

Interleukin-25 induces type 2 cytokine production in a steroid-resistant interleukin-17RB⁺ myeloid population that exacerbates asthmatic pathology

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Interleukin-25 (IL-25) is a cytokine associated with allergy and asthma that functions to promote type 2 immune responses at mucosal epithelial surfaces and serves to protect against helminth parasitic infections in the intestinal tract. This study identifies the IL-25 receptor, IL-17RB, as a key mediator of both innate and adaptive pulmonary type 2 immune responses. Allergen exposure upregulated IL-25 and induced type 2 cytokine production in a previously undescribed granulocytic population, termed type 2 myeloid (T2M) cells. *IL17rb*^{-/-} mice showed reduced lung pathology after chronic allergen exposure and decreased type 2 cytokine production in T2M cells and CD4⁺ T lymphocytes. Airway instillation of IL-25 induced IL-4 and IL-13 production in T2M cells, demonstrating their importance in eliciting T cell-independent inflammation. The adoptive transfer of T2M cells reconstituted IL-25-mediated responses in *IL17rb*^{-/-} mice. High-dose dexamethasone treatment did not reduce the IL-25-induced T2M pulmonary response. Finally, a similar IL-4- and IL-13-producing granulocytic population was identified in peripheral blood of human subjects with asthma. These data establish IL-25 and its receptor IL-17RB as targets for innate and adaptive immune responses in chronic allergic airway disease and identify T2M cells as a new steroid-resistant cell population.

Current therapies for asthma focus on reducing the frequency and severity of exacerbations by attenuating bronchiole inflammation and airway hyperreactivity. The limited efficacy of treatments, such as inhaled corticosteroids, to reduce the inflammatory response in some chronic asthmatics illustrates the need for further research into mechanisms underlying the pathophysiology of asthma^{1–3}. Three epithelial-derived type 2 inflammation associated cytokines, IL-25, thymic stromal lymphopoietin (TSLP) and IL-33, may represent targets for therapeutic treatment in severe asthma. IL-25, in particular, has been reported to enhance responses and further exacerbate allergic disease. In an original study⁴, the systemic injection of IL-25 into *Rag*^{-/-} mice induced type 2 cytokine expression and eosinophilia, demonstrating that T cell-independent mechanisms could drive type 2 inflammation.

The IL-17 family member IL-25 (IL-17E) regulates multiple aspects of mucosal immunity by promoting type 2 inflammation via production of IL-4, IL-5, and IL-13 (refs. 4–6). Pulmonary IL-25 is produced by eosinophils^{7,8}, mast cells and airway epithelial cells and stimulates asthma-like, allergic inflammation characterized by airway hyperreactivity, mucus production, airway eosinophilia and increased serum IgE⁹. The induction of these responses requires binding of a noncovalently bound IL-25 homodimer to the IL-17RA–IL-17RB heterodimer¹⁰, of which IL-17RB represents an IL-25-specific moiety in the lung¹¹. IL-25 is known to enhance T helper type 2 effector functions via Act1-dependent and TRAF6-dependent nuclear factor- κ B activation^{12–15}. Multiple cell types in the lung, including memory¹⁶

and effector^{14,16} T cells, invariant natural killer T cells¹⁷, antigen-presenting cells and airway smooth muscle cells, have been shown to express IL-17RB, whereas eosinophils do not⁷. Several studies have also identified additional IL-25-responsive, type 2 cytokine-producing non-B, non-T (NBNT) cell populations^{5,18–20}, and a recent investigation reported increased expression of IL-25 and its receptor in the airways of individuals with asthma after allergen provocation²¹.

This work reports that repeated allergen exposure upregulates both pulmonary IL-25 and IL-17RB in a mouse model of persistent allergic airway disease and induces the accumulation of a previously undescribed IL-4- and IL-13-producing IL-17RB⁺ T2M population in the lung. Cytokine production in T2M cells is promoted by IL-25, and these cells are both pathogenic and steroid resistant. Moreover, IL-4- and IL-13-producing T2M-like cells are present in the peripheral blood of human subjects with asthma.

RESULTS

Chronic allergen drives type 2 cytokine production in myeloid cells

Several reports have linked IL-25 expression to the severity of allergic asthma^{6,7,9,22,23}. C57BL/6J mice were sensitized via intraperitoneal and subcutaneous injection of cockroach allergen emulsified in incomplete Freund's adjuvant. The inflammatory response was localized to the lung via a series of six allergen challenges (four intranasal, and two intratracheal, given at 4-d intervals) starting 14 days post-sensitization. The induction of type 2 cytokine expression following the pulmonary instillation of allergen (Fig. 1) was accompanied by

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increased mRNA expression of *Il25* and *Il17rb* (Fig. 1c) and tracked with the severity of the developing disease as depicted by histology (Fig. 1a). Neither *Ifng* (encoding interferon- γ), other IL-17 family members (Fig. 1b,c), *Il22*, *Il33* nor the IL-33 receptor *Il1rl1* was upregulated in our model of cockroach allergen challenge (data not shown), indicating that type 2 inflammation represents the dominant response induced by this model.

We have previously identified a pathologically relevant population of IL-17RB⁺ myeloid cells with the capacity to produce IL-4 during chronic allergic airway disease⁷. Although CD4⁺ T cells are present following antigen sensitization, the most numerous IL-17RB⁺ IL-4⁺ and IL-13⁺-producing population in the lung was CD11b⁺ myeloid cells (Fig. 1d,e). We also examined the capacity of innate Lin⁻c-Kit⁺Sca-1⁺IL-17RB⁺ NBNT cells to produce type 2 cytokines. Lin⁻c-Kit⁺Sca-1⁺IL-17RB⁺ cells made up a relatively rare population in the lungs of both naive and allergen-challenged mice (range 250–1,000) and were not higher in number following allergen sensitization (Fig. 1d,e). Few myeloid IL-4⁺ and IL-13⁺-producing cells were present in the lungs of naive mice, and the IL-17RB⁺ cells preferentially produced type 2 cytokines. Myeloid IL-17RB⁺ cells represented the

major NBNT IL-4 and IL-13 cytokine-producing population in the lungs of allergen-challenged mice, outnumbering cytokine-producing CD4⁺ T cells 68:1 (Supplementary Fig. 1).

Despite marked increases in IL-4⁺ and IL-13⁺-producing myeloid cells in environments with elevated levels of IL-25, not all pulmonary IL-17RB⁺ myeloid populations seemed to produce these cytokines. Analysis of IL-4 and IL-13 production in IL-17RB⁺ myeloid subsets, on the basis of levels of Gr-1 expression, allowed for the identification of two distinct IL-17RB⁺ myeloid populations, Gr-1^{mid} and Gr-1^{hi} (Fig. 1f). A comparison of total cell numbers between naive and allergic mice identified significant increases in both Gr-1^{mid} (Fig. 1g) and Gr-1^{hi} (data not shown) subsets in the lungs of allergic mice; however, the IL-17RB⁺CD11b⁺Gr-1^{mid} population produced IL-4 and IL-13, whereas the Gr-1^{hi} population did not (data not shown). Isolation of the Gr-1^{mid} subset by FACS identified a granulocytic population with a circular, partially segmented nucleus and relatively high nucleus-to-cytoplasm ratio (Fig. 1h and Supplementary Fig. 2). Next, we sought to investigate the involvement of IL-17RB in the production of type 2 cytokines in this granulocytic IL-17RB⁺ population.

