

Divergent functions for airway epithelial matrix metalloproteinase 7 and retinoic acid in experimental asthma

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The innate immune response of airway epithelial cells to airborne allergens initiates the development of T cell responses that are central to allergic inflammation. Although proteinase allergens induce the expression of interleukin 25, we show here that epithelial matrix metalloproteinase 7 (MMP7) was expressed during asthma and was required for the maximum activity of interleukin 25 in promoting the differentiation of T helper type 2 cells. Allergen-challenged *Mmp7*^{-/-} mice had less airway hyper-reactivity and production of allergic inflammatory cytokines and higher expression of retinal dehydrogenase 1. Inhibition of retinal dehydrogenase 1 restored the asthma phenotype of *Mmp7*^{-/-} mice and inhibited the responses of lung regulatory T cells, whereas exogenous administration of retinoic acid attenuated the asthma phenotype. Thus, MMP7 coordinates allergic lung inflammation by activating interleukin 25 while simultaneously inhibiting retinoid-dependent development of regulatory T cells.

Proximal and distal airway epithelial cells directly communicate with the outside environment, and thus their response to inhaled allergens could be integral to the initiation of allergic lung inflammation. Identification of the critical epithelium-derived mediators that drive allergic lung responses is at an early stage but is essential to complete understanding of the molecular mechanisms that underlie diseases such as asthma. After being inhaled, proteinase allergens (such as airborne allergens with active proteinase properties) rapidly induce a powerful inflammatory response that initiates the recruitment of allergic immune cells into the lung^{1,2}. Although the function of cytokines expressed in the airway epithelium in asthma has been studied extensively^{3,4}, the mechanism by which allergens initiate the differentiation of airway T helper type 2 (T_H2) cells is poorly understood. Interleukin 25 (IL-25; also called IL-17E) is a member of the IL-17 family of cytokines that is produced mainly in the airway epithelium in response to proteinase allergens^{5,6}. Transgenic expression of human and mouse IL-25 induces T_H2 immune responses marked by considerable lung eosinophilia, mucus hyperproduction and higher expression of IL-4, IL-5, IL-13 and CCL11 (eotaxin 1) in the airway^{7,8}. Neutralization of IL-25 by blocking antibodies has been shown to reverse airway hyper-reactivity, a pathognomonic feature of asthma, in a mouse model of asthma⁹. Moreover, IL-25-deficient mice are unable to develop a strong T_H2-biased immune response and fail

to clear gut parasitic infection with *Nippostrongylus brasiliensis* or *Trichuris muris*¹⁰. Such studies suggest a critical function for epithelial cells in providing the IL-25 signals that may be required for effective T_H2 responses. Although the function of IL-25 in allergic lung disease is well known, a broader understanding of how IL-25 interacts with other airway epithelial factors to coordinately regulate allergic responses is needed.

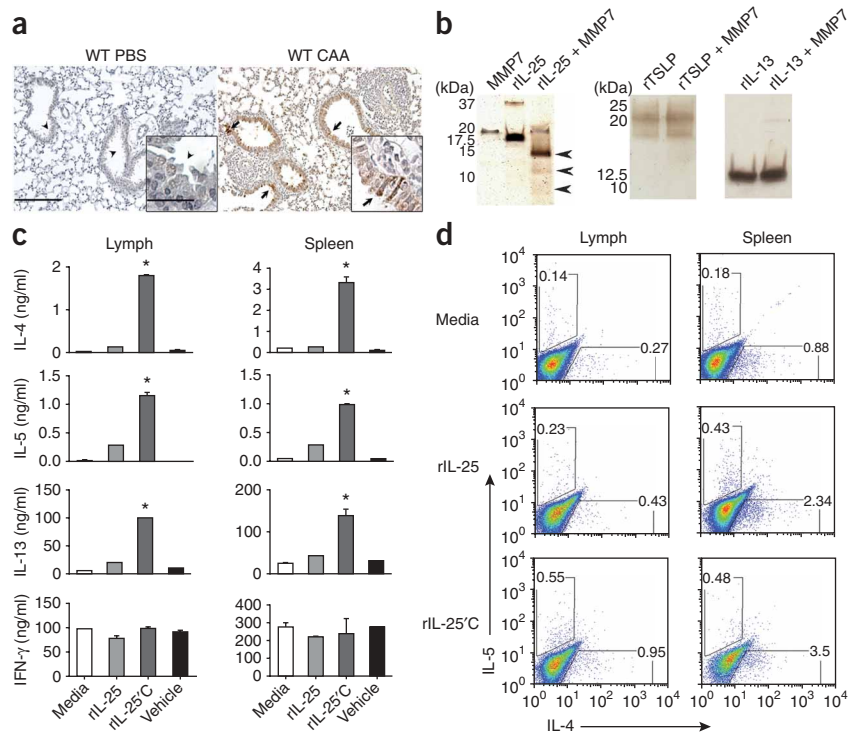
Matrix metalloproteinases (MMPs) are zinc-dependent enzymes that are induced in response to many stimuli. Although MMPs were originally shown to mediate the turnover of extracellular matrix molecules, it is now apparent that they control diverse biological processes unrelated to matrix degradation¹¹. Although the exact function of MMPs in chronic models of lung inflammation is debatable, it is clear that several members of this family are acutely induced either locally or systemically at the onset of allergen challenge^{12–16}. MMP2 and MMP9 are not required for the development of allergic lung disease but serve key functions in the clearance of inflammatory cells from lung parenchyma by establishing the necessary trans-epithelial chemokine gradients^{15,16}. However, whereas neither MMP2 nor MMP9 is expressed in the airway epithelium¹⁷, MMP7 (also called matrilysin; A001477), an epithelial cell-specific MMP, is induced in the lung and gut during inflammation^{18,19}. MMP7 has been shown to modify pro- α -defensin, the ligand for the

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Figure 1 MMP7 is induced in allergic inflammation and modulates IL-25 function.

(a) Immunohistochemistry of MMP7 in lungs of PBS-treated (WT PBS) and complete *Aspergillus* allergen (CAA)-immunized (WT CAA) C57BL/6 mice. Arrows and arrowheads indicate MMP7 expression; insets, $\times 40$ magnification of the airway. Scale bars, 100 μ m (main images) and 25 μ m (insets). Data are representative of at least three independent experiments. (b) Silver staining of rIL-25 (5 μ g), rIL-13 (5 μ g) and rTSLP (5 μ g) left untreated or incubated for 2 h at 37 $^{\circ}$ C with 4-aminophenylmercuric acetate-activated MMP7 (0.5 μ g; + MMP7) and then separated by electrophoresis through a 16.5% tricine gel. Arrowheads indicate MMP7-cleaved fragments of rIL-25. Results are representative of at least three independent experiments. (c) ELISA of IL-4, IL-5, IL-13 and interferon- γ (IFN- γ) in supernatants of naive lymph node cells (left) and spleen cells (right) isolated from C57BL/6 mice and stimulated for 2 d with plate-bound anti-CD3 (2 μ g/ml) in the presence of equal amounts of native rIL-25 (250 ng/ml) or rIL-25'C (250 ng/ml). Media alone and the 4-aminophenylmercuric acetate-containing buffer used to activate MMP7 (Vehicle) serve as controls. *, $P < 0.05$, versus rIL-25 (one-way ANOVA). Data are representative of three different experiments (mean and s.d.; $n = 3$ samples). (d) Flow cytometry of the expression of IL-4 and IL-5 by lymphocytes and spleen cells cultured for 3 d in the conditions described in c, then restimulated for 5 h with ionomycin (500 ng/ml) and phorbol 12-myristate 13-acetate (50 ng/ml) in the presence of GolgiStop, followed by intracytoplasmic staining. Numbers adjacent to outlined areas indicate percent IL-5 $^{+}$ IL-4 $^{+}$ cells (top left) or IL-5 $^{+}$ IL-4 $^{+}$ cells (bottom right). Data are representative of three independent experiments.



cell surface receptor Fas (FasL; CD95L) and tumor necrosis factor in the gut during defense against enteric pathogens, but its function in allergic inflammation has yet to be explored^{19,20}.

