Dual blockade of IL-4 and IL-13 with dupilumab, an IL-4Rα antibody, is required to broadly inhibit type 2 inflammation

Audrey Le Floc’h | Jeanne Allinne | Kirsten Nagashima | George Scott | Dylan Birchard | Seblewongel Asrat | Yu Bai | Wei Keat Lim | Joel Martin | Tammy Huang | Terra B. Potocky | Jee H. Kim | Ashique Rafique | Nicholas J. Papadopoulos | Neil Stahl | George D. Yancopoulos | Andrew J. Murphy | Matthew A. Sleeman | Jamie M. Orengo

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Abstract

Background: Dupilumab, a fully human monoclonal antibody that binds IL-4Rα and inhibits signaling of both IL-4 and IL-13, has shown efficacy across multiple diseases with underlying type 2 signatures and is approved for treatment of asthma, atopic dermatitis, and chronic sinusitis with nasal polyposis. We sought to provide a comprehensive analysis of the redundant and distinct roles of IL-4 and IL-13 in type 2 inflammation and report dupilumab mechanisms of action.

Methods: Using primary cell assays and a mouse model of house dust mite–induced asthma, we compared IL-4 vs IL-13 vs IL-4Rα blockers.

Results: Intranasal administration of either IL-4 or IL-13 confers an asthma-like phenotype in mice by inducing immune cell lung infiltration, including eosinophils, increasing cytokine/chemokine expression and mucus production, thus demonstrating redundant functions of these cytokines. We further teased out their respective contributions using human in vitro culture systems. Then, in a mouse asthma model by comparing in head-to-head studies, either IL-4 or IL-13 inhibition to dual IL-4/IL-13 inhibition, we demonstrate that blockade of both IL-4 and IL-13 is required to broadly block type 2 inflammation, which translates to protection from allergen-induced lung function impairment. Notably, only dual IL-4/IL-13 blockade prevented eosinophil infiltration into lung tissue without affecting circulating eosinophils, demonstrating that tissue, but not circulating eosinophils, contributes to disease pathology.

Abbreviations: Ab, antibody; AD, atopic dermatitis; AHR, airway hyperresponsiveness; APC, antigen-presenting cell; DC, dendritic cell; EdE, Eosinophilic esophagitis; FEV, forced expiratory volume; GCM, goblet cell metaplasia; HDM, house dust mite; HUVEC, human umbilical vein endothelial cell; IS, immunological synapse; KC/GRO, keratinocyte chemoattractant/human growth-regulated oncogene; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; moDC, monocyte-derived dendritic cell; NGS, next-generation sequencing; qPCR, quantitative polymerase chain reaction; RLU, relative luminescence unit; SEB, Staphylococcus enterotoxin B; TARC, thymus and activation-regulated chemokine; Th2 cell, T helper 2 cell.
**INTRODUCTION**

Type 2 inflammation has emerged as a unifying feature of both classically defined allergic diseases and a range of other inflammatory disorders, such as atopic dermatitis (AD) and asthma. This specialized immune response promotes barrier immunity on mucosal surfaces and includes activity of the cytokines IL-4, IL-5, IL-13, and IL-9. Given the pleiotropic roles of IL-4 and IL-13 in orchestrating type 2 responses, as well as their genetic association and increased expression in type 2 diseases, a number of therapeutic molecules targeting this pathway were evaluated in clinical trials for severe asthma over the last 20 years and failed to robustly demonstrate clinical efficacy. Renewed appreciation for the essential role of the two cytokines, IL-4 and IL-13, in driving type 2 disease has been resurrected based on clinical findings with dupilumab, a potent fully human IgG4-based monoclonal antibody specific for IL-4Rα. By targeting the shared receptor subunit IL-4Rα, dupilumab blocks both IL-4 and IL-13 signaling and has shown robust clinical efficacy across multiple diseases with underlying type 2 signatures, perhaps supporting the idea that potent, dual cytokine blockade is required for efficacy and emphasizing important contributions of each in disease pathologies.

**Conclusions:** Overall, these data support IL-4 and IL-13 as key drivers of type 2 inflammation and help provide insight into the therapeutic mechanism of dupilumab, a dual IL-4/IL-13 blocker, in multiple type 2 diseases.

**KEYWORDS**
cytokines, dupilumab, IL-13, IL-4, type 2 inflammation

**GRAPHICAL ABSTRACT**
The IL-4Rα antibody, dupilumab, binds IL-4Rα with high affinity, directly blocks IL-4 and IL-13/IL-13Rα1 complex binding to IL-4Rα, and thereby prevents IL-4Rα-mediated signaling induced by both IL-4 and IL-13. IL-4 and IL-13 play distinct and overlapping roles and blockade of both cytokines is required to broadly block type 2 inflammation, which translates to protection from allergen-induced lung function impairment. Only dual IL-4/IL-13 blockade with dupilumab prevents eosinophil infiltration into lung tissue without affecting circulating eosinophils, demonstrating that tissue, but not circulating eosinophils contribute to disease pathology.
and limited efforts have been put toward comprehensively delineating the exact role each cytokine plays in type 2 diseases.

