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

Bryan C. Petersen[1], **Alison L. Budelsky**[2], **Alan P. Baptist**[3], **Matthew A. Schaller**[1], and **Nicholas W. Lukacs**[1]

[1]The University of Michigan Department of Pathology, Ann Arbor, MI 48109

[2]Department of Inflammation, Amgen, Seattle, WA 98119

[3]The University of Michigan Department of Internal Medicine, Ann Arbor, MI 48109

Abstract

This study identifies the IL-25 receptor, IL-17RB, is an important mediator of both innate and adaptive pulmonary type 2 immune responses. Allergen exposure upregulated IL-25 and induced type 2 cytokine production in a novel granulocytic population, termed Type 2 Myeloid (T2M) cells. *Il17rb*$^{-/-}$ mice exhibited reduced lung pathology following chronic allergen exposure and decreased cytokine production in T2M cells and CD4$^+$ T-lymphocytes. Airway instillation of IL-25 induced IL-4 and IL-13 production exclusively in T2M cells demonstrating their importance in generating 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/IL-13 producing granulocytic population was identified in peripheral blood of asthmatics. These data establish IL-25/IL-17RB as targets for innate and adaptive immune responses in chronic allergic airways disease, and identify T2M cells as a novel 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 asthma's pathophysiology[1–3]. Three epithelial-derived type 2 inflammation associated

Corresponding Author- Nicholas W. Lukacs, Ph.D., Professor of Pathology, University of Michigan, 109 Zina Pitcher Way; 4059 BSRB, Ann Arbor, MI 48109-2200, nlukacs@umich.edu, 734-764-5135.

cytokines, IL-25, 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 study[4], the systemic injection of IL-25 into $Rag^{-/-}$ mice induced type 2 cytokine expression and eosinophilia, demonstrating 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[4–6]. Pulmonary IL-25 is produced by eosinophils[7,8], mast cells, and airway epithelial cells and stimulates asthma-like inflammation characterized by airway hyperreactivity, mucus production, airway eosinophilia, and increased serum IgE[9]. The induction of these responses requires binding of a noncovalently bound IL-25 homodimer to the IL-17RA/IL-17RB heterodimer[10], of which IL-17RB represents an IL-25 specific moiety[11]. IL-25 is known to enhance Th2 effector functions via Act1 and TRAF6 dependent NFκB activation[12–15]. Multiple cell types in the lung, including memory[16] and effector[14,16] T cells, invariant NKT cells[17], APCs, and airway smooth muscle, have been characterized as expressing IL-17RB, whereas eosinophils do not[7]. Several studies have also identified additional IL-25 responsive type 2 cytokine producing non-B, non-T cell (NBNT) populations[5,18–20], and a recent investigation reported increased expression of IL-25 and its receptor in the airways of asthmatics after allergen provocation[21].

The present study reports that repeated allergen exposure upregulated both pulmonary IL-25 and IL-17RB in a murine model of persistent allergic airway disease, and induced the accumulation of a novel IL-4 and IL-13 producing IL-17RB^{+} Type 2 Myeloid (T2M) population in the lung. T2M cells are granulocytes driven by IL-25, and are both pathogenic and steroid resistant. Moreover, IL-4 and IL-13 producing T2M-like cells were identified in the peripheral blood of asthmatics.

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]. The induction of type 2 cytokine expression following the pulmonary instillation of allergen (Fig. 1) was accompanied by 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 *Ifnγ*, other IL-17 family members, *Il33*, nor its receptor *Il1rl1* were upregulated with our model of cockroach allergen challenge (Fig. 1b,c), 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 disease[7]. While CD4^{+} T cells are present following antigen sensitization, the most numerous IL-17RB^{+} IL-4/IL-13 producing population in the lung were CD11b^{+} myeloid cells (Fig. 1d,e). We also examined the capacity of innate Lin^{−} ckit^{+} Sca1^{+} IL-17RB^{+} NBNT cells to produce type 2 cytokines. Lin[−] ckit[+] Sca1^{+} IL-17RB^{+} cells comprised a relatively rare population in the lungs of both naïve and allergen-challenged mice (range 250–1000), and were not increased following

allergen sensitization (Fig. 1d,e). Few myeloid IL-4 and IL-13 producing cells were present in the lungs of naïve mice, and the IL-17RB$^+$ cells preferentially produced type 2 cytokine. Myeloid IL-17RB$^+$ cells represented the major NBNT IL-4/13 cytokine producing population in the lung, outnumbering cytokine producing CD4$^+$ T cells 68:1 (see Supplemental Fig. 1).

Despite significant 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 appeared to produce these cytokines. Analysis of IL-4 and IL-13 production in IL-17RB$^+$ myeloid subsets, based on levels of Gr-1 expression, allowed for the identification of two distinct IL-17RB$^+$ myeloid populations (Fig. 1f). A comparison of total cell numbers between naïve and allergic animals identified significant increases in both Gr-1^{mid} (Fig. 1g) and Gr-1^{hi} subsets in the lungs of allergic mice, however the IL-17RB$^+$ CD11b$^+$ Gr-1^{mid} population produced IL-4 and IL-13 while 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, see Supplemental Fig. 2). These data inspired our next set of experiments, an investigation of IL-17RB's involvement in the production of type 2 cytokines in this granulocytic IL-17RB$^+$ population.

Il17rb$^{-/-}$ mice exhibit decreased type 2 inflammation

To further explore the overall role of IL-17RB in allergic asthma, *Il17rb*$^{-/-}$ mice were sensitized to allergen. The loss of the IL-25-specific receptor protected *Il17rb*$^{-/-}$ mice from allergen-induced inflammation (Fig. 2). *Il17rb*$^{-/-}$ allergic mice exhibited a dramatic reduction in peribronchial and perivascular inflammation, eosinophilic infiltrates, and mucus production (Fig. 2a). Pulmonary expression of type 2 cytokines as well as the eosinophil-associated chemokine CCL11 were 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 lymph node cells (Fig. 2c). Interestingly, a short-term model of allergen sensitization that utilized fewer allergen challenges revealed no phenotypic differences in *Il17rb*$^{-/-}$ mice (see Supplemental Fig. 3) suggesting that IL-17RB is most relevant during more chronic allergic responses when IL-25 production is significantly increased.

There was no detectable difference in total numbers of CD4$^+$ or CD8$^+$ T lymphocyte subsets between the lungs of allergen sensitized *Il17rb*$^{-/-}$ or WT mice. However, *in vitro* re-stimulation experiments, as well as *in vivo* adoptive transfer studies with *Rag2*$^{-/-}$ mice, demonstrated that CD4$^+$ T cell responses were altered in *Il17rb*$^{-/-}$ animals and could not efficiently transfer a type 2 immune response to the *Rag2*$^{-/-}$ recipients (see Supplemental Fig. 4). These data were similar to findings published by several investigators establishing a role of IL-25 in type 2 responses[9,10,16]. *Il17rb*$^{-/-}$ mice also exhibited significant reductions in the CD11b$^+$ Gr-1^{mid} myeloid cells previously identified as a source of type 2 cytokines in allergic animals (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. 3e). Reduced lymph node cytokine production, altered CD4$^+$ T cell function, the reduction in type 2 cytokines, and a dramatic 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 inflammation[4] to directly assess the effects of IL-25 on type 2 cytokine producing cells *in vivo*, thereby avoiding the confounding pro-inflammatory effects of antigen-specific activation. Recombinant murine IL-25 was instilled into the airways of IL-4-IRES-eGFP (4get) mice. 4get mice express GFP in cells in which the IL-4 promoter is transcriptionally active, and were used to identify cells poised to produce type 2 cytokines. As has been reported previously[4], the intratracheal administration of IL-25 induced a type 2 type inflammatory response, characterized by airway hyperreactivity, eosinophil infiltrates, mucus production, and the upregulation of inflammatory genes including *Il25* and *Il17rb* (see Supplemental Fig. 5). IL-25 instillation induced the expression of GFP/IL-4 in myeloid but not other cell subsets (Fig. 3a), with approximately 80% of GFP/IL-4+ cells being CD11b+. The CD11b+ Gr-1mid IL-17RB+ subset, termed T2M cells to describe their propensity for type 2 cytokine production, demonstrated particularly dramatic enrichment for GFP/IL-4 expression. While IL-25 treated mice showed no increase in other GFP/IL-4+ populations, IL-25 administration significantly increased CD11b+ Gr-1mid infiltrates (Fig. 3b). Figure 3c illustrates that among this population, all GFP+ cells were also IL-17RB+, indicating that IL-25 acts on IL-25 responsive myeloid cells in part by activating transcription at the IL-4 promoter. Flow cytometric analysis further confirmed that the predominant pulmonary source of IL-13 following IL-25 administration were T2M cells (Fig. 3d). Lin− ckit+ Sca-1+ IL-17RB+ cells, a population identified as a source of IL-25-induced IL-13 in the gut[19], were not altered by pulmonary IL-25 administration (Fig. 3f).