In this study, we examine how airway epithelial IL-25 coordinates proteinase-dependent allergic lung disease in the context of other potentially relevant airway epithelium-derived factors, including MMP7. We show that in response to proteinase allergen or recombinant IL-25 (rIL-25), mouse airway epithelial cells expressed MMP7. Activation of MMP7 was critical for IL-25 function because MMP7-cleaved rIL-25 (called 'rIL-25'C' here) enhanced T_H2 differentiation. Furthermore, we show that humans with chronic asthma also expressed MMP7 and IL-25 in their distal airspaces and, in response to ragweed extract, a potent allergenic stimuli, patients with a history of allergy had significantly more nasal secretion of MMP7. In the absence of MMP7, mice showed attenuated allergic responses to challenge with proteinase allergen and enhanced expression of retinal dehydrogenase 1 (RALDH-1), a rate-limiting enzyme for the production of retinoic acid. Moreover, *Mmp7* $^{-/-}$ mice had more regulatory T cells in the lung parenchyma. Our results collectively support the idea that MMP7 serves as a proinflammatory mediator that specifically enhances the function of IL-25 that is necessary for robust T_H2 responses. Finally, we demonstrate a tolerogenic mechanism initiated by airway epithelial cells in response to challenge with proteinase allergen in which RALDH-1 was induced to promote immunosuppressive regulatory T cells.

RESULTS

MMP7 mediates enhanced function of IL-25

MMP2 and MMP9 are not expressed in airway epithelial cells after allergen challenge¹⁷. In contrast, we found that a fungus-derived proteinase allergen strongly enhanced airway epithelial expression of

MMP7 (Fig. 1a). Whereas induction of MMP2 and MMP9 is IL-13 dependent¹⁵, MMP7 induction was independent of IL-13, as there was equivalent induction of MMP7 in wild-type mice and allergen-challenged mice deficient in STAT6, the principal mediator of the effects of IL-13 in the lung (data not shown). Because MMP7 is known to cleave and activate cytokines in the gut¹³, we next determined if MMP7 modifies cytokines critical for initiating allergic lung responses. In addition to IL-25, both IL-13 and thymic stromal lymphopoietin (TSLP) are critical participants in the molecular pathways that underlie allergic lung disease^{5,21}. We found that although rIL-13 and rTSLP were not substrates for activated MMP7, in the same conditions, rIL-25 was cleaved at multiple sites (Fig. 1b and Supplementary Table 1 and Fig. 1 online). To determine the function of cleaved IL-25, we examined the responses of naive spleen and lymph node cells after activation by crosslinking of T cell antigen receptors *in vitro*. We found that rIL-25'C induced significantly more secretion of T_H2 cytokines (IL-4, IL-5 and IL-13) than did native rIL-25 and it showed enhanced binding to a fusion protein of IL-17 receptor B and the Fc fragment *in vitro* (Fig. 1c,d and Supplementary Fig. 2a,b online). Indeed, native IL-25 had little effect on IL-4 secretion, but rIL-25'C enhanced IL-4 secretion tenfold, which suggested that the main active form of IL-25 is the MMP7-cleaved form. We found no effect of rIL-25 or rIL-25'C on the induction of interferon- γ , a canonical T_H1 cytokine (Fig. 1c). Collectively, these data indicate that IL-25 is a substrate for MMP7 and that cleavage of IL-25 by MMP7 is needed to drive robust T_H2 responses.

Attenuated asthma phenotype of *Mmp7* $^{-/-}$ mice

The greater potency of rIL-25'C in activating T_H2 cells *in vitro* raised the possibility of an essential function for MMP7 in mediating allergic inflammation *in vivo*. To investigate this, we compared the responses

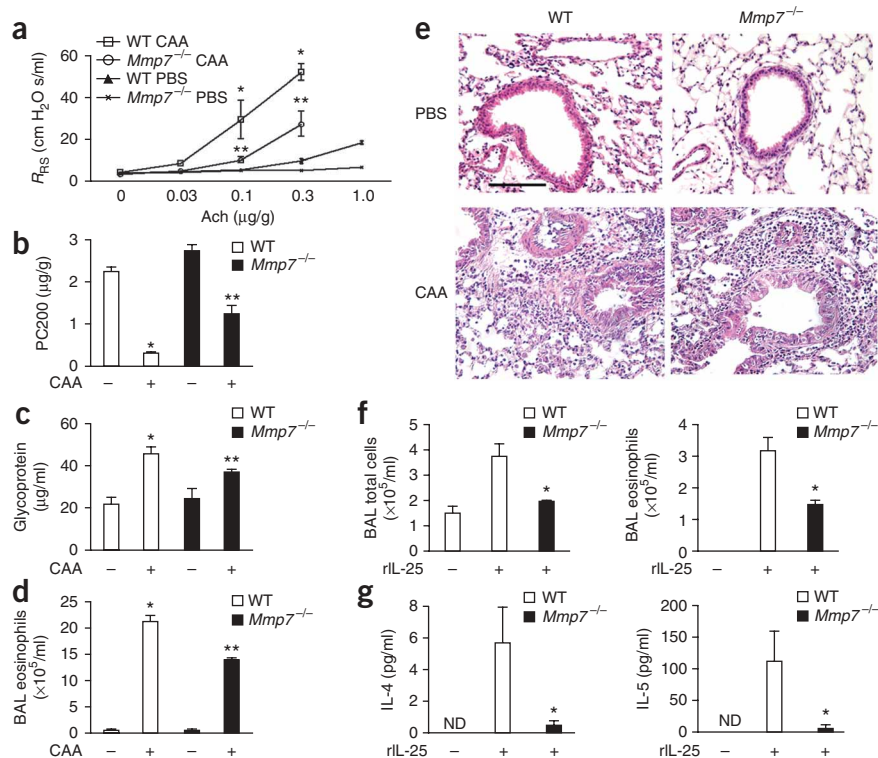


Figure 2 *Mmp7*^{-/-} mice have an attenuated asthma phenotype. (a,b) Airway hyper-reactivity in age- and sex-matched wild-type mice (WT; *n* = 4) and *Mmp7*^{-/-} mice (*n* = 4) immunized intranasally with PBS (-) or CAA (+) every 4 d for a total of five doses and assessed 24 h after the final immunization as respiratory system resistance (R_{RS}) in response to various doses of acetylcholine (Ach; a) or concentration of acetylcholine needed to elicit a 200% change from baseline respiratory system resistance (PC200; b). (c,d) ELISA of glycoproteins (c) and eosinophil cell counts (d) in BAL fluid from the mice in a,b. In a-d, **P* < 0.05, versus PBS-challenged wild-type; ***P* < 0.05, versus CAA-immunized wild-type (one-way ANOVA and *t*-test). Data are representative of at least three independent experiments (mean and s.d.). (e) Photomicrographs of bronchovascular bundles stained with hematoxylin and eosin (*n* = 4 mice per group). Scale bar, 100 μ m. Data are representative of at least three independent experiments. (f,g) Total cell and eosinophil counts (f) and ELISA of IL-4 and IL-5 (g) in BAL fluid of wild-type and *Mmp7*^{-/-} mice given PBS (-) or rIL-25 (+; 5 μ g) intranasally twice daily for 3 d, assessed 24 h after the final dose. **P* < 0.05, versus rIL-25-treated wild-type mice (one-way ANOVA). Data are representative of three independent experiments (mean and s.d.; *n* = 4 mice per group).