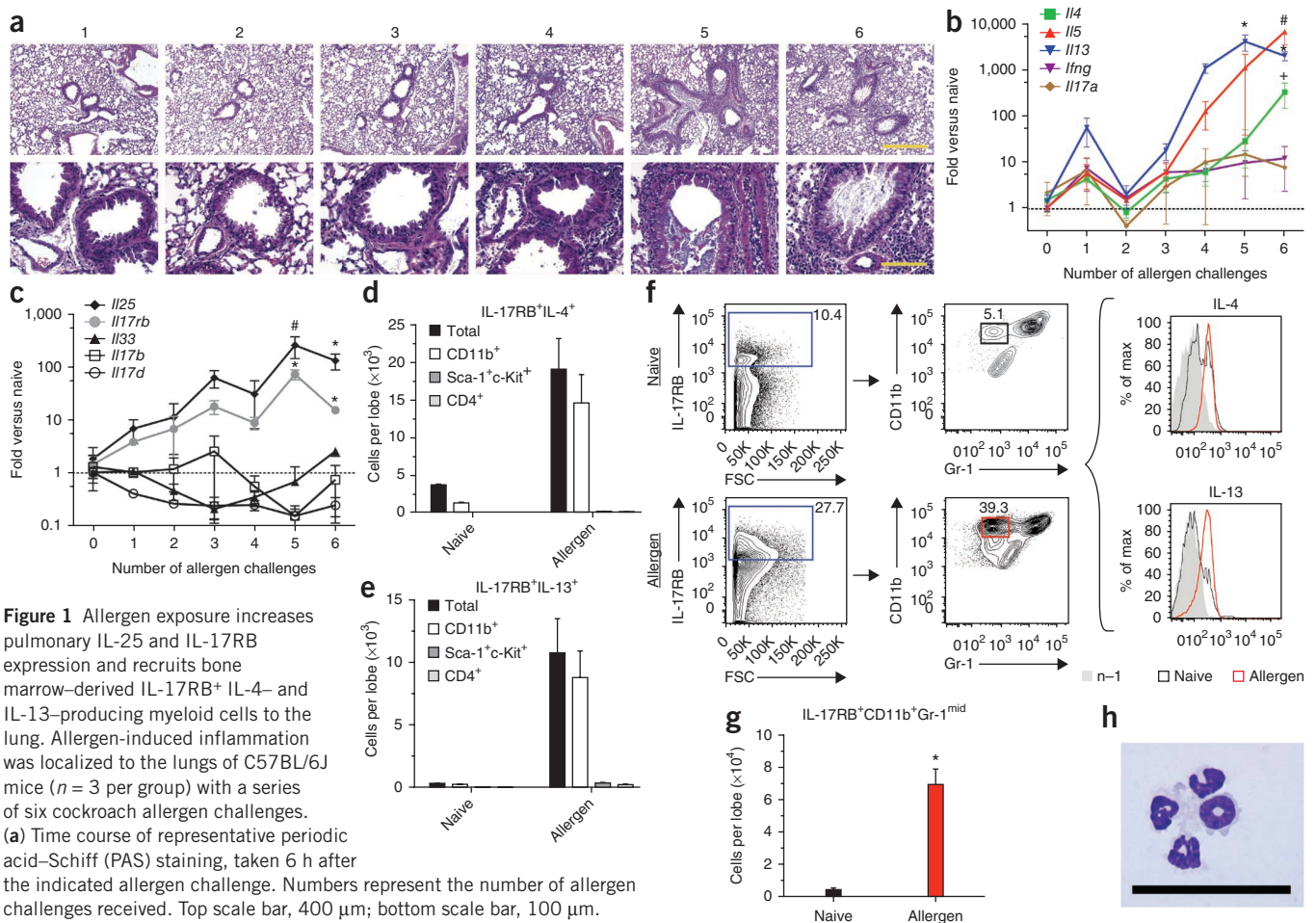


Figure 1 Allergen exposure increases pulmonary IL-25 and IL-17RB expression and recruits bone marrow-derived IL-17RB⁺ IL-4⁺ and IL-13⁺-producing myeloid cells to the lung. Allergen-induced inflammation was localized to the lungs of C57BL/6J mice ($n = 3$ per group) with a series of six cockroach allergen challenges.

(a) Time course of representative periodic acid–Schiff (PAS) staining, taken 6 h after the indicated allergen challenge. Numbers represent the number of allergen challenges received. Top scale bar, 400 μ m; bottom scale bar, 100 μ m.

(b) Time course of pulmonary *Il4*, *Il5*, *Il13*, *Ifng* and *Il17a* mRNA expression 6 h after the indicated number of allergen challenges. * $P < 8.44 \times 10^{-5}$, # $P = 0.0008$, + $P = 0.001$.

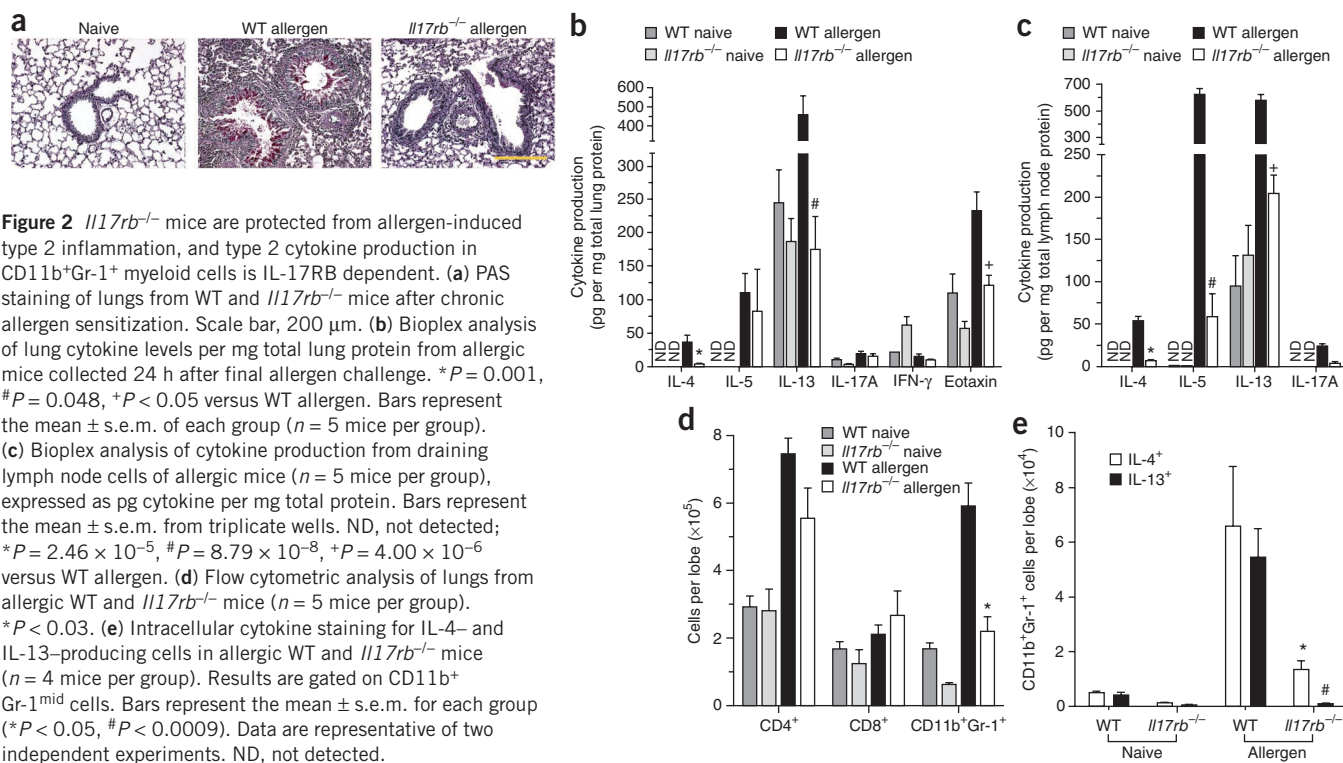
(c) Thirty-two-day time course of *Il25*, *Il17rb*, *Il33*, *Il17b* and *Il17d* mRNA expression 6 h after the indicated number of allergen challenges. * $P < 0.007$.

