

An asthma-associated *IL4R* variant exacerbates airway inflammation by promoting conversion of regulatory T cells to T_H17-like cells

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Mechanisms by which regulatory T (T_{reg}) cells fail to control inflammation in asthma remain poorly understood. We show that a severe asthma-associated polymorphism in the gene encoding the interleukin (IL)-4 receptor alpha chain (*IL4R*^{R576}) promotes conversion of induced T_{reg} (iT_{reg}) cells toward a T helper 17 (T_H17) cell fate. This skewing is mediated by the recruitment by IL-4Rα^{R576} of the growth-factor-receptor-bound protein 2 (GRB2) adaptor protein, which drives IL-17 expression by activating a pathway that involves extracellular-signal-regulated kinase, IL-6 and the transcription factor STAT3. T_{reg} cell-specific deletion of genes that regulate T_H17 cell differentiation, including *IL6ra* and RAR-related orphan receptor gamma (*Rorc*), but not of *IL4* or *IL13*, prevented exacerbated airway inflammation in mice expressing *IL4R*^{R576} (hereafter referred to as *IL4R*^{R576} mice). Furthermore, treatment of *IL4R*^{R576} mice with a neutralizing IL-6-specific antibody prevented iT_{reg} cell reprogramming into T_H17-like cells and protected against severe airway inflammation. These findings identify a previously unknown mechanism for the development of mixed T_H2–T_H17 cell inflammation in genetically prone individuals and point to interventions that stabilize iT_{reg} cells as potentially effective therapeutic strategies.

Asthma is characterized by airway hyper-responsiveness (AHR), chronic inflammation and tissue remodeling¹. Evidence points to reduced frequency and/or suppressive activity of FOXP3⁺ T_{reg} cells in asthma and atopic conditions^{2–4}. Intense inflammation in experimental allergic asthma, as well as in other conditions such as rheumatoid arthritis and multiple sclerosis, may lessen T_{reg} cell suppression or redirect T_{reg} cells to pro-inflammatory phenotypes^{5–9}; however, the molecular basis for impaired T_{reg} cell function in asthma and the effect of T_{reg} cell dysfunction on asthma severity remain to be fully elucidated.

The cytokines IL-4 and IL-13, which function via two distinct heterodimeric IL-4 receptor complexes, have a pivotal role in asthma pathogenesis. IL-4, which binds to both the IL-4R type I (composed of the IL-4Rα and the common cytokine receptor γc chains) and type II (composed of the IL-4Rα and IL-13Rα1 chains) directs T_H2 cell differentiation. In contrast, IL-13, which exclusively binds to the IL-4R type II, promotes airway inflammation and remodeling¹⁰. Increased signaling via IL-4Rα and the downstream transcription factor STAT6 exacerbates allergic airway inflammation and promotes food allergy; STAT6 has been shown to involve suppressed differentiation of allergen-specific T_{reg} cells and their re-programming into T_H2 cell-like cells^{9,11}.

Polymorphisms in the gene encoding IL-4Rα are associated with atopy and asthma¹². In particular, a glutamine (Q)-to-arginine (R) substitution at amino acid residue 576 of human IL-4Rα (IL-4Rα^{Q576R})

has been linked to asthma exacerbation and severity^{13–16}. The Gln576 residue is conserved in the murine IL-4Rα chain. Its replacement by Arg576 in *IL4R*^{tm2Tch} mice (also known as *IL4R*^{R576} mice) resulted in augmented allergic airway inflammation, severe eosinophilic infiltration and increased T_H2 responses^{17,18}. Gln576 lies immediately downstream of a STAT6-binding site at Tyr575, one of three such sites present in IL-4Rα (ref. 19), yet no discernable effect of the Arg576 mutation on STAT6 activation has been noted, indicating that Arg576 mobilizes alternative signaling pathways^{17,20}. We show here that the IL-4Rα^{R576} mutant enables recruitment of the adaptor GRB2 to promote airway inflammation by a mechanism involving IL-4-directed iT_{reg} cell differentiation toward the T_H17 cell lineage.

RESULTS

IL4R^{R576} T_{reg} cell phenotype in allergic airway inflammation

Whereas allergic asthma has classically been associated with a skewed T_H2 cell response, a subgroup of patients with severe disease manifest a mixture of T_H2 and T_H17 cell responses in their airways^{21–24}. To determine whether the IL-4Rα^{R576}-encoding mutation elicits T_H17 airway responses *in vivo*, we used a house dust mite (HDM)-induced model of airway inflammation¹⁸. *IL4R*^{R576} mice, which are homozygous for the *IL4R*^{R576} mutation, showed severe airway inflammation, as compared to control mice that were homozygous for the wild-type (WT) *IL4R*^{Q576} allele. The *IL4R*^{R576} mice had increased

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inflammatory cell infiltrates and mucus production, accentuated methacholine-induced AHR and robust lung tissue eosinophilia accompanied by neutrophilia, the latter of which was masked in earlier studies by the use of alum as an adjuvant (data not shown) (Fig. 1a–e)¹⁷. HDM treatment led to increased frequency and number of T_{reg} cells in lung tissues of WT mice, but this increase was tempered in *Il4ra*^{R576} mice (Fig. 1f). In contrast, HDM-treated *Il4ra*^{R576} mice had substantially increased frequencies and numbers of IL-17-, IL-13- and IL-6-expressing CD4⁺FOXP3[−] conventional T (T_{conv}) cells and CD4⁺FOXP3⁺ T_{reg} cells, as compared to HDM-treated WT control mice (Fig. 1g–i). Expression of IL-17 and IL-6, but not of IL-13, in *Il4ra*^{R576} T_{conv} and T_{reg} cells was largely overlapping (Supplementary Fig. 1a,b). Expression of IL-17, IL-13 and IL-5 was increased, whereas expression of interferon (IFN)- γ was decreased, in the bronchoalveolar lavage (BAL) fluid of HDM-treated *Il4ra*^{R576} mice as compared to that in the BAL fluid of HDM-treated WT control mice (Supplementary Fig. 1c). Furthermore, HDM exposure was associated with increased expression of the T_H17- and T_H2-promoting transcription factors ROR- γ t and GATA3, respectively, in lung T_{conv} and T_{reg} cells (Supplementary Fig. 1d–g). The frequencies and numbers of IL-4-producing T_{conv} and T_{reg} cells in lung tissues of HDM-treated *Il4ra*^{R576} mice tended to increase as compared to those in WT controls, whereas the frequencies and numbers of IFN- γ -producing cells were unchanged (Supplementary Fig. 1h,i).

Expression of the transcription factor Helios differentiates between natural T_{reg} (nT_{reg}) cells, which develop in the thymus and are biased toward recognition of self-antigens, and iT_{reg} cells, which arise *de novo* in the peripheral tissues and are biased toward recognition of foreign antigens²⁵. Analysis of the T_{reg} cells in lung tissue revealed decreased numbers of FOXP3⁺Helios^{low} T_{reg} cells in HDM-treated *Il4ra*^{R576} as compared to those in HDM-treated WT mice, indicating the existence of a reduced iT_{reg} cell population (Supplementary Fig. 2a,b). Expression of IL-17 and the T_H17 cell-associated chemokine receptor CCR6 was largely overlapping and highly enriched in T_{reg} and T_{conv} cells of HDM-treated *Il4ra*^{R576} mice, as compared to that in WT mice (Supplementary Fig. 2c,d). In *Il4ra*^{R576} T_{reg} cells, IL-17 and CCR6 were almost exclusively restricted to the FOXP3⁺Helios^{low} iT_{reg} cell population (Supplementary Fig. 2e,f)^{26,27}.

Il4ra^{R576} iT_{reg} cells express IL-13, IL-17 and IL-6

Treatment with transforming growth factor (TGF)- β 1 together with stimulation of the T cell receptor (TCR) converts naive CD4⁺FOXP3[−] T cells into FOXP3⁺ iT_{reg} cells²⁸. Given the increased production of IL-17 and IL-13 in lung T_{reg} cells of allergen-exposed *Il4ra*^{R576} mice, we examined *in vitro*-generated *Il4ra*^{R576} iT_{reg} cells for aberrant cytokine expression and functional competency in suppressing allergic airway inflammation. We used WT and *Il4ra*^{R576} DO11.10⁺Rag2^{−/−}Foxp3^{EGFP} mice, which express the DO11.10 TCR specific for an ovalbumin (OVA)_{323–339} peptide. These mice normally carry naive CD4⁺DO11.10⁺Foxp3[−], but not Foxp3⁺, T cells by virtue of being on a recombination activation gene 2 (Rag2)-deficient background. They also carry a *Foxp3* allele (*Foxp3*^{EGFP}) that enables expression in differentiated iT_{reg} cells of the enhanced green fluorescent protein (EGFP) under control of the germline *Foxp3* promoter. Naive WT and *Il4ra*^{R576} CD4⁺DO11.10⁺Rag2^{−/−}Foxp3^{EGFP} T cells were purified by cell sorting and differentiated into iT_{reg} cells in culture by stimulation with CD3- and CD28-specific monoclonal antibodies (mAbs) and TGF- β 1. *Il4ra*^{R576} CD4⁺ T cells showed decreased conversion to FOXP3⁺ T_{reg} cells as compared to WT controls, a deficit that was exacerbated by the addition of IL-4 to the cell cultures (Fig. 2a,b).

Notably, a sizeable fraction of *Il4ra*^{R576}, but not of WT, CD4⁺FOXP3⁺ iT_{reg} cells and CD4⁺FOXP3[−] T_{conv} cells expressed IL-17, which was further increased by exposure to IL-4 (Fig. 2c,d). Anti-CD3 and anti-CD28 stimulation of *Il4ra*^{R576} T cells induced elevated expression of IL-13 as compared to that in WT controls, independently of added TGF- β 1. IL-13 and IL-17 expression was largely non-overlapping. Addition of IL-4 induced increased IL-17 and IL-13 production in both CD4⁺FOXP3⁺ and CD4⁺FOXP3[−] populations (Fig. 2e,f). In line with the above studies, expression of ROR- γ t and GATA3 was upregulated in *Il4ra*^{R576} iT_{reg} cells relative to that in WT control cells (Fig. 2e,f and Supplementary Fig. 3a,b). IL-6 was co-expressed with IL-17 in *Il4ra*^{R576} T_{reg} cells (Fig. 2g,h); expression of both cytokines was IL-4 dependent, and IL-17 expression was also IL-6 dependent, consistent with autocrine and paracrine IL-4 production driving IL-6-dependent IL-17 expression (Fig. 2g,h). In contrast, IL-13 and IL-4 expression was increased in *Il4ra*^{R576} T_{reg} cells in an IL-4- but not IL-6-dependent manner (Supplementary Fig. 3c–e). IL-4 plus TGF- β 1 treatment induced a modest, equivalent increase in IL-9 expression in WT and *Il4ra*^{R576} iT_{reg} cells (Supplementary Fig. 3f–h).