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* exhibited dramatic 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 pulmonary effects of intratracheal IL-25 administration, T2M cells were also identified in the bone marrow of 4get mice following IL-25 treatments (see Supplemental Fig. 6). Thus, IL-25 had both local and systemic effects linked to development of T2M cells.

Pulmonary T2M cells are defined by a distinct combination of cell surface antigens (Fig. 4a). Similar expression patterns were observed 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-5rα or CCR3. FACS isolated T2Ms did not produce detectable transcripts of myeloperoxidase, major basic protein, or eosinophil peroxidase (**data not shown**), further supporting them as a separate granulocytic population. Microarray analysis of T2M cells compared to other isolated myeloid cell populations (Fig. 4b,c, see Supplemental 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, 4get animals were treated 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. QPCR analyses demonstrated significant increases in type 2 cytokines, mucus genes, and *Il17rb* that were unaffected by dexamethasone administration (Fig. 5c). Flow cytometric analysis measured equivalent numbers of IL-25-induced GFP/IL-4^{+} myeloid cells in animals treated with or without dexamethasone (Fig. 5d,e). To verify that dexamethasone treatment was effective, we examined splenic cell subsets (Supplementary Table 1). Dexamethasone significantly reduced total splenocytes, with a specific reduction in CD4^{+} and CD8^{+} T cells as well as eosinophils, but had no effect on splenic T2M cells. Overall these data present a striking finding that the IL-25-induced T2M cells are resistant to high dose glucocorticoid treatment.

In order to determine if IL-17RB^{+} T2M cells were sufficient to induce pulmonary inflammation, T2M cells from IL-25 treated mice were isolated and instilled into the airways of *Il17rb*^{−/−} mice 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*^{−/−} animals (Fig. 5f). Recipients of T2M cells significantly increased *Il13* transcripts, and the transfer of T2M cells with IL-25 further upregulated *Il13* expression (Fig. 5g) as well as 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 animals (see Supplemental Fig. 7). A similar pattern of increased mucus specific gene expression was observed following the adoptive transfer of T2M cells into allergen-sensitized recipients (see Supplemental Fig. 7). Therefore, in the context of increased pulmonary IL-25 levels, T2M cells are sufficient to induce pulmonary inflammation, IL-13, and mucus production; all hallmarks of allergic asthma.

T2M–like cells are increased in asthmatics

In order to assess whether a population analogous to T2M cells is present in humans and may be clinically relevant, asthmatic volunteers were recruited from the University of Michigan Asthma Clinic, and the expression of IL-17RB in peripheral blood was compared to non-asthmatic volunteers. Flow cytometric analysis identified significantly increased numbers of granulocytic IL-17RB^{+} cells in asthmatic patients (Fig. 6a,b). The granulocytic IL-17RB^{+} population co-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 HLA-DR, and were not CD4^{+} or CD8^{+}.

Based on the above findings, we focused our analysis upon IL-17RB^{+} CD11b^{+} CD16^{+} CD177^{+} cells and identified a significantly increased percentage of these cells in asthmatics (Fig. 6d) that was further elevated following *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. Thus, a

population with similar cell surface receptor expression and phenotype as murine 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. While previous studies have established that targeting IL-25 leads to the reduction of type 2 responses[22,23], 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[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 patients with severe allergic rhinitis exhibit increased IL-17RB[24], and polymorphisms in IL-17RB have been associated with increased risk for severe asthma[25]. Furthermore, a recent study has identified that allergen-induced expression of IL-25 and its receptor in atopic asthmatics correlates with disease severity[21].

Il17rb^{−/−} mice had reduced allergen-induced pathology, including a significant reduction in type 2 cytokines primarily associated with T2M cells that 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 appear to be steroid resistant *in vivo* and represent a distinct granulocytic population, which may have been identified in a model of pulmonary inflammation during *N. brasiliensis* infection[26]. 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.

While T lymphocytes are responsible for driving allergen-specific type 2 responses, multiple reports have identified critical roles for innate immune populations. Models of *N. brasiliensis* infection have identified novel IL-25 responsive cells in the gut, characterized as Lin[−] ckit[+] Sca-1^{+} IL-13^{+}, that play an important role in the clearance of enteric helminthes[18–20]. Recent studies have identified a similar innate lymphocytic cell population in the gut, lung, and nasal polyps of humans, further supporting their potential role in human disease[27]. In our studies, this population was present at low numbers in the lung, and did not increase following allergen or IL-25 administration. Previous reports have identified other non-lymphoid populations 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 significant IL-17RB expression in any of these populations in the lungs of allergen challenged animals. Thus, it appears that there are both Lin[+] and Lin[−] IL-25 responsive cells whose recruitment may depend upon the type of mucosal surface (gut versus lung), both with the capacity to produce type 2 cytokines in an antigen-independent manner.

Analysis of surface markers and presence in the bone marrow during allergen and IL-25-induced responses indicate that T2M cells are 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 accumulate in the lung during persistent or exacerbated disease. Because IL-17RB^{+} subsets could be distinguished based upon the 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 neutrophils[41]. Clinical studies of patients with steroid resistant asthma demonstrated that a neutrophilic inflammatory response is predominant, while animal studies have suggested that steroid resistant Th17 cells may explain neutrophil-mediated responses[42–50]. Since 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 likely depends upon an overall type 2 immune environment and perhaps IL-25 itself.

Our data also indicate that, based on the rapid accumulation of myeloid cells in lungs following 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 play an important role in the immediate response to an environmental allergen, priming the system for a type 2 response by producing cytokines prior to 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 asthmatics, as our studies identified increased numbers of T2M-like cells in circulation of asthmatics that may be recruited upon an exacerbation and accumulate in the lungs. The induction of IL-25 in airways by pathogens[51], 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 an intriguing biomarker and possible therapeutic target for the treatment of severe asthma.

Materials and Methods

Animals and allergen model

6–8 week old female C57BL/6J, BALBc, 4get, and $Rag2^{-/-}$ mice were purchased from The Jackson Laboratory. $Il17rb^{-/-}$ mice were provided by A.L. Budelsky (Amgen). Clinical skin test grade cockroach allergen (HollisterStier) was used for allergen sensitization as previously described[52]. Animals were immunized systemically (day 0) via intraperitoneal (IP) and subcutaneous injections of allergen emulsified with Incomplete Freund's Adjuvant. Mice were given 4 intranasal allergen challenges (1.5 μg in 15 μl PBS on days 14, 18, 22, and 26), followed by two intra-tracheal (IT) administrations (5 μg in 50 μl PBS on days 30 and 32). Tissues were analyzed 24 hours post final allergen challenge. All animal experiments were reviewed and approved by the University of Michigan University Committee on Care and Use of Animals.