In this study, we sought to further define how IL-4 and IL-13 cooperatively and independently contribute to type 2 disease pathology and report dupilumab mechanisms of action. We show that intranasal administration of either human IL-4 or IL-13 confers an asthma-like phenotype in mice highlighting redundancy. Then, using an animal model of allergic asthma and a head-to-head comparison of ligand blockers vs the dual IL-4/IL-13 receptor blocker, we show that IL-4 blockade potently prevents B-cell activation, IgE production, FcεRI-expressing innate cell priming, and pathogenic T-cell lung infiltration, whereas inhibition of IL-13 is required to prevent goblet cell metaplasia (GCM). However, dual IL-4/IL-13 blockade with dupilumab is required to potently block Th2 cell–induced antigen-presenting cell activation in the context of Th2 cell/APC interaction in vitro, as well as lung eosinophilia and expression of inflammatory cytokines and chemokines in the lungs in vivo. Overall, we demonstrate that dual blockade of IL-4 and IL-13 is required to broadly block type 2 inflammation, which translates to protection from allergen-induced lung function impairment in this model. These results help interpret some of the clinical benefits seen with the dual IL-4/IL-13 blocker dupilumab in the settings of multiple human type 2 diseases and provide insight into why blocking just one of these sister cytokines did not provide robust clinical benefit.24,25

2 | MATERIALS AND METHODS

2.1 | Antibody generation and binding measurements

Dupilumab was generated using the VelocImmune® technology.67,68 Biacore assays were performed to determine the binding kinetics for its interaction with human IL-4Rα, and its ability to block IL-4 and IL-13/IL-13Ra1.hFc complex binding to IL-4Ra. In vitro comparison of blocking properties, we also generated two IgG4-based comparator antibodies based on the published sequences of the humanized IL-4 antibody clone 8D4-8 variant 69 and lebrikizumab, a humanized IL-13 IgG4 monoclonal antibody (Roche).70 For in vivo mouse studies, the Regeneron-produced recombinant mouse IL-13Ra2-Fc fusion protein, which prevents binding and signaling of IL-13 via the Type 2 receptor, corresponds to the ectodomain of mouse IL-13Ra2 (amino acids E23-S333; NP_032382). The protein was produced in Chinese hamster ovary cells with a dimeric (mouse IgG2a Fc [mFc]) C-terminal tag.

2.2 | In vitro assays

The effect of recombinant in house generated IL-4 and IL-13 on signaling in Ramos.2G6,4C10/STAT3/Luc cells and HEK293/STAT6/Luc cells was quantified by measuring luminescence. The ability of IL-4 and IL-13 (R&D Systems) to mediate TARC release from eosinophils from healthy donors (BioreclamationIVT) was determined by ELISA (R&D Systems). Cell surface FccRιx expression on in vitro-generated human mast cells was determined by flow cytometry. Analysis of the effect of IL-4 and IL-13 on gene expression in mast cells upon IgE crosslinking was done by real-time qPCR and next-generation sequencing (NGS). CCL26, CCL2, and IL-6 release from human umbilical vein endothelial cells (HUVEC; Lonza) was determined by MSD multiplex. Human IL-4 and IL-13 were purchased from R&D Systems. For coculture assays, in vitro-generated human Th2 cells were incubated with human B cells (T cell:APC ratio = 2:1) or human moDCs (T cell:APC ratio = 8:1) in the presence of 1 ng/mL SEB superantigen (Toxin Technology). B-cell surface CD23 expression and cytokine secretion were assessed by flow cytometry and MSD multiplex, respectively.

2.3 | Mouse models of IL-4/IL-13 and allergen-induced lung inflammation model

Il4ra^hu/hu Il4 hu/hu mice (68.75% C57BL/6NTac 31.25% 129S6/SvEvTac), generated using VelociGene® technology, were exposed intranasally to either 10 µg human IL-4 or IL-13 (produced in house) for 12 days, or 50 µg house dust mite (HDM; Greer) 3 times per week for 4 consecutive weeks. HDM-exposed mice either received no antibody treatment, or twice-weekly subcutaneous injections of 10 or 25 mg/kg IL-4Ra Ab (dupilumab), IL-4 Ab, mouse IL-13Ra2-Fc or a corresponding isotype control antibody (human IgG4 and mouse IgG2a) starting 3 days before the first HDM exposure (or the day following the first HDM exposure for FlexiVent® experiments). At the end of the studies, mice were either killed, and lungs and/or spleens were harvested for RNA expression profiling of chemokines and type 2 cytokines (real-time qPCR and NGS), flow analysis of immune cell infiltrate by flow cytometry, histology analysis (PAS staining), or subjected to lung function testing using a FlexiVent® instrument (72-100 hours after final HDM exposure).

For flow cytometric analysis of circulating lung vs lung tissue inflammatory cell infiltrates, mice were injected intravenously with anti-CD45-BV650 antibody 5 minutes prior to kill to label immune cells that have infiltrated the lungs. Blood was also collected for determination of serum concentrations of total IgE and HDM-specific IgG1. All animal experiments were performed in accordance with the Guidelines for the Institutional Animal Care and Use Committee at Regeneron Pharmaceuticals, Inc.

2.4 | 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 $P < .05$.