The cell surface protein neuropilin 1 (NRP1) is highly expressed on nT_{reg} cells but not on iT_{reg} cells^{29,30}. To determine the effect of IL-4 signaling on *Il4ra*^{R576} nT_{reg} cells, splenic NRP1^{high} WT and *Il4ra*^{R576} T_{reg} cells, which are overwhelmingly nT_{reg} cells^{29,30}, were isolated to high purity by cell sorting (Supplementary Fig. 4a) and examined for their suppressive capacity in an *in vitro* T cell proliferation assay. IL-4 treatment did not affect the suppressive function of either WT or *Il4ra*^{R576} nT_{reg} cells (Supplementary Fig. 4b,c). Treatment with anti-CD3 and anti-CD28 in the presence of IL-4 induced expression of IL-17 but not IL-13 in a minor population of *Il4ra*^{R576}, but not WT, nT_{reg} cells that was on average tenfold less in frequency than that noted for IL-4-treated *Il4ra*^{R576} iT_{reg} cells (Supplementary Fig. 4d,e). Also, treatment of naive *Il4ra*^{R576} T_{conv} cells with IL-4 failed to induce IL-17 expression in the absence of TGF- β 1 (Supplementary Fig. 4f,g). These results indicated that the capacity of *Il4ra*^{R576} T_{reg} cells to express IL-17 in response to IL-4 treatment is largely restricted to the iT_{reg} cell population.

Impaired function of *Il4ra*^{R576} T_{reg} cells

To assess the suppressive capacities of *Il4ra*^{R576} versus WT iT_{reg} cells, we used an adoptive transfer model. iT_{reg} cells were derived from naive splenic CD4⁺ T cells of WT and *Il4ra*^{R576} *Thy1.1*⁺*DO10.11*⁺*Rag2*^{−/−}*Foxp3*^{EGFP} mice and adoptively transferred to OVA-sensitized *Il4ra*^{R576} *Thy1.2*⁺ mice, which were then challenged with aerosolized OVA and analyzed (Supplementary Fig. 5a). WT iT_{reg} cells almost completely abrogated OVA-induced tissue inflammation, goblet cell hyperplasia, AHR, eosinophilia–neutrophilia and lymphocytosis in the lungs of recipient *Il4ra*^{R576} mice. They also suppressed IL-17 and IL-13 expression in the recipient lung T_{conv} and T_{reg} cells. In contrast, *Il4ra*^{R576} iT_{reg} cells failed to do so (Supplementary Fig. 5b–f). Unlike the transferred WT iT_{reg} cells, up to a third of donor *Il4ra*^{R576} iT_{reg} cells lost their EGFP expression to become exiT_{reg} cells that expressed either IL-17 or, less frequently, IL-13, consistent with heightened instability (Supplementary Fig. 5g–j).

We used CCR6 as a marker of T_{reg} cells that were committed toward the T_H17 cell lineage to examine their functional, epigenetic and transcriptional profiles. CCR6⁺ *Il4ra*^{R576} T_{reg} cells isolated from OVA-sensitized and challenged mice showed decreased methylation of the *Foxp3* CNS2 enhancer region, indicative of decreased T_{reg} cell phenotypic stability (Fig. 3a,b). They also showed profoundly decreased suppressive function in an *in vitro* T cell proliferation assay,

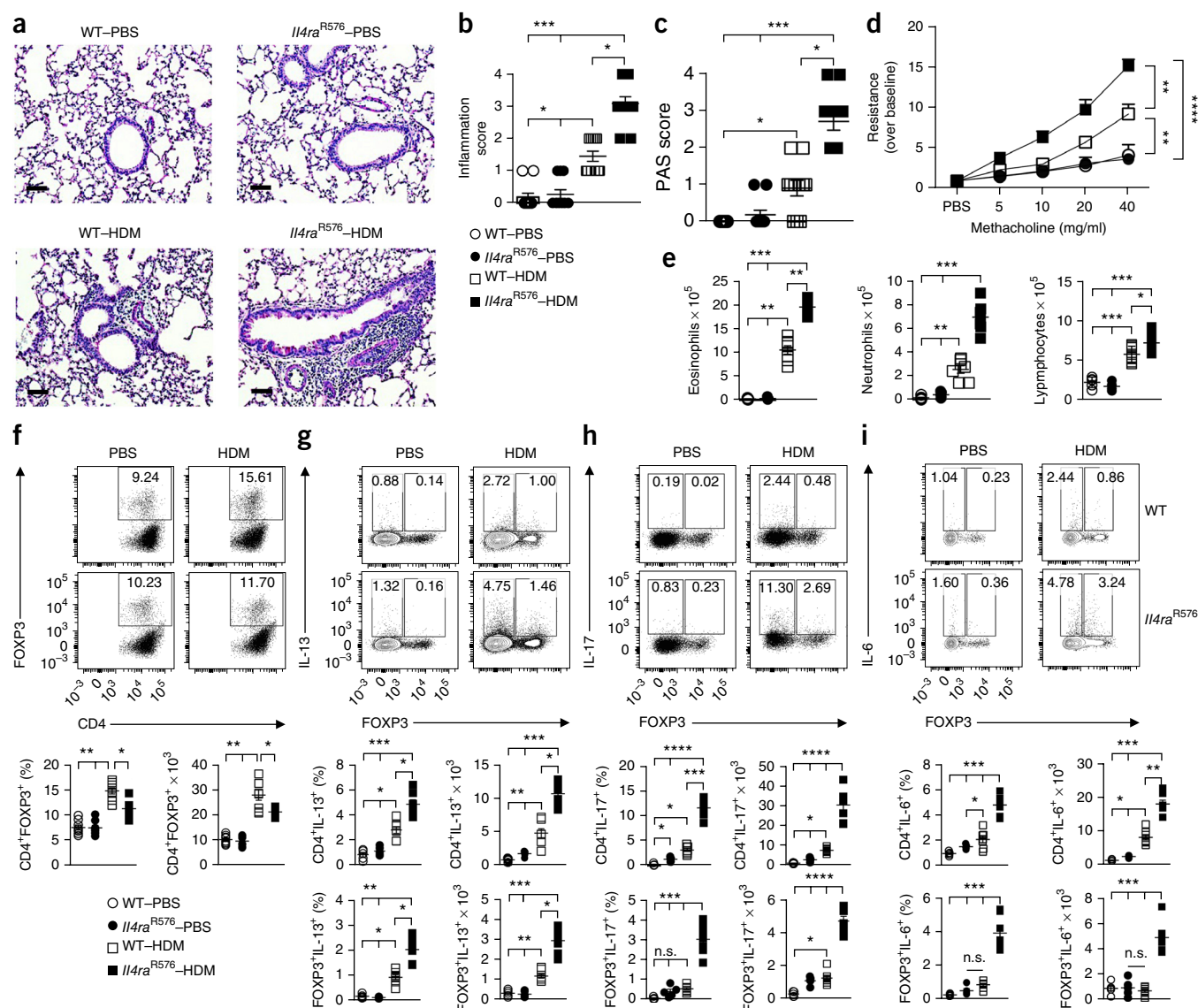


Figure 1 *Il4ra*^{R576} polymorphism promotes enhanced lung inflammation and AHR, and is associated with increased IL-17, IL-6 and IL-13 expression. (a) Representative PAS staining of lung sections isolated from WT (left) and *Il4ra*^{R576} (right) mice that were either sham-treated with PBS (top) or immunized and challenged with HDM (bottom) and analyzed 24 h after the last challenge ($n = 8$ mice per group). Scale bars, 50 μ m. (b,c) Inflammation (b) and PAS (c) scores from the groups of mice shown in a, as indicated in the key. (d) Methacholine-induced AHR for the same groups shown in a, as indicated in the key. (e) Absolute numbers of lung tissue eosinophils, neutrophils and lymphocytes in the respective mouse groups (PBS, $n = 5$ mice per group; HDM, $n = 7$ mice per group). (f) Representative flow cytometric analysis (top) and quantification of frequencies and absolute numbers (bottom) of CD4⁺FOXP3⁺ T_{reg} cells within lung tissue ($n = 8$ mice per group). (g–i) Representative flow cytometric analysis (top) and quantification (bottom) of IL-13 (g), IL-17 (h) and IL-6 (i) expression by CD4⁺FOXP3⁺ T_{conv} or CD4⁺FOXP3⁺ T_{reg} cells within the CD90.2⁺ gated cells (which represents all T lymphocytes) in lung tissues of WT and *Il4ra*^{R576} mice that were treated with PBS or HDM (PBS, $n = 5$ mice per group; HDM, $n = 7$ mice per group). Throughout, results represent mean $\pm$ s.e.m. from two independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; n.s., not significant; by one-way analysis of variance (ANOVA) with Bonferroni *post hoc* analysis. For AHR analysis (d), ** $P < 0.01$ and **** $P < 0.0001$ by two-way repeated measures ANOVA.

as compared to CCR6⁺ WT and CCR6⁺ *Il4ra*^{R576} T_{reg} cells (Fig. 3c). Transcriptional profiling revealed increased expression in CCR6⁺ *Il4ra*^{R576} T_{reg} cells of genes associated with a T_H17 cell signature, including *Rorc*, *Ccr6*, *Il23r*, *Il17a*, *Il17f*, *Il1r1*, *Nr1d1*, *CstII* and *Ahr* (Fig. 3d and Supplementary Data Set 1)^{26,31–33}. To determine whether the T_H17 cell-like T_{reg} cells in the lungs of allergen-treated *Il4ra*^{R576} mice gave rise to FOXP3⁺ T_H17 cells, we used a lineage-tracing approach in *Rosa26* Stop–flox yellow fluorescent protein (YFP) reporter (*R26*^{YFP}) mice that were crossed with *Foxp3*-directed Cre

recombinase (*Foxp3*^{EGFPCre}) mice on a WT or *Il4ra*^{R576} background. *Il4ra*^{R576}*Foxp3*^{EGFPCre}*R26*^{YFP} and control *Foxp3*^{EGFPCre}*R26*^{YFP} mice were either sham or OVA sensitized, and then challenged with aerosolized OVA. CCR6 and cytokine expression was examined in T_{reg} (EGFP⁺YFP⁺), exT_{reg} (EGFP⁺YFP⁺) and CD4⁺ T_{conv} (EGFP⁺YFP⁺) cells. We found markedly increased frequencies of EGFP⁺YFP⁺ exT_{reg} cells in the lungs of OVA-sensitized and challenged *Il4ra*^{R576}*Foxp3*^{EGFPCre}*R26*^{YFP} relative to those in the lungs of control *Foxp3*^{EGFPCre}*R26*^{YFP} mice, indicative of heightened T_{reg} cell instability

