Predicting probability of tolerating discrete amounts of peanut protein in allergic children using epitope-specific IgE antibody profiling

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ARTICLE SUMMARY

- Existing diagnostic testing is not predictive of severity or the threshold dose of clinical reactivity, and many patients still require an Oral Food Challenge (OFC). While OFCs are very useful for making an allergy diagnosis and determining clinical reactivity, they often cause anaphylaxis, which can increase patient anxiety. and are time and resource intensive.[1]
- An extensive validation was performed across 5 cohorts (all with confirmed oral food challenge results) across six different countries. Cohorts used: BOPI, OPIA, CAFETERIA, CoFAR6, and PEPITES with specimens from Australia, UK, US, Ireland, and Germany.
- This paper reports the first validated algorithm using two key peanut specific IgE epitopes to predict probabilities of reaction to different amounts of peanut in allergic subjects and may provide a useful clinical substitute for peanut oral food challenges.
- Using the algorithm, subjects were assigned into "high", "moderate", or "low" dose reactivity groups. On average, subjects in the "high" group were 4 times more likely to tolerate a specific dose, compared to the "low" group.[1] For example, 88% of patients in the high dose reactivity group were able to tolerate ≥ 144 mg of peanut protein whereas only 29% were able to tolerate the same amount in the low dose reactivity group.[1-2]

CLINICAL CONSIDERATIONS

- The new epitope test offers more granular information to help clinicians stratify treatment and peanut avoidance plans for their patients.
- See below for summary of clinical considerations based on threshold reactivity level.[1]

allergenis peanut diagnostic result	clinical considerations[1]
likely allergic – low dose reactor	• inform or avoid oral food challenge to reduce risk of anaphlyaxis • confirm strict avoidance of peanut • consider immunotherapy to reduce risk of reaction
likely allergic – moderate dose reactor	• consider a single oral food challenge (30 to 100 mg) to reduce anxiety and improve quality of life • less stringent avoidance of peanut regime • consider inclusions of precautionary labeled foods such as 'May contain peanut' • consider immunotherapy to reduce risk of reaction
likely allergic – high dose reactor	• consider a single oral food challenge (100 to 300 mg) to reduce anxiety and improve quality of life • less stringent avoidance of peanut regime • consider inclusions of precautionary labeled foods such as 'May contain peanut' • consider starting immunotherapy at higher doses to shorten time to maintenance dose
unlikely allergic	• oral food challenge to rule out the diagnosis of peanut allergy

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ORIGINAL ARTICLE

Asthma and Lower Airway Disease

A common IL-4 receptor variant promotes asthma severity via a T_{reg} cell GRB2-IL-6-Notch4 circuit

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Abstract

Background: The mechanisms by which genetic and environmental factors interact to promote asthma remain unclear. Both the IL-4 receptor alpha chain R576 (IL-4RαR576) variant and Notch4 license asthmatic lung inflammation by allergens and ambient pollutant particles by subverting lung regulatory T (T_{reg}) cells in an IL-6-dependent manner.

Objective: We examined the interaction between IL-4RαR576 and Notch4 in promoting asthmatic inflammation.

Methods: Peripheral blood mononuclear cells (PBMCs) of asthmatics were analyzed for T helper type 2 cytokine production and Notch4 expression on T_{reg} cells as a function of *IL4R*^{R576} allele. The capacity of IL-4RαR576 to upregulate Notch4 expression on T_{reg} cells to promote severe allergic airway inflammation was further analyzed in genetic mouse models.

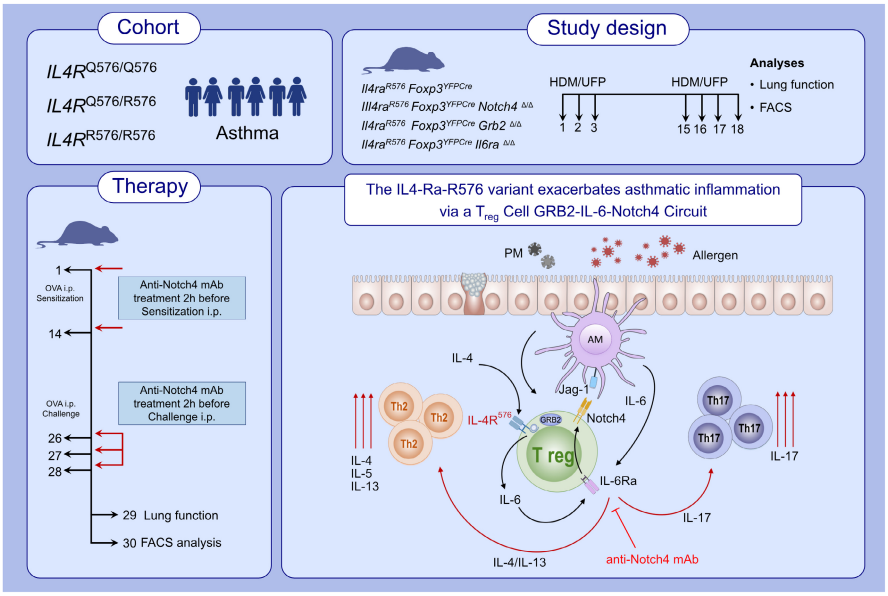
Results: Asthmatics carrying the *IL4R*^{R576} allele had increased Notch4 expression on their circulating T_{reg} cells as a function of disease severity and serum IL-6. Mice harboring the *Il4ra*^{R576} allele exhibited increased Notch4-dependent allergic airway inflammation that was inhibited upon T_{reg} cell-specific *Notch4* deletion or treatment with an anti-Notch4 antibody. Signaling via IL-4RαR576 upregulated the expression in lung T_{reg} cells of Notch4 and its downstream mediators Yap1 and beta-catenin, leading to exacerbated lung inflammation. This upregulation was dependent on growth factor receptor-bound protein 2 (GRB2) and IL-6 receptor.

Conclusion: These results identify an IL-4RαR576-regulated GRB2-IL-6-Notch4 circuit that promotes asthma severity by subverting lung T_{reg} cell function.

KEYWORDS
asthma, GRB2, interleukin 4 receptor, Notch4, regulatory T cells

Benamar, Harb and Chen contributed equally to the manuscript.

Crestani and Chatila contributed equally to the manuscript.



GRAPHICAL ABSTRACT

Asthmatics carrying the IL-4RαR576 variant have increased Notch4 expression on their circulating Treg cells as a function of disease severity and serum IL-6 concentrations. IL-4RαR576 acts via GRB2 and IL6R in Treg cells to upregulate Notch4 and its downstream signaling intermediates Yap1 and β-catenin, leading to exacerbated lung inflammation. Treatment with an anti-Notch4 neutralizing antibody inhibits allergic airway inflammation.