3 | RESULTS

3.1 Both IL-4 and IL-13 induce lung inflammation

To separate the contribution of human IL-4 and IL-13 in inducing airway inflammation in vivo, each cytokine was delivered intranasally to $Il4ra^{hu/hu}$ $Il4^{hu/hu}$ mice (Figure 1A). These mice were genetically modified by both the endogenous mouse IL-4 and the
The IL-4Rα antibody dupilumab blocks human IL-4 and IL-13/IL-13Rα1 complex binding to IL-4Rα and prevents IL-4 and IL-13 signaling through Type I and Type II receptors. A, Type I signaling: Ramos.2G6.4C10/STAT3/Luciferase cells were treated with increasing concentrations of IL-4, or serial dilutions of IL-4Rα Ab, IL-4 Ab, or an isotype control antibody (ctrl IgG) in the presence of 300 pmol/L IL-4. B and C, Type II signaling: HEK293/STAT6/Luciferase cells were treated with increasing concentrations of IL-4 (B), IL-13 (C), or serial dilutions of IL-4Rα Ab, IL-4 Ab, IL-13 Ab, or ctrl IgG in the presence of 10 pmol/L IL-4 (B) or 25 pmol/L IL-13 (C). Inhibition of IL-4- or IL-13-mediated signaling was quantified by measuring luminescence (RLU). Error bars represent SD from samples run in triplicate. Black arrows indicate cytokine concentrations used for the corresponding blocking assays. IC50 is shown. Data are representative of at least two independent experiments. D, Schematic diagram representing APC-Th2 coculture cell interactions in the presence of SEB. E, Cocultures of B cells with Th2 cells in the presence of SEB were coincubated with serial dilutions of IL-4Rα Ab, IL-4 Ab, IL-13 Ab, or control antibody (ctrl IgG) for 2 d. Inhibition of CD23 induction at the surface of B cells was monitored by flow cytometry. F, Cocultures of moDCs with Th2 cells in the presence of SEB were coincubated with serial dilutions of IL-4Rα Ab, IL-4 Ab, IL-13 Ab, or ctrl IgG for 2 d. Inhibition of IL-12p70 release from moDCs was determined by MSD. Error bars represent SD from samples run in duplicate. All data are representative of at least three independent experiments using different donors. G, To assess the ability of IL-4Rα Ab to block IL-4 and IL-13/IL-13Rα1 binding to IL-4Rα, human IL-4, human IL-13, human IL-13Rα1.hFc, or IL-13/IL-13Rα1.hFc complex was injected over human IL-4Rα surfaces that were prebound with IL-4Rα Ab or a control antibody (ctrl IgG). Graph presents surface-bound proteins in resonance units (RU). H, Schematic diagram showing dupilumab (IL-4Rα Ab) mechanism of action.
**FIGURE 3** IL-4 drives B-cell activation, class switching, and basophil priming in HDM-exposed mice. A, Il4ra^hu/hu Il4^hu/hu^ mice were exposed to saline or HDM for 4 weeks. Five groups of HDM-exposed mice received injections of an isotype control antibody (ctrl hIgG or ctrl mlG), IL-4Rα Ab, IL-4 Ab, or mouse IL-13Ra2-Fc. B and C, On day 30, serum concentrations of HDM-specific IgG1 (B) and total IgE (C) were determined by ELISA. No HDM-specific IgG1 antibodies were detectable in groups that were not exposed to HDM at the lowest dilution tested (1:100). Dotted line: lower limit of quantification. D, On day 30, mice were injected with anti-CD45 to label all circulating immune cells and killed after 5 min. Lungs were harvested and dissociated for flow cytometric analysis of circulating lung and lung tissue CD23^+ B cells. Circulating lung CD23^+ B cells were reported as frequency of circulating lung B cells (left panel), and lung tissue CD23^+ B cells were reported as frequency of lung tissue B cells (right panel). E and F, Analysis of IgG1^+ B cell (E) and IgE^+ plasma cell (F) frequencies in the spleen on day 30. IgG1^+ B cells and IgE^+ plasma cells were reported as frequency of live cells. G, Analysis of IgE surface expression on splenic basophils. Each symbol represents one mouse. n ≥ 5 mice per group. Saline (white boxes) or HDM (shaded boxes). †=P < .05; ††=P < .01; †††=P < .001 vs mice not exposed to HDM; #=P < .05; ##=P < .01; ###=P < .001 vs mice exposed to HDM; *=P < .05; **=P < .01; ***=P < .001 vs corresponding isotype group.
IL-4 dominates IgE-dependent human mast cell responses. A, In vitro differentiated human mast cells were treated for 2 d with increasing concentrations of IL-4 or IL-13, or serial dilutions of IL-4Rα Ab or a control (ctrl) IgG in the presence of 50 pmol/L IL-4 or 1 nmol/L IL-13, and FcεRIα surface expression was assessed by flow cytometry. Error bars represent SD from samples run in triplicate. Black arrows indicate cytokine concentrations used for the corresponding blocking assays. IC50 is shown. Data are representative of at least three independent experiments using different donors. B, Heatmap of cytokine- and chemokine-related genes upon IgE crosslinking (sensitization with two different Fel d 1-specific IgE followed by activation with Fel d 1 antigen) of human mast cells. 51 cytokine/chemokine-related genes were induced (FDR ≤ 0.05; |FC|≥2) by either IL-4, IL-13, Fel d 1-specific IgE (Fel d 1 IgE)+Fel d 1, IL-4 + Fel d 1 IgE + Fel d 1, or IL-13 + Fel d 1 IgE + Fel d 1. The color gradient represents fold-change values comparing each treatment to the control samples. C, mRNA expression levels measured by real-time qPCR and expressed relative to GAPDH mRNA expression; * = P < .05; ** = P < .01; *** = P < .001 between indicated conditions.