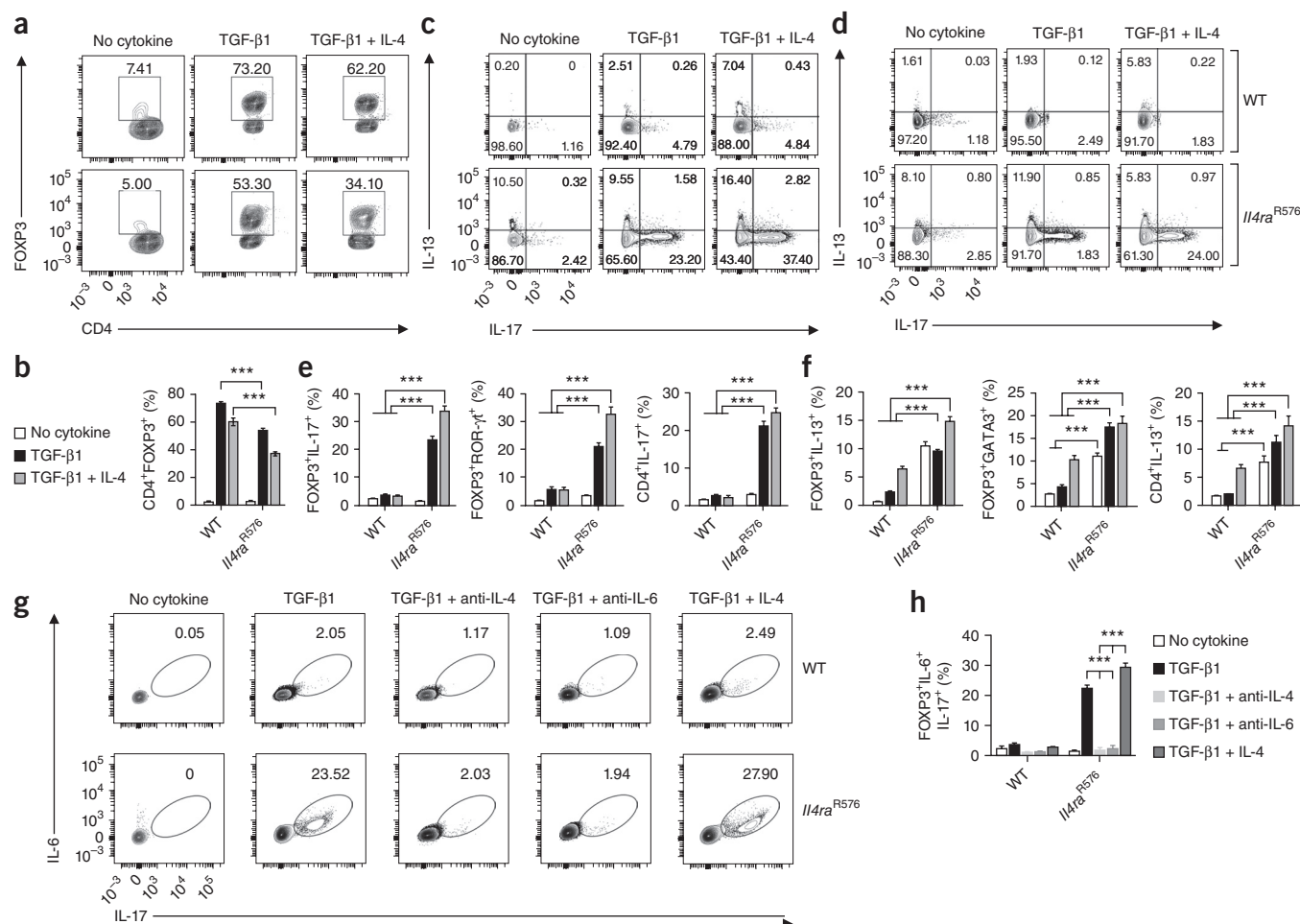


Figure 2 Defective formation and impaired suppressive function of *Il4ra*^{R576} iTreg cells. **(a,b)** Flow cytometric plots **(a)** and quantification **(b)** of iTreg cell generation *in vitro* from naive *DO11.10⁺Rag2^{-/-}Foxp3^{EGFP}* and *DO11.10⁺Il4ra^{R576}Rag2^{-/-}Foxp3^{EGFP}* CD4⁺ T_{conv} cells in the presence of TGF-β1 ± IL-4 for 5 d (*n* = 6 replicates per group). **(c,d)** Representative flow cytometric analysis of IL-17 and IL-13 expression by converted FOXP3⁺ iTreg cells **(c)** and CD4⁺FOXP3⁺ T_{conv} cells **(d)** in culture. **(e,f)** Frequencies of converted FOXP3⁺ iTreg cells expressing IL-17 (left) or ROR-γt (middle) **(e)**, FOXP3⁺ iTreg cells expressing IL-13 (left) or GATA3 (middle) **(f)**, and CD4⁺FOXP3⁺ T_{conv} cells expressing IL-17 **(e, right)** or IL-13 **(f, right)** (*n* = 6 replicates per group). **(g,h)** Representative flow cytometric analysis of dual IL-6 and IL-17 expression by converted iTreg cells under the conditions indicated **(g)** and quantification of IL-6- and IL-17-expressing converted iTreg cells **(h)** (*n* = 6 replicates per group). Each dot represents one replicate. Throughout, data represent mean ± s.e.m. from two independent experiments. ****P* < 0.001 by one-way ANOVA with Bonferroni *post hoc* analysis.

(Fig. 3e). Furthermore, about half of those exTreg cells expressed IL-17 and CCR6, consistent of their differentiation into T_H17 cells. In contrast, expression of IL-13 by exTreg cells was modest (Fig. 3f,g). Together, IL-17-producing Treg and exTreg cells accounted for 35–40% of the total CD4⁺IL-17⁺ and CD4⁺CCR6⁺ T cells in the lungs of OVA-treated *Il4ra*^{R576}*Foxp3^{EGFP}CreR26^{YFP}* mice but less than 3% of the cells in similarly treated WT controls (Fig. 3h). These results indicated that *Il4ra*^{R576}-driven Treg cell instability and T_H17 cell-like programming in the context of allergic airway inflammation ultimately leads to Treg cell transformation to T_H17 cells.

Recruitment of GRB2 to IL-4Rα phospho-Tyr575 activates MAPK

We noted that the Arg576 substitution rendered the sequence at phospho-Tyr575 (pY₅₇₅) (G₅₇₄-pY₅₇₅-R-E-F₅₇₈) homologous to a previously reported consensus sequence for high-specificity binding of the src homology 2 (SH2) domain of the adaptor protein GRB2 (pY-K/R-N-I/L)³⁴. Consistent with this prediction, GRB2 and the GRB2-associated binding protein 2 (GAB2) were detected by immunoblotting in IL-4Rα immunoprecipitates derived from IL-4-treated

Il4ra^{R576} T cells but not WT T cells (Fig. 4a). Furthermore, GRB2 was recovered from mouse splenocyte and human PBMC lysates by using biotinylated 15-residue murine and human IL-4Rα peptides containing phosphorylated Tyr575 and Arg576 (pY₅₇₅R₅₇₆) but not the Gln576 residue (pY₅₇₅Q₅₇₆) (Fig. 4b and Supplementary Fig. 6a). Both murine and human pY₅₇₅R₅₇₆ peptides showed a modest decrease in STAT6 binding as compared to that by pY₅₇₅Q₅₇₆. Together, these data indicated that the Arg576 substitution altered the specificity of pTyr575 toward binding GRB2 versus STAT6.

Receptor-bound GRB2 activates the guanine nucleotide exchange factor son of sevenless, which in turn activates the mitogen-activated protein kinase (MAPK) kinase cascade³⁵. Consistent with GRB2 recruitment and activation, IL-4 stimulation of *Il4ra*^{R576} splenocytes but not of WT splenocytes increased the phosphorylation of MAPKs, including the c-Raf, MKK3, MKK6, ERK1, ERK2 and p38 MAPKs (Fig. 4c and Supplementary Fig. 6b,c). Activation of these kinases was inhibited by a *Grb2*-specific small interfering RNA (siRNA) but not by a scrambled control siRNA (Fig. 4c). ERK1 and ERK2, but not p38 MAPK, activation was suppressed by treatment with a c-Raf-specific

