, X. Huang, M. Parikh, C. Kanegai, J.V. Fahy, J.A. Frank, Claudin-18 deficiency is associated with airway epithelial barrier dysfunction and asthma, J. Allergy Clin. Immunol. 139 (72–81) (2017) e71.
[66] M. Avdalovic, Pulmonary vasculature and critical asthma syndromes: a comprehensive review, Clin. Rev. Allergy Immunol. 48 (2015) 97–103.
[67] B.N. Feltis, D. Wignarajah, L. Zheng, C. Ward, D. Reid, R. Harding, E.H. Walters, Increased vascular endothelial growth factor and receptors: relationship to angiogenesis in asthma, Am. J. Respir. Crit. Care Med. 173 (2006) 1201–1207.
[68] C.M. Lilly, H. Tateno, T. Oguma, E. Israel, L.A. Sonna, Effects of allergen challenge on airway epithelial cell gene expression, Am. J. Respir. Crit. Care Med. 171 (2005) 579–586.
[69] L.F. Loffredo, H. Abdala-Valencia, K.R. Anekalla, L. Cuervo-Pardo, C.J. Gottardi, S. Berdnikovs, Beyond epithelial-to-mesenchymal transition: Common suppression of differentiation programs underlies epithelial barrier dysfunction in mild, moderate, and severe asthma, Allergy 72 (2017) 1988–2004.
[70] X. Chen, M. Miao, M. Zhou, J. Chen, D. Li, L. Zhang, A. Sun, M. Guan, Z. Wang, P. Liu, S. Zhang, X. Zha, X. Fan, Poly-L-arginine promotes asthma angiogenesis through induction of FGFBP1 in airway epithelial cells via activation of the mTORC1-STAT3 pathway, Cell Death Dis. 12 (2021) 761.
[71] A. Subramanian, P. Tamayo, V.K. Mootha, S. Mukherjee, B.L. Ebert, M.A. Gillette, A. Paulovich, S.L. Pomeroy, T.R. Golub, E.S. Lander, J.P. Mesirov, Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles, Proc. Nati. Acad. Sci. USA 102 (2005) 15545–15550.
[72] S. Asrat, N. Kaur, X. Liu, L.H. Ben, D. Kajimura, A.J. Murphy, M.A. Sleeman, A. Limnander, J.M. Orengo, Chronic allergen exposure drives accumulation of long-lived IgE plasma cells in the bone marrow, giving rise to serological memory, Sci. Immunol. (2020) 5.
[73] M.I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2, Genome Biol. 15 (2014) 550.