of *Mmp7*^{-/-} and wild-type mice to the proteinase allergen CAA (complete aspergillus allergen) extracted from fungi, a powerful allergen used before to elicit allergic lung disease in mice¹. This airway inflammation model is characterized by a predominantly eosinophilic influx and airway obstruction due to enhanced glycoprotein secretion, as well as more airway hyper-reactivity to secondary challenge with acetylcholine^{22,23}. *Mmp7*^{-/-} mice immunized with CAA had less airway hyper-reactivity than did wild-type mice, as shown by both their lower respiratory system resistance in response to many doses of acetylcholine and higher stimulating concentration of acetylcholine required to elicit a 200% change from the baseline respiratory system resistance. Moreover, *Mmp7*^{-/-} mice had less secretion of glycoproteins in bronchoalveolar lavage (BAL) fluid after CAA challenge (Fig. 2a-c).

Airway eosinophilia was also significantly lower in CAA-immunized *Mmp7*^{-/-} mice (Fig. 2d). Because the egress of lung parenchymal eosinophils into the airways is impaired in *Mmp2*^{-/-} and *Mmp9*^{-/-} mice^{15,16}, we assessed lung parenchymal eosinophils to rule out the possibility of a similar defect in *Mmp7*^{-/-} mice. Analysis of the total number of lung parenchymal inflammatory cells showed that the

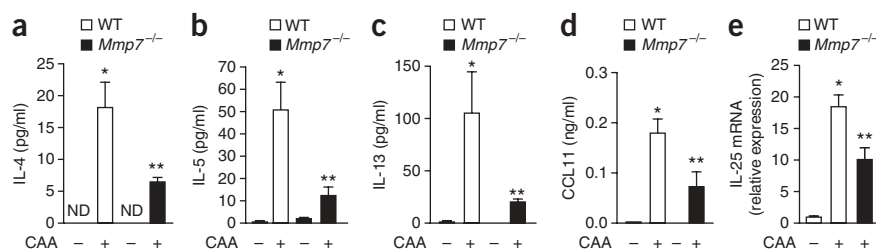
lungs of CAA-immunized *Mmp7*^{-/-} mice had fewer eosinophils than did those of wild-type mice (Fig. 2e and Supplementary Fig. 3 online), which indicated that the lower cell number in the airway was not due to less cell trafficking.

We next assessed whether MMP7-mediated modification of IL-25 was influential in determining allergic lung responses. We administered rIL-25 intranasally to wild-type and *Mmp7*^{-/-} mice and assessed eosinophil recruitment. In this assay, rIL-25 alone readily induced lung eosinophilia and new expression of MMP7 in airway epithelial cells of wild-type mice, but we found significantly fewer eosinophils and lower concentrations of IL-4 and IL-5 in the BAL fluid of *Mmp7*^{-/-} mice (Fig. 2f,g and Supplementary Fig. 4 online). Collectively, these findings support the idea that induction of MMP7 in response to allergens is critical for enhancing IL-25 function during initiation of the allergic immune response.

Diminished T_H2 responses of *Mmp7*^{-/-} mice

We next determined if the diminished airway hyper-reactivity and BAL inflammatory cells in *Mmp7*^{-/-} mice were secondary to lower

Figure 3 Attenuated T_H2 cytokines and chemokines in *Mmp7*^{-/-} mice. (a-c) Luminex assay of IL-4 (a), IL-5 (b) and IL-13 (c) in BAL fluid from age- and sex-matched wild-type and *Mmp7*^{-/-} mice (*n* = 5 mice per group) immunized intranasally with PBS (-) or CAA (+) every 4 d for a total of five doses and assessed 24 h after the final immunization. (d) ELISA of CCL11 in BAL fluid of mice treated as described in a-c. (e) Real-time RT-PCR analysis of the expression of *Il25* mRNA in the lungs of unimmunized (-) and CAA-immunized (+) wild-type and *Mmp7*^{-/-} mice, presented relative to *Actb* expression. **P* < 0.05, versus PBS-challenged wild-type; ***P* < 0.05, versus CAA-immunized wild-type (one-way ANOVA and *t*-test). Data are representative of three independent experiments (mean and s.d.).