Abbreviations: FACS, fluorescence-activated cell sorting; Foxp3, forkhead box P3; GRB2, growth factor receptor-bound protein 2; HDM, house dust mite; IL, interleukin; Jag-1, Jagged Canonical Notch Ligand 1; mAb, monoclonal antibodies; Notch4, Notch Receptor 4; OVA, ovalbumin; PM, particulate matter; Treg, regulatory T cell; UFP, ultrafine particles

1 | INTRODUCTION

The high prevalence of asthma across different societies is thought to reflect the interaction of genetic factors with environmental influences ushered by a shared modern lifestyle with altered microbial exposures, high air pollution, increased exposure to indoor allergens, and reduced physical activity.[1–4] Of particular interest is the impact of these changes on vulnerable populations, which suffer a disproportionate disease load and increased morbidity.[5–8] Such populations exhibit genetic variants that, in the context of specific environmental exposures, may act to increase asthma incidence and/or severity.[9,10] In that regard, we previously identified an interleukin 4 receptor (IL-4R) alpha chain variant with an arginine at position 576 (IL-4RαR576) that has been linked to asthma exacerbation and severity.[11–14] Notably, the IL-4RαR576 variant gives rise to robust mixed T helper type 2 (Th2) and type 17 (Th17) cell inflammation as compared to the classical Th2 cell inflammation seen in asthmatics.[15–17] The mechanism by which this variant promotes asthma was linked to its subversion of lung tissue regulatory *T* (T_{reg}) cell responses by destabilizing these cells towards a Th17 cell fate.[16] These results established the key role played by immune dysregulation in exacerbating asthmatic inflammation in predisposed subjects.[18–20]

Further studies in the interim highlighted the interaction of this pathway with ambient particulate matter pollutants (PM), particularly ultrafine particles (UFP), in aggravating asthmatic inflammation.[17,21] The uptake of PM by alveolar macrophages (AM) activates the aryl hydrocarbon receptor pathway in the latter cells to induce the expression of the Notch receptor ligand Jagged1 (Jag1),[17,21] which interacts with nascent induced T_{reg} (iT_{reg}) cells to drive their subversion. Recent studies have identified Notch4 as the Notch receptor on T_{reg} cells involved in these interactions, whose expression on T_{reg} cells is upregulated in an IL-6 and IL-33-dependent manner.[22] In this report, we demonstrate that T_{reg} cell-intrinsic signaling via the IL-4RαR576 variant amplifies the expression of Notch4 on T_{reg} cells by recruiting GRB2 to promote IL-6 expression. In turn, increased Notch4 expression mediates the exacerbated allergic inflammatory response effected by the IL-4Rα R576 variant. These results identified Notch4 as the central component of the immune dysregulatory circuit involved in the heightened asthmatic inflammation induced by the IL-4Rα R576 variant.

2 | RESULTS

2.1 | Association of the *IL4R*^{576} allele with asthma diagnosis and disease severity

The *IL4R*^{576} allele has previously been implicated in asthma severity and is highly prevalent in African Americans.[12,15,16,23] To investigate the relationship between genotype and asthma severity, we studied a group of 184 children and young adults, including 132 subjects with asthma and 52 non-atopic controls. Age ranged from 1 to 21 years (mean 8.6 ± 3.8 years), while gender distribution showed a preponderance of males ($n = 105$, 57.1%) vs females ($n = 79$, 42.9%) (Table S1). Participants were recruited in the Allergy clinic at Boston Children's Hospital and from inner-city cohort populations.[23,24]

Informed consent was obtained from adult participants and from legal guardians of minor subjects. Asthma severity was assigned based on EPR3 criteria and medication use. Asthmatic patients were grouped as having either intermittent/mild persistent asthma (n = 78) or moderate/severe persistent asthma (n = 54). Based on genotyping at the *IL4R* 576 codon, subjects were categorized as being either homozygous for the dominant allele (Q/Q, n = 67, 36.4%), heterozygous (R/Q, n = 69, 37.5%), or homozygous variant allele (R/R, n = 48, 26.1%). The demographic and clinical characteristics of asthmatic and control subjects and the ethnicity distribution among each individual genotype are summarized in Table S1 and Figure S1. Among asthmatic individuals, allergic rhinitis with evidence of sensitization to aeroallergens (including tree pollen, grass pollen, weed pollen, animal dander, dust mites, and mold) by either skin prick testing and/or specific IgE was present in 75.2% (98/125) individuals with available data (missing n = 7), while 31.8% (42/132) of asthmatic subjects carried a diagnosis of food allergy based on history of clinical reaction and positive IgE testing to the culprit food. Distribution of individual allergies is shown in Table S1, with most children suffering from multiple food and/or environmental allergies. Total IgE and circulating eosinophil data were available for 80 and 92 asthmatic children, respectively. Mean total IgE level was 825.2 kU/L (range 2–7491), with 65% (52/80) of children manifesting elevated IgE levels for age. Mean circulating eosinophil count was 334.5/mcL (range 0–1250), with age-adjusted peripheral eosinophilia detected in 47.8% (44/92) of subjects (Table S1). Taken altogether, these data suggest a high prevalence of allergic, Th2-driven asthma among our study subjects, consistent with previous reports of asthma in pediatric populations. In our population, individuals carrying either one (*IL4R*$^{Q576/R576}$) or two copies of the variant allele (*IL4R*$^{R576/R576}$) were more likely to have a diagnosis of asthma (OR 1.98, 95% C.I. 1.04–3.82) (Table S1). Furthermore, among Hispanic individuals with asthma, those who were either heterozygous or homozygous for the variant allele were more likely to present with moderate/severe asthma as compared to individuals who were homozygous for the WT allele (OR 5.76, 95% C.I. 1.30–25.51) (Table S3). No significant association was detected between genotype and asthma severity among Caucasian or African American asthmatics, suggesting that a possible contribution of the genotype at the *IL4R*^{576} allele to asthma severity may be at least in part dependent on the individual's ethnic background.

2.2 | *IL4R*^{R576} asthmatics exhibit increased TH2 and TH17 cytokine expression in their T_{eff} and T_{reg} cells

We analyzed the cytokine expression profiles of peripheral blood T_{eff} and T_{reg} cells as a function of *IL4R*$^{Q576/Q576}$ (*IL4R*^{Q576}) versus *IL4R*$^{Q576/R576}$ and *IL4R*$^{R576/R576}$ (*IL4R*^{R576}) genotypes and disease severity in the asthmatic and control subjects. Results revealed that patients either heterozygous or homozygous for *IL4R*^{R576} allele expressed higher levels of Th2 cytokines and markers (CRTH2) in their circulating T_{reg} and T_{eff} cells that progressively increased in relation to asthma severity (Figure 1A–B). They also present a higher level of Th17 cytokines and markers (CCR6) (Figure 1C–D).

We have previously established that the IL-4RαR576 variant promotes asthma severity and dysregulates Th2 and Th17 responses by destabilizing lung tissue induced T_{reg} cells in an IL-6-dependent manner. Moreover, IL-6 promotes NOTCH4 expression on lung tissue induced T_{reg} cell to increased asthma severity through a T_{reg} cells destabilization. To establish the relationship between the IL-4RαR576 variant and NOTCH4, we analyzed by flow cytometry the expression of NOTCH4 on circulating T_{reg} cells of human subjects with asthma as a function of *IL4R*^{R576} allele carriage and disease severity. NOTCH4 expression was increased on circulating T_{reg} cells of human asthmatics as a function of both disease severity and *IL4R*^{R576} allele carriage (Figure 1E; see Figure S2 for gating strategy). The *IL4R*^{R576} allele was associated with increased NOTCH4 expression on T_{reg} cells of asthmatic subjects in a gene dose-dependent manner (Figure 1F). Other Notch receptors are not upregulated in T_{reg} or T_{eff} as a function of either disease severity or *IL4R*^{R576} allele carriage (Figure S3). Consistent with the promotion by the IL4RαR576 variant of IL-6 production and the dependency of NOTCH4 expression in T_{reg} cells on IL-6 signaling,[16,22] we found a positive correlation between serum IL-6 levels and Notch4 expression in asthmatics that segregated as a function of *IL4R*^{R576} allele carriage and disease severity (Figure 1G, H). These results established the upregulation of the IL-6-Notch4 axis in *IL4R*$^{R576+}$ asthmatics in direct correlation with disease severity.