(d,e) IL-4 (d) and IL-13 (e) production among IL-17RB⁺ lung subsets from naive and allergen-sensitized C57BL/6J mice ($n = 5$ per group), as assessed by flow cytometry.

(f) Representative flow plots of intracellular cytokine staining in IL-17RB⁺CD11b⁺Gr-1^{mid} cells from naive and allergen-sensitized C57BL/6J mice. Gray: n-1 staining, black: naive IL-17RB⁺CD11b⁺Gr-1^{mid}, red: allergen-challenged IL-17RB⁺CD11b⁺Gr-1^{mid}. Numbers on the plots reflect the percentage of cells on each plot that are present within the gated area.

(g) Pulmonary IL-17RB⁺CD11b⁺Gr-1^{mid} populations following allergen sensitization in C57BL/6J mice. * $P = 0.0026$. Results are representative of two independent experiments.

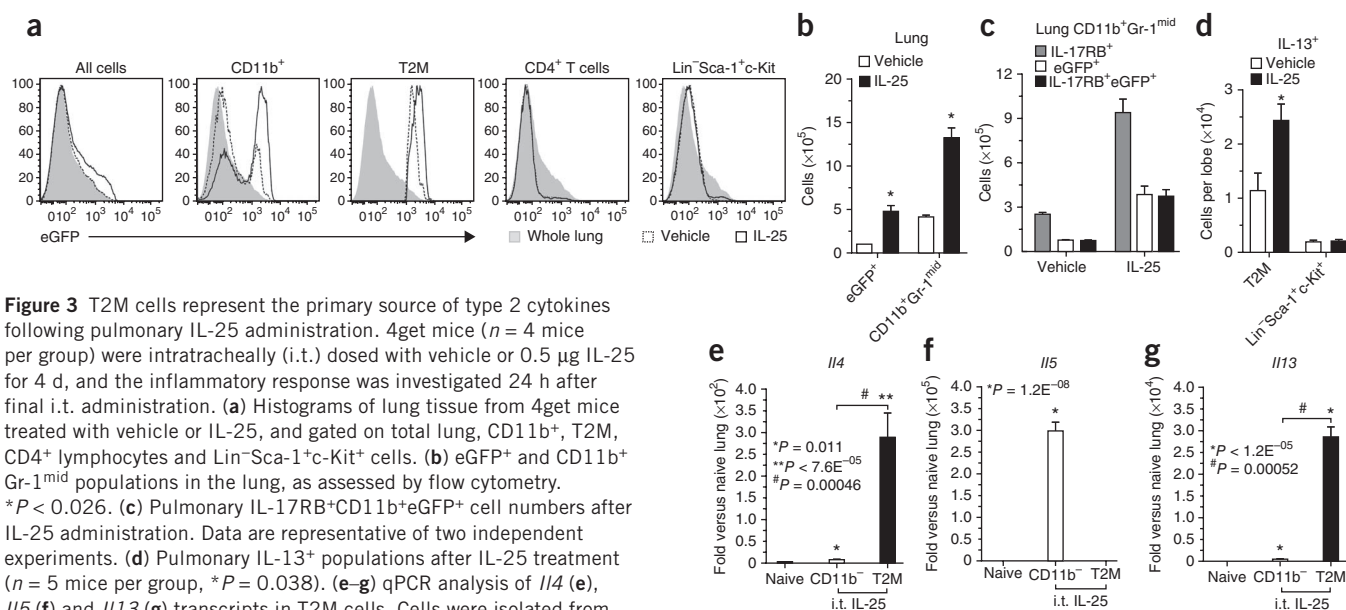
(h) Morphology of myeloid cells isolated from the lungs of allergen-sensitized mice. Cells were sorted as CD11b⁺Gr-1^{mid} Fc γ R⁺IL-17RB⁺CD4⁻CD8⁻B220⁻IL-7R α ⁻Sca-1⁻c-Kit⁻ and stained with H&E. Scale bar, 50 μ m. All data are presented as mean $\pm$ s.e.m.



Il17rb^{-/-} mice show decreased type 2 inflammation

To further explore the overall role of IL-17RB in allergic asthma, we sensitized *Il17rb*^{-/-} mice to cockroach allergen as previously described and induced allergic airway disease with six allergen challenges over a 32-d period. The loss of the IL-25-specific receptor protected *Il17rb*^{-/-} mice from allergen-induced inflammation (Fig. 2). *Il17rb*^{-/-} allergic mice showed a marked reduction in peribronchial and perivascular inflammation, eosinophilic infiltrates and mucus production (Fig. 2a). Pulmonary expression of type 2 cytokines and of the

eosinophil-associated chemokine CCL11 (Eotaxin) was significantly decreased in lungs of *Il17rb*^{-/-} mice (Fig. 2b). *Il17rb*^{-/-} draining lymph node cells re-stimulated with antigen produced significantly less type 2 cytokines than wild-type (WT) lymph node cells (Fig. 2c). Notably, a short-term model of allergen sensitization using only three allergen challenges revealed no phenotypic differences in *Il17rb*^{-/-} mice (Supplementary Fig. 3), suggesting that IL-17RB is most relevant during more chronic allergic responses when IL-25 production is substantially increased.