**FIGURE 4**
cells infiltrating the lung tissue (lung tissue immune cells; Figure S2A,B). Immune cell (CD45+) lung infiltration, and specifically CD4+ T-cell lung infiltration, was greatly increased by either IL-4 or IL-13 intranasal administration, with no effect of either cytokine observed on circulating immune cells (Figure 1B,C). IL-13 administration, and to a lesser extent IL-4, dramatically reduced the frequency of tissue alveolar macrophages, the most abundant cells in the alveolar spaces and conducting airways in healthy lungs and known to be involved in immune homeostasis in the respiratory tract (Figure 2D and Figure S2C). Similar to previous findings, we showed that both IL-4 and IL-13 significantly increased the recruitment of both neutrophils and eosinophils into the lungs (Figure 1E,F and Figure S2D,E).

Either IL-4 or IL-13 exposure resulted in elevated expression of the inflammatory cytokines IL6 and IL33, and the type 2 cytokines IL4, IL5, and IL13 (Figure 1G). Expression of chemokines involved in the recruitment of different subsets of immune cells was also increased upon IL-4 and IL-13 exposure: The eosinophil chemotactic proteins Ccl11 (eotaxin 1) and Ccl24 (eotaxin 2), the Th2 cell chemoattractant Ccl17 (TARC), the monocyte chemoattractant proteins Ccl2 (MCP-1) and Ccl9 (MIP-1), and the neutrophil chemotactant Cxcl1 (KC/GROα) were all increased in the lungs upon exposure to either cytokine. Consistent with previous publications, goblet cell metaplasia (GCM), that is, the increase in the number of mucus-producing cells, was also drastically increased in the lungs upon IL-13 exposure, and to a lesser extent upon IL-4 exposure (Figure 1H). These data highlight a role for both IL-4 and IL-13 in inducing an asthma phenotype, strongly suggesting that dual blockade of IL-4 and IL-13 is required to broadly prevent lung inflammation.

### 3.2 Dupilumab binds to IL-4Rα, inhibits both IL-4 and IL-13 signaling through Type I and Type II receptor complexes, and prevents IL-4/IL-13-induced signaling in Th2:APC interactions

Dupilumab binds to both monomeric and dimeric human IL-4Rα, with Kd values of 33pM and 12pM, respectively (Figure S3A-C). Blocking of IL-4 and IL-13-mediated signaling using IL-4 or IL-13 blocking antibodies, which bind with high affinity to their respective human ligands (Figure S3A), and IL-4Rα antibody was confirmed in vitro using cells endogenously expressing either the Type I receptor, consisting of the IL-4Ra and IL-2Rγ subunits (Ramos cells), specifically activated by IL-4 (Figure 2A); or the Type II receptor (HEK293 cells), consisting of IL-4Ra and IL-13Rα1 subunits and activated by both IL-4 and IL-13 (Figure 2B,C, respectively). Of note, dupilumab was as efficient as IL-4 and IL-13 blockers at inhibiting IL-4 and IL-13 signaling, respectively, in these assays. Reductions in TARC and IgE levels, two effector molecules implicated in type 2 diseases, were associated with dupilumab treatment in both asthma and AD patients. Here, we show that both IL-4 and IL-13 induced TARC release from human PBMCs and induction of the activation marker CD23 on B cells (Figure S4A-D), and that all these effects were efficiently blocked by either IL-4Ra antibody or the respective ligand blocker (Figure S4A-F).

Th2 cells are key effectors of type 2 inflammation and can be activated by direct interaction with antigen-presenting cells (APC), such as dendritic cells (DC) and B cells, through formation of an immunological synapse (IS). We hypothesized in the context of type 2 inflammation that IL-4 and IL-13 would be concomitantly secreted by Th2 cells and that IL-4Ra blockade may be needed to prevent Th2 cell-induced APC activation. B cells and monocyte-derived DC (moDC) expressed components of both Type I and Type II receptor complexes (IL-4Rx, IL-2Rγ, and IL-13Rα1 subunits) (Figure S5A and Figure S5B), suggesting responsiveness to both IL-4 and IL-13. As previously reported, Th2 cells only expressed the Type I receptor complex (Figure S5A and Figure S5B). We established an in vitro Th2 cell:APC system in which B cells or moDC were cocultured with Th2 cells in the presence of a superantigen (SEB) (Figure 2D and Figure S5C) to mimic an IS. Only in the presence of SEB did the coculture of Th2 cells with either B cells or moDC lead to robust secretion of both IL-4 and IL-13, demonstrating that cell:cell interaction is required for Th2 cell activation (Figure S5D and Figure S5E). Similarly, SEB was required in the coculture for activation of the B cells or the moDC, as revealed by upregulation of CD23 and release of IL-12p70, respectively, confirming the importance of robust Th2 cell:APC interaction for full APC activation (Figure S5F and Figure S5G). Strikingly, IL-4Ra blockade, but not IL-4 or IL-13 blockade, fully blocked CD23 upregulation on B cells (Figure 2E) and IL-12p70 secretion from moDC (Figure 2F), demonstrating that blocking both IL-4 and IL-13 signaling via the shared receptor subunit IL-4Ra efficiently blocks Th2 cell-mediated APC activation, where both cytokines are locally secreted. Finally, we demonstrated that dupilumab mechanistically prevented IL-4 and IL-13 signaling by blocking binding of both IL-4 and IL-13/IL-13Rα1 complex to IL-4Ra (Figure 2G).

Overall, these experiments show that the IL-4Ra antibody dupilumab binds with high affinity to the IL-4Rx subunit shared by the IL-4 and IL-13 signaling complexes, directly blocks IL-4 and IL-13/IL-13Rα1 complex binding to IL-4Rx, and thereby prevents IL-4Ra-mediated signaling induced by both IL-4 and IL-13 (Figure 2H). Also, in the context of a Th2 cell:APC interaction where IL-4 and IL-13 are concomitantly released, blocking IL-4Ra provides an advantage over blocking the individual ligands.