2.3 | Increased Notch4 expression on T_{reg} cells underlies heightened airway inflammation in *Il4ra*^{R576} mice

To further decipher the functional impact of Notch4^{+}T_{reg} cells in the exacerbated asthmatic inflammation imparted by the *IL4R*^{R576} allele, we employed an allergic airway inflammation mouse model using the previously described *Il4ra*^{R576} mice, whose interleukin 4 receptor alpha chain gene (*Il4ra*) encodes the R576 substitution at the same position as the human protein variant.[15,16] We further examined the impact of T_{reg} cell-specific deletion of *Notch4* in these mice on their allergic airway inflammatory response using a floxed *Notch4* allele and a Cre recombinase driven by the Foxp3 gene (*Foxp3*^{YFPCre}). Mice were sensitized with chicken egg ovalbumin (OVA) and then challenged with aerosolized OVA either without or together with intranasal treatment with Traffic-related ultrafine pollutant particles (UFP), as described.[17,21,22] In agreement with our previous results, the *Il4ra*^{R576}*Foxp3*^{YFPCre} mice showed increased airway hyperresponsiveness (AHR) and exacerbated tissue inflammation scores upon OVA sensitization and challenge as compared to *Foxp3*^{YFPCre} control mice. Both responses were further augmented by UFP co-treatment during the challenge phase (Figure 2A–C, Figure S4). T_{reg} cell-specific deletion of *Notch4* profoundly inhibited both AHR and tissue inflammation down to similar levels in both set of mice (*Il4ra*^{R576}*Foxp3*^{YFPCre}*Notch4*$^{\Delta/\Delta}$ and control *Foxp3*^{YFPCre}*Notch4*$^{\Delta/\Delta}$),

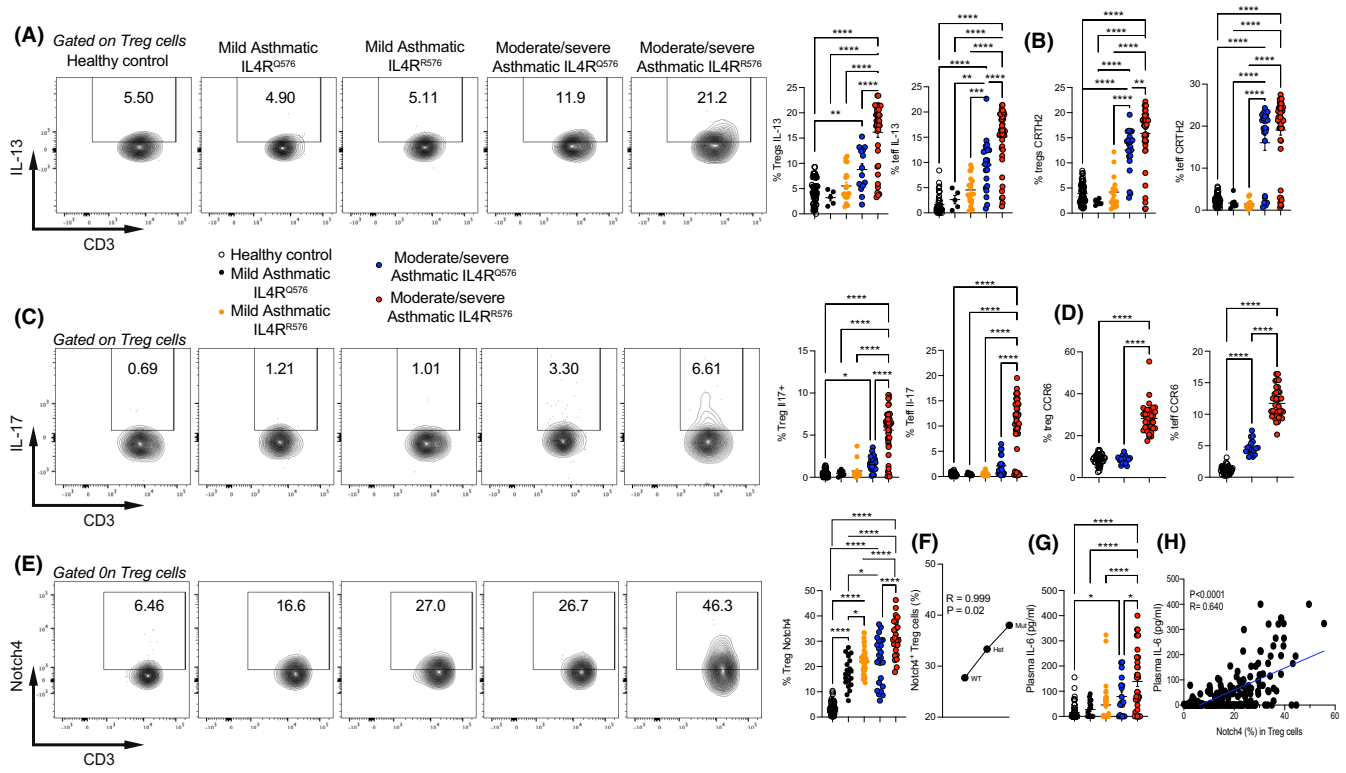


FIGURE 1 Notch4, Chemokine, and cytokine profiles of circulating CD4$^+$ T cells in asthmatics segregated by the *IL4R* genotype. A-B, flow cytometric analysis (A) and cell frequencies (B) of IL-13$^+$ and CRTH2 expression on circulating T_{reg} or T_{eff} cells (A, B) in healthy controls and subjects with moderate and severe asthma homozygous for the dominant *IL4R*^{Q576} allele or heterozygous or homozygous for the *IL4R*^{R576} allele. C-D, flow cytometric analysis (C) and cell frequencies (D) of IL17$^+$ and CCR6$^+$ expression on circulating T_{reg} or T_{eff} cells (C, D) in healthy controls and subjects with moderate and severe asthma homozygous for the dominant *IL4R*^{Q576R} allele or heterozygous or homozygous for the *IL4R*^{R576} allele. (E) Notch4 expression on T_{reg} cells of healthy controls and mild persistent, moderate persistent and severe persistent asthmatics segregated by *IL4R* genotype (QQ versus QR and RR). (F) Pearson correlation analysis of Notch4 expression of T_{reg} cells of asthmatics with IL4R alleles. (G) IL-6 concentrations in the serum of control and asthmatic subjects. (H) Pearson correlation between Notch4 expression of T_{reg} cells of asthmatic subject with serum IL-6 concentrations. Each symbol represents one patient. Numbers in flow plots indicate percentages. Error bars indicate SEM. Statistical tests: One-way ANOVA with Dunnett's post hoc analysis $^*p < .05$, $^{***}p < .001$, $^{****}p < .0001$. simple linear regression analysis (F, H); $^{***}p < .001$, $^{****}p < .0001$

indicating that the augmentation of AHR and allergic lung inflammation by the *Il4ra*^{R576} allele was Notch4-dependent (Figure 2A–C). Consistent with these results, flow cytometric analysis confirmed that allergic airway inflammation was associated with upregulation of *Notch4* expression on T_{reg} cell as a function of disease severity, which was completely abrogated upon Cre-mediated deletion of *Notch4*. (Figure 2D, E). In addition to suppressing AHR and tissue inflammation, *Notch4* deletion in T_{reg} cells also suppressed the OVA-specific IgE response, lung tissue eosinophilia and CD4$^+$ T cell lymphocytosis, and Th2 and Th17 cell cytokine expression (Figure 2D–H).

The above results were reproduced in a separate set of studies that employed house dust mite (HDM) as an inciting allergen in airway inflammation. Similar to the case with OVA, HDM-induced airway hyperresponsiveness and tissue inflammation, eosinophilia and Th2 and Th17 cell cytokine production were all upregulated in the *Il4ra*^{R576}*Foxp3*^{YFPCre} mice in a T_{reg} cell Notch4-dependent manner (Figure S5). Together, these findings confirmed the crucial role played by Notch4 expression on T_{reg} cells in the augmented allergic airway inflammation induced by the IL-4Rα-R576 variant.