Airway hyperreactivity (AHR)

AHR 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 periodic acid-Schiff (PAS).

Primary cell isolation

Lung tissue was processed via enzymatic digestion as previously described[7]. Draining LN and spleen cells were dispersed by mechanical disruption through a 40 μM filter. Bone marrow was isolated by flushing femurs and tibias with $1\times$ PBS and 1% FCS through a 40 μM filter. Cell numbers were quantified following RBC lysis.

LN restimulation

LN cells (4×10^5 per well, plated in triplicate) were restimulated with 10 μL mL^{-1} allergen, 10 ng mL^{-1} IL-25, or both. RNA was isolated after 2 hours in culture; supernatants were analyzed for protein production after 48 hours in culture.

mRNA and Protein Quantification

Whole lung, LN, 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 quantitative PCR 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 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 ionomycin and incubated for 6 hours at 37°C, 5% CO_2. 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 using 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 supplemental material.

Intra-tracheal IL-25 administration and dexamethasone treatments

6–8 week old female C57BL/6J, Balbc, 4get, or $Il17rb^{-/-}$ mice received daily intra-tracheal injections (0.5 μg recombinant IL-25 (R&D) in 50 μL PBS) for 4 days[10]. Dexamethasone (Sigma) was administered in 2 doses (3 mg per kg per day) via IP injection, 1 hour prior the first and third IL-25 injections. Tissues were analyzed 24 hours after final IL-25 administration.

T2M Isolation and Adoptive Transfers

T2M cells were isolated from the lungs of C57BL/6J mice treated with intra-tracheal 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 intratracheal injection in 50 μL PBS. Recipients received four transfers total at 24 hour intervals. Lung tissue was harvested 24 hours post 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 consent from adult volunteers. Subjects were recruited from the University of Michigan Asthma Clinic and had been diagnosed with asthma based on clinical assessment and pulmonary testing consistent with 2007 NIH/NAEPP guidelines. Patients ($n = 9$) were persistent asthmatics and 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 supplemental material.

Statistical analysis

All data are presented as mean ± s.e.m. Data were evaluated by one-way ANOVA 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 multi-array average (RMA). The Affymetrix package of bio-conductor implemented in the R statistical language was used to analyze probesets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Wang W, Li JJ, Foster PS, Hansbro PM, Yang M. Potential therapeutic targets for steroid-resistant asthma. Curr Drug Targets. 2010; 11:957–970. [PubMed: 20412045]

2. Ogawa Y, Calhoun WJ. Phenotypic characterization of severe asthma. Curr Opin Pulm Med. 2010; 16:48–54. [PubMed: 19996897]

3. Adcock IM, Ford PA, Bhavsar P, Ahmad T, Chung KF. Steroid resistance in asthma: mechanisms and treatment options. Curr Allergy Asthma Rep. 2008; 8:171–178. [PubMed: 18417060]

4. Fort MM, et al. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. Immunity. 2001; 15:985–995. [PubMed: 11754819]

5. Fallon PG, et al. Identification of an interleukin (IL)-25-dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. J Exp Med. 2006; 203:1105–1116. [PubMed: 16606668]

6. Tamachi T, et al. IL-25 enhances allergic airway inflammation by amplifying a TH2 cell-dependent pathway in mice. J Allergy Clin Immunol. 2006; 118:606–614. [PubMed: 16950278]

7. Dolgachev V, Petersen BC, Budelsky AL, Berlin AA, Lukacs NW. Pulmonary IL-17E (IL-25) production and IL-17RB+ myeloid cell-derived Th2 cytokine production are dependent upon stem cell factor-induced responses during chronic allergic pulmonary disease. J Immunol. 2009; 183:5705–5715. [PubMed: 19828636]

8. Terrier B, et al. IL-25: a cytokine linking eosinophils and adaptative immunity in Churg-Strauss syndrome. Blood.

9. Sharkhuu T, et al. Mechanism of interleukin-25 (IL-17E)-induced pulmonary inflammation and airways hyper-reactivity. Clin Exp Allergy. 2006; 36:1575–1583. [PubMed: 17177681]

10. Rickel EA, et al. Identification of functional roles for both IL-17RB and IL-17RA in mediating IL-25-induced activities. J Immunol. 2008; 181:4299–4310. [PubMed: 18768888]

11. Lee J, et al. IL-17E, a novel proinflammatory ligand for the IL-17 receptor homolog IL-17Rh1. J Biol Chem. 2001; 276:1660–1664. [PubMed: 11058597]

12. Claudio E, et al. The adaptor protein CIKS/Act1 is essential for IL-25-mediated allergic airway inflammation. J Immunol. 2009; 182:1617–1630. [PubMed: 19155511]

13. Swaidani S, et al. The critical role of epithelial-derived Act1 in IL-17- and IL-25-mediated pulmonary inflammation. J Immunol. 2009; 182:1631–1640. [PubMed: 19155512]

14. Wong CK, Li PW, Lam CW. Intracellular JNK, p38 MAPK and NF-kappaB regulate IL-25 induced release of cytokines and chemokines from costimulated T helper lymphocytes. Immunol Lett. 2007; 112:82–91. [PubMed: 17719653]

15. Maezawa Y, et al. Involvement of TNF receptor-associated factor 6 in IL-25 receptor signaling. J Immunol. 2006; 176:1013–1018. [PubMed: 16393988]

16. Wang YH, et al. IL-25 augments type 2 immune responses by enhancing the expansion and functions of TSLP-DC-activated Th2 memory cells. J Exp Med. 2007; 204:1837–1847. [PubMed: 17635955]

17. Stock P, Lombardi V, Kohlrautz V, Akbari O. Induction of airway hyperreactivity by IL-25 is dependent on a subset of invariant NKT cells expressing IL-17RB. J Immunol. 2009; 182:5116–5122. [PubMed: 19342692]

18. Moro K, et al. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. Nature. 463:540–544. [PubMed: 20023630]

19. Neill DR, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. Nature. 464:1367–1370. [PubMed: 20200518]

20. Saenz SA, et al. IL25 elicits a multipotent progenitor cell population that promotes T(H)2 cytokine responses. Nature. 464:1362–1366. [PubMed: 20200520]

21. Corrigan CJ, et al. Allergen-induced expression of IL-25 and IL-25 receptor in atopic asthmatic airways and late-phase cutaneous responses. J Allergy Clin Immunol. 2011; 128:116–124. [PubMed: 21570719]

22. Angkasekwinai P, et al. Interleukin 25 promotes the initiation of proallergic type 2 responses. J Exp Med. 2007; 204:1509–1517. [PubMed: 17562814]

23. Ballantyne SJ, et al. Blocking IL-25 prevents airway hyperresponsiveness in allergic asthma. J Allergy Clin Immunol. 2007; 120:1324–1331. [PubMed: 17889290]

24. Wang H, et al. Allergen challenge of peripheral blood mononuclear cells from patients with seasonal allergic rhinitis increases IL-17RB, which regulates basophil apoptosis and degranulation. Clin Exp Allergy. 40:1194–1202. [PubMed: 20545698]

25. Jung JS, et al. Association of IL-17RB gene polymorphism with asthma. Chest. 2009; 135:1173–1180. [PubMed: 19118269]

26. Voehringer D, Reese TA, Huang X, Shinkai K, Locksley RM. Type 2 immunity is controlled by IL-4/IL-13 expression in hematopoietic non-eosinophil cells of the innate immune system. J Exp Med. 2006; 203:1435–1446. [PubMed: 16702603]