### 3.3 IL-4 drives B-cell activation and IgE-associated allergic inflammation

Respiratory exposure to HDM in mice recapitulates pathological features of type 2 inflammation observed in asthma and has been shown to significantly increase circulating IgE and HDM-specific IgG1. Having established the effects of IL-4/IL-13 inhibition with dupilumab on B-cell activation in vitro, we first examined the impact of IL-4, IL-13, and dual IL-4/IL-13 inhibition on B-cell activation, class switching, and IgE-associated inflammation in a HDM-induced asthma model (Figure 3A). To specifically block mouse IL-13 signaling
in \(il4ra^{hu/hi}\) mice exposed to HDM, we used a soluble IL-13Ra2-Fc fusion protein,\(^{29}\) since the IL-13 antibody does not bind to mouse IL-13.

HDM exposure in \(il4ra^{hu/hi}\) mice induced B-cell activation and class switching, which translated to increased circulating HDM-specific IgG1 and total IgE levels (Figure 3B,C). Dual IL-4/IL-13 blockade with an IL-4R\(\alpha\) antibody as well as IL-4 blockade alone (IL-4 antibody), but not IL-13 inhibition alone (soluble IL-13Ra2-Fc fusion protein), effectively blunted HDM-specific IgG1 and total IgE production (Figure 3B,C), demonstrating the major role of IL-4 in IgG1 and IgE production. After HDM exposure, the frequency of activated CD23\(^+\) B cells increased in both the lung circulation and lung tissue (Figures S2B, S6A and 3D). Dual IL-4/IL-13 blockade and IL-4 blockade, but not IL-13 inhibition, prevented this increase in both compartments. Similarly, both IL-4 blockade and dual IL-4/IL-13 blockade, but not IL-13 inhibition, prevented the HDM-induced increase of splenic IgG1\(^+\) B-cell and IgE\(^+\) plasma cell frequencies (Figure 3E,F; Figure S6B).\(^{41}\) Together, these data demonstrate that IL-4, but not IL-13, drives the activation of both circulating and lung tissue B cells, as well as isotype class switching to IgG1 and IgE.

Binding of antigen-specific IgE to the high affinity IgE receptor Fc\(\epsilon\)RI on basophils and mast cells is fundamental to the initiation and propagation of IgE-associated allergic responses.\(^{52}\) HDM exposure led to an increase in IgE-bound surface Fc\(\epsilon\)RI expression on splenic basophils, as measured by surface IgE levels, and this was prevented by
IL-4 and dual IL-4/IL-13 blockades, but not IL-13 inhibition (Figure 3G and Figure S6C). Together with basophils, mast cells are the main effectors of allergic inflammation by binding IgE through FcεRI and both IL-4 and IL-13 have been reported to directly upregulate FcεRI expression at their surface.⁴³,⁴⁴ In vitro stimulation of human bone marrow–derived mast cells revealed that IL-4, and IL-13 marginally (which correlated with low IL-13Rxm1 mRNA expression, Figure S7A), increased FcεRI expression at the surface of mast cells even in the absence of IgE, which was completely blocked by IL-4Rx antibody (Figure 4A). This confirms that IL-4, and to a lesser extent IL-13, can directly affect FcεRI expression, potentially priming these cells for allergen-induced activation. We further investigated the role of these two cytokines on IgE-dependent mast cell activation and found that pretreatment with either IL-4 or IL-13 induced minimal but notable expression of genes in mast cells (Figure 4B). Consistent with the surface expression observation, IL-4, and marginally IL-13, induced FCER1A expression, which was confirmed by qPCR (Figure 4C).Remarkably, these two cytokines also induced the expression of genes involved in chemotaxis, such as CCL18 (MIP-4) and the eotaxins CCL24 and CCL26. To look at IgE-dependent mast cell activation, two different Fel d 1-specific IgE were used as the IgE source to sensitize mast cells, with Fel d 1 as the allergen to induce IgE crosslinking. While IgE crosslinking in the absence of cytokine pretreatment led to induction of several chemokine- and cytokine-related genes, mast cell priming with either IL-4 or IL-13 altered IgE-dependent mast cell activation by inducing more cytokine/chemokine genes (Figure 4B). qPCR confirmed that pretreatment with IL-4, and to some extent IL-13, induced IgE-dependent expression of a panel of cytokines, including the type 2 cytokines IL5 and IL13, and the proinflammatory cytokine TNF (Figure 4C). Interestingly, pretreatment with IL-4, and marginally IL-13, also induced IgE-dependent expression of other cytokines including the mast cell growth factor IL9, thymic stromal lymphopoietin (TSLP), and the pruritus-inducing cytokine IL31 (Figure 4B,C). Overall, IL-4 was more potent at inducing inflammatory gene expression alone or in combination with IgE crosslinking compared to IL-13, demonstrating a more dominant role for IL-4 on mast cell activation and priming.

In summary, these data highlight a major role for IL-4 in driving IgE-associated allergic inflammation both locally and systemically, by inducing B-cell activation, isotype class switching to IgG1 and IgE, and priming of FcεRI-expressing innate cells by inducing immune genes involved in type 2/inflammatory diseases.

