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A Common IL-4 Receptor Variant Promotes Asthma Severity via a T_{reg} Cell GRB2-IL-6-Notch4 Circuit

Mehdi Benamar[1,2,*], **Hani Harb**[1,2,3,*], **Qian Chen**[1,2,*], **Muyun Wang**[1,2], **Tsz Man Fion Chan**[1,2], **Jason Fong**[1,2], **Wanda Phipatanakul**[1,2], **Amparito Cunningham**[1,2], **Deniz Ertem**[1,2], **Carter R. Petty**[4], **Amirhosein J. Mousavi**[5], **Constantinos Sioutas**[5], **Elena Crestani**[1,2,†], **Talal A. Chatila**[1,2,†,$]

[1]Division of Immunology, Boston Children's Hospital, Boston, MA, USA.

[2]Department of Pediatrics, Harvard Medical School, Boston, MA, USA;

[3]Institute for Medical Microbiology and Virology, Technical University Dresden, Germany

[4]Institutional Centers for Clinical and Translational Research, Boston Children's Hospital, Boston, Massachusetts;

[5]Department of Civil and Environmental Engineering, University of Southern California, CA, USA.

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 Treg cells as a function of *IL4R*^{R576} allele. The capacity of IL-4RαR576 to upregulate Notch4 expression on Treg 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 Treg 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.

[$]To whom correspondence should be addressed: Talal A. Chatila, M.D., M.Sc., Division of Immunology, Boston Children's Hospital, Department of Pediatrics, Harvard Medical School, Boston, MA, USA. talal.chatila@childrens.harvard.edu.

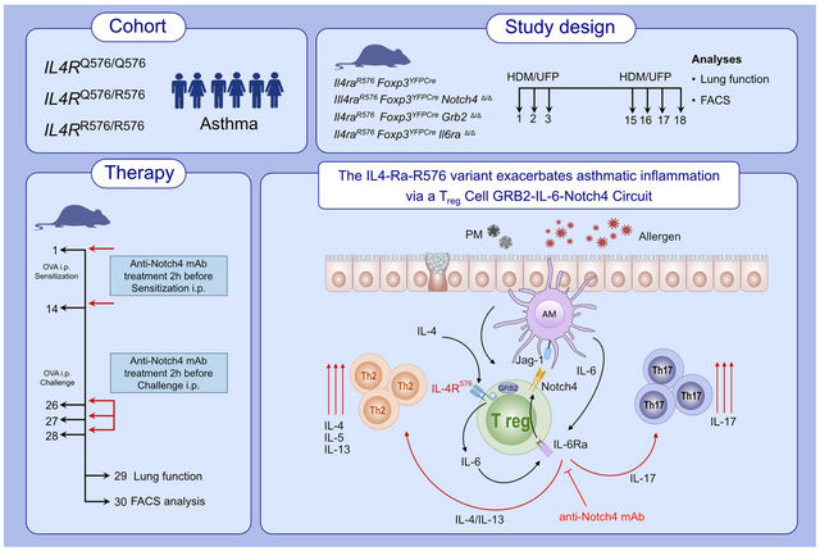
[*]Both authors contributed equally to the manuscript.

[†]Both authors contributed equally to the manuscript.

Authors 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.

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

Graphical Abstract



Keywords

Asthma; interleukin 4 receptor; Notch4; GRB2; Regulatory T cells

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 life style 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

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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.

Results

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, 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 S2). Furthermore, among Hispanic individuals with asthma, those who

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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.

IL4R^{R576} Asthmatics exhibit increased T_H2 and T_H17 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 with asthma severity (Fig. 1, A–B). They also present a higher levels of Th17 cytokines and markers (CCR6) (Fig. 1, C–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 (Fig. 1E; see Fig. 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 (Fig. 1F). Other Notch receptors are not upregulated in T_{reg} or T_{eff} as a function of either disease severity and *IL4R*^{R576} allele carriage (Fig. 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 (Fig. 1, G and H). These results established the upregulation of the IL-6-Notch4 axis in *IL4R*$^{R576+}$ asthmatics in direct correlation with disease severity.

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

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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 (Fig. 2, A – C, Fig.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*$^{\Delta/\Delta}$), indicating that the augmentation of AHR and allergic lung inflammation by the *Il4ra*^{R576} allele was Notch4-dependent (Fig. 2, A – 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*. (Fig. 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 (Fig. 2, D–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 (Fig. 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-4RαR576 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 (Fig. 3, A and B), OVA-specific IgE (Fig. 3C) $CD4^+$ T cell lymphocytosis, tissue eosinophilia and neutrophilia (Fig. 3, D – F) and tissue Th cell infiltration (Fig.3, G and H).