27. Mjosberg JM, et al. Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. Nat Immunol. 2011; 12:1055–1062. [PubMed: 21909091]

28. Paul WE. Interleukin-4 production by Fc epsilon R+ cells. Skin Pharmacol. 1991; 4(Suppl 1):8–14. [PubMed: 1837220]

29. Seder RA, et al. Production of interleukin-4 and other cytokines following stimulation of mast cell lines and in vivo mast cells/basophils. Int Arch Allergy Appl Immunol. 1991; 94:137–140. [PubMed: 1834578]

30. van Panhuys N, et al. Basophils Are the Major Producers of IL-4 during Primary Helminth Infection. J Immunol. 2011

31. Torrero MN, Hubner MP, Larson D, Karasuyama H, Mitre E. Basophils amplify type 2 immune responses, but do not serve a protective role, during chronic infection of mice with the filarial nematode Litomosoides sigmodontis. J Immunol. 2010; 185:7426–7434. [PubMed: 21057084]

32. Steinfelder S, et al. The major component in schistosome eggs responsible for conditioning dendritic cells for Th2 polarization is a T2 ribonuclease (omega-1). J Exp Med. 2009; 206:1681–1690. [PubMed: 19635859]

33. Yoshimoto T, et al. Basophils contribute to T(H)2-IgE responses in vivo via IL-4 production and presentation of peptide-MHC class II complexes to CD4+ T cells. Nat Immunol. 2009; 10:706–712. [PubMed: 19465908]

34. Perrigoue JG, et al. MHC class II-dependent basophil-CD4+ T cell interactions promote T(H)2 cytokine-dependent immunity. Nat Immunol. 2009; 10:697–705. [PubMed: 19465906]

35. Wang HB, Ghiran I, Matthaei K, Weller PF. Airway eosinophils: allergic inflammation recruited professional antigen-presenting cells. J Immunol. 2007; 179:7585–7592. [PubMed: 18025204]

36. Bandeira-Melo C, et al. IL-16 promotes leukotriene C(4) and IL-4 release from human eosinophils via CD4- and autocrine CCR3-chemokine-mediated signaling. J Immunol. 2002; 168:4756–4763. [PubMed: 11971026]

37. Holtzman MJ, et al. Immune pathways for translating viral infection into chronic airway disease. Adv Immunol. 2009; 102:245–276. [PubMed: 19477323]

38. Kim EY, et al. Persistent activation of an innate immune response translates respiratory viral infection into chronic lung disease. Nat Med. 2008; 14:633–640. [PubMed: 18488036]

39. Peranzoni E, et al. Myeloid-derived suppressor cell heterogeneity and subset definition. Curr Opin Immunol. 2010; 22:238–244. [PubMed: 20171075]

40. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol. 2009; 9:162–174. [PubMed: 19197294]

41. Garley M, Jablonska E, Grabowska SZ, Piotrowski L. IL-17 family cytokines in neutrophils of patients with oral epithelial squamous cell carcinoma. Neoplasma. 2009; 56:96–100. [PubMed: 19239321]

42. Hansbro PM, Kaiko GE, Foster PS. Cytokine/anti-cytokine therapy - novel treatments for asthma? Br J Pharmacol. 2011

43. Bullens DM, et al. IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? Respir Res. 2006; 7:135. [PubMed: 17083726]

44. Drews AC, et al. Neutrophilic airway inflammation is a main feature of induced sputum in nonatopic asthmatic children. Allergy. 2009; 64:1597–1601. [PubMed: 19392986]

45. Kikuchi S, Nagata M, Kikuchi I, Hagiwara K, Kanazawa M. Association between neutrophilic and eosinophilic inflammation in patients with severe persistent asthma. Int Arch Allergy Immunol. 2005; 137(Suppl 1):7–11. [PubMed: 15947478]

46. Fukakusa M, et al. Oral corticosteroids decrease eosinophil and CC chemokine expression but increase neutrophil, IL-8, and IFN-gamma-inducible protein 10 expression in asthmatic airway mucosa. J Allergy Clin Immunol. 2005; 115:280–286. [PubMed: 15696082]

47. Linden A, Hoshino H, Laan M. Airway neutrophils and interleukin-17. Eur Respir J. 2000; 15:973–977. [PubMed: 10853869]

48. Jatakanon A, et al. Neutrophilic inflammation in severe persistent asthma. Am J Respir Crit Care Med. 1999; 160:1532–1539. [PubMed: 10556116]

49. Vazquez-Tello A, et al. Induction of glucocorticoid receptor-beta expression in epithelial cells of asthmatic airways by T-helper type 17 cytokines. Clin Exp Allergy. 2010; 40:1312–1322. [PubMed: 20545708]

50. McKinley L, et al. TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. J Immunol. 2008; 181:4089–4097. [PubMed: 18768865]

51. Kaiko GE, Phipps S, Angkasekwinai P, Dong C, Foster PS. NK Cell Deficiency Predisposes to Viral-Induced Th2-Type Allergic Inflammation via Epithelial-Derived IL-25. J Immunol. 185:4681–4690. [PubMed: 20855881]

52. Berlin AA, Hogaboam CM, Lukacs NW. Inhibition of SCF attenuates peribronchial remodeling in chronic cockroach allergen-induced asthma. Lab Invest. 2006; 86:557–565. [PubMed: 16607380]

53. Campbell EM, Kunkel SL, Strieter RM, Lukacs NW. Temporal role of chemokines in a murine model of cockroach allergen-induced airway hyperreactivity and eosinophilia. J Immunol. 1998; 161:7047–7053. [PubMed: 9862742]

54. Campbell E, Hogaboam C, Lincoln P, Lukacs NW. Stem cell factor-induced airway hyperreactivity in allergic and normal mice. Am J Pathol. 1999; 154:1259–1265. [PubMed: 10233863]

55. Lukacs NW, et al. Respiratory virus-induced TLR7 activation controls IL-17-associated increased mucus via IL-23 regulation. J Immunol. 2010; 185:2231–2239. [PubMed: 20624950]

56. Kallal LE, Hartigan AJ, Hogaboam CM, Schaller MA, Lukacs NW. Inefficient lymph node sensitization during respiratory viral infection promotes IL-17-mediated lung pathology. J Immunol. 2010; 185:4137–4147. [PubMed: 20805422]