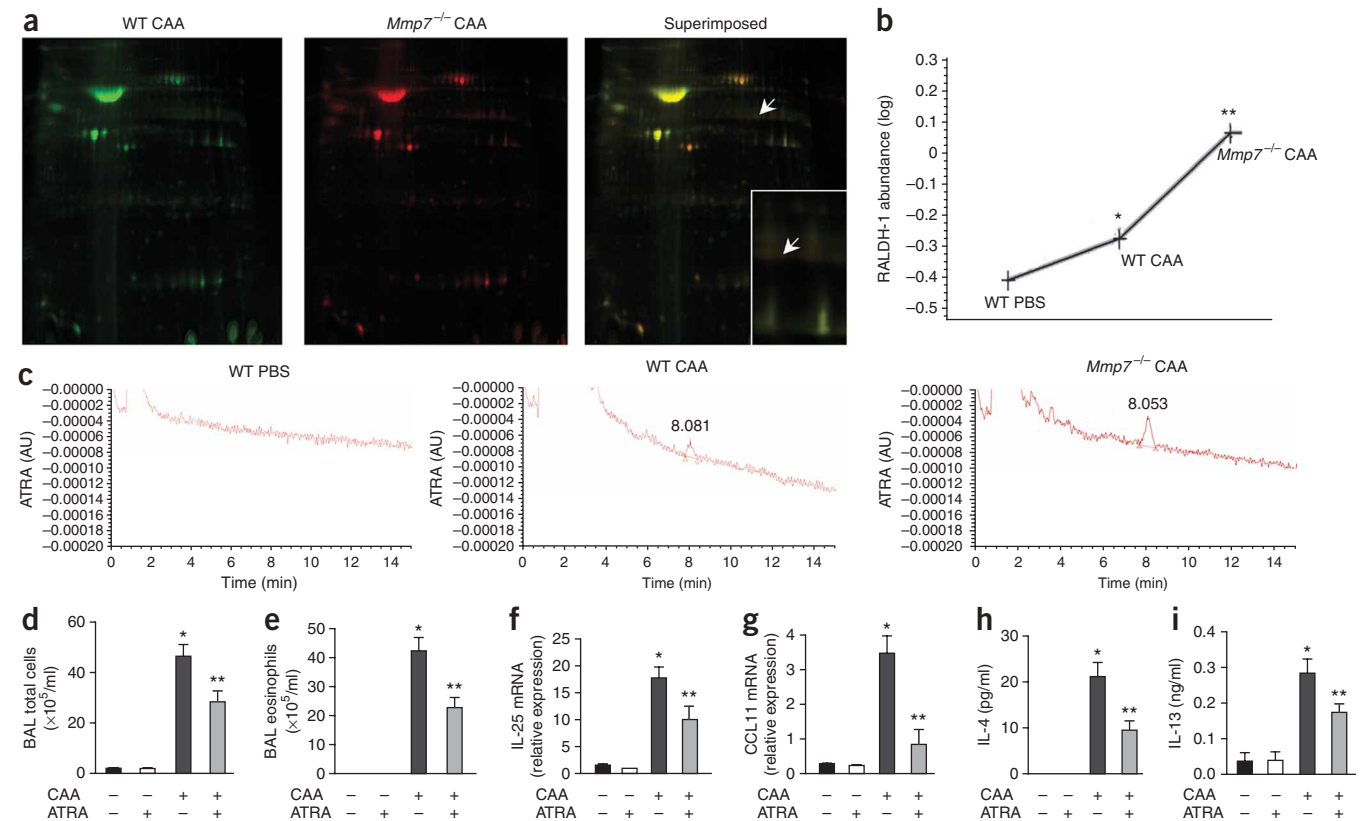


Figure 4 Proteomics analysis of BAL fluid from CAA-immunized wild-type and *Mmp7*^{-/-} mice. (a) Two-dimensional gel electrophoresis assessing the processing of proteins in pooled BAL fluid from CAA-immunized wild-type mice (green; indocarbocyanine) and *Mmp7*^{-/-} mice (red; indocarbocyanine; **Supplementary Table 2**). Superimposed images (right) show differences in proteins: yellow, no change; red spots, more abundant in *Mmp7*^{-/-}; green spots, more abundant in wild-type; inset, enlargement of the area with RALDH-1. Arrows indicate RALDH-1 protein. Data are representative of two independent experiments (*n* = 3 mice per group). (b) RALDH-1 abundance in BAL fluid from wild-type and *Mmp7*^{-/-} mice immunized with PBS or CAA, assessed as spot volumes standardized and log-transformed with DeCyder software, Biological Variation Analysis. Each point represents the spot volume of a pooled sample (*n* = 3 mice). *, *P* < 0.05, versus saline-challenged wild-type; **, *P* < 0.05, versus CAA-immunized wild-type mice (*t*-test). Data are representative of at least three independent experiments (mean ± s.d.). (c) HPLC of ATRA in BAL fluid from wild-type and *Mmp7*^{-/-} mice (*n* = 3) immunized with PBS or CAA, presented as absorbance units (AU). Numbers in graphs indicate time of ATRA peak; area under the curve for each peak is 344 (WT CAA) and 755 (*Mmp7*^{-/-} CAA). (d,e) Total cells (d) and eosinophils (e) in BAL fluid from wild-type mice treated with liposomal ATRA (5 μg per g body weight) 1 h before intranasal immunization with CAA. Negative control, liposomal ATRA and PBS (*n* = 5); positive control, CAA alone (*n* = 5). (f,g) Real-time RT-PCR analysis of the expression of *Il25* mRNA (f) and *Ccl11* mRNA (g) in the lungs of the mice in d,e, presented relative to the expression of *Actb* (f) or 18S RNA (g). (h,i) Luminex assay of IL-4 (h) and IL-13 (i) in BAL fluid. *, *P* < 0.05, versus PBS-challenged wild-type; **, *P* < 0.05, versus CAA-immunized wild-type (one-way ANOVA). Data are representative of three independent experiments (mean and s.d.).

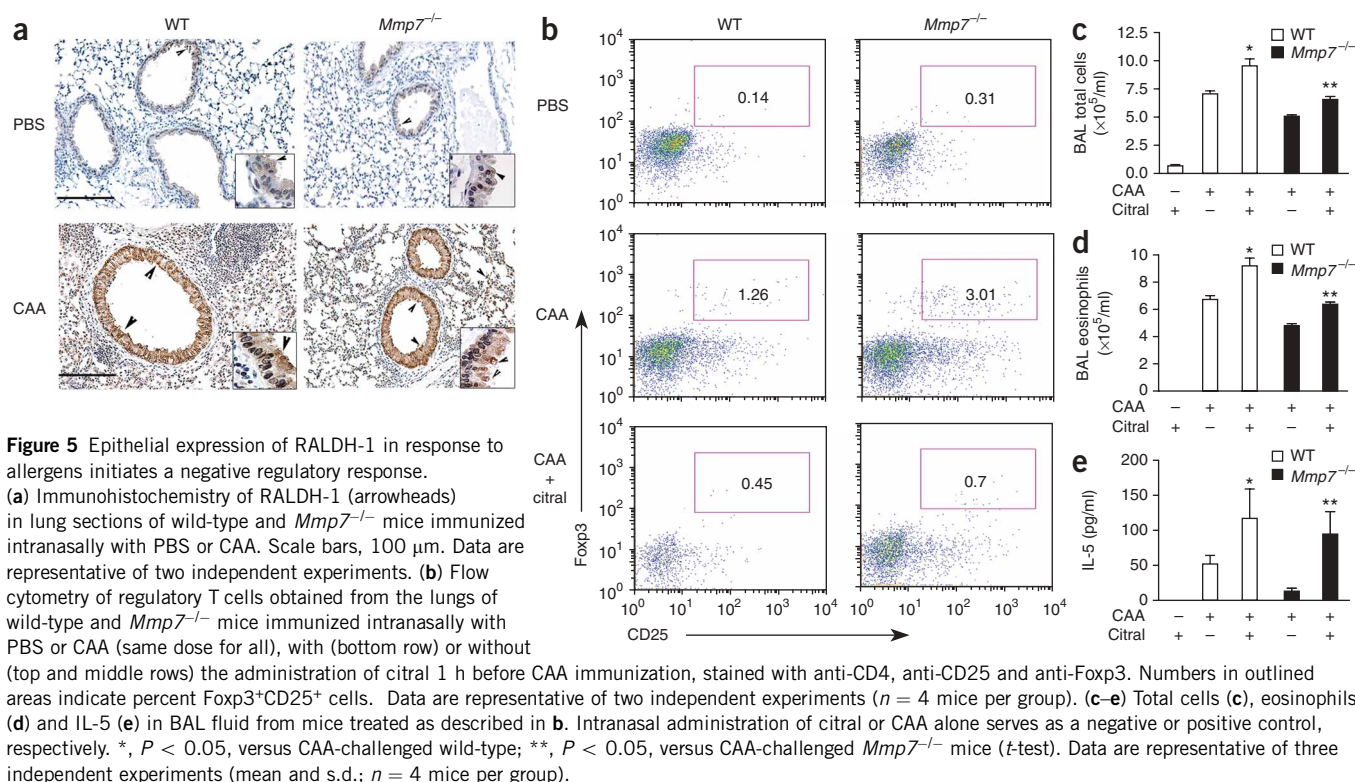
T_H2 responses in *Mmp7*^{-/-} mice. As expected, CAA-challenged *Mmp7*^{-/-} mice had less IL-4, IL-5, IL-13, IL-6 and tumor necrosis factor in BAL fluid than did wild-type mice (**Fig. 3a–c** and **Supplementary Fig. 5** online), whereas we found no significant differences in the concentrations of CCL3, IL-9 and IL-1β (data not shown). Consistent with the diminished lung eosinophilia, we found significantly lower CCL11 expression in CAA-immunized *Mmp7*^{-/-} mice than in CAA-immunized wild-type mice, but we detected no significant differences the concentrations of CCL7 and CCL17 (**Fig. 3d**, and data not shown). Furthermore, whole-lung analysis of RNA showed that immunized *Mmp7*^{-/-} mice had lower expression of *Il25* than did wild-type mice (**Fig. 3e**), which suggested that in addition to proteolytic modification of IL-25, activation and function of MMP7 in the lung is required for the transcriptional expression of *Il25* in this model of allergic airway disease.