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

Our previous studies have demonstrated that the IL-4RαR576 variant uniquely recruits the intracellular signaling intermediate Grb2 to 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*$^{\Delta/\Delta}$) failed to exhibit the

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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}) (Fig. 4, A–F). Importantly, deletion *of Grb2* in control mice bearing the wild-type *Il4ra*^{Q576} allele (*Foxp3*^{YFPCre}*Grb2*$^{\Delta/\Delta}$) 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 (Fig. 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.

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

To determine the role of the IL-4RαR576-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*$^{\Delta/\Delta}$, respectively), compared to those of *Il4ra*^{R576} mice (*Il4ra*^{R576}*Foxp3*^{YFPCre} and *Il4ra*^{R576}*Foxp3*^{YFPCre}*Grb2*$^{\Delta/\Delta}$, respectively). The respective mouse groups were subjected to HDM-induced allergic airway inflammation, as shown in Fig. 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 (Fig. 5A; see Fig. 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-4RαR576 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 (Fig. 5B). The role of IL-6 in the upreglation of Notch4 by IL-4RαR576 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*$^{\Delta/\Delta}$ and *Il4ra*^{R576}*Foxp3*^{YFPCre}*Il6ra*$^{\Delta/\Delta}$, respectively) and abrogated differential disease severity in the latter mice (Fig. S7A–H). T_{reg} cell-specific *Grb2* deletion also abrogated 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 (Fig. 5, C and D). These results are in agreement with the abrogation in HDM-treated *Il4ra*^{R576}*Foxp3*^{YFPCre}*Grb2*$^{\Delta/\Delta}$ mice of the augmented Th2 and Th17 cells responses typically associated with the *Il4ra*^{R576} allele. Together, these results indicated that the

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destabilization of lung T_{reg} cells upon signaling via IL-4RαR576 variant in allergic airway inflammation proceeded by a Grb2-IL-6-Notch4 circuit.

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 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

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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 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].

Materials and Methods

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.

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*

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(*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*^{YFPCre}*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

Particles.

UFP (≤0.18 μm) were 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.

Isolation of Human peripheral blood mononuclear cells (PBMCs).

Human 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 hours in the presence of GolgiPlug monensin (1 μg/ml, BD Biosciences) at 37°C in a humidified, 5% CO_2 atmosphere.

IL-6 ELISA.

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

Allergic sensitization and challenge.

Mice were sensitized to OVA by intraperitoneal (i.p.) injection of 100 μg OVA in 100 μl PBS, then boosted two 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 minutes 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/100μl 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]

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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.

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.

Anti-Notch4 antibody treatment.

The anti Notch4 neutralizing antibodies (InVivoMAb anti-mouse Notch4, HMN4-14) were introduced at the concentration of 10 ug/ml of mAb i.P. with a final volume of 100μl 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.

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 (147295 1:200) and β-Catenin (196624, 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.).

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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 <0.05 was considered statistically significant.

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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Competing interests:

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.

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

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UFP	ultrafine particles

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- Asthmatics carrying the IL4Ra^{R576} variant have increased Notch4 expression on their circulating Treg cells as a function of disease severity and serum IL-6 concentrations.
- IL-4Ra^{R576} acts via GRB2 and IL6R in Treg cells to upregulate Notch4 and its downstream signaling intermediates Yap1 and b-catenin, leading to exacerbated lung inflammation
- Treatment with an anti-Notch4 neutralizing antibody inhibits allergic airway inflammation