### 3.4 Dual IL-4/IL-13 blockade is required to fully prevent infiltration of pathogenic cells at the site of inflammation

HDM exposure significantly reduced the frequency of alveolar macrophages in the lung tissue, and dual IL-4/IL-13 inhibition using dupilumab, as well as either IL-4 or IL-13 inhibition, prevented this decrease (Figure S8A), suggesting that both IL-4 and IL-13 participate in disrupting alveolar integrity and that blocking this pathway could preserve homeostasis in the respiratory tract. Pathogenic CD4⁺ T cells are associated with allergic disorders and represent a subset of tissue-resident pathogenic memory Th2 cells that express the IL-33 receptor, ST2, and release high amounts of IL-5 and IL-13.⁴⁵-⁴⁷ HDM exposure significantly increased the frequency of ST2⁺ CD4⁺ T cells in the lung tissue as observed by flow cytometry, with a trend toward an increase in the lung circulation (Figure S8B) that was only inhibited by both IL-4 blockade and dual IL-4/IL-13 blockade, but not IL-13 inhibition (Figure S8B).

HDM exposure significantly increased lung tissue eosinophil frequency, with no effect on circulating lung eosinophils (Figure 5A and Figure S2A). Since terminal differentiation of eosinophils occurs in the bone marrow, this tissue increase is likely due to massive eosinophil recruitment into the lungs. Strikingly, dual IL-4/IL-13 blockade was required to fully prevent lung eosinophil infiltration of HDM-exposed mice, as either IL-4 or IL-13 inhibition alone showed trends toward reduced eosinophil infiltration (Figure 5A). Of note, dual IL-4/IL-13 blockade did not impact eosinophils in the bone marrow (Figure S9), suggesting that generation of eosinophils or their egress from the bone marrow is not affected. It is noteworthy that lung tissue eosinophils in HDM-exposed mice expressed higher CD11c at their surface compared to circulating lung eosinophils (Figure 5A, plots), and this has been previously associated with an activated phenotype.⁴⁸ Thus, we next examined whether IL-4 and IL-13 could

### FIGURE 6 Dual IL-4/IL-13 blockade broadly inhibits chemokine and type 2 proinflammatory cytokine expression in the lungs of HDM-exposed mice. A. Volcano plot of HDM signature. After 4 wk of HDM exposure, significant changes were detected in 873 transcripts that were differentially expressed in the lungs of HDM-exposed mice compared with the lungs of mice not exposed to HDM (adjusted P-value < .05; [fold change]≥2). B and D, Venn diagrams of HDM signature vs IL-4Rx Ab (B), IL-4 Ab (C), and mIL-13Rx2-Fc (D) signatures (adjusted P-value < .05; [fold change]≥2). Five groups of HDM-exposed mice received injections of an isotype control antibody (ctrl hlgG or ctrl mlgG), IL-4Rx Ab, IL-4 Ab, or mouse IL-13Rx2-Fc. Treatment signatures correspond to statistically significant changes in gene expression compared to mice receiving corresponding ctrl IgG. †, treatment-responsive genes inversely differentially regulated in the HDM signature. E, Heatmap of cytokine/chemokine-related genes. 29 cytokine/chemokine-related genes from the HDM signature were inversely regulated by IL-4Rx Ab, IL-4 Ab, or mouse IL-13Rx2-Fc. The color gradient represents z-score of the gene expression values in various treatment and control samples. F, IL4Rx/hu/IL13Rx/hu mice were exposed to saline (white boxes) or HDM (shaded boxes) for 4 wk. Five groups of HDM-exposed mice received injections of an isotype control antibody (ctrl hlgG or ctrl mlgG), IL-4Rx Ab, IL-4 Ab, or mouse IL-13Rx2-Fc. At the end of the study, lung tissue mRNA expression levels were measured by real-time qPCR and are expressed relative to β-actin (ACTB) mRNA expression. Each symbol represents one mouse. n ≥ 5 mice per group. ††P < .01 vs corresponding isotype group.
(E) Cytokine and chemokine gene heatmap

(F) Lung tissue mRNA levels of inflammatory cytokines and chemokines

(G) Effect of IL-4 and IL-13 on endothelial cell activity
directly affect eosinophil activation by measuring TARC secretion upon cytokine stimulation in vitro.\(^{49}\) Human eosinophils express the components of both Type I and Type II receptors (IL-4R\(\alpha\), IL-2R\(\gamma\), and IL-13R\(\alpha_1\)) (Figure S7B). Both IL-4 and IL-13 induced TARC release from human eosinophils, which was prevented by IL-4R\(\alpha\) antibody treatment (Figure 5B). Therefore, upon IL-4/IL-13 exposure, eosino-

3.5 | Dual IL-4/IL-13 blockade broadly prevents chemokine and type 2 proinflammatory cytokine expression in the lungs

The “HDM signature” was defined as transcripts differentially expressed in the lungs of HDM-exposed mice compared with the lungs of unexposed mice (873 genes, Figure 6A). Dual IL-4/IL-13 blockade, IL-4 blockade, and IL-13 inhibition resulted in 357, 155, and 406 statistically significant changes in gene expression, respectively, compared to mice receiving a corresponding control antibody. Interestingly, among those treatment-responsive genes, 86% (308 genes), 63% (97), and 74% (301), for dual IL-4/IL-13 blockade, IL-4 blockade, and IL-13 inhibition, respectively, were inversely differentially regulated in the HDM signature (Figure 6B-D). Notably, when further examined by looking specifically at chemokine- and cytokine-related genes (Figure 6E), HDM exposure resulted in elevated expression of the proinflammatory cytokines Il6, Il13 and Il11, and the type 2 cytokines Il4 and Il13 in the lungs. Of note, the Il5 gene did not pass the QC filter in this experiment and was excluded from differential expression analysis. Lung expression of chemokines involved in the recruitment of immune cell subsets was also increased upon HDM exposure. The eosinophil chemotact-

3.6 | Dual IL-4/IL-13 blockade prevents HDM-induced impairment of lung function

HDM exposure increased GCM in the lung (Figure 7A), and this was strongly reduced by dual IL-4/IL-13 blockade (Figure 7B). While IL-4 blockade has no significant effect on GCM, IL-13 inhibition...
prevented GCM as efficiently as dual IL-4/IL-13 blockade, highlighting IL-13 as the key driver of GCM.