57. Smit JJ, et al. The balance between plasmacytoid DC versus conventional DC determines pulmonary immunity to virus infections. PLoS One. 2008; 3:e1720. [PubMed: 18320041]
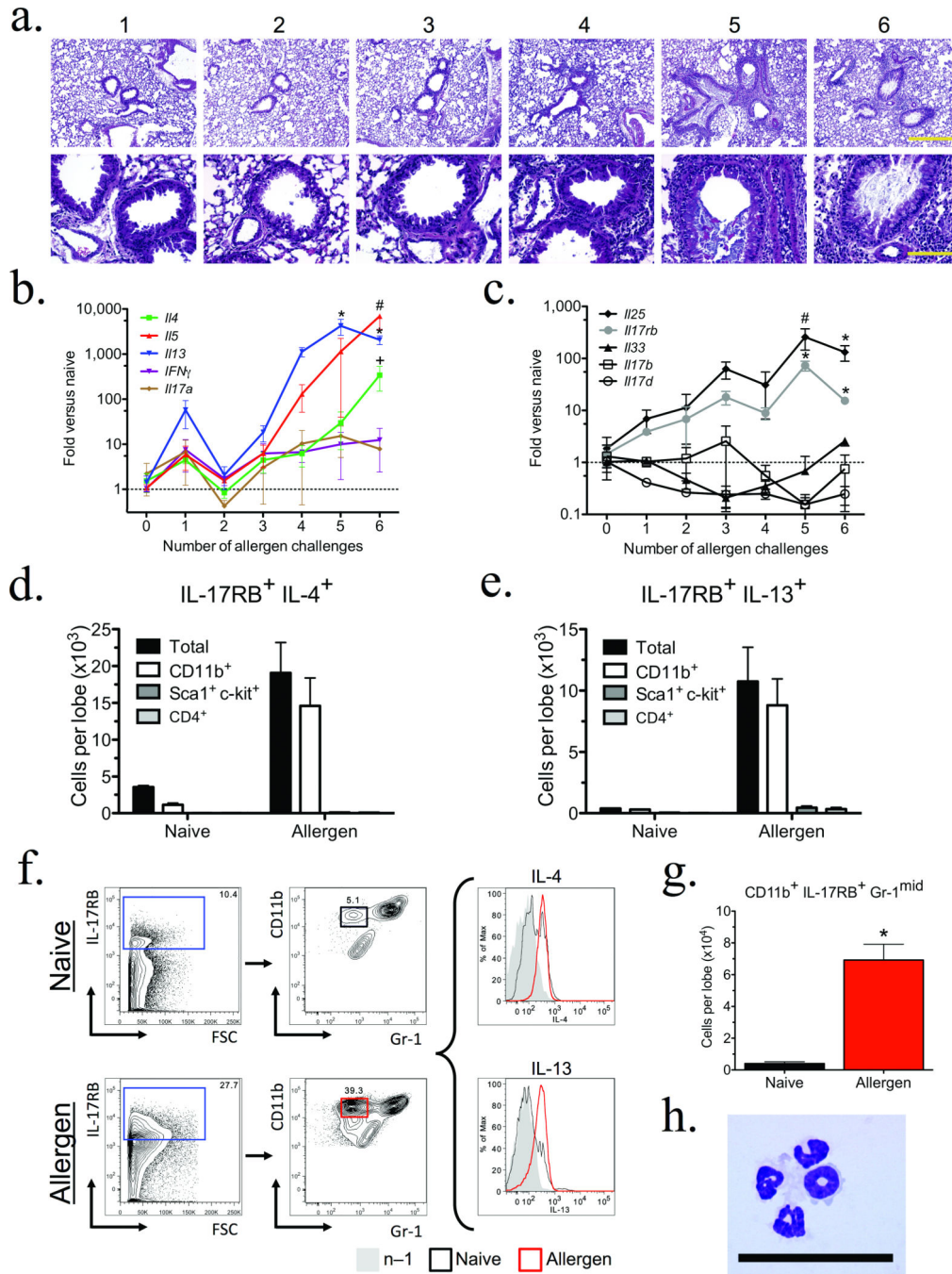
Figure 1. Allergen exposure increases pulmonary IL-25 and IL17RB, and recruits bone marrow-derived IL17RB^{+} IL-4 and IL-13 producing myeloid cells to the lung

Allergen-induced inflammation was localized to the lungs of C57BL/6J mice ($n = 3$ per group) via a series of 6 allergen challenges. (**a**) Time course of representative PAS staining, taken 6 hrs post indicated allergen challenge. Upper row scale bar, 400 μm; lower row, 100 μm. (**b**) Time course of pulmonary IL-4, IL-5, IL-13, IFN-γ and IL-17a mRNA expression 6 hours post indicated allergen challenge. (*$P < 8.44E^{-05}$, #$P = 0.0008$, +$P = 0.001$). (**c**) Time course of IL-25, IL17RB, IL-33, IL-17b, and IL17d mRNA expression 6 hours post

indicated allergen challenge. ST2 and IL-22 transcripts were not detectable. (*$P < 0.007$). (**d** and **e**) IL-17RB$^+$ lung subsets from naïve and allergen sensitized C57BL/6J mice ($n = 5$ per group) were assessed by flow cytometry for IL-4 and IL-13 production. (**f**) Representative flow plots of intracellular cytokine staining in IL-17RB$^+$ CD11b$^+$ Gr-1^{mid} cells from naïve and allergen sensitized C57BL/6J mice. Gray: n-1 staining, black: naïve IL-17RB$^+$ CD11b$^+$ Gr-1^{mid}, red: allergen challenged IL-17RB$^+$ CD11b$^+$Gr-1^{mid} (**g**) Pulmonary IL-17RB$^{-/-}$ CD11b$^+$ Gr-1^{mid} populations are significantly increased following allergen sensitization. (*$P = 0.0026$). 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} FcγR$^+$ IL-17RB$^+$ CD4$^-$ CD8$^-$ B220$^-$ IL-7Rα^- Sca1$^-$ c-kit$^-$ and stained with H+E. Scale bar 50 μm. All data are presented as mean ± s.e.m.
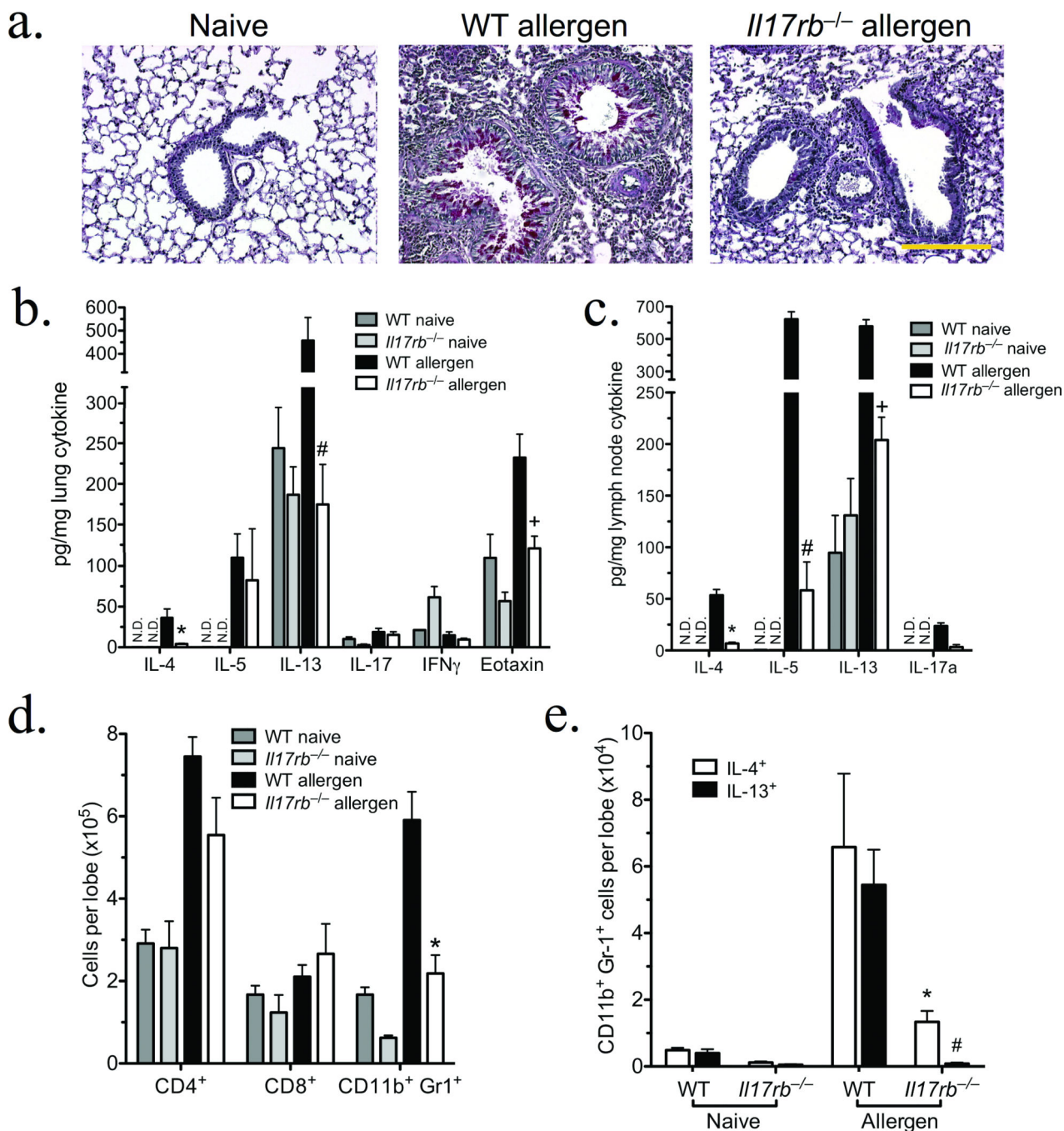
Figure 2. *Il17rb*⁻/⁻ **mice are protected from allergen induced type 2 inflammation, and type 2 cytokine production in CD11b⁺ Gr-1⁺ myeloid cells is IL-17RB dependent**