More retinoic acid in allergen-challenged *Mmp7*^{-/-} mice

To identify additional events that might regulate the airway epithelial response to allergen, we did proteomic analyses of BAL fluid from

CAA-immunized wild-type and *Mmp7*^{-/-} mice¹⁷. Among the proteins that were significantly and differently modulated in CAA-immunized *Mmp7*^{-/-} mice (*P* < 0.05; **Supplementary Table 2** online), we found that they had more RALDH-1 (**Fig. 4a,b** and **Supplementary Fig. 6** online). RALDH-1 is the rate-limiting enzyme for the conversion of retinaldehyde to all-*trans* retinoic acid (ATRA), which is critical for maintenance of the tolerogenic environment of mucosal surfaces²⁴. In support of our proteomic-based finding of more RALDH-1, analysis of BAL fluid showed that CAA-immunized *Mmp7*^{-/-} mice had a higher ATRA concentration than did CAA-immunized wild-type mice (**Fig. 4c**).

Next, we determined if more production of ATRA in the lungs of *Mmp7*^{-/-} mice might account in part for their attenuated response to allergen. We delivered liposomal ATRA by means of respiratory aerosol to wild-type mice 1 h before each immunization with CAA, then assessed lung allergic responses. Mice that received ATRA had an attenuated asthma phenotype, as demonstrated by fewer total cells in BAL fluid, especially eosinophils, and lower expression of *Il25* mRNA than that of mice given aerosol vehicle (empty liposome; **Fig. 4d–f**).



Consistent with lower expression of inflammatory markers, we also detected less CCL11, IL-4 and IL-13, which act ‘downstream’ of IL-25 signaling (Fig. 4g–i and Supplementary Fig. 7 online). These data confirm that ATRA has potent anti-inflammatory activity when administered to the lung and that this activity can be enhanced through inhibition of *Il25* expression.

Epithelial RALDH-1 and ATRA induce regulatory T cells

We found expression of RALDH-1 protein in airway epithelial cells and alveolar macrophages of CAA-immunized wild-type and *Mmp7*^{-/-} mice (Fig. 5a). Higher expression of RALDH-1 and concentrations of ATRA have been linked to enhanced immune tolerance and more production of inducible regulatory T cells²⁵. Therefore, we determined if higher expression of RALDH-1 acted as a negative regulator in response to allergens and increased the abundance of regulatory T cells in the lungs of wild-type and *Mmp7*^{-/-} mice after immunization with CAA. CAA-immunized wild-type and *Mmp7*^{-/-} mice had more

CD25⁺Fopx3⁺ regulatory T cells in lungs than did naive mice (Fig. 5b and Supplementary Fig. 8 online). To further examine the function of RALDH-1 in inducing the production of regulatory T cells, we administered citral, an inhibitor of RALDH-1, to mice immunized with CAA²⁶. Inhibition of the synthesis of retinoic acid by citral resulted in fewer CD25⁺Fopx3⁺ regulatory T cells in the lung (Fig. 5b and Supplementary Fig. 9 online), which suggested possible involvement of RALDH-1 and ATRA in the development of this inhibitory subset of lung T cells. Moreover, wild-type and *Mmp7*^{-/-} mice immunized with CAA and challenged with citral had more eosinophils, IL-5 and CCL11 in BAL fluid than did mice immunized with CAA alone (Fig. 5c–e and data not shown).

MMP7 expression in response to allergen in human asthma

To address the relevance of MMP7 expression to human asthma, we studied bronchial biopsy specimens from human volunteers with and without asthma. Notably, whereas we detected IL-25 in airway

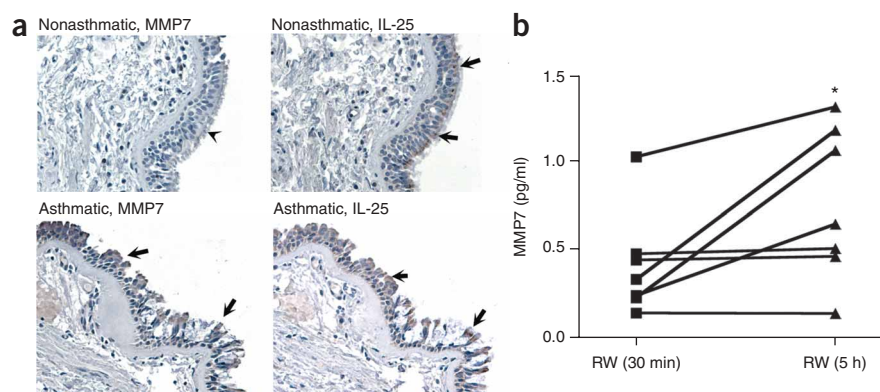


Figure 6 Expression of MMP7 and IL-25 in humans with asthma. (a) Serial photomicrographs of bronchial biopsies from a nonasthmatic subject (top row) and an asthmatic patient (bottom row), stained with anti-MMP7 (left) or anti-IL-25 (right). Arrows indicate positive immunoreactivity; arrow-head indicates lack of MMP7 expression. Data are representative of at least three independent experiments (*n* = 5 subjects per group). (b) ELISA of MMP7 responses in nasal washings from seven atopic-allergic volunteers (*n* = 7) challenged intranasally with increasing doses of ragweed (RW) or saline (data not shown). *, *P* < 0.05, versus 30-minute ragweed challenge (two-tailed paired *t*-test). Data are representative of at least three independent experiments.

epithelia of subjects with and without asthma and thus it seemed to be constitutively present, we found MMP7 expression exclusively in the airways of asthmatic subjects (Fig. 6a). Consistent with those findings, analysis of nasal washings collected from volunteers with allergic rhinitis 30 min and 5 h after intranasal challenge with ragweed (a potent proteinase allergen) showed significantly more MMP7 secretion in response to ragweed (Fig. 6b) but not in response to the normal saline control (data not shown). We did not detect secretion of IL-25 in nasal washes. Nonetheless, these data suggest that constitutive IL-25 was activated by inducible expression of MMP7 during allergen challenge of the human airway.

DISCUSSION

We have shown here how proteinase allergens simultaneously activated pro- and anti-inflammatory programs in airway epithelial cells. Allergens enhanced MMP7 expression in airway epithelial cells to maximize allergic lung inflammation; conversely, in the absence of MMP7, mice developed an attenuated asthmatic phenotype. The mechanisms responsible for the diminished allergic inflammation in *Mmp7*^{-/-} mice seemed to be in part related to lower expression of IL-25, which has been shown to be critical in initiating robust T_H2 responses^{5,7,27,28}. We found that in addition to modulating *Il25* expression, MMP7 mediated IL-25 cleavage that was required for most of the biological activity of IL-25 in allergic inflammation.