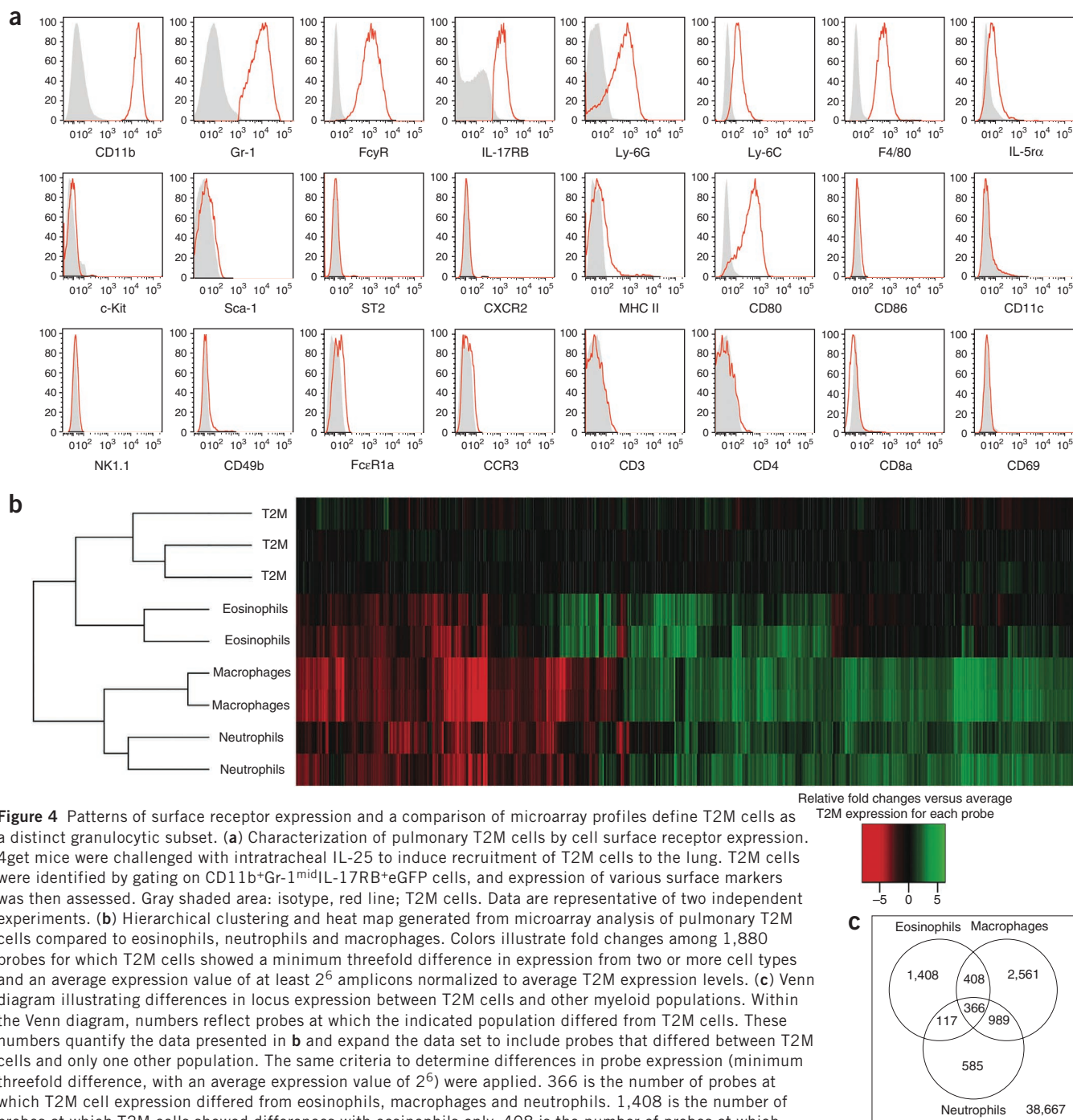


Figure 4 Patterns of surface receptor expression and a comparison of microarray profiles define T2M cells as a distinct granulocytic subset. **(a)** Characterization of pulmonary T2M cells by cell surface receptor expression. 4get mice were challenged with intratracheal IL-25 to induce recruitment of T2M cells to the lung. T2M cells were identified by gating on CD11b⁺Gr-1^{mid}IL-17RB⁺eGFP cells, and expression of various surface markers was then assessed. Gray shaded area: isotype, red line; T2M cells. Data are representative of two independent experiments. **(b)** Hierarchical clustering and heat map generated from microarray analysis of pulmonary T2M cells compared to eosinophils, neutrophils and macrophages. Colors illustrate fold changes among 1,880 probes for which T2M cells showed a minimum threefold difference in expression from two or more cell types and an average expression value of at least 2^6 amplicons normalized to average T2M expression levels. **(c)** Venn diagram illustrating differences in locus expression between T2M cells and other myeloid populations. Within the Venn diagram, numbers reflect probes at which the indicated population differed from T2M cells. These numbers quantify the data presented in **b** and expand the data set to include probes that differed between T2M cells and only one other population. The same criteria to determine differences in probe expression (minimum threefold difference, with an average expression value of 2^6) were applied. 366 is the number of probes at which T2M cell expression differed from eosinophils, macrophages and neutrophils. 1,408 is the number of probes at which T2M cells showed differences with eosinophils only. 408 is the number of probes at which eosinophils and macrophages showed differential expression from T2M cells. 38,667 is the number of probes whose expression did not differ between the four cell types. 1,880 probes differed between T2M cells and at least two of the comparison populations.

There was no detectable difference in total numbers of CD4⁺ or CD8⁺ T lymphocyte subsets between the lungs of allergen-sensitized *Il17rb*^{-/-} and WT mice. However, *in vitro* re-stimulation experiments, as well as *in vivo* adoptive transfer studies with *Rag2*^{-/-} mice lacking B and T cells demonstrated that CD4⁺ T cell responses were altered in *Il17rb*^{-/-} mice and that *Il17rb*^{-/-} CD4⁺ T cells could not efficiently transfer a type 2 immune response to the *Rag2*^{-/-} recipients (**Supplementary Fig. 4**). Despite the ability of both WT and *Il17rb*^{-/-} T cells to induce a type 2 inflammatory response, we observed a marked reduction in overall pulmonary inflammation

and subsequent response to allergen in *Rag2*^{-/-} recipients of *Il17rb*^{-/-} T cells compared to WT recipients (**Supplementary Fig. 4**). This altered response affected the recruitment of other cells via a reduction in transcripts of allergen-associated chemokines such as *Tarc* (CCL17) (**Supplementary Fig. 4**). It also led to a significant ($P < 0.03$) decrease in myeloid infiltrates following allergen challenge, indicating that the absence of IL-17RB in CD4⁺ T cells led to a systemic deficiency in inducing a type 2 cytokine-mediated inflammatory environment. These data were similar to findings published by several groups establishing a role of IL-25 in type 2 responses^{9,10,16}. *Il17rb*^{-/-} mice also