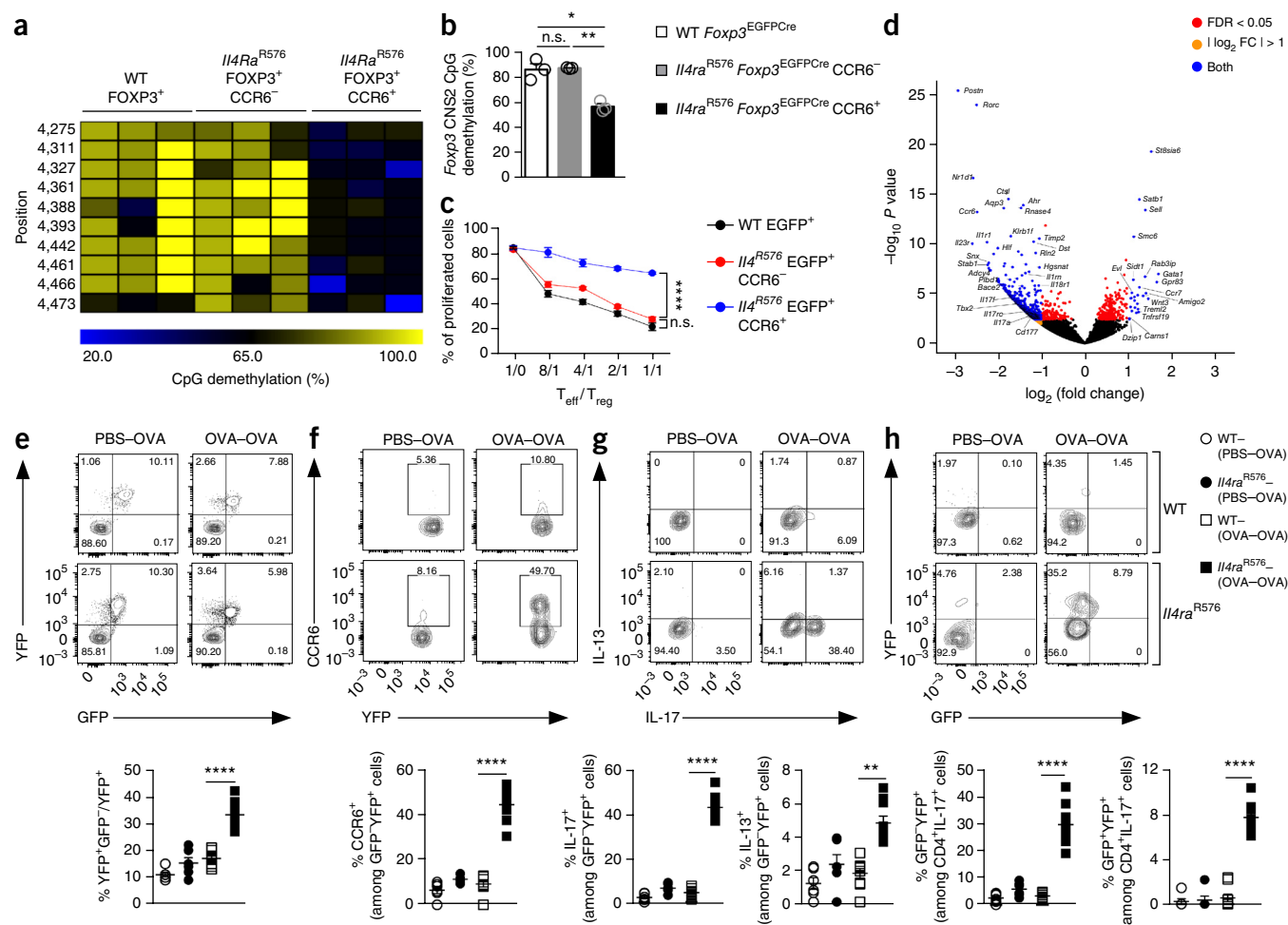


Figure 3 CCR6⁺ IL-17⁺ *IIRaR576* T_{reg} cells show instability and compromised suppressive activity. **(a)** Methylation status of CpG motifs (as defined by the colored bar) in T_{reg} cells isolated from lung tissue of OVA-sensitized and challenged mice. Individual CpG motifs are numbered, with reference to the transcription initiation site of *Foxp3*, on the left side. **(b)** Global methylation status of *Foxp3* CNS2 in the respective T_{reg} cell populations ($n = 3$ mice per group, with 7–12 clones per mouse). **(c)** *In vitro* suppression of the proliferation of WT responder CD4⁺ T cells (T_{eff} cells) by the respective T_{reg} cell populations ($n = 3$ replicates per group). **** $P < 0.0001$; n.s., not significant; by repeated-measures two-way ANOVA. **(d)** Gene expression profiles (volcano plot) of EGFP+CCR6⁺ T_{reg} cells ($n = 4$ mice) versus EGFP+CCR6⁺ T_{reg} cells ($n = 3$ mice) that were isolated by FACS from lung digests of OVA-sensitized and challenged *Foxp3*^{EGFP} *IIRaR576* mice. FDR, false discovery rate; log₂FC, log₂(fold change). **(e)** Flow cytometric analysis (top) and frequencies (bottom) of exT_{reg} (GFP-YFP⁺) cells, plotted as a fraction of exT_{reg} to total T_{reg} cells in lung tissue of PBS-sensitized and OVA-challenged (PBS-OVA) or of OVA-sensitized and OVA-challenged (OVA-OVA) mice. **(f,g)** Flow cytometric analysis (top) and frequencies (bottom) of CCR6-producing (among GFP-YFP⁺ cells) and IL-17- and IL-13-producing (among GFP-YFP⁺ cells) exT_{reg} cells in lung tissues. **(h)** Flow cytometric analysis (top) and frequencies (bottom) of exT_{reg} and T_{reg} cells among CD4⁺IL-17⁺ T_{conv} cells in lung tissues of the respective mouse groups. In **e–h**, $n = 6$ mice for PBS-treated groups, and $n = 9$ mice for OVA-treated groups. Data represent mean $\pm$ s.e.m. from two independent experiments. Unless otherwise indicated, * $P < 0.05$, ** $P < 0.005$, **** $P < 0.0001$; n.s., not significant; by one-way ANOVA with Bonferroni *post hoc* analysis.

inhibitor, pointing to two downstream cascades branching out from GRB2, one involving c-Raf-ERK1-ERK2 and the other involving p38 MAPK and its activator MKK3-MKK6 (Supplementary Fig. 6d). IL-4-activated *Il6* transcription in several cell types, including bone marrow (BM)-derived macrophages (BMDM), splenocytes and purified T cells in a GRB2-ERK1-ERK2-cascade-dependent manner, as evidenced by inhibition of *Il6* expression by a *Grb2*-specific siRNA and by treatment with a MAPK kinase (MEK) inhibitor (which inhibits ERK1 and ERK2 activation), but not by a p38 MAPK inhibitor (Fig. 4d,e and Supplementary Fig. 6e). IL-4 induced ERK1- and ERK2-dependent phosphorylation of NF- κ B p65, known transcriptional activators of *Il6*, and mobilized p65 and C/EBP- β to the *Il6* promoter^{36,37} (Fig. 4f and Supplementary Fig. 6f). The activation of NF- κ B was inhibited by MEK inhibitor treatment (Supplementary Fig. 6g).

IL-6-dependent STAT3 activation in *IIRaR576* cells

IL-6 skews iT_{reg} cell differentiation toward the T_H17 cell lineage in a STAT3-dependent manner^{38,39}. Consequent to IL-4R α ^{R576}-mediated IL-6 production, differentiating *IIRaR576* iT_{reg} cells showed increased STAT3 activation as compared to that in WT controls, and this STAT3 activation was IL-4 and IL-6 dependent (Fig. 4g). Analysis of IL-4-induced STAT3 activation in naive *IIRaR576* CD4⁺ T cells revealed delayed phosphorylation of STAT3 that started at 30 min after stimulation and that progressively increased over time. It was completely abrogated by treatment with an IL-6-specific mAb, indicating that IL-4-induced STAT3 phosphorylation proceeded by IL-4-mediated IL-6 production. In contrast, activation by IL-4 of STAT6, and by IL-6 of STAT3, was indistinguishable between WT and *IIRaR576* T cells (Supplementary Fig. 7a–c).

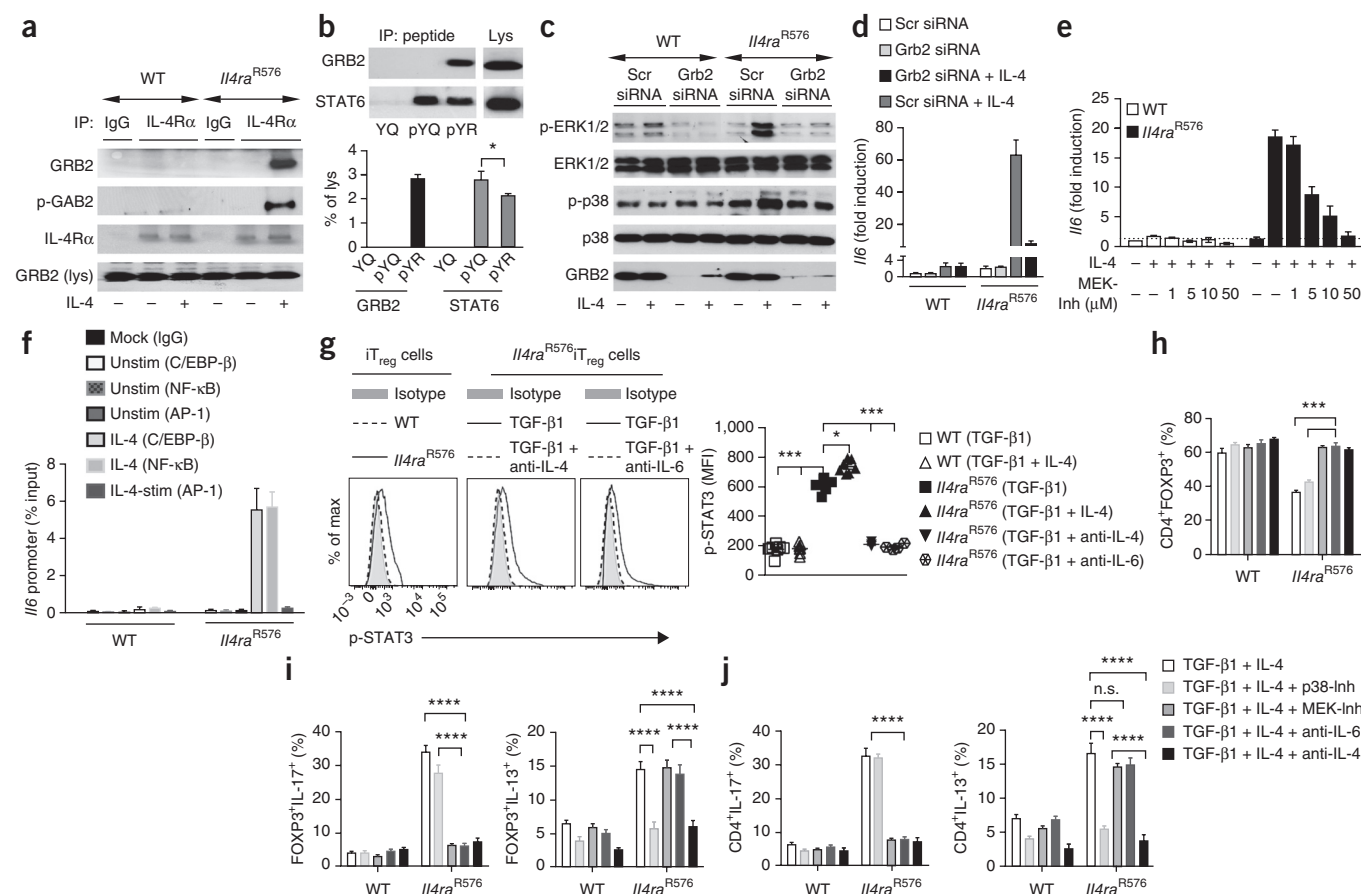


Figure 4 IL-4R α^{R576} activates GRB2-coupled MAPK. **(a)** Representative western blot analysis for immunoprecipitates (IP) from medium- or IL-4-treated WT and *Il4ra*^{R576} splenocytes, using IgG or anti-IL-4R α . Lys, lysates. Data is representative of three independent experiments. **(b)** Binding of GRB2 and STAT6 in splenocyte lysates to biotinylated nonphosphorylated (YQ) or phosphorylated (pYQ or pYR) murine IL-4R $\alpha^{Y575,Q576}$ or IL-4R $\alpha^{Y575,R576}$ peptides. Immunoblotted proteins (top) were quantified by densitometry ($n = 3$ replicates per group; bottom graph). **(c)** Immunoblot analysis for phosphorylated and total ERK1 and ERK2 (p-ERK1/2 and ERK1/2, respectively) and p38, and for GRB2 in medium- or IL-4-treated WT and *Il4ra*^{R576} BMDMs that were transfected with a scrambled siRNA (Scr) or a *Grb2*-specific siRNA (*Grb2* siRNA). Data is representative of three independent experiments. **(d)** RT-PCR analysis of *Il6* transcripts in the same cells in **(c)**. **(e)** *Il6* transcripts in splenocytes treated with medium or IL-4 and the indicated concentrations of the MEK inhibitor (MEK-Inh). The dashed line represents a fold-induction value of 1. **(f)** ChIP analysis of C/EBP- β , NF- κ B and AP-1 binding at the *Il6* promoter in medium- (unstim) or IL-4-treated WT and *Il4ra*^{R576} splenocytes ($n = 3$ replicates per group). **(g)** Flow cytometric analysis (left) and quantification of mean fluorescence intensity (MFI, right) of p-STAT3 in WT and *Il4ra*^{R576} iT_{reg} cells differentiated with anti-CD3, anti-CD28 mAbs, and TGF- β 1 without or with IL-4 (all $n = 7$ replicates per group), anti-IL-4 ($n = 4$ replicates per group) or anti-IL-6 ($n = 4$ replicates per group). **(h–j)** Frequencies of iT_{reg} cells ($n = 4$ replicates per group) **(h)**, IL-17- or IL-13-expressing FOXP3⁺ iT_{reg} cells ($n = 6$ replicates per group) **(i)** and CD4⁺FOXP3⁺ T_{conv} cells ($n = 6$ replicates per group) **(j)** treated with the indicated combinations of cytokines, inhibitors and mAbs. Throughout, Data represent mean $\pm$ s.e.m. from three independent experiments. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$; n.s., not significant; by one-way ANOVA with Bonferroni *post hoc* analysis.