(**a**) PAS staining of lungs from WT and *Il17rb*⁻/⁻ mice following chronic allergen sensitization. Scale bar 200 μm. (**b**) Lungs from allergic mice (*n* = 5 animals per group) were harvested 24 h post final allergen challenge and whole lung homogenates were analyzed for cytokine production by bioplex. (*P* = 0.001, #*P* = 0.048, +*P* < 0.05 versus WT allergen). (**c**) Cytokine production from draining lymph nodes cells of allergic mice (*n* = 5 animals per group). Bars represent the mean ± s.e.m. from triplicate wells. (N.D. = not

detected, $*P = 2.46\text{E}^{-05}$, $\#P = 8.79\text{E}^{-08}$, $+P = 4.00\text{E}^{-06}$ versus WT allergen). (**d**) Flow cytometric analysis of lungs from allergic WT and $Il17rb^{-/-}$ mice ($n = 5$ animals per group), ($*P < 0.03$). (**e**) Intracellular cytokine staining for IL-4 and IL-13 producing cells in allergic WT and $Il17rb^{-/-}$ mice ($n = 4$ animals per group). Results are gated on CD11b$^+$ Gr-1$^{\text{mid}}$ cells. Bars represent the mean ± s.e.m. for each group ($*P < 0.05$, $\#P < 0.0009$). Data are representative of two independent experiments.
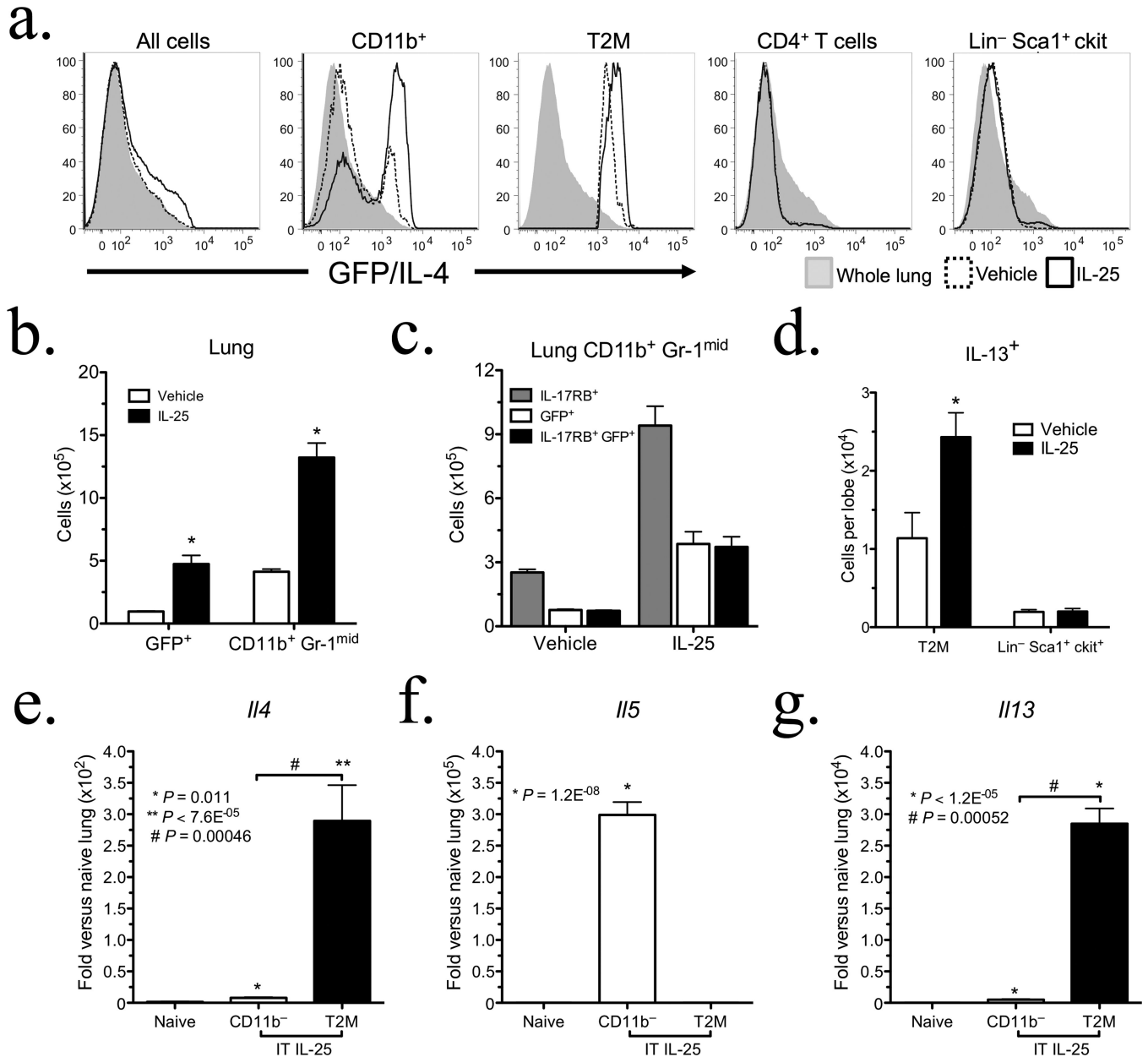
Figure 3. T2M cells represent the primary source of type 2 cytokines following pulmonary IL-25 administration