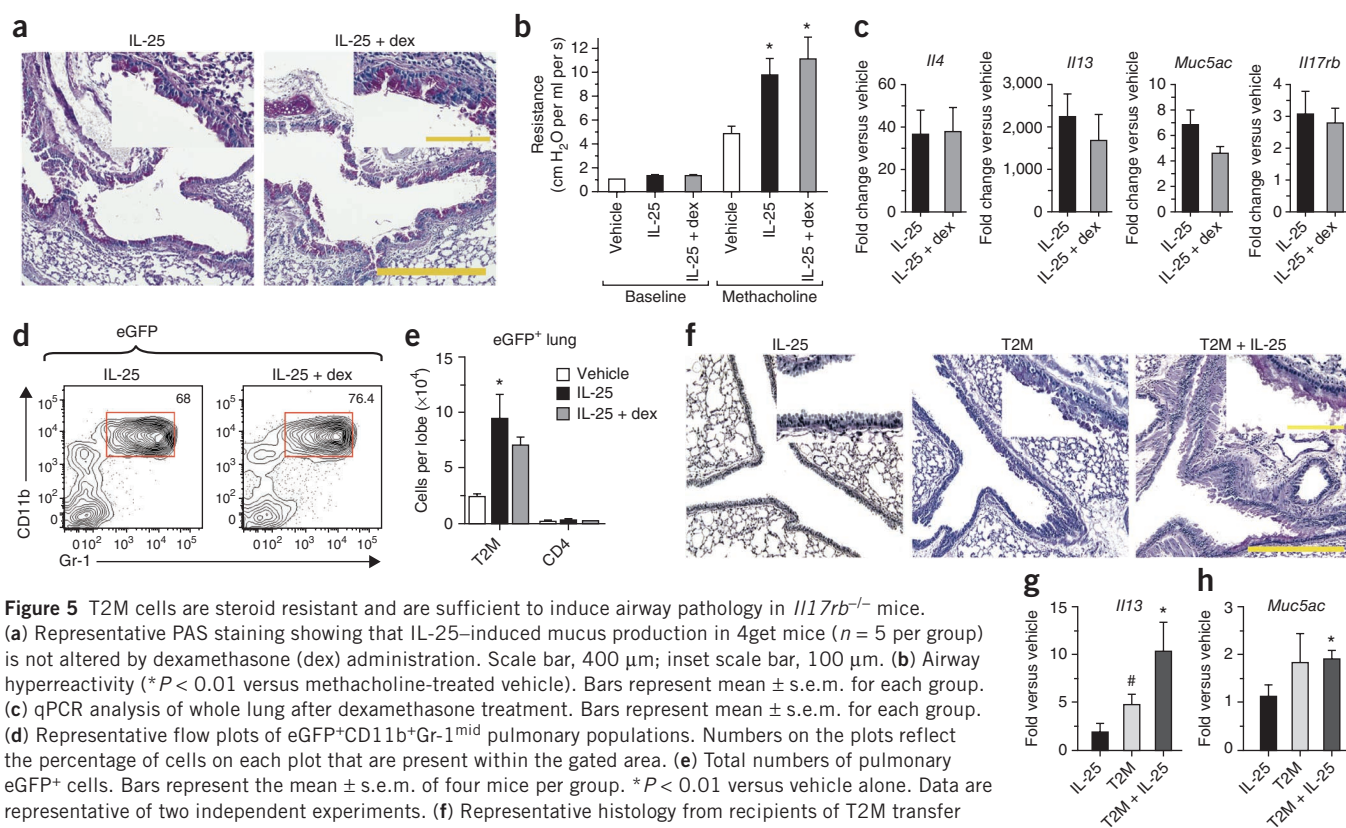


Figure 5 T2M cells are steroid resistant and are sufficient to induce airway pathology in *Il17rb*^{-/-} mice.

(a) Representative PAS staining showing that IL-25-induced mucus production in 4get mice ($n = 5$ per group) is not altered by dexamethasone (dex) administration. Scale bar, 400 μ m; inset scale bar, 100 μ m. (b) Airway hyperreactivity ($*P < 0.01$ versus methacholine-treated vehicle). Bars represent mean $\pm$ s.e.m. for each group. (c) qPCR analysis of whole lung after dexamethasone treatment. Bars represent mean $\pm$ s.e.m. for each group. (d) Representative flow plots of eGFP⁺CD11b⁺Gr-1^{mid} pulmonary populations. Numbers on the plots reflect the percentage of cells on each plot that are present within the gated area. (e) Total numbers of pulmonary eGFP⁺ cells. Bars represent the mean $\pm$ s.e.m. of four mice per group. $*P < 0.01$ versus vehicle alone. Data are representative of two independent experiments. (f) Representative histology from recipients of T2M transfer stained with PAS, 24 h after final IL-25 administration. Scale bar, 400 μ m; inset scale bar, 100 μ m. T2M cells were isolated by MACS enrichment and FACS, and 2.0×10^5 cells were instilled into the airways of *Il17rb*^{-/-} mice. Mice received four total treatments, consisting of 0.5 μ g IL-25, T2M cells alone or IL-25 + T2M cells. (g) qPCR expression of IL-13 after T2M transfer ($*P = 0.017$, $^{\#}P = 0.042$). Bars represent the mean $\pm$ s.e.m. of four mice per group. Results are representative of three independent experiments. (h) qPCR expression for the mucus-specific gene *Muc5ac* ($*P < 0.026$). Bars represent the mean $\pm$ s.e.m. of four mice per group.