We next analyzed the role of GRB2-activated MAPK cascades in iT_{reg} cell skewing toward a T_H17 versus T_H2 cell phenotype. Treatment of differentiating *Il4ra*^{R576} iT_{reg} cells with the MEK inhibitor or an IL-6-specific mAb, but not with a p38 MAPK inhibitor, rescued the decreased iT_{reg} cell differentiation, decreased IL-6 levels in culture and blocked IL-17 expression in differentiating iT_{reg} cells and in CD4⁺ T_{conv} cells present in the same co-cultures. In contrast, IL-13 expression was not affected by treatment with the MEK inhibitor or an IL-6-specific mAb, (Fig. 4h–j and Supplementary Fig. 6h–j). Treatment with the p38 MAPK inhibitor blocked IL-13 expression but not IL-17 expression, consistent with the role of p38 in the upregulation of *Il13* gene transcription by a GATA3-dependent mechanism⁴⁰, whereas treatment with anti-IL-4 blocked both (Fig. 4i,j and Supplementary Fig. 6i,j). Thus the two branches of MAPK signaling downstream of IL-4R α^{R576} mediate activation of distinct T_H cell cytokine programs.

Whereas T cells express only the type I IL-4R, which responds to IL-4, BMDMs also express the type II receptor, which responds to IL-4 and IL-13. Stimulation of *Il4ra*^{R576} BMDMs but not WT BMDMs with IL-4 or IL-13 induced a similar profile of delayed, sustained STAT3 activation. In contrast, STAT3 was similarly activated in IL-6-treated WT and *Il4ra*^{R576} BMDMs (Supplementary Fig. 7d,e).

Expression of transcripts encoding the chemokine CCL11, which mediates tissue eosinophil recruitment, is markedly upregulated in the airways of allergen-challenged *Il4ra*^{R576} mice¹⁷. *Ccl11* transcripts were greatly increased in IL-4-treated *Il4ra*^{R576} BMDMs as compared to those in similarly treated WT BMDMs (Supplementary Fig. 7f). *Ccl11* transcriptional activation by IL-4 and IL-13 is normally mediated by STAT6, whereas that by IL-17 and IL-9 is mediated by STAT3 (refs. 41–43). Chromatin immunoprecipitation (ChIP) assays revealed that whereas STAT6 bound equally well to the *Ccl11* promoter in

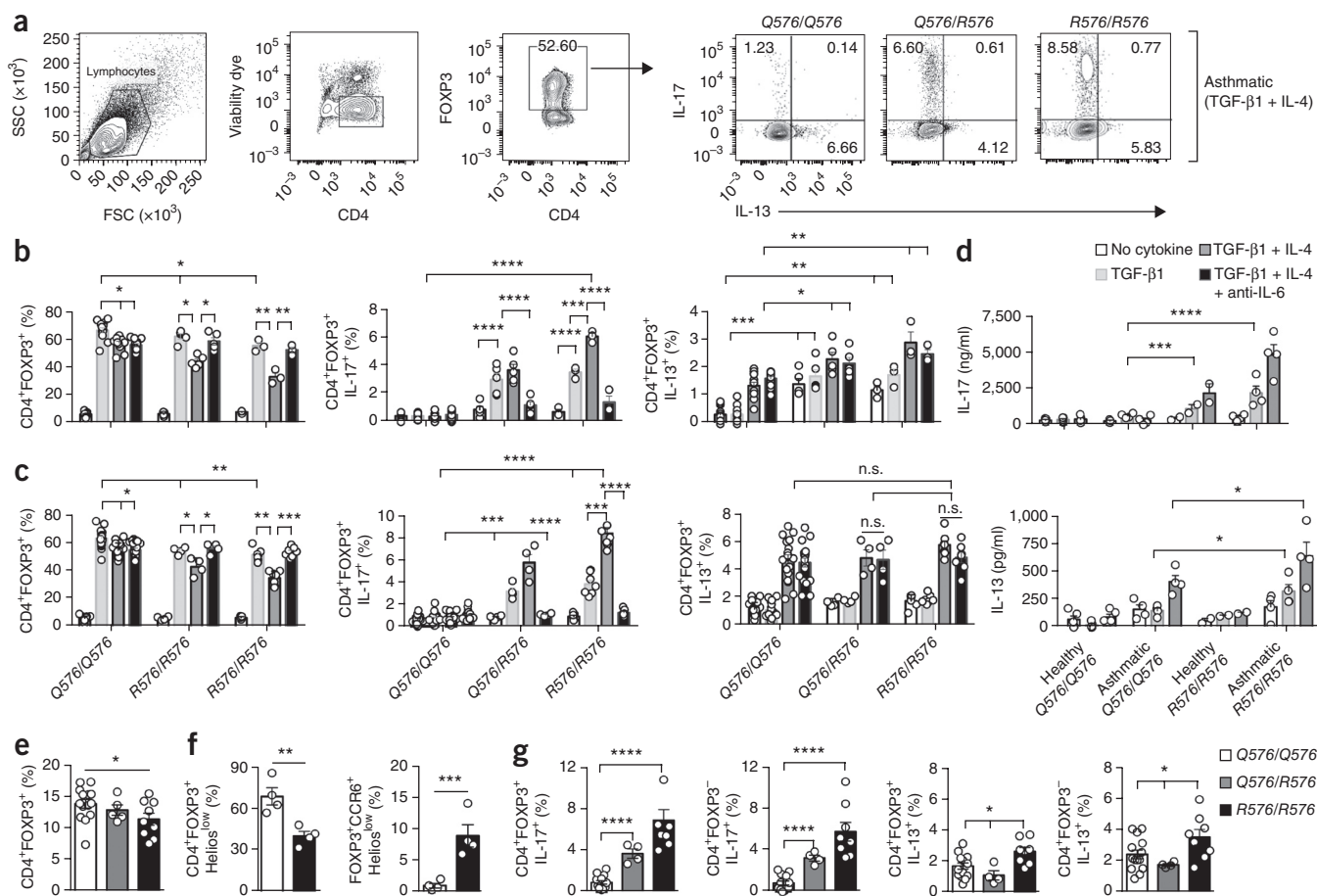


Figure 5 Naive CD4⁺ T_{conv} cells from subjects with asthma bearing the *IL4R*^{R576} mutation show defective induction of iT_{reg} cells and their skewing toward a T_H17-like phenotype. **(a)** Representative flow cytometric analysis of CD4⁺FOXP3⁺ iT_{reg} cells induced from purified naive CD4⁺ T_{conv} in the presence of anti-CD3 + anti-CD28 and TGF-β1 + IL-4. Q576/Q576, *IL4R*^{Q576/Q576}, Q576/R576, *IL4R*^{Q576/R576}, R576/R576; *IL4R*^{R576/R576}. **(b,c)** Cumulative frequencies of CD4⁺FOXP3⁺ iT_{reg} cells and IL-17- and IL-13-producing FOXP3⁺ iT_{reg} cells within converted iT_{reg} cells of subjects (with the indicated *IL4R*α-variant-encoding allele) who are not (R576/R576, *n* = 3; Q576/R576, *n* = 5; Q576/Q576, *n* = 13) **(b)** or are (R576/R576, *n* = 7; Q576/R576, *n* = 4; Q576/Q576, *n* = 19) **(c)** asthmatic, differentiated in the absence or presence of TGF-β1, TGF-β1 + IL-4, or TGF-β1 + IL-4 + anti-IL-6. **(d)** IL-17 and IL-13 cytokine levels in the supernatants of differentiated iT_{reg} cells in the treated groups mentioned in **b** (asthmatic R576/R576, *n* = 4; healthy R576/R576, *n* = 2; asthmatic Q576/Q576, *n* = 3; healthy Q576/Q576, *n* = 5). **(e–g)** Frequencies of total CD4⁺FOXP3⁺ T_{reg} cells (R576/R576, *n* = 9; Q576/R576, *n* = 5; Q576/Q576, *n* = 14) **(e)**, CD4⁺FOXP3⁺Helios^{low} and FOXP3⁺CCR6⁺Helios^{low} T_{reg} cells (*n* = 4) **(f)**, and of IL-17- and IL-13-producing FOXP3⁺ T_{reg} and CD4⁺ T_{conv} cells in respective groups within PBMCs of subjects with asthma (R576/R576, *n* = 8; Q576/R576, *n* = 4; Q576/Q576, *n* = 14). Each dot represents one subject. Data represent mean ± s.e.m. For subject characteristics, see **Supplementary Tables 1 and 2**. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001; n.s., not significant; by one-way ANOVA with Bonferroni *post hoc* analysis.

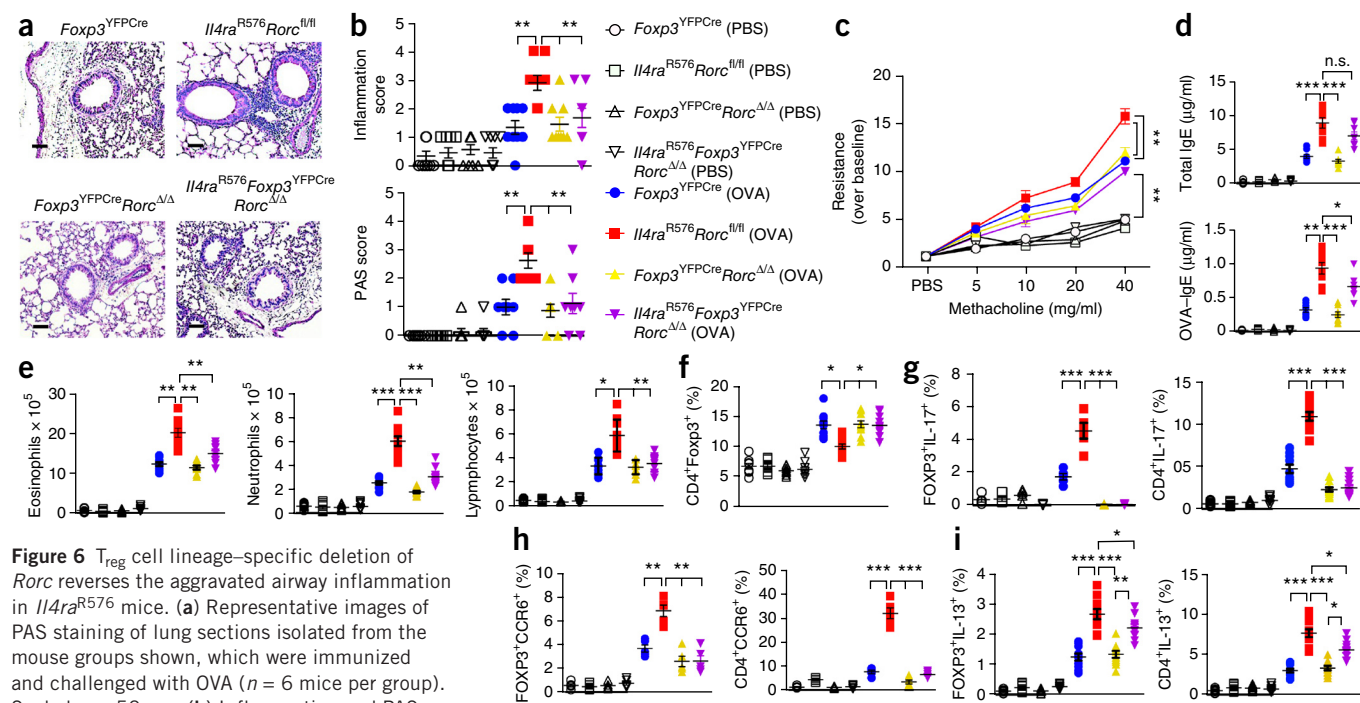
IL-4-treated WT and *Il4ra*^{R576} BMDMs, STAT3 was selectively recruited to the *Ccl11* promoter in *Il4ra*^{R576} BMDMs in an IL-6-dependent manner (**Supplementary Fig. 7g**). Knockdown of *Stat3* expression by siRNA treatment abrogated the increase in *Ccl11* transcription in IL-4-treated *Il4ra*^{R576} BMDMs, reducing it to levels similar to those of WT controls (**Supplementary Fig. 7h,i**). Thus dual STAT6–STAT3 activation by IL-4Rα^{R576} may promote tissue eosinophilia by super-inducing *Ccl11* transcription.