4 get mice (*n* = 4 animals per group) were IT dosed with vehicle or 0.5 μg IL-25 for 4 days, and the inflammatory response was investigated 24 h post final IT. (**a**) Histograms of lung tissue from 4get mice treated with vehicle or IL-25, gated on total lung, CD11b^{+}, T2M, CD4^{+} Lymphocytes, and Lin^{−} Sca^{+} c-kit^{+} cells respectively. (**b**) GFP^{+} and CD11b^{+} Gr-1[mid] populations in the lung were assessed by flow cytometry, (*$P < 0.026$). (**c**) Pulmonary IL-17RB^{+} CD11b^{+} GFP/IL-4^{+} cell numbers following IL-25 administration. Data are representative of two independent experiments. (**d**) Pulmonary IL-13^{+} populations following IL-25 treatment (*n* = 5 animals per group, *$P = 0.038$). (**e**, **f**, and **g**) QPCR analysis of IL-4, IL-5, and IL-13 transcripts in T2M cells. Cells were isolated from C57BL/6J mice dosed
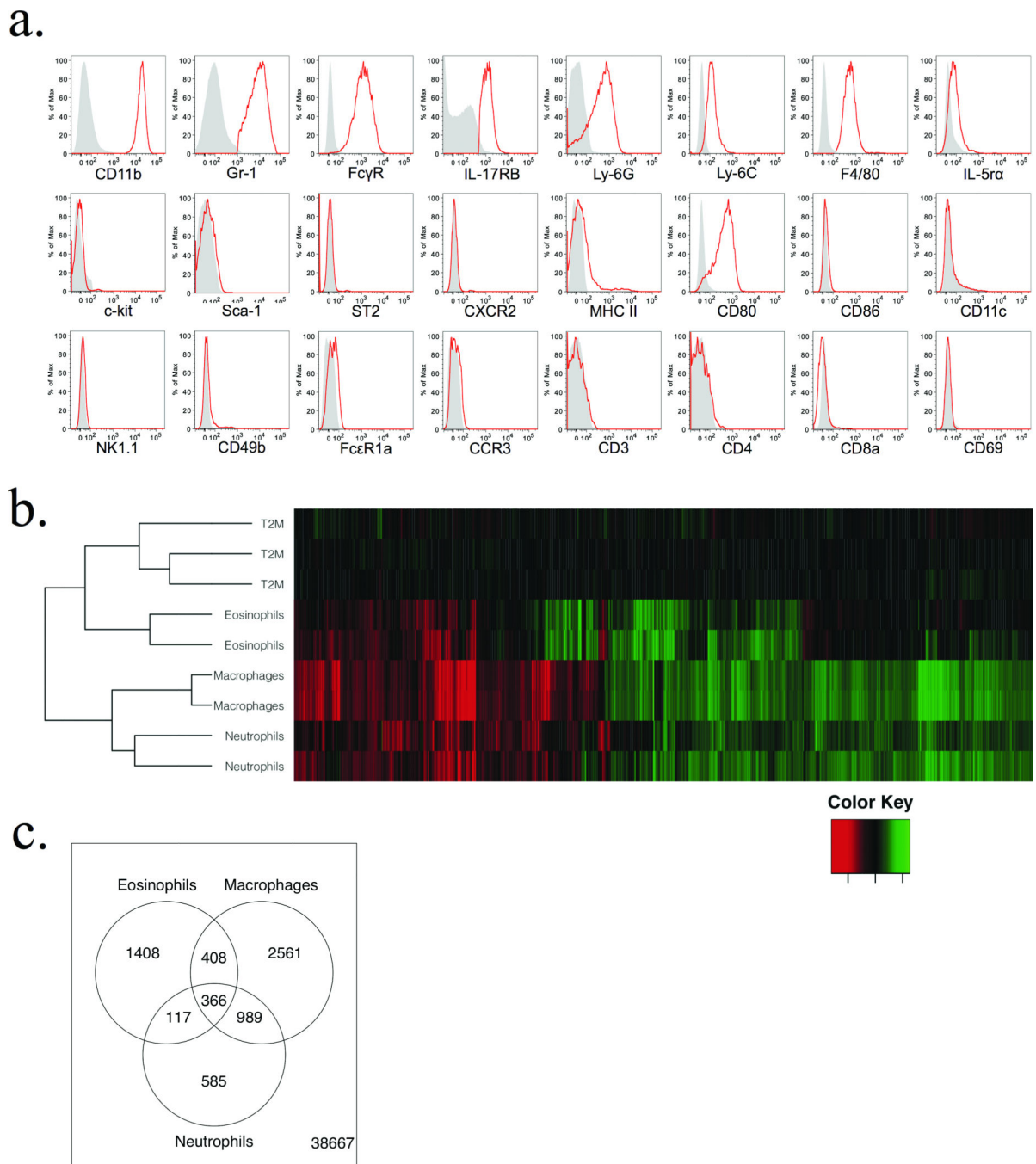
with 0.5 μg IL-25 for 4 days (n = 5 animals per group), and plated in triplicate. T2M cells were isolated using MACS magnetic bead enrichment followed by FACS. mRNA was isolated from naïve C57BL/6J mice, CD11b depleted lung from IL-25 treated mice, and T2M cells isolated from IL-25 treated mice. All data are presented as mean ± s.e.m.

a.

b.

Color Key

c.

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 (as previously described) with intratracheal IL-25 to induce recruitment of T2M cells to the lung. T2M cells were identified by gating on CD11b+ Gr1mid IL-17RB+ GFP/IL-4+ cells, and expression of various surface markers was then assessed. Gray shaded area: isotype, red line; T2M cells. Data are representative of 2 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 exhibited a minimum 3 fold difference in expression from 2 or more cell types, and an average expression value of at least 2^6, normalized to average T2M expression levels. (**c**) Venn diagram illustrating differences in locus expression between T2M cells and other myeloid populations. 366 probes were differentially expression between T2M cells and all cell types analyzed, 1,880 probes differed between T2M cells and at least 2 of the comparison populations.
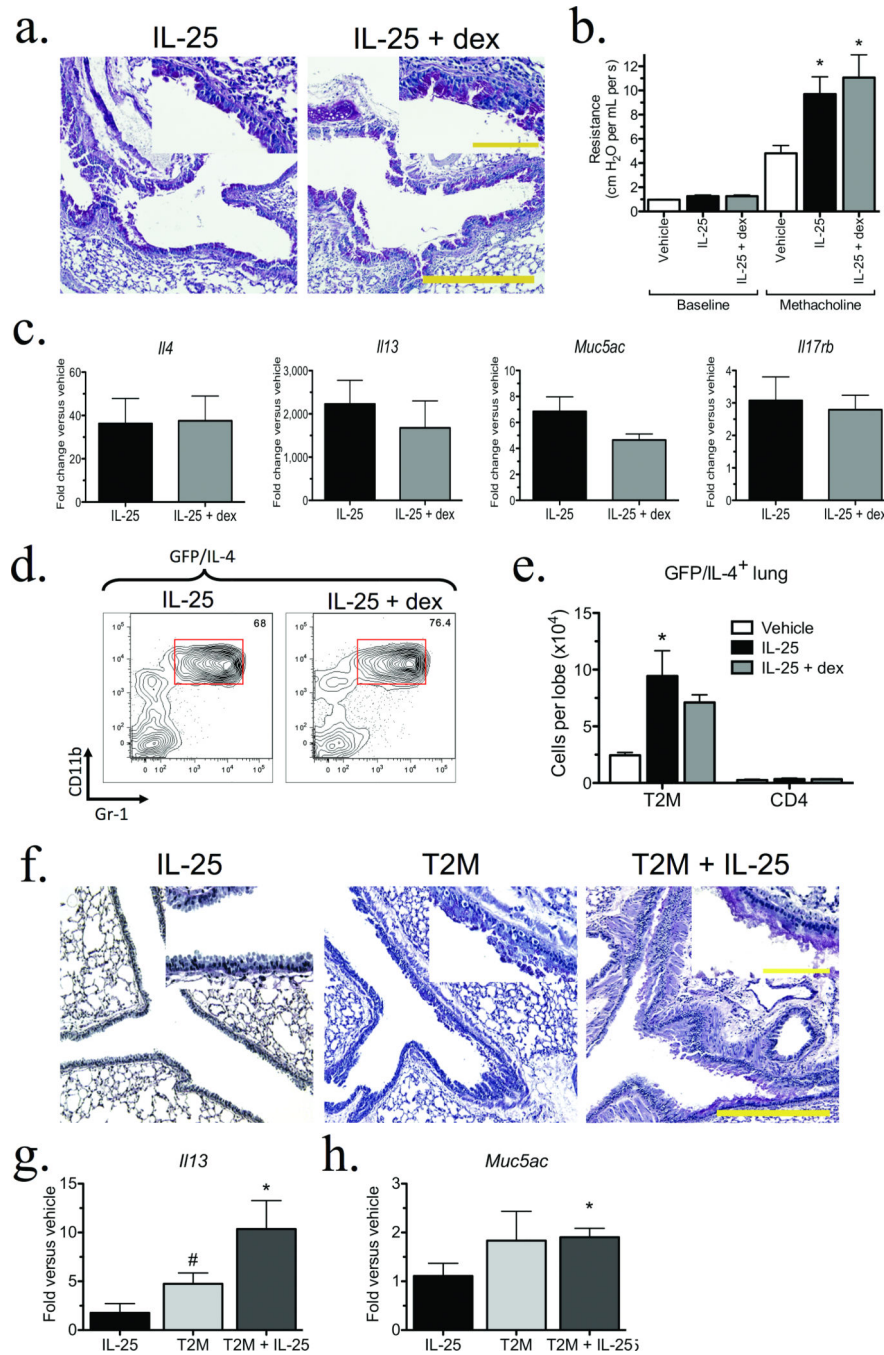
Figure 5. T2M cells are steroid resistant, and are sufficient to induce airway pathology in IL-17RB$^{-/-}$ mice