To determine whether acute Notch4 inhibition would rescue disease phenotype, we examined the capacity of therapy with a blocking Notch4 antibody to inhibit OVA-induced allergic airway inflammation in *Il4ra*^{R576}*Foxp3*^{YFPCre} mice. Results showed that treatment with the anti-Notch4 antibody at the time of allergen sensitization and challenge suppressed both OVA and OVA+UFP induced allergic airway inflammatory responses to levels approaching those achieved with the T_{reg} cell-specific *Notch4* deletion. Parameters inhibited by the antibody therapy included AHR (Figure 3A, B), OVA-specific IgE (Figure 3C) CD4$^+$ T cell lymphocytosis, tissue eosinophilia and neutrophilia (Figure 3D–F), and tissue Th cell infiltration (Figure 3G, H).

2.4 | T_{reg} cell-specific *Grb2* deletion abrogates the augmentation of allergic airway inflammation by *Il4ra*^{R576}

Our previous studies have demonstrated that the IL-4Rα-R576 variant uniquely recruits the intracellular signaling intermediate Grb2 to

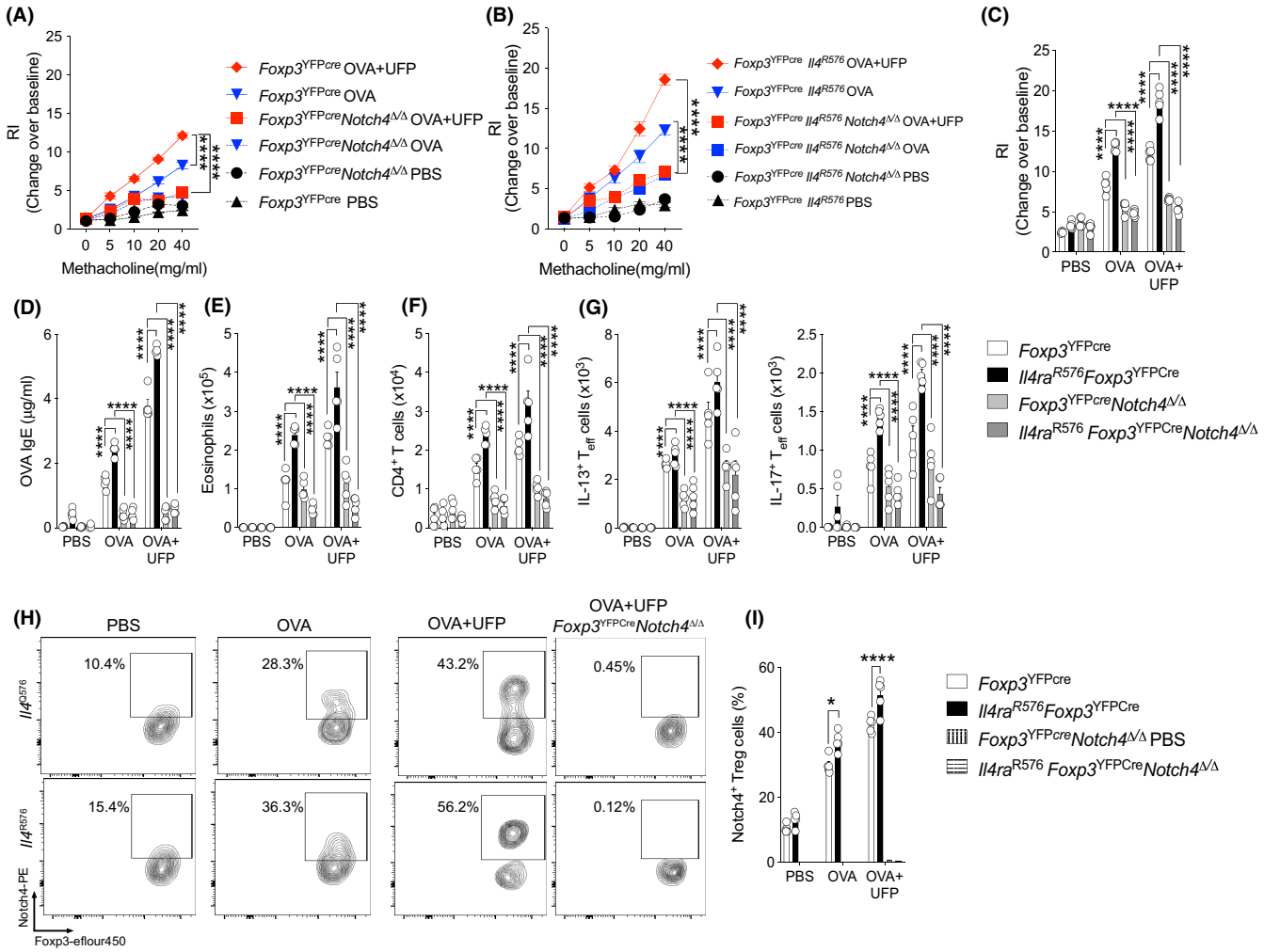


FIGURE 2 Notch4 expression on lung T_{reg} cells licenses allergic airway inflammation in *Il4Rα*^{R576} mice. (A, B, and C) AHR in the respective mouse groups in response to methacholine. (D) serum OVA-specific IgE concentrations. (E and F) absolute numbers of lung eosinophils and CD4$^+$ T cells. (G) IL-13 and IL-17 expression in lung T_{eff} cells (H and I) Notch4 expression in T_{reg} cells of the respective mouse groups. Each symbol represents an independent sample. Numbers in flow plots indicate percentages. Error bars indicate SEM. Statistical tests: two-way ANOVA with Sidak's post hoc analysis (A–I). *p <0.05, ***p <0.001, ****p <0.0001. Data representative of two or three independent experiments

mediate microtubule-associated protein kinase (MAPK) activation and IL-6 production.[16] This process was incriminated in corrupting lung tissue allergen-specific induced T_{reg} cell formation towards a Th17 fate. Accordingly, we examined the capacity of T_{reg} cell-specific deletion of *Grb2* to reverse the augmentation in allergic airway inflammation induced in *Il4ra*^{R576} mice. Analysis revealed that *Il4ra*^{R576} mice whose T_{reg} cells lacked GRB2 (*Il4ra*^{R576}*Foxp3*^{YFPCre}*Grb2*$^{Δ/Δ}$) failed to exhibit the expected augmentation in attributes of airway inflammatory normally associated with the *Il4ra*^{R576} allele upon sensitization and challenge with HDM. Measures including AHR, total IgE, and tissue eosinophilia and neutrophilia were all normalized to levels achieved in similarly treated Grb2-sufficient control mice bearing the wild-type *Il4ra*^{Q576} allele (*Foxp3*^{YFPCre}) (Figure 4A–F). Importantly, deletion *of Grb2* in control mice bearing the wild-type *Il4ra*^{Q576} allele (*Foxp3*^{YFPCre}*Grb2*$^{Δ/Δ}$) did not affect the allergic airway inflammatory response induced by HDM as compared to *Grb2*-sufficient *Foxp3*^{YFPCre} mice. Further analysis revealed that Grb2 deletion completely abrogated the augmentation in Th17, cell responses associated with the *Il4ra*^{R576} allele (Figure 4G–H). Grb2 deletion suppressed the increased in IgE production and eosinophilia associated with the *Il4ra*^{R576} allele but had a more modest impact on the Th2 response, suggesting additional mechanisms mobilized by IL-4RαR576 variant in promoting Th2 skewing.