Impaired lung function and airway hyperresponsiveness (AHR) are defining features of asthma. Airway narrowing due to mucus hypersecretion and other structural and immunological changes results in impaired lung function, which can be measured as a decline in FEV$_1$ in humans, or FEV$_{0.1}$ in mice. HDM exposure caused a significant decline in FEV$_{0.1}$, indicating reduced lung function (Figure 7C,D). Notably, HDM-exposed mice treated with dupilumab exhibited an FEV$_{0.1}$ similar to mice not exposed to HDM (Figure 7D), showing that dupilumab prevented the decline in lung function induced by HDM exposure. Finally, AHR was assessed by measuring FEV$_{0.1}$ in response to increasing doses of inhaled methacholine. Dupilumab efficiently protected mice against HDM-induced AHR, with dupilumab-treated mice showing no significant response to increasing doses of methacholine, similarly to mice not exposed to HDM (Figure 7E). Overall, these experiments demonstrate that dupilumab effectively protects from allergen-induced lung function impairment.
by preventing pathogenic immune cell-induced lung inflammation as well as IL-13-driven GCM (ie, mucus production).

4 | DISCUSSION

Here, we provide a comprehensive analysis of how the type 2 cytokines IL-4 and IL-13 contribute to disease pathology by driving distinct and overlapping effects. Administration of either IL-4 or IL-13 in mice causes lung inflammation by increasing immune cell infiltration, including eosinophils, increasing lung cytokine/chemokine expression and GCM, thus demonstrating redundant functions of these pleiotropic cytokines. In a mouse asthma model, we demonstrate that blockade of both IL-4 and IL-13 is required to broadly block type 2 inflammation, which translates to protection from allergen-induced lung function impairment. Herein, we also describe for the first time the detailed mechanism of action of dupilumab: By targeting the IL-4Ra subunit, this single blocking antibody simultaneously inhibits the two major type 2 cytokines IL-4 and IL-13 and has translated to clinical efficacy across a number of type 2 conditions.3,5,7,16

Th2 effector cells are major players in classic allergic responses by directly interacting with APC, such as B cells and DC. In the context of a T helper cell-B cell interaction, IL-4 produced by Th2 cells is thought to provide antigen-specific help to B cells, promoting CD23 induction and isotype switching.51 We show that only the dual ligand blocker (IL-4Ra antibody) prevents B-cell activation, as measured by increased cell surface of the low affinity receptor for IgE, CD23. Importantly, it has been shown that IgE-CD23 interaction contributes to allergic inflammation through IgE-facilitated allergen presentation by B cells to T cells.52 During T cell-DC interactions, IL-4 produced by Th2 cells promotes DC to release the type 1 cytokine IL-12p70.38,39 Interestingly, we observed that IL-4 and IL-13 redundantly drive DC production of this type 1 cytokine, as dual ligand blockade with dupilumab was required to fully prevent Th2 cell–driven DC activation and subsequent release of IL-12. These findings are consistent with clinical observations suggesting that dupilumab treatment may impact a type 1 response, as decreased expression of type 1/IFNγ-related genes including IFN-γ, MX1, and CXCL10 was observed in skin biopsies from AD patients treated with dupilumab.31

Acute allergic symptoms and anaphylaxis are initiated by allergen-induced crosslinking of allergen-specific IgE bound to FccR on effector cells, such as mast cells. In addition to IgE-dependent activation, mast cells can also be activated by IgE-independent mechanisms.53 and play a role in asthma and the development of AHR.54 IL-4 has been shown to enhance chemokine/cytokine production by human mast cells.43 and both IL-4 and IL-13 increase FccRlx expression at their surface.43,44 but less is known about the mast cell transcriptome profile in direct response to IL-4 or IL-13 or the effects of either cytokine upon IgE crosslinking. Here, we report direct effects of IL-4 and IL-13 on human mast cell transcriptome, independent of IgE crosslinking, and also show that each cytokine can potentiate the effects of IgE crosslinking and alter the gene expression profile.

Either IL-4 or IL-13 stimulation, together with IgE crosslinking, leads to increase in expression of cytokine genes including Il31, Il5, Il13, Tnf, and Il9, whereas either cytokine alone does not. We also show that these cytokines induce the expression of genes involved in chemotaxis in mast cells, such as CCL18 (MIP-4), and the eotaxins CCL24 and CCL26. While IL-4 and IgE have been shown to synergize to induce FcεRIIa expression at the cell surface of mast cells,55 we found that only IL-4, not IgE, drives FCER1A RNA expression in these cells, suggesting that IgE rather stabilizes FcεRIIa at the cell surface. Altogether, these data strongly suggest that dampening IgE-dependent effects on mast cells and/or FcεR receptor expression with anti-IgE therapies is not enough to prevent mast cell activation, as IL-4/IL-13-induced IgE-independent effects on mast cells could still lead to effector cell activation and the downstream allergic cascade. Overall, the observed IgE-dependent and IgE-independent effects of both IL-4 and IL-13 on mast cell activation and priming support the requirement of dual blockade to inhibit mast cell functions in the context of type 2 inflammation.