(**a**) Representative PAS staining indicates IL-25-induced mucus production in 4get mice (*n* = 5 per group) is not altered by dexamethasone administration. Scale bar 400 μm; inset 100 μm. (**b**) Airway hyperreactivity (*$P < 0.01$ versus methacholine treated vehicle). (**c**) QPCR analysis of whole lung following dexamethasone treatment. (**d**) Representative flow plots of GFP/IL-4$^+$ CD11b$^+$ Gr-1^{mid} pulmonary populations. (**e**) Total numbers of pulmonary GFP/IL-4$^+$ cells. Bars represent the mean ± s.e.m. of 4 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 hours post final IL-25 administration. Scale bar 400 μm; inset 100 μm. 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 4 total treatments, consisting of 0.5 μg IL-25, T2M cells alone, or IL-25 + T2M cells. (**g**) QPCR expression of IL-13 following T2M transfer (*P = 0.017, #P = 0.042). (**h**) QPCR expression for the mucus specific gene *muc5ac* (*P < 0.026). Bars represent the mean ± s.e.m. of 4 mice per group. Results are representative of 3 independent experiments.
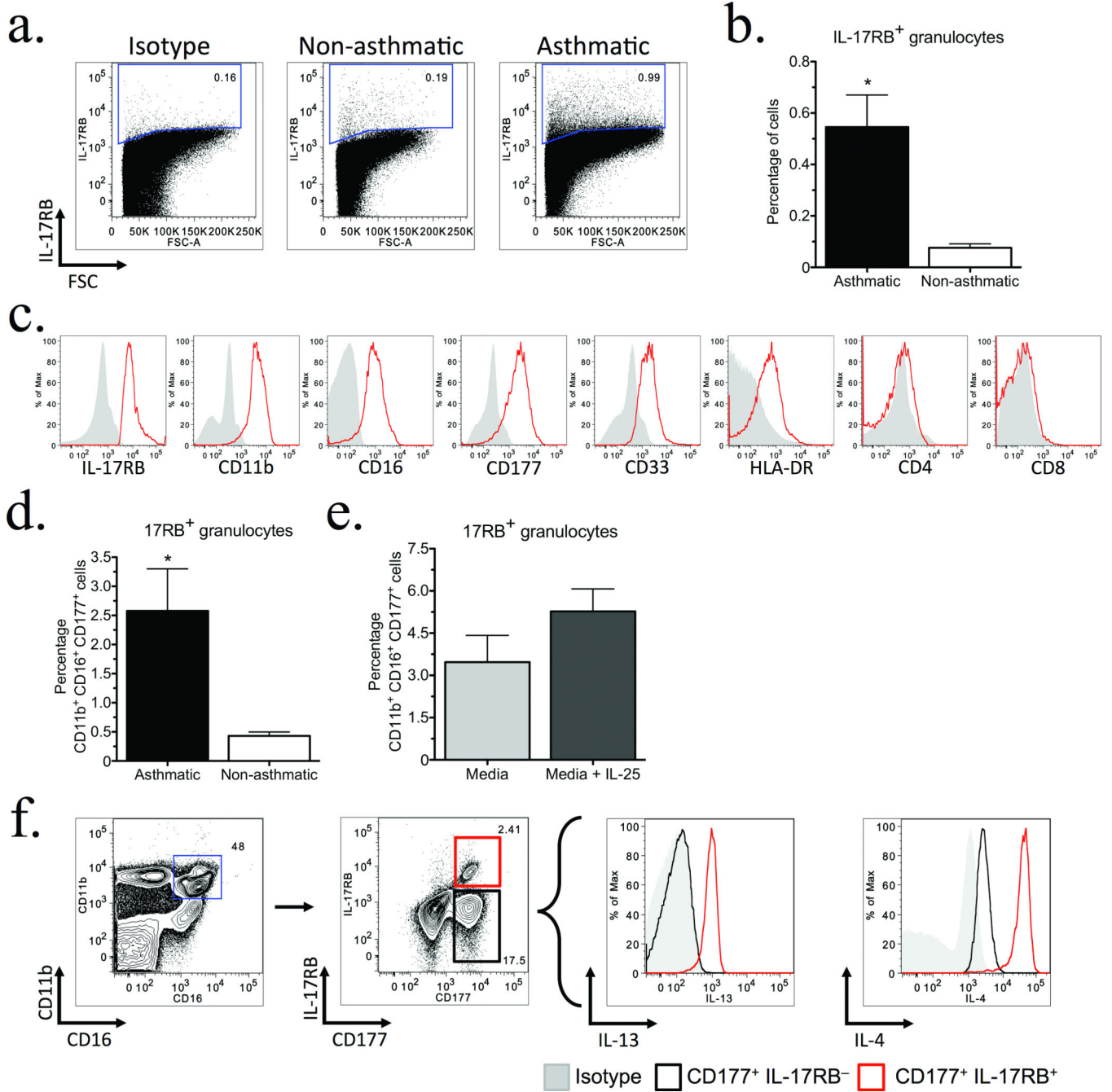
Figure 6. An IL-4 and IL-13 producing population analogous to T2M cells is identifiable in peripheral blood and significantly increased in Asthmatics

Granulocytes were isolated from peripheral blood samples donated by asthmatic (n = 9) or non-asthmatic (n = 8) volunteers and analyzed by flow cytometry. (**a**) Representative dot plots of granulocytes stained for IL-17RB, normalized to 400k events. (**b**) Percent total IL-17RB+ granulocytes isolated from peripheral blood of volunteer donors. Bars represent the mean ± s.e.m. for each group, (*P = 0.0031). (**c**) Representative histograms of IL-17RB+ granulocytes from an asthmatic donor indicate the majority of IL-17RB+ granulocytes are CD11b+ CD16+ CD177+. Cells were gated on total IL-17RB+ cells and assessed for surface

marker expression. (**d**) Percent total CD11b$^+$ CD16$^+$ CD177$^+$ IL-17RB$^+$ granulocytes from volunteer donors. Bars represent the mean ± s.e.m. for each group, (*P = 0.023). (**e**) Percent total CD11b$^+$ CD16$^+$ CD177$^+$ IL-17RB$^+$ cells from whole blood of volunteer asthmatic donors, cultured *in vitro* for 2 hours ± 50 ng mL^{-1} IL-25. (**f**) Representative intracellular cytokine staining for IL-4 and IL-13 from whole blood from an asthmatic volunteer, cultured for 2 hours with RPMI 1640.