iT_{reg} cells of humans expressing *IL4R*^{R576} produce IL-17

We examined *in vitro* iT_{reg} cell differentiation in prospectively recruited healthy and asthmatic subjects who were either WT (*IL4R*^{Q576/Q576}), heterozygous (*IL4R*^{Q576/R576}) or homozygous (*IL4R*^{R576/R576}) for the mutated allele (**Fig. 5**). The flow cytometric gating strategy used in these studies is outlined in **Figure 5a**. In healthy subjects, whose demographics are detailed in **Supplementary Table 1**, naive heterozygous

and homozygous mutant T_{conv} cells differentiated to iT_{reg} cells at lower frequencies as compared to WT T cells, especially in the presence of IL-4 (**Fig. 5b**). The deficit in iT_{reg} conversion was rescued by treatment with an IL-6-specific mAb. The differentiated iT_{reg} cells and CD4⁺ T_{conv} cells that were present in the same co-cultures produced increased amounts of IL-17, as assessed by flow cytometric analysis and by ELISA, which was further upregulated by IL-4 treatment but abrogated by anti-IL-6 treatment (**Fig. 5b,d**). IL-13 expression was significantly increased in the activated T cells of subjects carrying the mutated allele, independently of their ability to differentiate into iT_{reg} cells, mirroring the findings in murine *Il4ra*^{R576} T cells (**Fig. 5b,d**).

We also examined the differentiation and cytokine expression of iT_{reg} cells in prospectively recruited subjects with asthma as a function of *IL4R* genotype. The demographics, *IL4R* genotype and disease severity classification of the subjects with asthma are described in **Supplementary Table 2**. Although there were no differences in age



and sex distribution between asthmatics bearing the WT allele versus those who were heterozygous or homozygous for the mutated allele, the latter subjects showed increased asthma severity, in agreement with previously published reports (Supplementary Table 2)^{13–16}. Heterozygous and homozygous mutant, but not WT, iT_{reg} cells of asthmatics also showed IL-6-dependent decreased differentiation and increased IL-17 production (Fig. 5c,d). IL-13 expression was upregulated in activated T_{conv} and in iT_{reg} cells of asthmatics regardless of their genotype, although ELISA assays revealed its increased production by cells carrying the mutated allele (Fig. 5c,d). Analysis of peripheral blood mononuclear cells (PBMCs) revealed a modest but significant decrease in circulating total T_{reg} cells in asthmatics who were homozygous for the *IL4R*^{R576} allele. This decrease was restricted to the FOXP3⁺Helios^{low} (iT_{reg}) cell population, which also manifested a selective increase in CCR6 expression, echoing the results found in *Il4ra*^{R576} mice (Fig. 5e,f and Supplementary Fig. 8). Notably, the frequency of IL-17-expressing CD4⁺ T_{conv} and T_{reg} cells was selectively increased in subjects who were either heterozygous or homozygous for *IL4R*^{R576}. IL-13 expression was modestly increased in CD4⁺ T_{conv} and T_{reg} cells from the PBMCs of subjects homozygous for *IL4R*^{R576}, consistent with heightened T_H17 and T_H2 responses (Fig. 5g). These results established that IL-4-driven acquisition by iT_{reg} cells of a T_H17 cell-like phenotype is an attribute that segregates with the *IL-4R*^{R576}-encoding mutation.

Il4ra^{R576} T_{reg} cell phenotype change affects airway inflammation

To determine whether the acquisition by lung *Il4ra*^{R576} T_{reg} cells of a T_H17 cell-like phenotype contributed to exaggerated airway

inflammation, we examined the consequences of a T_{reg} cell-specific deletion of *Rorc*, which encodes the T_H17 cell-promoting transcription factor ROR- γ t, on airway inflammation⁴⁴. The T_{reg} cell-specific deletion was effected by crossing mice expressing a loxP-flanked (floxed) *Rorc* allele with mice expressing *Foxp3*^{YFP-Cre}, a Cre recombinase under the control of the *Foxp3* locus (Supplementary Fig. 9a,b). T_{reg} cell-specific deletion of *Rorc* in *Il4ra*^{R576} mice (*Il4ra*^{R576}*Foxp3*^{YFP-Cre}*Rorc* ^{Δ/Δ}) decreased airway inflammation, mucus production and AHR after OVA sensitization and challenge such that they resembled those same responses in WT control mice whose T_{reg} cells were either sufficient or deficient in *Rorc* (*Foxp3*^{YFP-Cre} and *Foxp3*^{YFP-Cre}*Rorc* ^{Δ/Δ} , respectively) and whose own previously mentioned responses were not significantly different (Fig. 6a–c). It also led to a sharp decrease in lung tissue neutrophilia, lymphocytosis and, to a lesser extent, eosinophilia. In contrast, total and OVA-specific serum IgE concentrations were marginally decreased (Fig. 6d,e). *Rorc* deletion rescued the decrease in lung tissue T_{reg} cells in OVA-sensitized and challenged *Il4ra*^{R576} mice (Supplementary Fig. 9c). It abolished IL-17 expression and normalized CCR6 expression in *Il4ra*^{R576} T_{reg} cells, and normalized IL-17 and CCR6 expression in CD4⁺ T_{conv} cells. In contrast, IL-13 expression was unaffected in *Il4ra*^{R576}*Foxp3*^{YFP-Cre}*Rorc* ^{Δ/Δ} T_{reg} cells and marginally affected in CD4⁺ T_{conv} cells (Fig. 6f–i and Supplementary Fig. 9d–h). Almost identical results were obtained after *Il4ra*^{R576} T_{reg} cell-specific deletion of *Il6ra*, which encodes the IL-6R α chain, consistent with the requirement for IL-4-induced IL-6 production in programming T_H17 cell-like T_{reg} cells (Supplementary Fig. 10). Similarly, treatment with anti-IL-6 suppressed airway inflammation and AHR in HDM-treated *Il4ra*^{R576} mice, abrogated airway

neutrophilia and profoundly inhibited the airway eosinophilia. It normalized lung tissue T_{reg} cell numbers and prevented iT_{reg} cell reprogramming into T_H17 -like cells, and it inhibited T_H2 and T_H17 cytokine production and the increased IgE response (**Supplementary Fig. 11**). In contrast, treatment with an IL-17-specific mAb was less effective in suppressing HDM-induced airway inflammation in $Il4ra^{R576}$ mice. It partially decreased tissue inflammation and AHR, normalized airway neutrophilia and inhibited IL-17 production; however, it only marginally decreased airway eosinophilia. It failed to suppress IgE or T_H2 cytokine production or to rescue reduced T_{reg} cell numbers in lung tissues (**Supplementary Fig. 12**). Although it inhibited the dysregulated airway eosinophilia and CCL11 production observed in HDM-treated $Il4ra^{R576}$ mice, treatment with a CCL11-specific mAb did not suppress tissue inflammation, airway neutrophilia, T_H2 and T_H17 cytokine or IgE production, nor did it rescue ineffective T_{reg} cell generation in lung tissues (**Supplementary Fig. 13**).

Unlike the case of *Rorc* and *Il6ra*, combined T_{reg} cell-specific deletion of *Il4* and *Il13* had no significant effect on airway inflammation, AHR or lung tissue neutrophilia in OVA-sensitized and challenged $Il4ra^{R576}$ mice, despite decreased total eosinophilia and total, and OVA-specific, IgE responses. It failed to rescue the decrease in lung tissue T_{reg} cells in inflamed $Il4ra^{R576}$ mice. Although T_{reg} cell-specific deletion of *Il4* and *Il13* completely suppressed IL-13 expression in T_{reg} cells and decreased it in $CD4^+ T_{conv}$ cells, it left IL-17 expression unaltered in both cell populations (**Supplementary Fig. 14**).

For comparison, we used $Il4ra^{F709}$ mice, which are homozygous for a Tyr709Phe substitution that inactivates the immunotyrosine inhibitory motif of the IL-4R α chain and consequently augments STAT6 activation¹¹. This mutation, which models polymorphisms that encode human IL-4R α variants that promote STAT6 activation, results in exaggerated allergic airway inflammation due to dysregulated T_H2 immunity^{11,45,46}. $Il4ra^{F709}$ T_{reg} cells undergo T_H2 cell-like reprogramming that has a fundamental role in mediating the exaggerated allergic phenotype⁹. Unlike $Il4ra^{R576}$ mice, T_{reg} cell-specific deletion of *Il4* and *Il13* in $Il4ra^{F709}$ mice normalized their airway inflammation, AHR and tissue eosinophilia, and decreased their total and OVA-specific IgE responses. It reversed T_{reg} cell T_H2 cell-like reprogramming, as evidenced by abolishment of their heightened IL-13 expression, and reduced IL-13 production by $CD4^+ T_{conv}$ cells, without affecting the low IL-17 expression in $CD4^+ T_{conv}$ and T_{reg} cells (**Supplementary Fig. 15**). Thus, the $Il4ra^{R576}$ and $Il4ra^{F709}$ augment airway inflammation by two distinct mechanisms—one that elicits a T_H17 cell-like reprogramming of T_{reg} cells and the other that elicits a T_H2 cell-like reprogramming of T_{reg} cells.

DISCUSSION

Our studies elucidate a previously unknown mechanism for the development of mixed T_H2 and T_H17 cell responses that are relevant to asthma and other allergic inflammatory diseases. By activating an additional branch of IL-4R signaling that is initiated by the adaptor protein GRB2, a mutant-IL-4R α -encoding polymorphism that is associated with asthma exacerbations and severity potentiates T_H17 and, to a lesser extent, T_H2 responses. Notably, IL-4-induced GRB2 recruitment mediates MAPK-dependent autocrine IL-6 signaling in T cells, which redirects TGF- β -dependent iT_{reg} cell differentiation toward the T_H17 cell lineage. Deletion of *Il6ra* or *Rorc* in T_{reg} cells or treatment with an IL-6-specific mAb protected against the deleterious effects of IL-4R α^{R576} on airway inflammation, prevented iT_{reg} cell reprogramming toward the T_H17 lineage and normalized the T_H17 cell response, confirming the centrality

of iT_{reg} cell acquisition of a T_H17 cell-like phenotype in mediating disease exacerbation by this polymorphism.