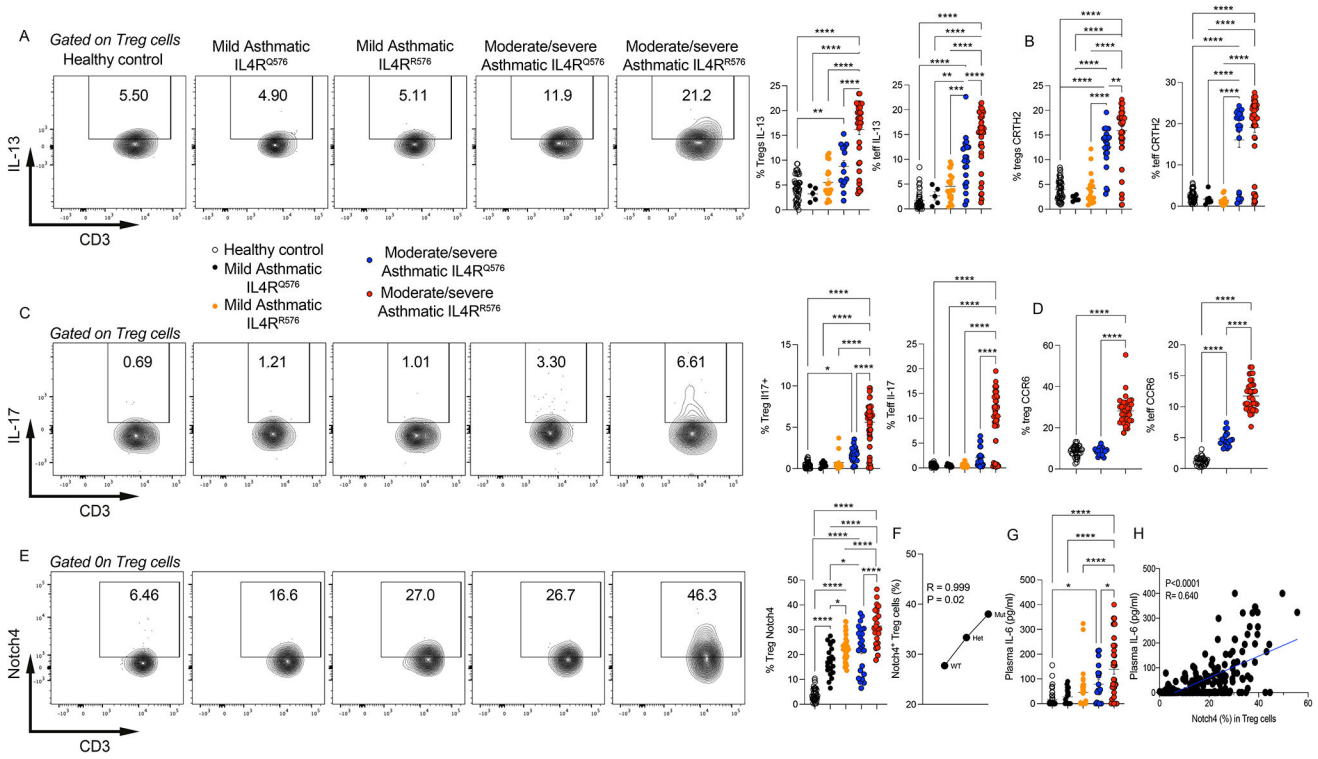

Fig.1, Notch4, Chemokine and cytokine profiles of circulating CD4^{+} T cells in asthmatics segregated by the *IL4R* genotype.

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<0.05, ***P<0.001, ****P<0.0001. simple linear regression analysis (**F,H**); ***P<0.001, ****P<0.0001.