2.5 | The upregulation of Notch4 signaling in *Il4ra*^{R576} T_{reg} cells is *Grb2*-dependent

To determine the role of the IL-4Rα-R576-coupled Grb2 pathway in the upregulation of Notch4 signaling in *Il4ra*^{R576} T_{reg} cells, we analyzed Notch4 expression in Grb2-sufficient and deficient lung T_{reg} cells of mice bearing the WT *Il4ra* allele (*Foxp3*^{YFPCre} and *Foxp3*^{YFPCre}*Grb2*$^{Δ/Δ}$, respectively), compared with those of *Il4ra*^{R576} mice (*Il4ra*^{R576}*Foxp3*^{YFPCre} and *Il4ra*^{R576}*Foxp3*^{YFPCre}*Grb2*$^{Δ/Δ}$,

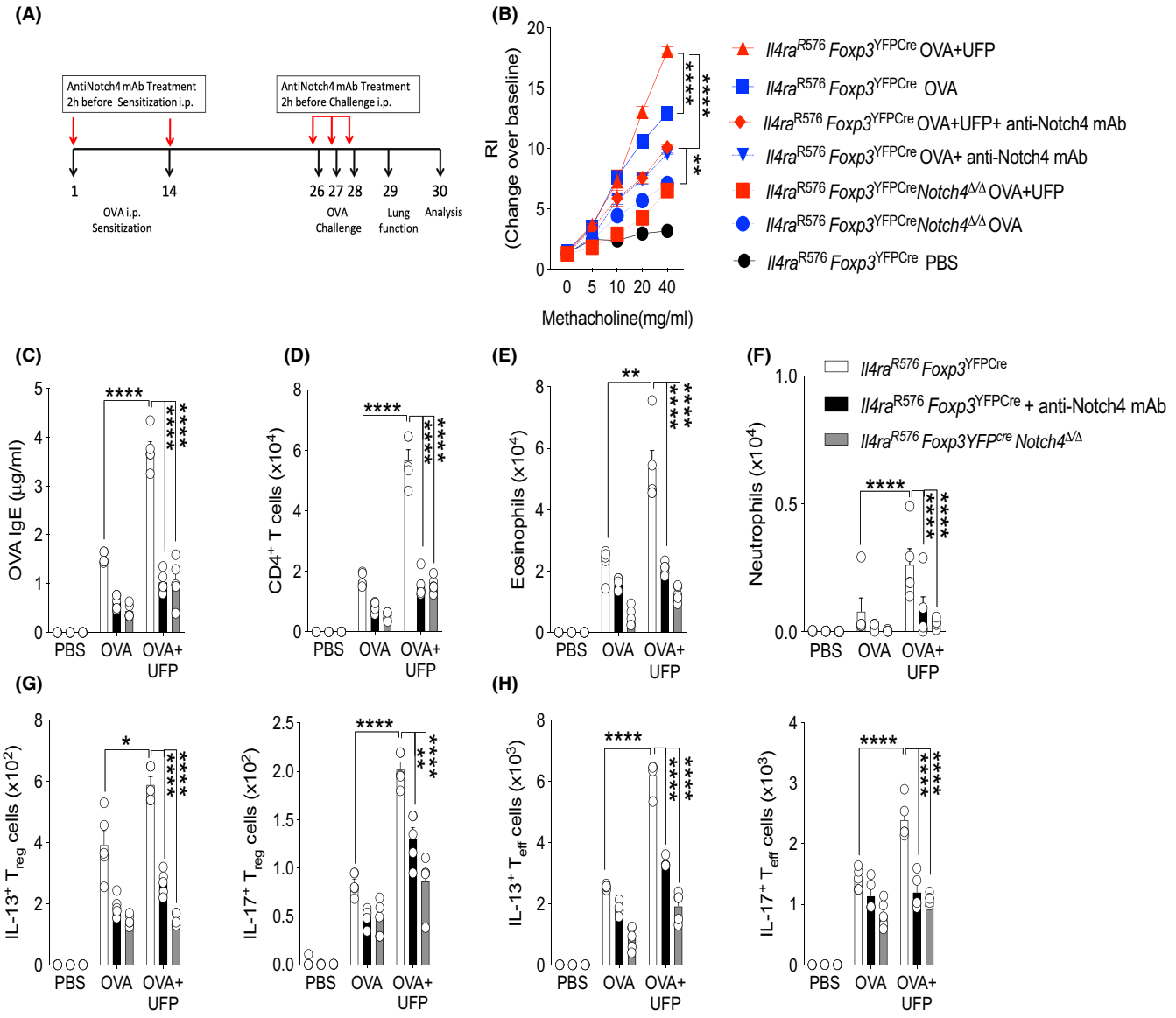


FIGURE 3 Anti-Notch4 neutralizing mAb inhibit allergic airway inflammation in *Il4Rα*^{R576} mice. (A) scheme of antibody therapy (10 µg) and airway sensitization and challenge. (B) airway hyperresponsivness in the respective mouse groups in response to methacholine. (C) serum OVA-specific IgE concentrations. (D–F) absolute numbers of lung CD4$^+$ T cells, eosinophils, and neutrophils. (G) IL-13 and IL-17 expression in lung T_{reg} cells. (H) IL-13 and IL-17 expression in lung T_{eff} cells. Each symbol represents an independent sample. Numbers in flow plots indicate percentages. Error bars indicate SEM. Statistical tests: two-way ANOVA with Sidak's post hoc analysis (B–G). $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$

respectively). The respective mouse groups were subjected to HDM-induced allergic airway inflammation, as shown in Figure 4, and their lung T_{reg} cells were analyzed for Notch4 and IL-6 expression. The T_{reg} cells were also analyzed for the expression of the Notch4 downstream mediators Yap1 and β-catenin, effector proteins of the Hippo and Wnt pathways, respectively, which control the lung tissue Th17 and Th2 cell responses.[22] Results showed that the increased Notch4 expression in the lung T_{reg} cells of *Il4ra*^{R576}*Foxp3*^{YFPCre} mice was completely abrogated upon T_{reg} cell-specific deletion of *Grb2*, both at baseline and especially after HDM sensitization and challenge (Figure 5A; see Figure S6 for gating strategy).

In view of our previous demonstration that Notch4 induction on lung T_{reg} cells proceeds by an IL-6-dependent mechanism,[22] we analyzed IL-6 production in *Grb2*-sufficient and deficient T_{reg} cells of sham and HDM-treated mice bearing the WT and *Il4ra*^{R576} allele. T_{reg} cell-specific *Grb2* deletion abrogated the increase in IL-6 production mediated by the IL-4Rα-R576 variant,[16] consistent with a mechanistic autocrine loop involving Grb2-dependent IL-6 production mediating the super-induction of Notch4 on the T_{reg} cells of *Il4ra*^{R576} mice (Figure 5B). The role of IL-6 in the upregulation of Notch4 by IL-4Rα-R576 variant was further established using mice with T_{reg} cell-specific deletion of *Il6ra*, encoding the IL-6 receptor alpha chain. *Il6ra* deletion in T_{reg} cells inhibited Notch4 expression in both control and *Il4ra*^{R576} mice (*Foxp3*^{YFPCre}*Il6ra*$^{Δ/Δ}$ and *Il4ra*^{R576}*Foxp3*^{YFPCre}*Il6ra*$^{Δ/Δ}$, respectively) and abrogated differential disease severity in the latter mice (Figure S7A–H). T_{reg} cell-specific *Grb2* deletion also abrogated