In mouse models, IL-17 induces steroid-resistant airway inflammation and neutrophilic infiltration^{27,47,48}. However, although an IL-17-specific mAb treatment suppressed lung tissue neutrophilia and neutralized IL-17 in the airways of $Il4ra^{R576}$ mice, it only partially ameliorated the augmented AHR and tissue inflammation, and it failed to normalize the T_{reg} cell response or suppress T_H17 – T_H2 polarization. Thus, the efficacy of interventions such as T_{reg} cell-specific deletion of *Rorc* or *Il6ra*, or treatment with anti-IL-6 relates not simply to their suppression of IL-17 production but more fundamentally to their stabilization of the iT_{reg} cell response, resulting in better control of airway inflammation.

Studies on $Il4ra^{R576}$ and $Il4ra^{F709}$ mice revealed two distinct modes of pathogenic iT_{reg} cell programming relevant to airway inflammation. Whereas IL-4R α^{R576} mediated GRB2-dependent iT_{reg} cell programming toward a T_H17 cell-like phenotype, IL-4R α^{F709} mediated STAT6-dependent programming toward a T_H2 cell-like phenotype⁹. T_{reg} cell-specific deletion of *Il6ra* or *Rorc* in $Il4ra^{R576}$ mice and of *Il4* and *Il13* in $Il4ra^{F709}$ mice reduced airway inflammation in the respective strain back to levels that were associated with the WT *Il4ra* allele. $Il4ra^{R576}$ and $Il4ra^{F709}$ mice model mixed T_H2 – T_H17 and T_H2 cell-high asthma inflammatory mechanisms, or endotypes, respectively^{49–51}. Both models indicate that acquisition of iT_{reg} cells of a T_H17 or a T_H2 cell-like program has a fundamental role in the pathogenicity of the respective allele in airway inflammation. Accordingly, pro-asthmatic IL-4R-encoding polymorphisms may promote distinct asthma endotypes (T_H2 cell-high versus mixed T_H2 – T_H17 cell inflammation) by directing iT_{reg} cells differentiation toward the respective T_H cell program.

Our results indicate that IL-4-directed and IL-6-dependent subversion of $IL4R^{R576}$ iT_{reg} cells into T_H17 -like cells may have a critical role in the asthma severity associated with this allele. Blocking the IL-4R or IL-6R pathways may prevent the subversion of allergen-specific iT_{reg} cells into T_H2 - and/or T_H17 -like cells, respectively, and provide personalized therapeutic approaches to tolerance re-establishment in asthma^{52,53}.

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Accession codes. Gene Expression Omnibus: RNA-seq data are available under accession number [GSE80804](#).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

T.A.C. conceived of the project and directed the research; A.H.M. and T.A.C. designed the experiments and evaluated the data; W.P. provided blood samples from subjects with asthma and discussed results; A.M. performed the experiments and prepared the figures; L.-M.C. performed epigenetic studies; D.L. and M.P. performed gene expression profiling studies; and A.M. and T.A.C. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals. BALB/cByJ (WT) and all of the following strains, except where indicated, were obtained from, or re-derived at, the Jackson Lab. C.129X1-*Il4ra*^{tm2.1Tch} (*Il4ra*^{R576}), C.129X1-*Il4ra*^{tm3.1Tch} (*Il4ra*^{F709}), and C.Cg-*Foxp3*^{tm2Tch/J} (*Foxp3*^{EGFP}) have been previously described^{11,17,54}. NOD/ShiLt-Tg(*Foxp3*^{EGFP-Cre})1cJbs/J (*Foxp3*^{EGFP-Cre}) were backcrossed 12 generations on BALB/cBYJ⁵⁵. C.129P2(Cg)-*Il4-Il13*^{tm1.1Lky} (*Il4-Il13*^{fl/fl}) mice were crossed with *Il4ra*^{R576} and *Il4ra*^{R576}*Foxp3*^{EGFP-Cre} mice⁵⁶. DO11.10+*Rag2*^{-/-} mice were obtained from Taconic Farms (Hudson, NY) and were crossed with Thy1.1+*Foxp3*^{EGFP} and Thy1.1+*Il4ra*^{R576} mice to generate Thy1.1+DO11.10+*Rag2*^{-/-}*Foxp3*^{EGFP} and Thy1.1+*Il4ra*^{R576}DO11.10+*Rag2*^{-/-}*Foxp3*^{EGFP} mice. ROR- γ t/fB6(Cg)-*Rorc*^{tm3Litt/J} (*Rorc*^{fl/fl}), B6;SJL-*Il6ra*^{tm1.1Drew/J} (*Il6ra*^{fl/fl}), B6.129(Cg)*Foxp3*^{tm4(YFP/cre)Ayr/J} (*Foxp3*^{YFP-Cre}) and *Il4ra*^{R576}C57BL/6 congenic were crossed to generate *Il4ra*^{Q576}*Foxp3*^{YFP-Cre}, *Foxp3*^{YFP-Cre}*Rorc*^{Δ/Δ} and *Il4ra*^{R576}*Foxp3*^{YFP-Cre}*Rorc*^{Δ/Δ}. BALB/c *Il4ra*^{R576} mice were crossed to congenic *Foxp3*^{EGFP-Cre}*Rosa26*^{YFP-Cre} mice to generate *Il4ra*^{R576}*Foxp3*^{EGFP-Cre}*Rosa26*^{YFP-Cre} (refs. 57,58). Male and female mice, age 5–7 weeks, were used in experiments in equal proportions. Mice were bred and maintained under specific-pathogen-free conditions and used under protocols approved by the Institutional Animal Care and Use Committee at Boston Children's Hospital.

Flow cytometry. 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), Phospho-Stat3 (pY705, 1:50), Phospho-Stat6 (pY641, 1:50) (BD Biosciences), Thy1.2 (30-H12, 1:500), *Foxp3* (FJK-16S, 1:300), DO11.10 (KJ1-26, 1:500), IFN- γ (XMGI.2, 1:300), IL-13 (eBio13a, 1:300), GATA3 (TWAJ, 1:200), ROR- γ t (AFKJS-9, 1:200), Helios (22F6, 1:200), CD25 (PC61.5, 1:500), CTLA4 (UC10-4B9, 1:200), 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), IL-6 (MP5-20F3, 1:200), GR-1 (RB6-8C5, 1:500), CCR6 (29-2L17, 1:300) and CD45 (30-F11, 1:300) and CD304 (neuropilin-1; 3E12, 1:200) (BioLegend). Antibodies against the following human antigens were used: CD4 (RPA-T4, 1:300), *Foxp3* (236A/E7, 1:200), IL-13 (JES10-5A2, 1:200), IL-4 (MP4-25D2, 1:400) (eBioscience), IL-17A (BL168, 1:200), helios (22F6, 1:200), CCR6 (G034E3, 1:200) (BioLegend). The specificity and optimal dilution of each antibody was validated by testing on appropriate negative and positive controls or was otherwise provided on the manufacturer's website. Intracellular cytokine staining was performed as previously described⁹. Cells were routinely excluded from the analysis based on the staining of eFluor 780 Fixable Viability Dye (eBioscience). Stained cells were analyzed on a BD LSRFortessa cell analyzer (BD Biosciences), and data were processed using FlowJo (Tree Star Inc.).

Cell purification. Cell suspensions were enriched in CD4⁺ T cells by magnetic-activated cell sorting (MACS) negative selection (Miltenyi Biotec) and further sorted by fluorescence-activated cell sorting (FACS) on a FACSaria II (BD Biosciences), based on CD4 and EGFP (*Foxp3*) expression.

Mouse allergic sensitization and antibody treatment. Mice were sensitized to ovalbumin (OVA) (Grade V; Sigma-Aldrich) by intraperitoneal injection of 100 μ g of OVA in 100 μ l of PBS and then boosted 2 weeks later with a second intraperitoneal 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%, delivered through a Schuco 2000 nebulizer (Allied Health Care Products) for 30 min daily for 3 d. Mice were euthanized on day 32 post sensitization and analyzed¹⁸. For house dust mite (HDM)-induced allergic airway inflammation, mice received 20 μ g of lyophilized *Dermatophagoides pteronyssinus* (DP) extract in 100 μ l of PBS intranasally for 3 d at the start of the protocol and were then challenged with the same dose of DP extract on days 15 to 17. Mice were euthanized on day 18 and analyzed for measures of airway inflammation¹⁸. In some experiments, at day 15 to 17 monoclonal anti-IL-6 (MP5-20F3, BioXCell) or anti-IL-17 (17F3, BioXCell) antibodies or the isotype-matched control antibodies (BioXCell) were administered intratracheally (100 μ g/d), 30 min before allergen challenge.

Preparation of cell suspensions from lung tissues. Lungs were removed, minced and incubated for 45 min at 37 °C in collagenase (Sigma-Aldrich)

(0.5 mM in PBS) and passed through a 40- μ m strainer. Single-cell suspensions were resuspended in RPMI medium (Invitrogen).

Measurement of airway tissue and functional responses. Paraffin-embedded lung sections were stained with periodic acid–Schiff (PAS) as previously described⁵⁹. Lung inflammation was scored for cellular infiltration around the 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; and 4, a ring of inflammatory cells greater than four cells deep¹¹. The number and distribution of goblet cells was assessed by using PAS staining of mucin granules. Individual airways (bronchi and bronchioles) were scored for goblet cell hyperplasia according to the following scale: 0, no PAS-positive cells; 1, less than 5% PAS-positive cells; 2, 5% to 10% PAS-positive cells; 3, 10% to 25% PAS-positive cells; and 4, greater than 25% PAS-positive cells¹¹. Allergen-induced airway hyper-reactivity (AHR) was measured, as previously described¹⁸. 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.

In vitro iT_{reg} cell differentiation. Sorted naive CD4⁺CD62L⁺*Foxp3*^{EGFP}-T cells (1 $\times$ 10⁶/ml) were cultured with plate-bound anti-CD28 (5 μ g/ml, BioLegend), anti-CD3 (5 μ g/ml, BioLegend), recombinant TGF- β 1 (5 ng/ml, R&D Systems), with or without murine recombinant IL-4 (10 ng/ml) (Peprotech), anti-IL-4 (11B11, 10 μ g/ml) (BioLegend) or anti-IL-6 mAb (MP5-20F3, 10 μ g/ml) (BioLegend), mitogen-activated protein kinase kinase (MEK) inhibitor PD98059 (50 μ M, Sigma-Aldrich) or p38 inhibitor IV (10 μ M, Sigma-Aldrich). After 4 d, the induced iT_{reg} cells were analyzed by flow cytometry for *Foxp3* expression and intracellular cytokine production and/or re-sorted on the basis of EGFP fluorescence.

Differentiation of BM-derived macrophages (BMDMs). BMDMs generated from BM cells that were collected from femurs were cultured in complete RPMI medium supplemented with 10 ng/ml recombinant murine M-CSF (PeproTech) for 10 d. The medium was changed every other day. Adherent BMDMs were harvested at day 10. The purity of the BMDMs was verified by using flow cytometry and was greater than 90%, as determined by using CD11c and F/480 staining.