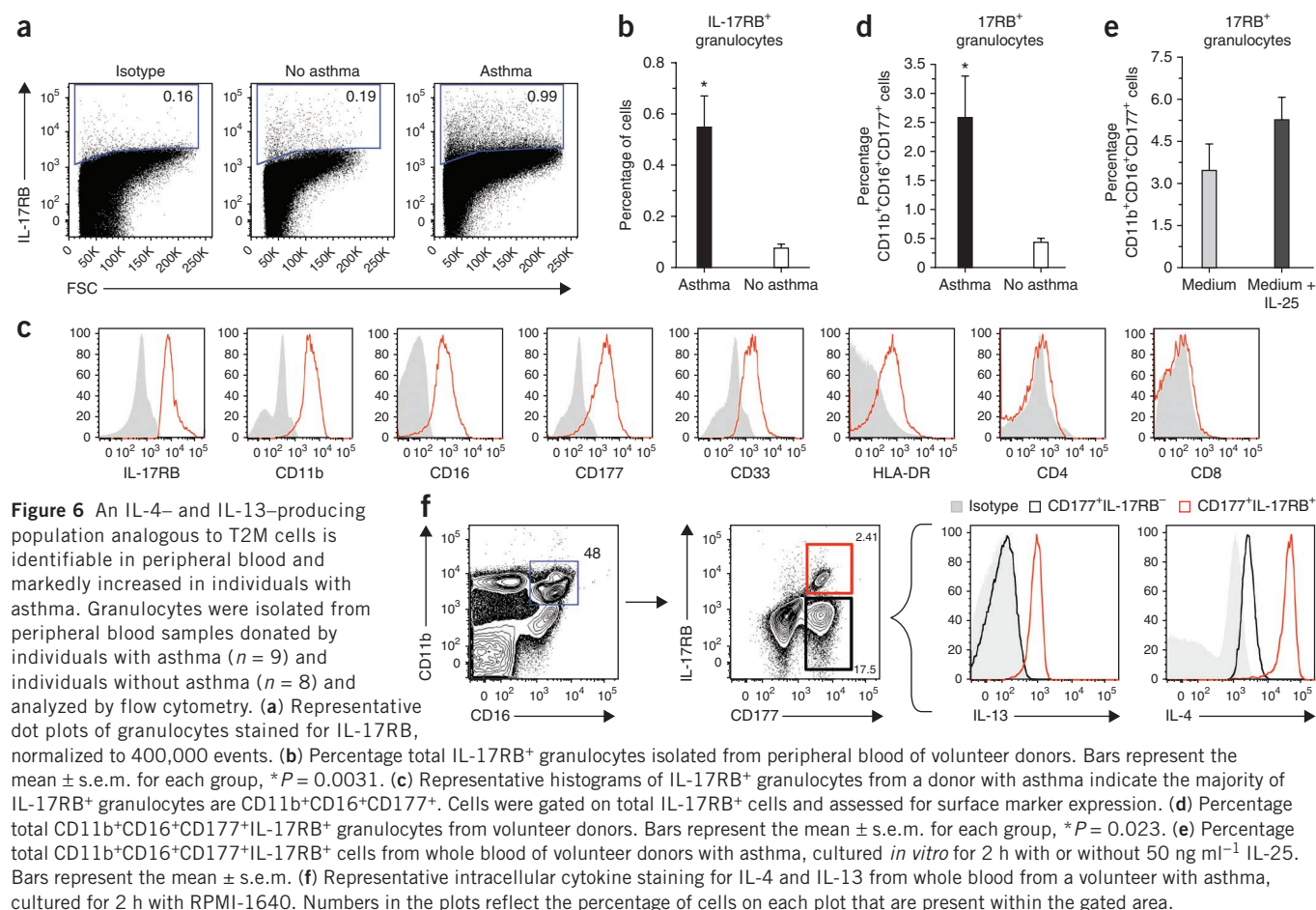
showed significant reductions in the frequency of CD11b⁺Gr-1^{mid} myeloid cells per lobe that we previously identified as a source of type 2 cytokines in allergic mice (Fig. 2d). Intracellular cytokine staining of the CD11b⁺Gr-1^{mid} population verified that *Il17rb*^{-/-} mice had a significant reduction in type 2 cytokine-producing cells relative to WT mice (Fig. 2e). Reduced cytokine production in lymph nodes, altered CD4⁺ T cell function, a reduction in type 2 cytokines in the lungs and in CD11b⁺Gr-1^{mid} myeloid cells, and a marked decrease in pulmonary myeloid cell infiltrates indicated that multiple cell types were affected by the absence of IL-17RB.

IL-25-induced inflammation is T2M cell dependent

We adapted a model of antigen-independent, IL-25-induced pulmonary inflammation⁴ to directly assess the effects of IL-25 on type 2 cytokine-producing cells *in vivo*, thereby avoiding the confounding proinflammatory effects of antigen-specific activation. Recombinant mouse IL-25 was instilled into the airways of IL-4-IRES-eGFP (4get) mice. 4get mice express eGFP in cells in which the *Il4* promoter is transcriptionally active, and we used them to identify cells poised to produce type 2 cytokines. As has been reported previously⁴, the intratracheal administration of IL-25 induced a type 2 inflammatory response, characterized by airway hyperreactivity, eosinophil infiltrates, mucus production and the upregulation of inflammatory genes, including *Il25* and *Il17rb* (Supplementary Fig. 5). IL-25 instillation induced the expression of eGFP in myeloid but not other cell subsets (Fig. 3a), with approximately 80% of eGFP cells being CD11b⁺. The CD11b⁺Gr-1^{mid}IL-17RB⁺ subset, termed T2M cells

to describe their propensity for type 2 cytokine production, showed particularly marked enrichment for eGFP expression. Whereas IL-25-treated mice showed no increase in other eGFP populations, IL-25 administration significantly increased CD11b⁺Gr-1^{mid} infiltrates (Fig. 3b). Among this population, all eGFP⁺ cells were also IL-17RB⁺ (Fig. 3c), indicating that IL-25 acts on IL-25-responsive myeloid cells in part by activating transcription at the *Il4* promoter. Flow cytometric analysis further confirmed that the predominant pulmonary source of IL-13 following IL-25 administration was T2M cells (Fig. 3d). Lin⁻c-Kit⁺Sca-1⁺IL-17RB⁺ cells, a population identified as a source of IL-25-induced IL-13 in the gut¹⁹, were not altered by pulmonary IL-25 administration (Fig. 3d).

To verify that myeloid cells were producing type 2 cytokines in response to IL-25 administration, we isolated T2M cells from the lungs of IL-25-treated mice and assessed type 2 transcripts in this population. T2M cells exposed to IL-25 *in vivo* showed marked increases in *Il4* and *Il13* transcripts, whereas *Il5* was derived from a CD11b⁻ cell population that remains ill-defined in our studies (Fig. 3e–g). In addition to the increase in pulmonary T2M cells that we observed after intratracheal IL-25 administration, we also identified T2M cells in the bone marrow of 4get mice after IL-25 treatments. The pulmonary instillation of recombinant IL-25 increased numbers of bone marrow T2M cells and induced IL-4 expression by the bone marrow T2M population (Supplementary Fig. 6). Thus, IL-25 exerts both local and systemic effects by increasing numbers of T2M cells in lung and bone marrow and by stimulating cytokine production in these populations.



Pulmonary T2M cells are defined by a distinct combination of cell surface antigens (**Fig. 4a**). We observed similar expression patterns of cell surface antigens in T2M cells derived from other tissues, including spleen, bone marrow and peripheral blood (data not shown). T2M cells did not express the neutrophil-specific receptor CXCR2, nor did they express eosinophil protein markers such as IL-5 α and CCR3. FACS-isolated T2Ms did not produce detectable transcripts encoding myeloperoxidase, major basic protein or eosinophil peroxidase (data not shown), further supporting the claim that they are a separate granulocytic population. Microarray analysis of T2M cells compared to other isolated myeloid cell populations (**Fig. 4b,c** and **Supplementary Fig. 2**) provides further evidence that this population represents a distinct granulocytic subset most closely related to eosinophils.

T2M cells are steroid resistant and pathologically relevant

To examine potential clinical implications of type 2 cytokine production in the T2M population, we next examined how steroid treatment affected IL-25-induced pulmonary inflammation. To focus on the T2M response, we treated 4get mice with IL-25 as in previous experiments, with or without dexamethasone. Histologic examination (**Fig. 5a**) and measurements of airway hyperreactivity (**Fig. 5b**) indicated that IL-25-induced responses were not significantly altered by dexamethasone. Quantitative PCR (qPCR) analyses demonstrated marked increases in the expression of type 2 cytokine genes, mucus genes and *Il17rb* that were not suppressed after dexamethasone administration (**Fig. 5c**). Flow cytometric

analysis revealed equivalent numbers of IL-25-induced eGFP⁺ myeloid cells in mice treated with or without dexamethasone (**Fig. 5d,e**). To verify that the dexamethasone treatment was effective, we examined splenic cell subsets (**Supplementary Table 1**). Dexamethasone significantly reduced the number of total splenocytes, with a specific reduction in CD4⁺ and CD8⁺ T cells as well as eosinophils, but it had no effect on splenic T2M cells. Overall, these data indicate that IL-25-induced T2M cells are resistant to high-dose glucocorticoid treatment.