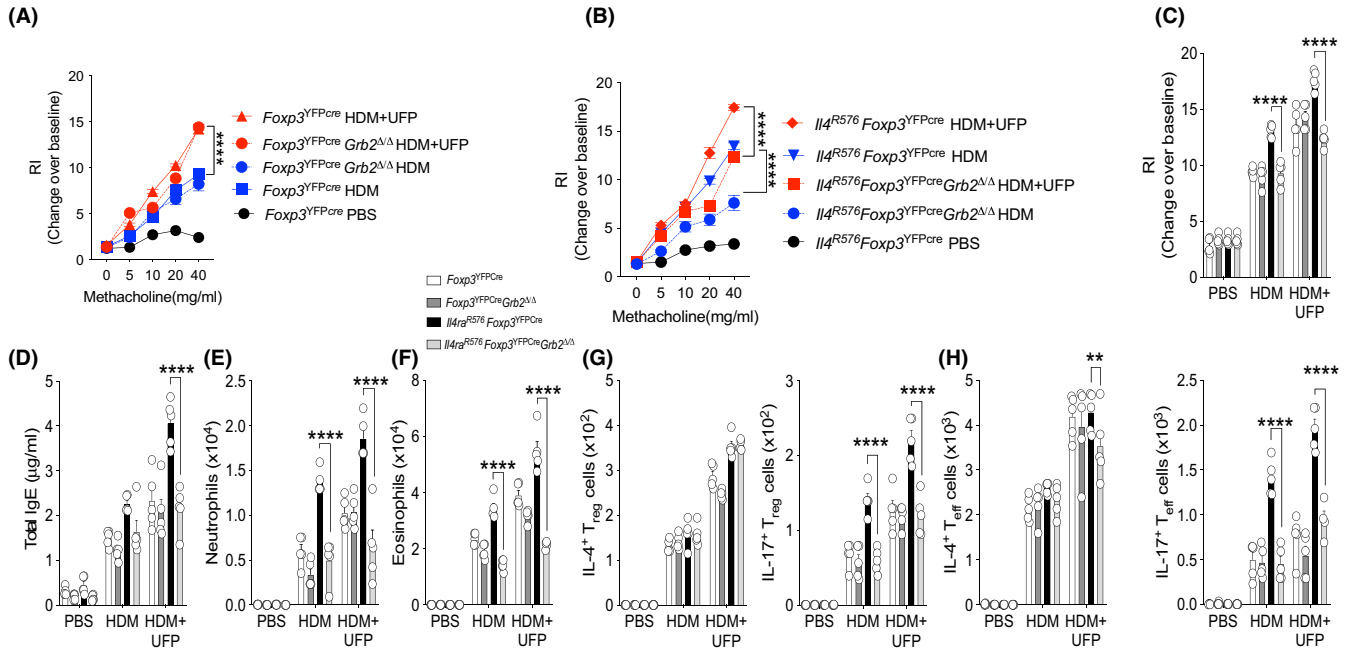


FIGURE 4 GRB2 drives the super-induction of allergic airway inflammation in *Il4Ra*^{R576} mice. (A and B) airway hyperresponsivness in the respective mouse groups in response to methacholine. (C) peak airway resistance in the respective groups of panels A and B. (D) serum OVA-specific IgE concentrations. (E–F) absolute numbers of lung neutrophils and eosinophils. (G) IL-4 and IL-17 expression in lung T_{reg} cells (H) IL-4 and IL-17 expression in lung T_{eff} cells. Each symbol represents an independent sample. Error bars indicate SEM. Statistical tests: two-way ANOVA with Sidak's post hoc analysis (A–H). $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$

the super-induction of the Notch4 downstream mediators Yap1 and β-catenin in the T_{reg} cells of HDM-treated *Il4ra*^{R576}*Foxp3*^{YFPCre} mice (Figure 5C, D). These results are in agreement with the abrogation in HDM-treated *Il4ra*^{R576}*Foxp3*^{YFPCre}*Grb2*$^{Δ/Δ}$ mice of the augmented Th2 and Th17 cells responses typically associated with the *Il4ra*^{R576} allele. Together, these results indicated that the destabilization of lung T_{reg} cells upon signaling via IL-4Rα-R576 variant in allergic airway inflammation proceeded by a Grb2-IL-6-Notch4 circuit.

3 | DISCUSSION

The increased prevalence and severity of asthma is thought to reflect the interaction of genetic risk factors and environmental exposures relevant at-risk groups. Our studies have previously demonstrated that the *IL4R*^{R576} allele increases the severity of asthmatic inflammation by virtue of its destabilization of allergen-specific T_{reg} cells towards Th2 and Th17 cell phenotypes.[15,16,23] In this report, we employed a pediatric and young adult asthma cohort and relevant mouse models to establish the precise mechanisms by which such a destabilization takes place, involving a novel GRB2-IL-6-Notch4 molecular circuit that destabilizes T_{reg} cells to promote Th2 and Th17 cell responses. Our results emphasize the fundamental role of T_{reg} cell subversion in dictating asthma severity as a function of genetic risk factors such as the *IL4R*^{R576} allele and environmental exposures such as traffic-related particulate matter.[18,25,26]

Analysis of this asthmatic cohort revealed evidence of *T* cell dysregulation as function of asthma severity, including the increased frequencies among moderate and severe asthmatics of circulating CD4$^+$ T_{reg} and T_{eff} cells expressing Th2 and Th17 cell cytokines and chemokine receptors. Importantly, our studies identified three pathways independently implicated in asthma disease severity, namely *IL4R*^{R576}[16] *Notch4*[27] and *IL6/IL6R*[28] as mechanistically intersecting in our patient population. Thus, moderate and severe asthmatics exhibited higher expression of Notch4 on their T_{reg} cells that segregated with the *IL4R*^{R576} allele and serum IL-6 levels. We further demonstrate using relevant mouse genetic models that these intersecting pathways are mechanistically integrated in one genetic circuit that acts to subvert T_{reg} cell control of allergic lung tissue inflammation to result in dysregulated Th2 and Th17 cell responses.

A critical component of the immune dysregulatory circuit described above is the IL-4RαR576-coupled signaling intermediate GRB2. As a result, T_{reg} cell-specific deletion of *Grb2* reset the increased airway inflammation associated with the mouse *Il4ra*^{R576} allele. Grb2 uniquely links the IL-4RαR576 variant to downstream MAPK activation, leading to activation of *Il6* gene transcription.[16] Thus, T_{reg} cell-specific *Grb2* deletion completely reversed the increase in IL-6 production observed in lung T_{reg} cells of allergen sensitized and challenged *Il4ra*^{R576} mice. Given the dependency of Notch4 expression on IL-6, and the normalization of Notch4 expression in lung T_{reg} cells of *Il4ra*^{R576} mice upon *Grb2* deletion, these results are consistent with an IL-6-Notch4 loop operative in the lung T_{reg} cells of *Il4ra*^{R576} mice which upregulates their Notch4 expression and further subverts their function.