Enhanced expression of MMP family members is a frequent manifestation of active or chronic inflammatory process, but the precise function of MMPs in these conditions remains poorly understood¹³. Studies have documented that proteinase allergens are able to induce an innate immune response that further modulates the adaptive immune response during allergic inflammation^{1,2}. We have shown here that airway epithelial expression of MMP7 was critical for the development of asthma-like disease, a finding in contrast to the functions of MMP2 and MMP9, two gelatinases that are also upregulated in the same model, as absence of these enzymes leads to exaggerated lung allergic inflammation^{15–17}. Furthermore, we have shown that MMP7 was expressed in the distal lung parenchyma of untreated asthmatics and that in response to a proteinase allergen derived from ragweed, MMP7 expression was significantly induced. Although the secretion of IL-25 in the same conditions was below the detection limit of our enzyme-linked immunosorbent assay (ELISA), we found IL-25 expression in the distal lung space in normal and asthmatic volunteers. Collectively, these findings demonstrate a temporal relation between exposure to allergens and induction of MMP7 in human allergic disease and may indicate involvement of activation of IL-25 by MMP7 in conditions of allergen exposure. It is important to note that during allergic inflammation, many endogenous proteinases, including members of the serine and cysteine family, are abundantly present in the airway^{1,29}. However, our finding that MMP7 was required for the amplification of T_H2 responses emphasizes the nonredundant functions that MMPs may serve in the course of inflammation. The specificity of this response was exemplified by the specific proteolysis of IL-25, but not of other allergy-related cytokines such as TSLP and IL-13, by MMP7. Similarly, MMP7-mediated proteolytic modification of defensins, apoptotic ligands and cytokines has been shown to be important in diverse biological functions, such as innate immunity in the gut to cancer cell metastasis^{19,30–32}. The findings that rIL-25 induced expression of lung MMP7 and intranasal administration of rIL-25 to *Mmp7*^{-/-} mice only weakly induced allergic inflammation suggest that the bioactivity of IL-25 depends mainly on proteolysis by MMP7. These data further suggest the existence of a feedback loop in which IL-25 upregulates lung MMP7 expression,

followed by cleavage and activation of IL-25 that further enhances both MMP7 expression and allergic lung disease.

Although regulation of gene expression has been studied for other members of the IL-17 family, little is known about IL-25 regulation¹². We found that although there was little or no RALDH-1 in the airways of naive mice, this enzyme had prominent expression in the airway epithelial cells of both wild-type and *Mmp7*^{-/-} mice in response to a proteinase allergen. By high-throughput proteomic analysis, we found excess RALDH-1 enzyme in CAA-immunized *Mmp7*^{-/-} mice that was functionally relevant because it resulted in higher BAL fluid concentrations of ATRA and less allergic inflammation. The function of ATRA in the regulation of adaptive immunity, especially T_H2 responses, is controversial. For example, IL-4-induced eotaxin production, eosinophil lineage commitment and IL-5 receptor expression are all inhibited by the addition of ATRA *in vitro*^{33,34}. Furthermore, retinoic acid in the presence of transforming growth factor-β1 inhibits the binding of STAT6 to the promoter of the gene encoding the transcription factor Foxp3 and effectively inhibits IL-4 signaling in T cells³⁵. In other models of allergic lung disease, deficiency in vitamin A results in lower expression of the muscarinic M2 receptor, which results in more airway hyper-reactivity³⁶. In contrast, intranasal administration of high concentrations of ATRA (over 1,500 μg) has been shown to fail to alter T_H2 responses in another asthma model³⁷, and vitamin A deficiency has been shown to attenuate the production of T_H2 cytokines in mice³⁸. In our study, allergen-challenged mice that received 100 μg ATRA showed much lower allergic inflammation and attenuation of the asthmatic phenotype. Such divergent findings are not unexpected, given the known idiosyncrasies of retinoid, which can act as a hormone and, depending on the physiological or pharmaceutical context, can mediate diverse effects on cells of the immune system³⁹.

Studies have indicated involvement of ATRA in the development and differentiation of inducible regulatory T cells, an anti-inflammatory subset of T cells that are important in the homeostasis of lymphocytes in various organs⁴⁰. In particular, dendritic cells in the lamina propria have been found to provide ATRA that is critical for the differentiation of regulatory T cells and homing of these cells to the intestinal mucosa^{41,42}. Although we did not identify the cellular origin of ATRA, we found that RALDH-1, the rate-limiting enzyme that converts retinaldehyde to ATRA, was upregulated in response to allergic stimulation in the airway epithelium, which indicates a possible site for ATRA production in the lung. Consistent with the requirement for ATRA in the differentiation of regulatory T cells *in vivo*^{25,41,43}, we found more CD25⁺Foxp3⁺ T cells in the lungs of *Mmp7*^{-/-} mice immunized with CAA than in the lungs of CAA-immunized wild-type mice. Moreover, we found that inhibition of the synthesis of retinoic acid by citral inhibited the development of regulatory T cells. The higher percentage of regulatory T cells after allergen challenge in both wild-type and *Mmp7*^{-/-} mice suggested that RALDH-1 is a negative regulator of allergic inflammation, a plausible protective response regulated by airway epithelial cells that we found to be enhanced in the absence of MMP7. What remains unclear is the mechanism responsible for the higher expression and function of RALDH-1 in the lung in the absence of MMP7. Our finding of RALDH-1 expression in airway epithelial cells and macrophages in response to allergen challenge is in agreement with published reports indicating an anti-inflammatory function for these cells in allergic inflammation⁴⁴. Furthermore, depletion of alveolar macrophages results in an exaggerated allergic response that can be inhibited by adoptive transfer of alveolar macrophages in brown Norway rats⁴⁵. Our data suggest that alveolar macrophages that mediate anti-inflammatory responses during allergic inflammation might be acting

through RALDH-1, an idea that is also consistent with the tolerogenic function of RALDH-1 in gut lamina propria^{41,42}.

In summary, we have described here a proinflammatory function of MMP7 that was activated in response to proteinase allergens and involved proteolytic modification of the expression and function of IL-25. In this model, we found activation of RALDH-1 was 'downstream' of allergen-induced inflammation and that MMP7 actively inhibited the function of this enzyme *in vivo*. This unique mechanism involved, at least in part, inhibition of regulatory T cells that inhibit lung inflammation. Our findings thus substantially expand the understanding of the regulation of IL-25, a critical innate cytokine in allergic responses, and further identify pro- and anti-inflammatory targets that can be explored for therapeutic benefit.

METHODS

Mice. *Mmp7*^{-/-} mice backcrossed nine generations with C57BL/6 mice were provided by P.W. Park and were bred in the transgenic animal facility at Baylor College of Medicine (accredited by the Association for Assessment and Accreditation of Laboratory Animal Care). All experiments were in accordance with protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine. Information on the genotyping of *Mmp7*^{-/-} mice and description of control mice are in the **Supplementary Methods** online. *Stat6*^{-/-} mice (BALB/c background) and *Il13*^{-/-} mice (C57BL/6 background) from the Jackson Laboratories were bred for ten to twelve generations onto the BALB/c background.

Experimental model of asthma. CAA, composed of *Aspergillus oryzae* proteinase (1 mg/ml; Sigma) and ovalbumin (0.5 mg/ml; Sigma) in a volume of 50 μ l, was administered intranasally every 4 d for a total of five doses as described before^{1,16}. In some experiments, rIL-25 (5 μ g in 50 μ l PBS) was administered intranasally twice daily for 3 d. In some experiments, liposomal ATRA or citral was given by aerosol administration 1 h before CAA challenge. Additional information on the liposome formulation and administration of ATRA is in the **Supplementary Methods**.