We also found that intranasal administration of either IL-4 or IL-13 can induce lung eosinophilia and demonstrated that both cytokines contribute to eosinophil lung infiltration in a mouse asthma model. In fact, a phase 2 clinical trial with tralokinumab for asthmatic patients demonstrated that the IL-13-specific antibody did not reduce eosinophilic airway inflammation,56 and this was associated with a lack of clinical efficacy in phase 3 studies.44 However, in eosinophilic esophagitis (EoE), a disease characterized by eosinophil infiltration into the esophagus, dual IL-4/IL-13 blockade with dupilumab inhibited eosinophil infiltration into the tissue, further support the dual role of IL-4 and IL-13 in driving eosinophilic inflammation.57 We found that dupilumab did not impact bone marrow eosinophil levels suggesting that generation of eosinophils or their egress from the bone marrow into the blood is not impacted, but rather prevented from infiltrating the lung tissue. In fact, a trend toward a modest increase in lung circulating eosinophils was also observed in some mice, which is consistent with the temporary changes in the blood observed in some patients treated with dupilumab.8,12,13 Interestingly, IL-4Ra blockade with dupilumab showed a dramatic reduction in exacerbations and improvement in lung function in asthmatic patients, demonstrating that blood eosinophil levels are not necessarily reflective of tissue eosinophil levels and that tissue, but not circulating eosinophils, contribute to disease pathology.

IL-4 and IL-13 are known to increase expression of adhesion molecules on endothelium to promote diapedesis of immune cells into tissues.58,59 In our model, dupilumab blocked the expression of key proinflammatory cytokines (IL4, IL5, IL13, IL6, IL33, and IL1a) and chemokines (CCL11, CCL24, CCL17, CCL2, and CXC11), suggesting that IL-4 and IL-13 may also promote immune cell infiltration into the lungs of HDM-exposed mice by inducing the expression of potent chemokines/cytokines that attract pathogenic immune cells, such as eosinophils, to the lung parenchyma. Interestingly, eosinophils recruited into the lungs of mice exposed to HDM display an activated phenotype, and both IL-4 and IL-13 directly induce human eosinophils to secrete TARC. We also show that both IL-4 and IL-13
directly affect human endothelial cells by inducing the secretion of CCL26, CCL2, and IL-6, and that IL-4 induces CCL26 expression in human mast cells. These data highlight a direct role for the two type 2 cytokines on both the endothelium and immune cells at inducing the release of chemokines and inflammatory cytokines involved in the recruitment/activation of eosinophils and other inflammatory immune cells into inflamed tissues. And consistent with these observations, dupilumab treatment reduces both TARC (CCL17) and eotaxin 3 (CCL26) levels in patients with asthma.\textsuperscript{10} While novel strategies to block multiple type 2 cytokines are emerging, such as IL-4Ra/IL-5 bispecific antibody,\textsuperscript{60} this approach might not be required. Indeed, in our model, dupilumab completely inhibited all features of type 2 inflammation analyzed including increased IL5 expression in the lungs, lung eosinophilia, and lung function impairment, suggesting that dual IL-4/IL-13 blockade is sufficient to prevent IL-5 signaling.

Overall, the above studies, together with the clinical findings with dupilumab, support both IL-4 and IL-13 as key initiators and central drivers of type 2 inflammation. By blocking the central drivers of the type 2 immune response, IL-4 and IL-13, dupilumab affects multiple downstream effector cells and cytokines (both IgE and eosinophils, but also IL-5, IL-13, FcεRIα, pathogenic Th2 cells, alveolar macrophages, etc). In asthma, effective dual IL-4/IL-13 blockade with dupilumab (in contrast to IL-13, IL-5, or IgE blockade) not only reduces exacerbations, but also improves lung function.\textsuperscript{3,5,61-66} A corollary to this is that mediators in addition to eosinophils and IgE are critical for impacting lung function in asthma, and simply targeting eosinophils or IgE may only manage the signs and symptoms. All too frequently, patients have manifestations in multiple tissues and suffer from numerous related comorbidities (eg, AD, asthma, allergic rhinitis, nasal polyposis, etc). Emerging data regarding the central role of both IL-4 and IL-13 across all provides important new hope to such patients—that is, that a single therapeutic, attacking the fundamental systemic drivers of their immune deviation may benefit and correct all of their disease manifestations whether in skin, gastrointestinal tract, or respiratory systems.

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CONFLICT OF INTERESTS

All authors are employees of Regeneron Pharmaceuticals, Inc, and may hold stock and/or stock options in the company.

AUTHOR CONTRIBUTIONS

AL, JA, JM, TM, TBP, JHK, AR, TH, AJM, NJP, NS, GGY, and JMO conceived the studies and analyzed and interpreted the data. AL, GS, DB, KN, and SA acquired, analyzed, and interpreted the data. YB and WKL analyzed the NGS data. AL, JA, MAS, NJP, NS, GDY, AJM, and JMO drafted the manuscript.

DATA AVAILABILITY STATEMENT

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.

ORCID

Jamie M. Orengo https://orcid.org/0000-0002-2695-6508

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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