We have previously described two Notch4 downstream pathways operative in lung T_{reg} cells of asthmatics and allergic airway

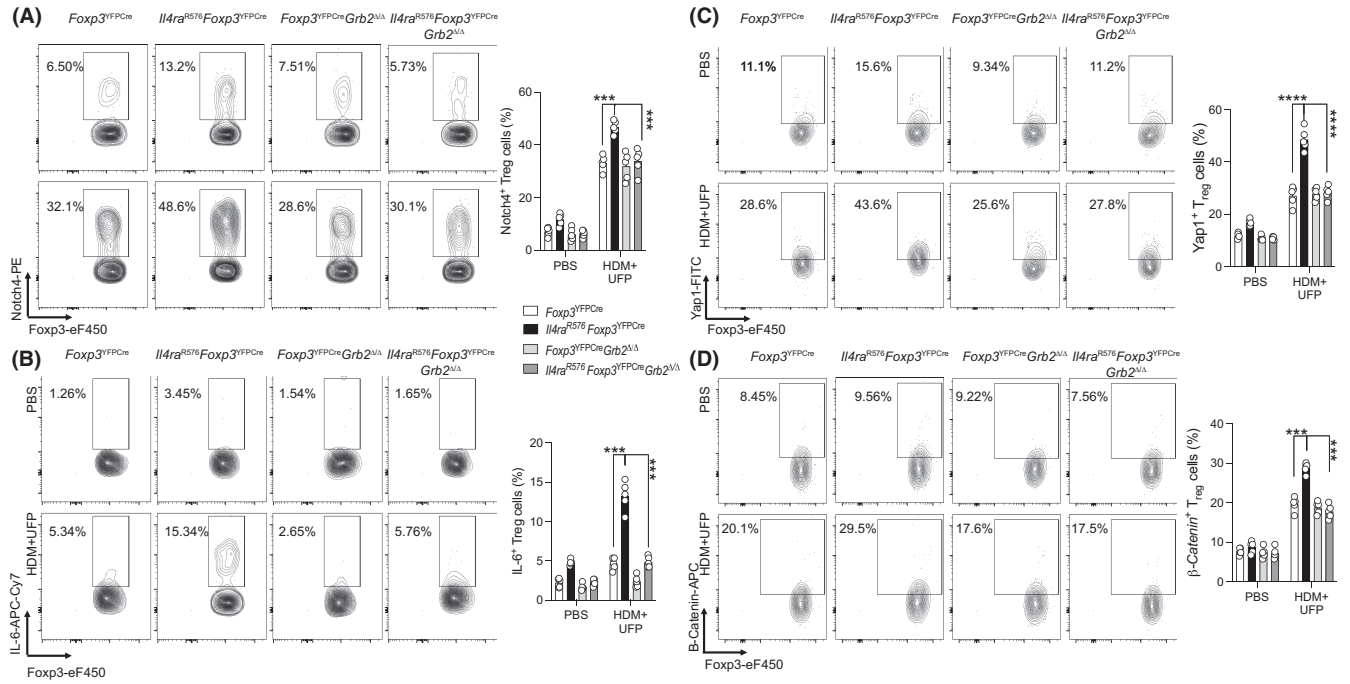


FIGURE 5 GRB2 drives the super-induction of Notch4 and downstream molecules expression in *Il4Ra*^{R576} mice. (A) Notch4 expression in T_{reg} cells in the respective mouse groups in Sham and HDM sensitized and challenged mice. (B) IL-6 expression in T_{reg} cells in the respective mouse groups in Sham and HDM sensitized and challenged mice. (C) Yap1 expression in T_{reg} cells in the respective mouse groups in Sham and HDM sensitized and challenged mice. (D) β-Catenin expression in T_{reg} cells in the respective mouse groups in Sham and HDM sensitized and challenged mice. Each symbol represents an independent sample. Numbers in flow plots indicate percentages. Error bars indicate SEM. Statistical tests: two-way ANOVA with Sidak's post hoc analysis (A-D). ***$p < .001$, ****$p < .0001$

inflammation models in mice.[22] The first, the Hippo pathway, destabilizes the T_{reg} cells towards a Th17 cell fate,[22,29] while the second, the Wnt pathway, controls their Th2 cell-like skewing.[22] T_{reg} cell-specific deletion of effector genes of the respective pathway (*Yap1* and *Wwtr1* for the Hippo pathway and *Ctnnb1* for the Wnt pathway) suppressed the allergic airway inflammatory Th17 and Th2 responses, respectively. Expression of Notch4 and of the Hippo and the Wnt pathway effectors Yap and β-catenin were all upregulated in lung T_{reg} cells of *Il4ra*^{R576} mice undergoing allergic airway inflammation and were concordantly down-regulated back to WT T_{reg} cell levels upon T_{reg} cell-specific *Grb2* deletion in *Il4ra*^{576} mice. Furthermore, deletion of *Il6ra* specifically in T_{reg} cells inhibited Notch4 expression and suppressed allergic airway inflammation in both control and *Il4ra*^{576} mice. Together, these results delineate the components of the genetic circuit operative in lung T_{reg} cells of *Il4ra*^{R576} mice that directs disease severity, starting with GRB2 recruitment to the IL-4RαR576 variant, leading to T_{reg} cell IL-6 production, and further upregulation of Notch4 and its downstream pathways Hippo and Wnt, altogether resulting in heightened mixed Th2/Th17 cell responses in the airway.

The role of traffic-related particulate matter, most notably UFP, in augmenting airway inflammation in synergy with the *IL4R*^{R576} allele is of special interest, given the increased exposure of inner-city minority populations to traffic-related pollution.[30–32] We have previously demonstrated that in mice particulate matter is overwhelmingly taken up by alveolar macrophages, where it upregulates Jag1 expression to aggravate airway inflammation in synergy with the *Il4ra*^{R576} allele.[17,21] Deletion of *Jag1* in alveolar macrophages abrogated the increased airway inflammation induced by UFP. Our current results further clarify this gene-by-environment interaction, indicating that the increased Jag1 expression in alveolar macrophages induced by UFP would amplify Notch4 signaling intensity, leading to more robust inflammation in subjects carrying the *IL4R*^{R576} allele.[17]

In conclusion, our studies reconcile two key mechanisms for increased asthma severity, one involving the *IL4R*^{R576} allele and the second involving the IL-6, Notch4-Hippo-Wnt axis by showing that the two pathways are linked by the recruitment and activation of GRB2 by IL-4RαR576 variant. These results reveal convergence of pathways hitherto thought of as independently operating to foster increased asthma severity. They also highlight the interaction of genetic and environmental factor in disease pathogenesis and provide novel opportunities for preventive and therapeutic interventions in disease management, including those targeting the Notch4 and IL-6 receptor pathway.[33]

4 | MATERIALS AND METHODS

4.1 | Human subjects

Asthmatic children and young adults and age-matched control subjects were recruited in the Allergy clinic at Boston Children's Hospital and from previously described inner-city cohort populations.[23,24]

Demographics, including age, self-identified ethnicity and gender, and clinical information were obtained from the subjects' clinical electronic records. Informed consent was obtained from adult participants and from legal guardians of minor subjects.

4.2 | Mice

The following mouse strains were obtained from the JAX Laboratories: *Foxp3*^{YFPCre} (B6.129(Cg)-*Foxp3*$^{tm4(YFP/icre)Ayr}$/J),[34] *Il4ra*^{R576} (C.129X1-Il4ra^{tm2Tch}/J),[15] floxed *Il6ra* (*Il6ra*$^{fl/fl}$; B6;SJL-Il6ratm1.1Drew/J) floxed *Grb2* (*Grb2*$^{fl/fl}$; B6.C(Cg)-Grb2$^{tm1.1Lnit}$/J)[35] and floxed Notch4 (*Notch4*$^{fl/fl}$; *Notch4*$^{tm1c[NCOM]Mfgc}$) were obtained from the Canadian mutant mouse repository. *Foxp3*^{YFPCre}*Il6ra*$^{\Delta/\Delta}$, *Il4ra*^{R576}*Foxp3*^{YFPCrev}*Il6ra*$^{\Delta/\Delta}$, *Foxp3*^{YFPCre}*Notch4*$^{\Delta/\Delta}$, *Il4ra*^{R576}*Foxp3*^{YFPCre}*Notch4*$^{\Delta/\Delta}$, *Foxp3*^{YFPCre}*Grb2*$^{\Delta/\Delta}$, and *Il4ra*^{R576}*Foxp3*^{YFPCre}*Grb2*$^{\Delta/\Delta}$ were generated by crossing the respective component strains. Mice were maintained in at Boston Children's Hospital Animal facility under breeding and research protocols approved by the institutional animal care and use committee (IACUC). All experiments were approved by Boston Children's Hospital IACUC.