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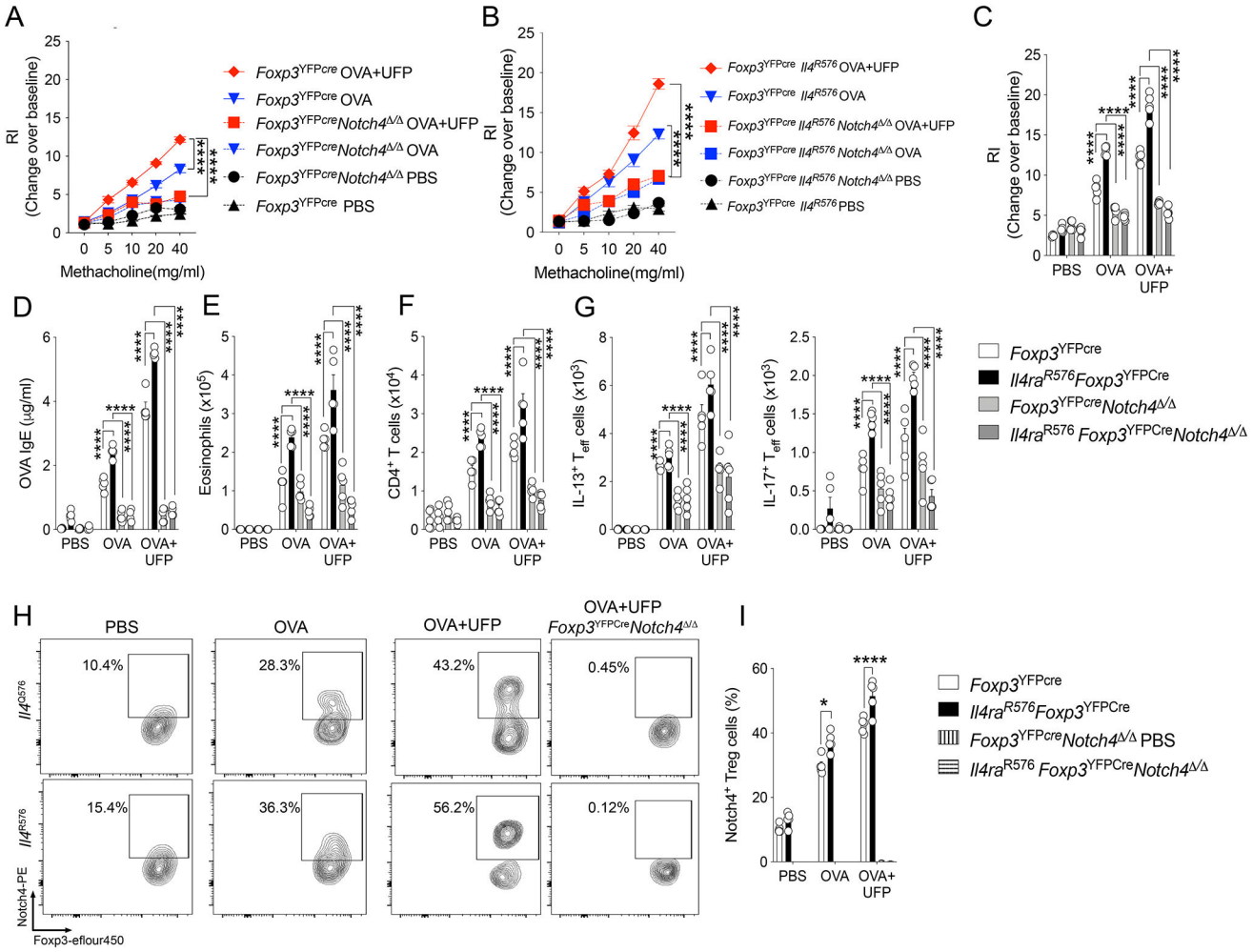

Fig. 2. Notch4 expression on lung T_{reg} cells licenses allergic airway inflammation in *Il4Rα*^{R576} mice.

(**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.