To determine whether IL-17RB⁺ T2M cells are sufficient to induce pulmonary inflammation, we isolated T2M cells from IL-25-treated mice and instilled them into the airways of *Il17rb*^{-/-} mice together with recombinant IL-25. The transfer of T2M cells from IL-25-treated WT mice into *Il17rb*^{-/-} recipients, coupled with instillations of IL-25, induced mucus production and inflammation in otherwise IL-25-insensitive *Il17rb*^{-/-} mice (**Fig. 5f**). Recipients of T2M cells had significantly higher *Il13* transcript levels, and the transfer of T2M cells with IL-25 further upregulated expression of *Il13* (**Fig. 5g**) and the mucus-specific gene *Muc5ac* (**Fig. 5h**). In separate experiments, T2M transfer exacerbated the inflammatory response in WT recipients and increased *Muc5ac* transcripts to levels comparable to those observed in IL-25-treated WT mice (**Supplementary Fig. 7**). We observed a similar pattern of increased mucus-specific gene expression after the adoptive transfer of T2M cells into allergen-sensitized recipients (**Supplementary Fig. 7**). Therefore, in the context of high pulmonary IL-25 levels, T2M cells are sufficient to induce pulmonary inflammation, IL-13 expression and mucus production.

T2M-like cells are increased in individuals with asthma

To assess whether a population analogous to T2M cells is present in humans and may be clinically relevant, we recruited volunteer subjects with asthma from the University of Michigan Asthma Clinic and compared their expression of IL-17RB in peripheral blood to that of nonasthmatic volunteers. Flow cytometric analysis identified significantly higher numbers of granulocytic IL-17RB⁺ cells in subjects with asthma (Fig. 6a,b). The granulocytic IL-17RB⁺ population concurrently expressed CD11b, CD16, and the Ly6 family member/myeloid progenitor/neutrophil marker CD177 (Fig. 6c). In addition, most IL-17RB⁺ cells were CD33⁺, weakly expressed human leukocyte antigen-DR and were not CD4⁺ or CD8⁺.

Given the above findings, we focused our analysis on IL-17RB⁺ CD11b⁺CD16⁺CD177⁺ cells and identified a significantly higher percentage of these cells in subjects with asthma (Fig. 6d) that was further elevated after *in vitro* stimulation with IL-25 (Fig. 6e). This IL-17RB⁺ subset produced both IL-4 and IL-13, whereas IL-17RB⁺ cells did not (Fig. 6f). Thus, a population with similar cell surface receptor expression and phenotype as mouse T2M cells can be identified in peripheral blood, is significantly elevated in asthmatics, and represents a source of both IL-4 and IL-13.

DISCUSSION

IL-25 has been established as a regulator of type 2 inflammation, and multiple reports have described its ability to exacerbate inflammatory responses at mucosal epithelia, including those in allergic asthma. The present study used a mouse model of chronic allergic asthma to identify both T and non-T IL-25-responsive cells involved in pulmonary inflammation. Although previous studies have established that targeting IL-25 leads to the reduction of type 2 responses^{22,23}, to our knowledge this study is the first to characterize how deficiency in IL-17RB reduces the pathology of allergic asthma induced by a common environmental allergen. Other reports, including one from our laboratory, have demonstrated that eosinophils produce IL-25 (refs. 7,8), thus linking IL-25 production to eosinophilia induced by the allergic response. These data are consistent with clinical studies, as peripheral blood mononuclear cells from individuals with severe allergic rhinitis show increased IL-17RB expression²⁴, and polymorphisms in *IL17RB* have been associated with increased risk for severe asthma²⁵. Furthermore, a recent study has identified that allergen-induced expression of IL-25 and its receptor in individuals with atopic asthma correlates with disease severity²¹.

Il17rb^{-/-} mice had reduced allergen-induced pathology, including a marked reduction in type 2 cytokines primarily associated with T2M cells, which were most prominent during persistent allergen-induced disease. The transfer of IL-25-induced T2M cells recapitulated lung pathology in IL-25-treated *Il17rb*^{-/-} mice, demonstrating the sufficiency of T2M cells to mediate pathogenic responses. T2M cells seem to be steroid resistant *in vivo* and represent a distinct granulocytic population, which may have been identified in a model of pulmonary inflammation during *Nippostrongylus brasiliensis* infection²⁶. T cell function was also altered in *Il17rb*^{-/-} mice, demonstrating that in chronic allergic disease both T cell and non-T cell populations are contributors to type 2 cytokine-mediated pathophysiology.

Although T lymphocytes are responsible for driving allergen-specific type 2 responses, multiple reports have identified critical roles for innate immune populations. IL-25-responsive lymphoid cells have been identified in the gut in models of *N. brasiliensis* infection and have a key role in the clearance of enteric helminths^{18–20}. Recent studies have identified a similar innate lymphocytic cell population in the gut, lung and nasal polyps of humans, further supporting

this population's potential role in human disease²⁷. In our studies, this population was present at low numbers in the lung and did not increase after allergen or IL-25 administration. Other nonlymphoid populations have also been identified as sources of type 2 cytokines that can contribute to the allergic environment, including basophils, mast cells, eosinophils and macrophages^{28–38}. We did not detect substantial IL-17RB expression in any of these populations in the lungs of allergen-challenged mice. Thus, it seems that there are both Lin⁺ and Lin⁻ IL-25-responsive cells whose recruitment may vary depending on the mucosal surface (gut versus lung), with each cell type maintaining the capacity to produce type 2 cytokines in an antigen-independent manner.

On the basis of an analysis of T2M surface markers as well as the identification of T2M cells in the bone marrow during allergen- and IL-25-induced responses, T2M cells seem to be derived from the bone marrow. Their presence in peripheral blood of humans with asthma may represent an induced cell population that may be recruited and may accumulate in the lung during persistent or exacerbated disease. Because IL-17RB⁺ subsets could be distinguished on the basis of their intensity of Gr-1 expression, and in light of the differing capacity for IL-4 and IL-13 production between Gr-1^{mid} and Gr-1^{hi} populations, IL-17RB⁺Gr-1^{hi} cells may have functions that overlap with other CD11b⁺Gr-1⁺ populations, such as myeloid suppressor cells^{39,40}. A recent study reported that both IL-17RA and IL-17RB can be expressed on the surface of human neutrophils⁴¹. Clinical studies of patients with steroid-resistant asthma demonstrated that a neutrophilic inflammatory response is predominant, and mouse studies have suggested that steroid-resistant T helper type 17 cells may explain neutrophil-mediated responses^{42–50}. As IL-17RA is required for functional IL-17A and IL-25 signaling, these cytokines may share downstream signals induced following ligand binding that provide a common link for steroid resistance. The development of T2M cells probably depends on an overall type 2 immune environment and perhaps IL-25 itself.