Experimental ragweed allergen challenge in human volunteers. Nasal 'provocation' with ragweed pollen extract was done as described⁴⁶. The Institutional Review Board at the University of Texas Medical Branch reviewed and approved the protocol and consent forms. The challenge procedure and analysis of nasal lavage fluid and bronchial biopsies are described in the **Supplementary Methods**.

Analysis of asthmatic phenotype. All data were collected 24 h after the final allergen challenge. Airway hyper-reactivity was assessed by estimation of the concentration of acetylcholine (in mg per g body weight) that caused a 200% increase in airway resistance over the baseline, calculated by linear interpolation of the appropriate dose-response curves as described¹⁵. Collection and analysis of BAL fluid and lung tissue is described in the **Supplementary Methods**.

Immunohistochemistry. A standard published protocol was used for immunohistochemistry⁴⁷. Deparaffinized sections were washed with 3% (vol/vol) H₂O₂ and 2% (vol/vol) Triton X-100 in PBS, followed by antigen retrieval (Target Retrieval Solution; Dako). Slides were incubated overnight at 4 °C with rabbit polyclonal antibody to mouse ALDH1A1 (24343; Abcam), rat monoclonal antibody to mouse MMP7 (377314; R&D Systems), mouse monoclonal antibody to human MMP7 (377314 (R&D Systems) or ID2 (Calbiochem)) and mouse monoclonal antibody to human IL-17E. Additional information on staining and analysis is in the **Supplementary Methods**.

Measurement of cytokines and chemokines in BAL fluid. CCL11, CCL7 and CCL17 in BAL fluid were measured by standard antibody-based ELISA and/or a multiplex bead-based cytokine detection kit (BioRad) as described¹⁶. Reagents and the LINCplex assay are described in the **Supplementary Methods**.

Proteomics analysis of BAL protein. Proteomics analysis of BAL fluid was done as described¹⁷. BAL samples from wild-type and *Mmp7*^{-/-} mice were

pooled and were concentrated with AMICON filters, and protein concentrations were measured by BCA protein assay (bicinchoninic acid; Pierce). An equal amount of protein from each pooled sample (50 μ g) was labeled with 400 pmol fluorescent dye (CyDye; Amersham Biosciences) matched by charge and molecular size and was brought to a final volume of 250 μ l with sample buffer that also contained 0.5% (wt/vol) ampholytes (pH 3–11, non-linear), 0.1% (wt/vol) bromophenol blue and DeStreak reagent (12 μ l/ml; GE Healthcare). Additional information on proteomics analysis is in the **Supplementary Methods**.

Quantitative RT-PCR. The RNeasy mini kit (Qiagen) was used for extraction of total cellular RNA from mouse lung tissues stabilized with the RNA Later reagent (Qiagen). One-step real-time quantitative RT-PCR (Real-Time PCR system 7300; Applied Biosystems) was used to assess the expression of *Ccl11* (encoding CCL11 (eotaxin 1)) and *Aldh1a1* (encoding RALDH-1). The primer and probe mixtures of target genes (*Ccl11*, Mm00441238_m1; *Raldh-1*, Mm01194995_mH; Applied Biosystems identifiers) and 18S rRNA were from Applied Biosystems. Additional information on RT-PCR is in the **Supplementary Methods**.

MMP activation and *in vitro* cleavage assay. Carrier-free mouse rIL-25 (5 μ g; R&D), mouse rIL-13 (Peprotech) and mouse rTSLP (R&D Systems) were incubated for 2 h at 37 °C with 0.5 μ g 4-aminophenylmercuric acetate-activated MMP7 in the presence or absence of the MMP inhibitor 1,10-phenanthroline (10 mM; Sigma). After incubation, equal volumes of each sample (16.5 μ l) were reduced and were resolved by separation through a 16.5% tricine gel. Cleaved and uncleaved proteins were visualized with the Proteosilver Plus Silver Stain kit according to the manufacturer's instructions (Sigma-Aldrich). Additional information on these assays is in the **Supplementary Methods**. Amino-terminal sequencing of IL-25 was done by standard protein-sequencing methods (ProCise 492cLC; Applied Biosystems).

Cell culture. Lymph node cells and splenocytes isolated from C57BL/6 mice were stimulated with plate-bound antibody to CD3 (anti-CD3; 2 μ g/ml) and human IL-2 (50 U/ml) in the presence of mouse rIL-25 (250 ng/ml; R&D Systems) or rIL-25' (250 ng/ml). After 2 d, culture supernatants were collected and analyzed for IL-4, IL-5, IL-13 and interferon- γ by ELISA as described⁵. On day 3, cells were restimulated for 5 h with ionomycin (500 ng/ml) and phorbol 12-myristate 13-acetate (50 ng/ml) in the presence of GolgiStop (BD Biosciences). Cells were made permeable with a Cytofix/Cytoperm kit (BD Biosciences) and were analyzed for intracellular expression of IL-4 (with phycoerythrin-conjugated anti-IL-4; 11B11) and IL-5 (with allophycocyanin-conjugated anti-IL-5; TRFK5; both from BD Biosciences).

Flow cytometry. Total lung eosinophils were quantified by bead-enhanced flow cytometry as described⁴⁸. Total mouse lung cells were stained with fluorescein isothiocyanate-conjugated antibody to major histocompatibility class II (2G9; 553623; BD Biosciences) and phycoerythrin-conjugated anti-Siglec-F (1 μ g per 10⁶ cells; E50-2440; BD Biosciences), followed by incubation with fluorescent beads (Flow-Check Fluorospheres; Beckman Coulter). Samples were acquired with a flow cytometer (XL2; Beckman Coulter) and cell and bead counts were measured. Absolute numbers of eosinophils were calculated with formulas as described⁴⁸. Additional information on flow cytometry is in the **Supplementary Methods**.

High-performance liquid chromatography (HPLC). Pooled BAL samples from wild-type and *Mmp7*^{-/-} mice were evaporated under nitrogen and the dry residue was dissolved in 100 μ l acetonitrile (Fisher). Aliquots of 25 μ l were then analyzed with the Waters HPLC system, consisting of a WISP autosampler (model 717 Plus), dual-absorbance detector (model 2487), pump (model 515) and Nova-Pak C18 columns (3.9 mm $\times$ 150 mm). ATRA was separated with a solvent system of 57.5% (vol/vol) acetonitrile, 25% (vol/vol) acetic acid (2% solution) at a flow rate of 1.3 ml/min. Data were analyzed with Waters Millennium software (version 3.2).

Binding assay. Analysis of the binding of IL-25 to the fusion protein of IL-17 receptor B and Fc fragment is described in the **Supplementary Methods**.

Statistics. All data were analyzed with Prism 4.0a statistical analysis software. Significant differences ($P < 0.05$) between treatment groups were identified with a *t*-test, one-way analysis of variance (ANOVA) or Bonferroni multiple-comparison test.

Accession code. UCSD-Nature Signaling Gateway (<http://www.signaling-gateway.org>): A001477.

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

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AUTHOR CONTRIBUTIONS

S.G. did animal experiments, *in vitro* cleavage assay, immunohistochemistry, flow cytometry, RT-PCR, ELISA and Luminex assays; P.A. did *in vitro* T cell experiments; W.T.B. and S.P. did airway physiology experiments; K.J.G. did proteomics analysis; A.S. made liposomal ATRA and helped with HPLC; M.S., L.S., D.R., B.S. and S.S. did the ragweed challenges of humans and ELISA of samples from allergic volunteers; P.W. obtained bronchial biopsies of asthmatic and control volunteers; S.G., F.K., D.B.C. and C.D. designed experiments; S.G. and F.K. wrote the manuscript; and P.A., D.B.C. and C.D. critically reviewed the manuscript.