4.3 | Particles

UFP (≤0.18 μm) was collected in an urban area of downtown Los Angeles, as previously reported.[21] The respective particles were suspended in an aqueous solution, with the hydrophilic components becoming part of the solution, while the solid non-soluble UFP cores are left in suspension. The entire mixture was administered intranasally, as indicated below.

4.4 | Isolation of Human peripheral blood mononuclear cells

Human peripheral blood mononuclear cells (PBMCs) were isolated from full blood from either healthy control, mild asthmatics, moderate asthmatics, or severe asthmatics probands via density gradient using Ficoll (GE Healthcare). PBMCs were then stored frozen in Fetal Calf Serum (FCS) (Sigma Aldrich) and 15% Dimethyl sulfoxide (DMSO) (Sigma Aldrich). The cells were later thawed for flow cytometry analysis. For intracellular cytokine staining, cells were stimulated with PMA (100 ng/ml) and ionomycin (1 μg/ml) for 4 h in the presence of GolgiPlug monensin (1 μg/ml, BD Biosciences) at 37°C in a humidified, 5% CO_2 atmosphere.

4.5 | IL-6 ELISA

EDTA Plasma from 88 probands (Healthy controls, mild, moderate, and severe persistent asthma patients) was used to measure IL-6 using enzyme-linked immunosorbent assay (ELISA) (Invitrogen) according to manufacturer's protocol.

4.6 | Allergic sensitization and challenge

Mice were sensitized to OVA by intraperitoneal (i.p.) injection of 100 μg OVA in 100 μl PBS, then boosted 2 weeks later with a second i.p. injection of OVA in PBS. Control mice were sham sensitized and boosted with PBS alone. Starting on Day 29, both OVA and sham-sensitized mice were challenged with aerosolized OVA at 1%, for 30 min daily for 3 days. Two hours before each OVA aerosol exposure, subgroups of mice were given intranasally (i.n.) either PBS or UFP at 10 μg/100 μl PBS/instillation. Mice were euthanized on Day 32 post sensitization and analyzed. For dust mite-induced allergic airway inflammation, mice received 5 μg of lyophilized D. Pteronyssinus extract (Greer) in 100 μl PBS intranasally for 3 days at the start of the protocol then challenged with the same dose of D. Pteronyssinus extract on days 15–17 with or without UFP at the same concentration as before. Mice were euthanized on Day 18 and analyzed for measures of airway inflammation. Bronchoalveolar lavage (BAL) fluid and lung tissues were obtained and analyzed for cellular components and T cell cytokine expression as described.[16]

4.7 | Measurement of airway functional responses

Allergen-induced airway hyperreactivity (AHR) was measured, as previously described.[17] Anesthetized mice were exposed to doubling concentrations of aerosolized acetyl-β-methacholine (Sigma-Aldrich) by using a Buxco small-animal ventilator (Data Sciences International). The relative peak airway resistance for each methacholine dose, normalized to the saline baseline, was calculated.

4.8 | Lung histopathology staining

Paraffin-embedded lung sections were stained with hematoxylin and eosin (H&E) or Paraffin-acid-Schiff staining (PAS). The lung pathology was scored by blinded operators. Inflammation was scored separately for cellular infiltration around blood vessels and airways: 0, no infiltrates; 1, few inflammatory cells; 2, a ring of inflammatory cells 1 cell layer deep; 3, a ring of inflammatory cells 2–4 cells deep; 4, a ring of inflammatory cells of >4 cells deep.[15] A composite score was determined by the adding the inflammatory scores for both vessels and airways.

4.9 | Anti-Notch4 antibody treatment

The anti Notch4 neutralizing antibodies (InVivoMAb anti-mouse Notch4, HMN4-14) were introduced at the concentration of 10 μg/ml of mAb i.p. with a final volume of 100 μl two hours prior to

sensitizations or challenge on days 1, 14, and 26,27,28, respectively. On Day 30, the mice were analyzed for airway hyperresponsiveness and then sacrificed for further analysis.

4.10 | Flow cytometric analysis of mouse and human cells

Antibodies against the following murine antigens were used for flow cytometric analyses: IL-4 (clone 11B11, 1:300 dilution), Siglec-F (E50-2440, 1:300), Foxp3 (FJK-16S, 1:300), IFN-γ (XMG1.2, 1:300), IL-13 (eBio13a, 1:300), CD11c (30-f11, 1:500), CD11b (M1/70, 1:500) (eBioscience), CD4 (RM4-5, 1:500), CD3 (145-2C11, 1:500), IL-17 (TC11-18H10.1, 1:200), GR-1 (RB6-8C5, 1:500), CD45 (30-F11, 1:300), Notch4 (HMN4-14 1:200) (Biolegend), IL-6 (MP5-20F3, 1:200, Biolegend), Yap1 (147,295 1:200), and β-Catenin (196,624, 1:500). Antibodies against the following human antigens were used: CD3 (HIT3a 1:300), CD4 (RPA-T4, 1:300), Foxp3 (236A/E7, 1:200), Notch1 (HMN1-519 1:100), Notch2 (HMN2-25 1:100), Notch3 (HMN3-21 1:100), Notch4 (HMN4-2 1:50), IL-4 (MP4-25D2, 1:250), IL-13 (JES10-5A2, 1:200), IL-17 (BL168, 1:200), CCR6 (G034E3, 1:250), CRTH2 (BM16, 1:300), CD127 (A019D5, 1:200) (Biolegend) The specificity and optimal dilution of each antibody was validated by testing on appropriate negative and positive controls or otherwise provided on the manufacturer's website. Intracellular cytokine staining was performed as previously described.[36] Cytokines were stained overnight as previously prescribed in.[36] Dead cells were routinely excluded from the analysis based on the staining of eFluor 780 Fixable Viability Dye (1:1000 dilution) (eBioscience). Stained cells were analyzed on a BD LSR Fortessa cell analyzer (BD Biosciences) and data were processed using Flowjo (Tree Star Inc.).

4.11 | Statistical analysis

Logistic regression and Chi-square statistics were used to investigate the independent effect of race and genotype on asthma severity. Student's two-tailed t-test, one- and two-way ANOVA and repeat measures two-way ANOVA with Sidak post-test analysis of groups were used to compare test groups, as indicated. A p-value $<.05$ was considered statistically significant.

4.12 | Study approval

Recruitment of human subjects was approved by the Institutional Review Board at Boston Children's Hospital. All animal studies were reviewed and approved by the Boston Children's Hospital office of Animal Care Resources.

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AUTHOR CONTRIBUTIONS

M.B, H.H., Q.C, and T.A.C. designed experiments. H.H., M.B., Q.C, M.W, T.M.F.C, J.F., and D.E. performed experiments and developed experimental models. E.C., A.C., and W.P. recruited patients. E.C and P.R.C. analyzed the demographics of the human asthmatic and control populations. C.S. and A.J.M provided UFP. M.B, H.H., Q.C, and T.A.C. wrote the manuscript.

CONFLICT OF INTEREST

T.A.C., H.H., and A.M. are inventors on published US patent application No. WO2019178488A1 submitted by The Children's Medical Center Corporation, titled "Method for treating asthma or allergic disease". T.A.C. and H.H. are scientific co-founders of and hold equity in Alcea Therapeutics. Wanda Phipatanakul is a Consultant for Genentech, Novartis, Regeneron, Sanofi Genzyme, and Glaxo Smith Kline, and receives clinical trial support from Genentech, Novartis, Regeneron, Circassia, Thermo Fisher, Monaghan, Lincoln Diagnositcs, Alk Abello, and Glaxo Smith Kline.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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