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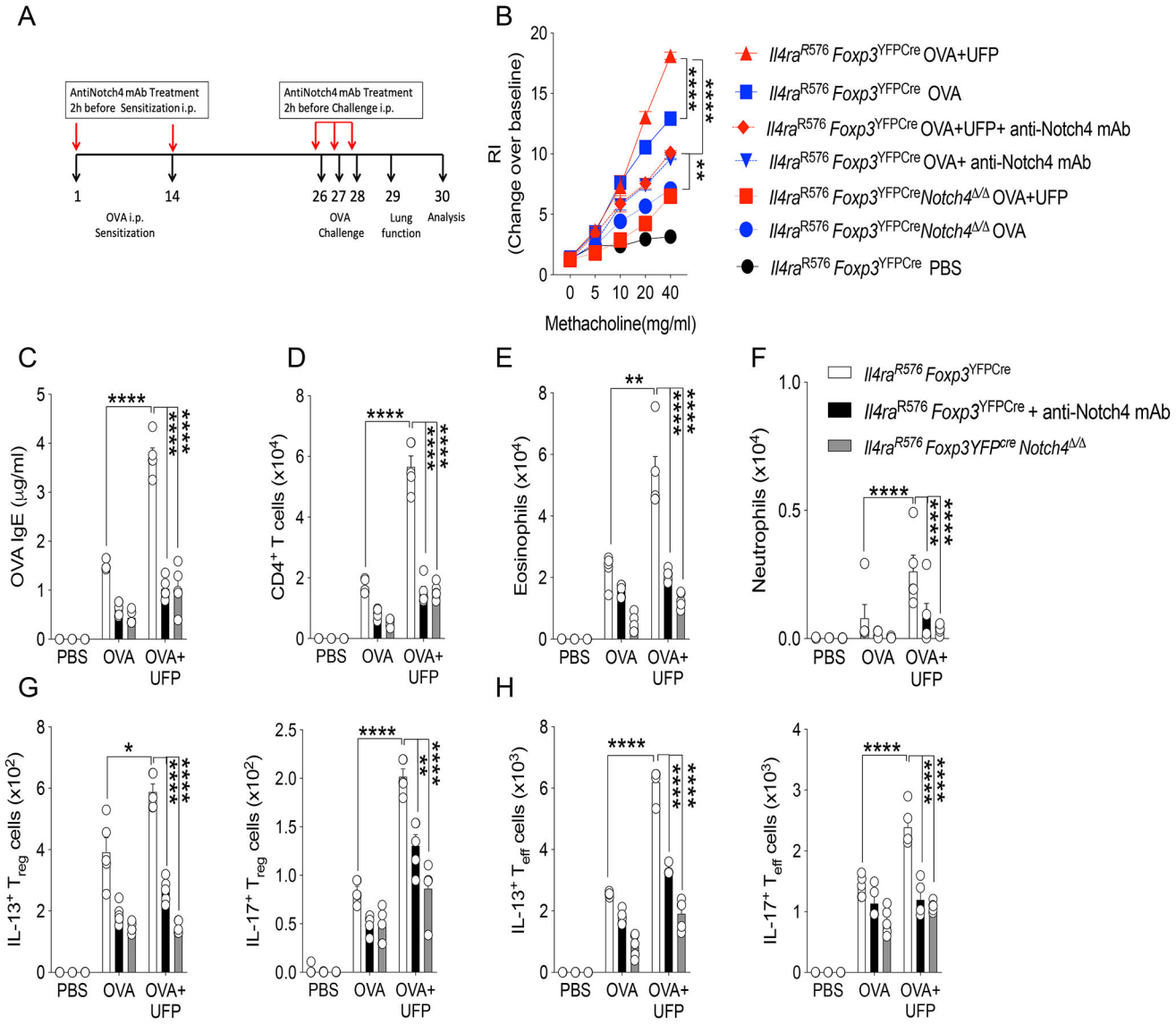

Fig. 3. Anti-Notch4 neutralizing mAb inhibit allergic airway inflammation in *Il4Rα*^{R576} mice.

(**A**) scheme of antibody therapy (10µg) 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<0.01, ***P<0.001, ****P<0.0001.