Quantitative real-time PCR. RNA was extracted from treated cells, as mentioned in the figure legends, using Quick-RNA MiniPrep kit (Zymo Research) according to the manufacturer's protocol. Reverse-transcription was performed with the SuperScript III RT-PCR system (Invitrogen), and quantitative real-time RT-PCR was performed with Taqman Fast Universal PCR master mix, internal housekeeping gene mouse (GAPDH VIC-MGB dye) and specific target gene primers for murine *Il6*, *Ccl11*, *Rorc* or *Il4* genes, as indicated (FAM Dye) (Applied Biosystems) on a Step-One-Plus machine. Relative expression was normalized to that of *Gapdh* and was calculated as fold change relative to that in unstimulated WT cells.

Immunoblotting and immunoprecipitation. Cells were treated with medium alone or with medium plus IL-4 (10 ng/ml) for the indicated times, lysed with the lysis buffer (25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA (pH 8), 1 mM Na₃VO₄, 0.5% NP-40, 1 $\times$ diluted protease inhibitor cocktail (Sigma-Aldrich)). Whole-cell lysates were resolved by SDS-PAGE and transferred to PVDF membranes (Thermo Scientific) for immunoblotting analysis⁵⁹. Immunoprecipitates were derived from cell lysates by using the indicated antibodies and protein G superparamagnetic beads (Life Technologies). Following overnight incubation, the immunoprecipitates were eluted from the beads by boiling in gel-loading buffer and subjected to immunoblotting as previously described⁵⁹. Antibodies to the following proteins were used: GRB2 (clone 4C6-H6, 1:1,000 dilution), p-GAB2 (Ser159, EP369Y, 1:1,000), p-ERK1/2 (Thr202/Tyr204, D13.14.4E, 1:1,000), ERK1/2 (p44/42 MAP Kinase, 137F5, 1:1,000), STAT6 (D3H4, 1:1,000), STAT3 (D3Z2G, 1:1,000) p-c-Raf (Ser338, 56A6, 1:1,000), c-Raf (D5X6R, 1:1,000), p-p38 (Thr180/Tyr182, D3F9, 1:1,000), p38 (D13E1, 1:1,000), p-MKK3/6 (Ser189/Ser207, 22A8, 1:1,000), MKK3 (D4C3, 1:1,000), NF- κ B p-p65 (Ser536, 93H1, 1:1,000), NF- κ B p65 (D14E12, 1:1,000)

(all obtained from Cell Signaling Technology), C/EBP- β (C-190, 1:1,000) (Santa Cruz Biotechnology) and IL-4R- α (CD124) (mIL-4R-M1, 1:1,000) (BD Pharmingen). The specificity and optimal dilution of each antibody was validated by testing on appropriate negative and positive controls or was otherwise provided on the manufacturer's website.

Chromatin immunoprecipitation (ChIP). ChIP was performed with the Agarose ChIP Kit (Pierce) and antibodies to STAT3 (D3Z2G, 1:100), STAT6 (D3H4, 1:100), c-Jun (AP-1, 1:100) and NF- κ B p65 (D14E12, 1:100) (all obtained from Cell Signaling Technology), C/EBP- β (C-190, 1:100) (Santa Cruz Biotechnology) or the respective isotype control antibodies on 2×10^6 cells starved for 3 h and treated with medium alone or with IL-4 (10 ng/ml, 2 h). The DNA from sample and input was amplified by RT-PCR (40 cycles), using the following primers specific for the *Il6* promoter: 5'-AGTGGTGAAGAGACTC AGTG-3' (forward) and 5'-GGCAGAATGAGCCTCAGA-3' (reverse) and specific primers for the *Ccl11* promoter: 5'-CTTCATGTTGGAGGCTGAAG-3' (forward) and 5'-GGATCTGGAATCTGGTCAGC-3' (reverse). Results presented as the ratio of the cycling threshold value of immunoprecipitated DNA to that of input DNA.

siRNA-mediated knockdown of *Grb2*. Cells were transfected for 72 h with scrambled small interfering RNA (siRNA) or *Grb2* siRNA I (Cell Signaling Technology) for inhibition of GRB2 expression (50 nM/10⁶ cells). Stat3 siRNA I (Cell Signaling Technology) was used for inhibition of STAT3 expression. Lipofectamine 3000 reagent (Life Technologies) was used to increase the efficiency of transfection (0.5 nmol/liter).

IgE and cytokine ELISA assays. Total mouse serum IgE was measured by ELISA using Mouse IgE ELISA Ready-SET-Go kit (eBioscience). OVA-specific IgE was measured by a modified assay such that the plates were coated overnight with 200 μ g/ml OVA rather than anti-IgE capture antibody, and the rest of the test was similar to the total serum IgE ELISA assay. Mouse IL-4, IL-5, IL-13, IL-17, IFN- γ and eotaxin cytokines were analyzed in BAL fluids collected from mice using eBioscience ELISA kits, according to the manufacturer's protocol. Human IL-13 and IL-17 were analyzed in culture supernatants of human peripheral blood cells activated with anti-CD3 + anti-CD28 mAbs in the absence or added presence of TGF- β or TGF- β + IL-4 using eBioscience ELISA kits.

Peptide-affinity pulldown experiment. The following biotinylated peptides, which correspond to amino acids 569–583 and 569–584 of the human and mouse IL-4R α , respectively, and which encompass either WT (Gln) or mutant (Arg) amino acid residues at position 576 were used: Y₅₇₅Q₅₇₆: biotin-SAPTSQYQEFVHAVE (human), biotin-PAPAGGYQEFVQAVKQ (mouse); pY₅₇₅Q₅₇₆: pY₅₇₅R₅₇₆: biotin-SAPTSQpYQEFVHAVE (human), PAPAGGpYQEFVQAVKQ (mouse); pY₅₇₅R₅₇₆: biotin-SAPTSQpYREFVHAVE (human), PAPAGGpYREFVQAVKQ (mouse). The peptides were incubated with whole-splenocyte lysates and avidin-conjugated magnetic beads overnight. The beads were then separated by magnetic sorting and washed. The bound proteins were eluted and analyzed using SDS-PAGE and immunoblotting^{11,60}.

Methylation analysis. The methylation status of the FOXP3 T_{reg} cell-specific de-methylation region (CNS2) of WT FOXP3⁺, *Il4ra*^{R576} FOXP3⁺CCR6⁺ and *Il4ra*^{R576} FOXP3⁺CCR6⁺ lung T_{reg} cells of OVA-sensitized and challenged mice was assessed by bisulfite sequence analysis, as described⁶¹. The T_{reg} cell-specific demethylation region of converted DNA was amplified with methylation-specific primer sequences: *Foxp3* CNS2, 5'-TATTTTTTTGGGTTTTGGGATA TTA-3' (forward) and *Foxp3* CNS2 5'-AACCAACCAACTCTCTACACTAT CTAT-3' (reverse). The PCR product was subcloned and sequenced. BLAST analyses were done by comparison of the resulting sequences with converted *Foxp3* sequences.

In vitro suppression assays. Total CD4⁺ T cells were isolated by using a CD4 negative isolation kit (Miltenyi Biotec) followed by cell sorting on a FACSaria instrument. Isolated cells were labeled with CellTrace Violet Cell Proliferation dye according to the manufacturer's instructions (Life Technologies) and were used as responder cells. T_{reg} cells were isolated on a FACSaria on the basis of

CD4, EGFP and/or CCR6 expression, and they were used as suppressor cells. Responder cells were co-cultured with T_{reg} cells, at the indicated ratios, and stimulated for 3 d with 2 μ g/ml of soluble anti-CD3 and 5 μ g/ml of soluble anti-CD28 in 96-well, round-bottomed plates in triplicates. The cells were then analyzed for CellTrace dye dilution by flow cytometry.

Transcriptome profiling. CCR6⁺ or CCR6⁺ CD4⁺EGFP⁺ T_{reg} cells were isolated by FACS from total lung digests of OVA-sensitized and challenged *Il4ra*^{R576} mice. Total RNA was extracted using TRIzol reagent (Invitrogen) and converted into double-stranded DNA (dsDNA), using SMART-Seq v4 Ultra Low Input RNA kit (Clontech). dsDNA was then fragmented to 200- to 300-bp-sized fragments, using M220 Focused-ultrasonicator (Covaris), and these were used for the construction of libraries for Illumina sequencing, using the KAPA Hyper Prep Kit (Kapa Biosystems). Libraries were then quantified using Qubit dsDNA HS (high-sensitivity) Assay Kit on Agilent High Sensitivity DNA Bioanalyzer.

RNA sequencing data was demultiplexed by using perfect matches to indices and was quality-inspected using FastQC. The sequencing data was aligned to the mm10 build (Gencode annotation) of the mouse genome using STAR⁶², and counts were quantified using HTSeq⁶³. Raw counts were filtered for non-mitochondrial protein-coding genes with at least three counts in one sample, and were normalized using the DESeq2 package in R⁶⁴. Pairwise comparisons of differential gene expression were computed using DESeq2.

Human cell studies. Subjects with asthma and healthy controls were recruited under protocols approved by the Institutional Review Board at Boston Children's Hospital. The demographic details of the respective groups are described in **Supplementary Tables 1** and **2**. Asthmatic subjects, aged between 6 and 47 years, were identified as having intermittent asthma or persistent mild, moderate to severe asthma following the classification of The National Asthma Education and Prevention Program: expert panel report 3 (ref. 65). Inclusion criteria for asthma included current doctor's diagnosis of asthma, and $\geq 12\%$ FEV1 bronchodilator reversibility or airway hyper-responsiveness reflected by a methacholine PC20 ≤ 16 mg/ml. Healthy control subjects who were recruited had no medical history of asthma. Informed consent was obtained from all of the participants before blood collection. Blood samples were used for isolation of DNA, using DNeasy Blood & Tissue Kit (Qiagen) for genotyping. Mutation detection was carried out using amplification resistance mutation screen (ARMS) PCR method with the following primers: common oligo: 5'-CCAGAGTCCAGACAACCTGACT TGCAAGAGAC-3'; Q576-specific: 5'-TGGGTGCCACCCTGCTCCACCGCA TGTACAAACTCAT-3'; R576-specific: 5'-TGGGTGCCACCCTGCTCCACC GCATGTACAAACTCAC-3' (ref. 66). For iT_{reg} cell differentiation, peripheral blood mononuclear cells (PBMCs) were isolated and naive CD4⁺CDRA⁺CDRO⁺ T cells were derived by magnetic separation (Miltenyi Biotec). The isolated T cells were differentiated into iT_{reg} cells, as described for the murine study, and directly used for intracellular cytokine and FOXP3 expression analysis, using flow cytometry.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 6 software (GraphPad) and using the statistical tests indicated in the individual figure legends. Samples size was selected based on previous experiments. No samples were excluded. The investigators were blinded as to scoring the lung histopathology in individual experiments. *P* values of <0.05 were considered significant; n.s. indicates 'not significant'. All error bars represent s.e.m. as noted in the individual figure legends. Unless otherwise stated, ≥ 2 independent experiments were carried out for all assays, and the displayed figures are representative of the obtained results. Comparisons between the average severity scores and ages between subject with asthma who were homozygous for *IL4R*^{Q576} versus those heterozygous and homozygous for the *IL4R*^{R576} allele were carried out using Student's unpaired two-tailed *t*-test. The sex distribution between those two groups was compared using Fisher's exact test.

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