Our data also indicate that, given the rapid accumulation of myeloid cells in lungs after IL-25 administration, there is a pool of cytokine-producing, IL-25-responsive cells capable of amplifying a type 2 response. In allergic individuals, T2M cells may have a key role in the immediate response to an environmental allergen, priming the system for a type 2 response by producing cytokines before T lymphocyte activation. T2M cells could also contribute to chronic disease, as airway epithelial damage stimulates IL-25 secretion. This concept may be especially relevant in individuals with asthma, as our studies identified high numbers of T2M-like cells in the circulation of individuals with asthma that may be recruited upon an exacerbation and accumulate in the lungs. The induction of IL-25 in airways by pathogens⁵¹, allergens or other noxious stimuli may amplify the severity of the response by activating steroid-resistant T2M cells, especially in patients with underlying pulmonary disease. A complete understanding of the development and function of T2M cells will require further investigation; however, our findings suggest that they represent a useful biomarker and possible therapeutic target for the treatment of severe asthma.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturemedicine/>.

Accession codes. Expression data for T2M cells, eosinophils, neutrophils and macrophages from IL-25-treated 4get mice have been deposited in the Gene Expression Omnibus with accession number GSE36392.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

B.C.P. conceived of the study, performed experiments and data analyses and wrote the manuscript. A.L.B. and M.A.S. provided intellectual contributions through technical advice and experimental design. A.P.B. coordinated recruitment of subjects with asthma. N.W.L. conceived of and supervised the study and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals and allergen model. Six- to eight-week-old female C57BL/6J, BALB/c, 4get and *Rag2*^{-/-} mice were purchased from the Jackson Laboratory. *Il17rb*^{-/-} mice were provided by A.L. Budelsky. Clinical-skin-test-grade cockroach allergen (HollisterStier) was used for allergen sensitization as previously described⁵². Mice were immunized systemically (day 0) via intraperitoneal and subcutaneous injections of allergen emulsified with incomplete Freund's adjuvant. Mice were given four intranasal allergen challenges (1.5 µg in 15 µl PBS on days 14, 18, 22 and 26), followed by two i.t. administrations (5 µg in 50 µl PBS on days 30 and 32). Tissues were analyzed 24 h after final allergen challenge. All mouse experiments were reviewed and approved by the University of Michigan University Committee on Care and Use of Animals.

Airway hyperreactivity. Airway hyperreactivity was measured using direct ventilation mouse plethysmography, specifically designed for low tidal volumes (Buxco Research Systems), as previously described^{53,54}.

Lung histology. Serial 6-µm sections were obtained from paraffin-embedded, formalin-fixed left lungs stained with H&E or PAS.

Primary cell isolation. Lung tissue was processed via enzymatic digestion as previously described⁷. Draining lymph nodes and spleen cells were dispersed by mechanical disruption through an 18-gauge needle. Bone marrow was isolated by flushing femurs and tibias with 1× PBS and 1% FCS through a 40-µm filter. Cell numbers were quantified following red blood cell lysis.

Lymph node re-stimulation. Lymph node cells (4×10^5 per well, plated in triplicate) were re-stimulated with 10 µl ml⁻¹ allergen, 10 ng ml⁻¹ IL-25 or both in complete medium. RNA was isolated after 2 h in culture; supernatants were analyzed for protein production after 48 h in culture.

mRNA and protein quantification. Whole-lung, lymph node and bone marrow RNA was isolated using TRIzol (Invitrogen). RNA from cells sorted by FACS was isolated with a microprep kit (Qiagen), DNase treated (Invitrogen) and reverse transcribed with Superscript III (Invitrogen). mRNA was assessed using qPCR analysis (TaqMan) with primers and probe sets from Applied Biosystems. Expression of genes of interest was normalized to *Gapdh*. Protein was quantified by Bioplex (Bio-Rad) according to the manufacturer's instructions.

Flow cytometry. Populations were assessed using standard techniques. Samples for intracellular staining were treated with 0.5 µl mL⁻¹ brefeldin A, 0.5 µl mL⁻¹ monensin, 0.5 ng mL⁻¹ PMA and 500 ng mL⁻¹ ionomycin in complete medium and incubated for 6 h at 37 °C, 5% CO₂. Cells were stained according to manufacturer's instructions (BD Biosciences fix/perm kit). Data were collected on a BD Biosciences LSR II flow cytometer and on a BD Biosciences FACSaria and analyzed with FlowJo software (Tree Star). Cellular surface markers of spleen cell controls were not altered by collagenase treatment as compared to untreated cells, consistent with previous studies^{55–57}. All antibodies used in these studies are listed in the **Supplementary Methods**.

Intratracheal IL-25 administration and dexamethasone treatments. Six- to eight-week-old female C57BL/6J, BALB/c, 4get or *Il17rb*^{-/-} mice received daily i.t. injections (0.5 µg recombinant IL-25 (R&D) in 50 µl PBS) for 4 d¹⁰.

Dexamethasone (Sigma) was administered in 2 doses (3 mg per kg body weight per day) via intraperitoneal injection, 1 h before the first and third IL-25 injections. Tissues were analyzed 24 h after final IL-25 administration.

T2M Isolation and adoptive transfers. T2M cells were isolated from the lungs of C57BL/6J mice treated with i.t. IL-25, as described above, by enriching for CD11b⁺ cells with MACS (Miltenyi), followed by FACS. Isolated cells (2.0×10^5 per recipient) were instilled into the airways of *Il17rb*^{-/-} mice via i.t. injection in 50 µl PBS. Recipients received four transfers total at 24-h intervals. Lung tissue was collected 24 h after final adoptive transfer.

Microarrays. Pooled cells from IL-25-treated 4get mice ($n = 4$ mice per sample) were sorted by FACS and RNA isolated for microarray analysis. Affymetrix Mouse 430 2.0 microarrays were processed in the University of Michigan DNA Sequencing Core using the WT-Pico V.2 kit.

Clinical studies. All human studies were performed in accordance with an approved University of Michigan Institutional Review Board protocol after legal informed consent from adult volunteers. Subjects were recruited from the University of Michigan Asthma Clinic and had been diagnosed with asthma on the basis of clinical assessment and pulmonary testing consistent with 2007 US National Institutes of Health National Asthma Education and Prevention Program guidelines. Subjects ($n = 9$) had persistent asthma and were on daily controller medication. Healthy control subjects ($n = 8$) had not previously been diagnosed with asthma. The protocol used to assess inflammatory subsets is described in the **Supplementary Methods**.

Statistical analyses. All data are presented as means $\pm$ s.e.m. Data were evaluated by one-way analysis of variance and, where appropriate, further evaluated with the parametric Student-Newman-Keuls test for multiple comparisons or the nonparametric Mann-Whitney rank-sum test. For microarray analysis, expression values for each gene were assessed using a robust multiarray average. The Affymetrix package of Bioconductor implemented in the R statistical language was used to analyze probe sets.

Additional methods. Detailed methodology is described in the **Supplementary Methods**.

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