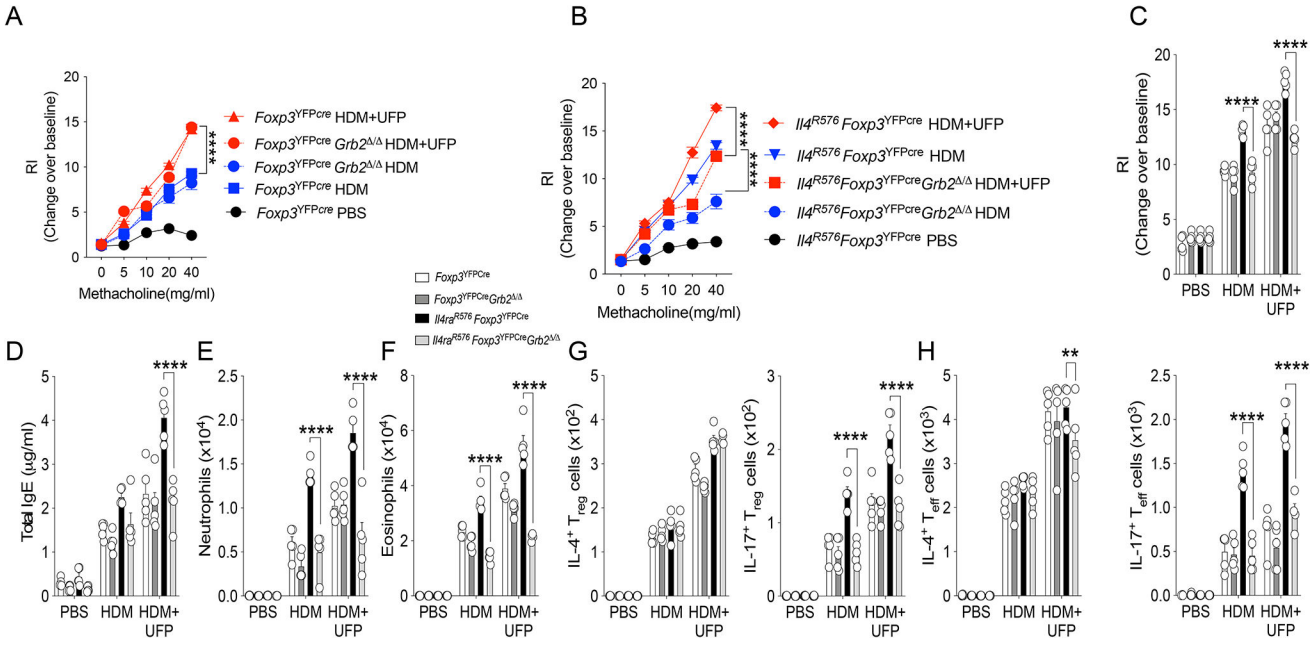

Fig. 4. GRB2 drives the super induction of allergic airway inflammation in *Il4Ra*^{R576} mice.

(**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<0.01, ***P<0.001, ****P<0.0001.

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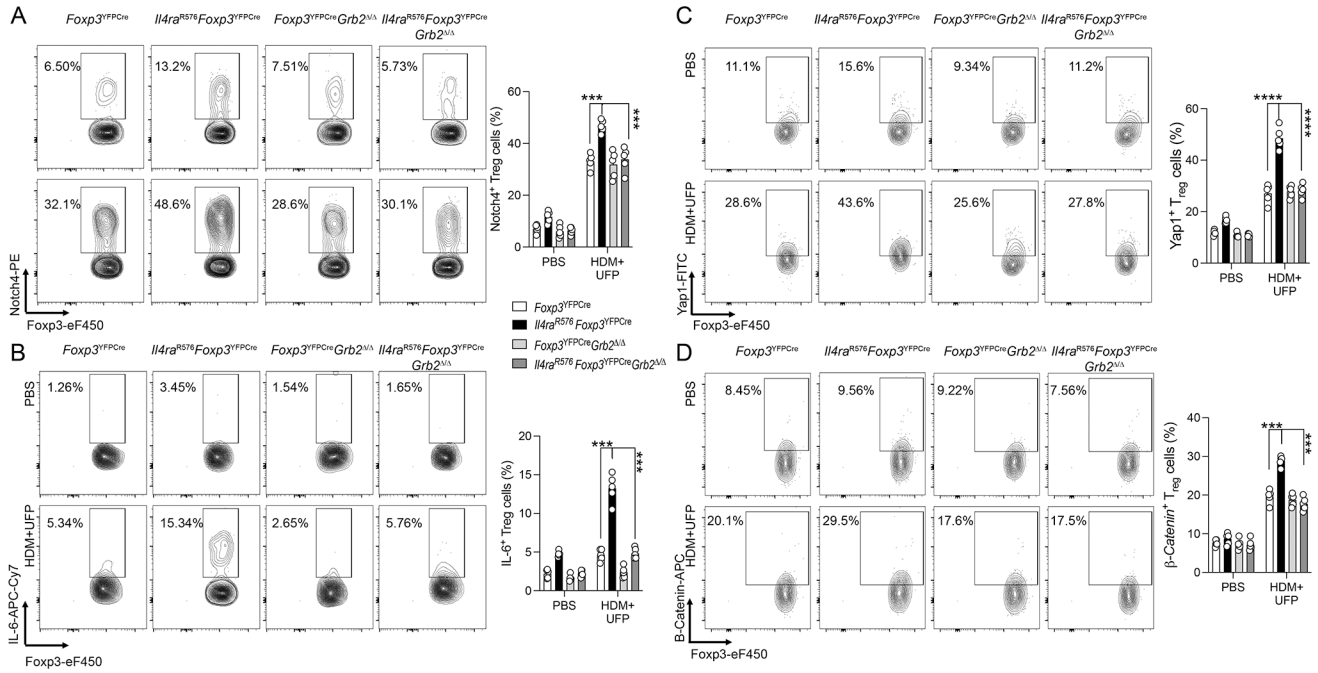

Fig. 5. GRB2 drives the super induction of Notch4 and downstream molecules expression in *Il4Ra*^{R576} mice.

(**A**) Notch4 expression in Treg cells in the respective mouse groups in Sham and HDM sensitized and challenged mice. (**B**) IL-6 expression in Treg cells in the respective mouse groups in Sham and HDM sensitized and challenged mice. (**C**) Yap1 expression in Treg cells in the respective mouse groups in Sham and HDM sensitized and challenged mice. (**D**) β-Catenin expression in Treg 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<0.001, ****P<0.0001.

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