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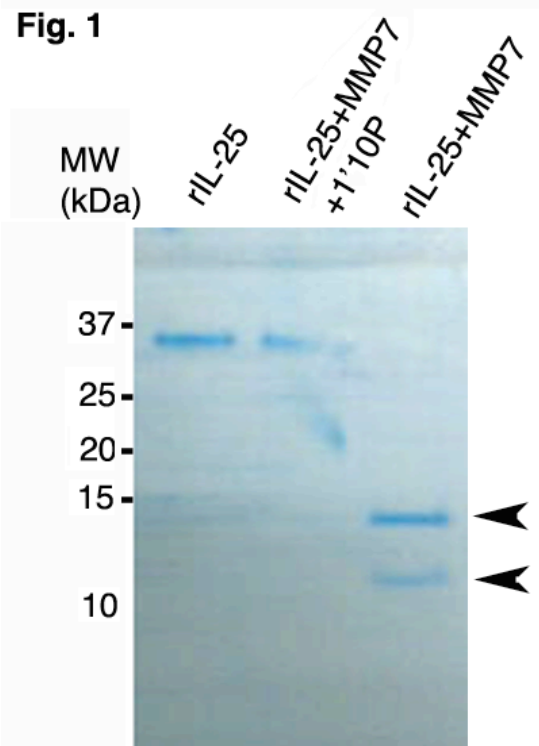
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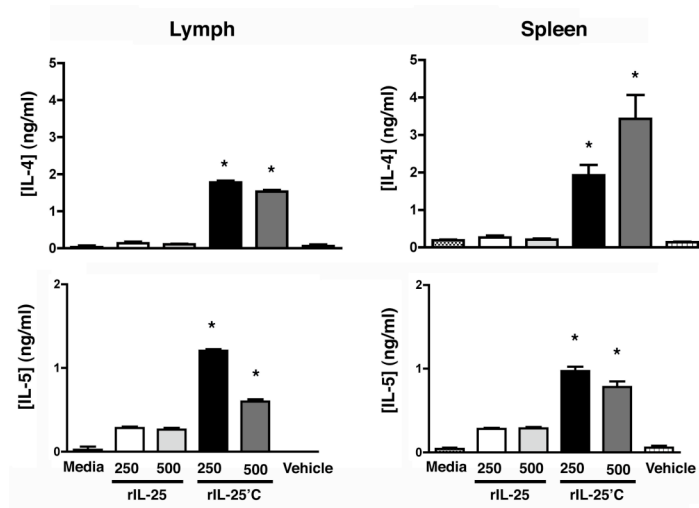
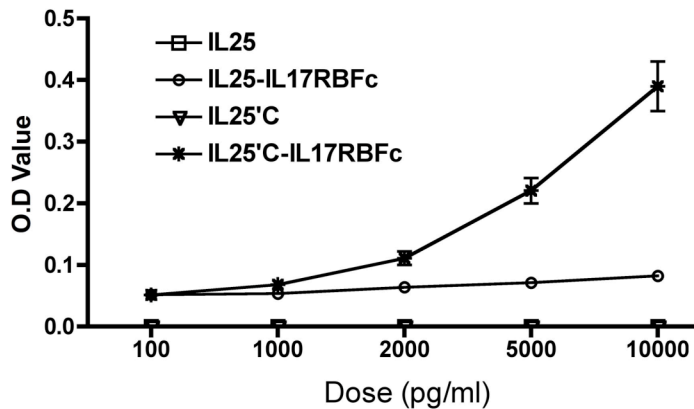
Divergent Roles for Airway Epithelial MMP7 and Retinoic Acid in Experimental Asthma

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Fig. 1

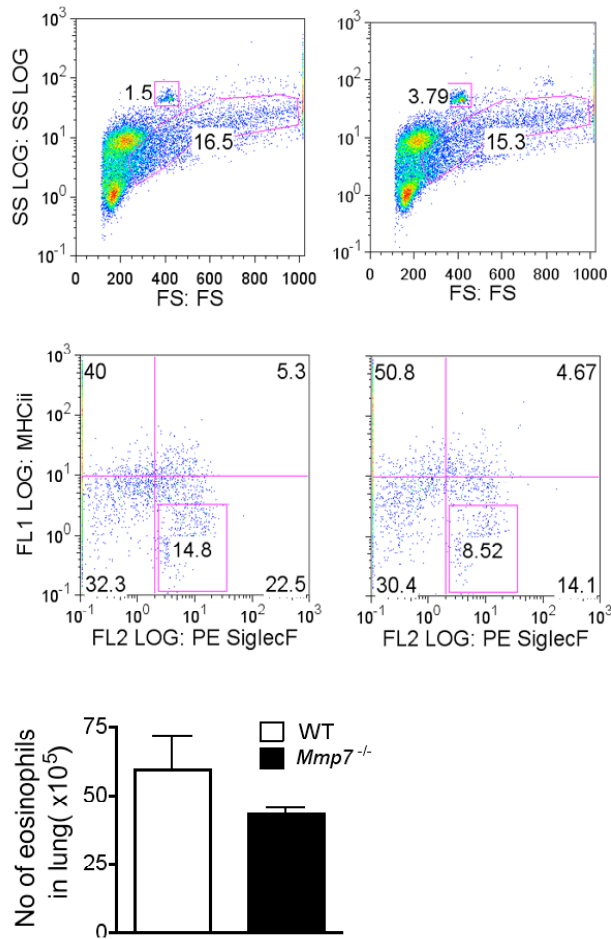


Supplementary Figure 1. MMP7 cleaves IL-25 at distinct sites. Recombinant (r)IL-25 (5 μ g), was incubated for 2 hours at 37°C with APMA-activated MMP7 (0.5 μ g), in the presence or absence of 10 mM 1' 10-phenantroline (1' 10P; MMP inhibitor), and were resolved on 16.5% Tricine gel followed by detection using coomassie blue. Arrows indicate bands corresponding to fragments of rIL-25 that were isolated and sequenced as shown in Table 1.

Fig. 2a**Fig 2b**

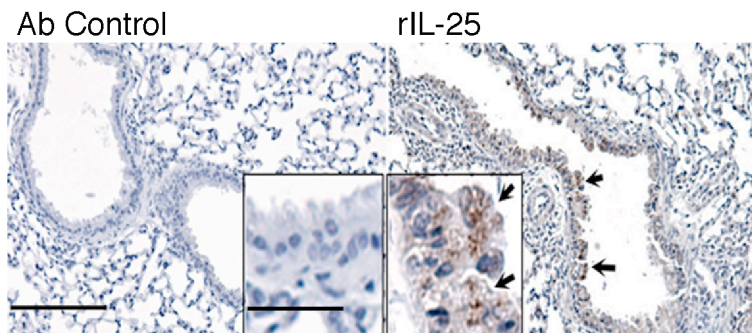
Supplementary Figure 2a-b. a) Lymphocyte dose response to rIL-25'C. Naïve lymph node cells (left column) and spleen cells (right column) isolated from C57BL/6 mice, were stimulated with 2 µg/ml of plate-bound anti-CD3 in the presence of equal amount of native rIL-25 (250 and 500 ng/ml) or MMP7 cleaved rIL-25 (IL-25'C; 250, and 500 ng/ml). On Day 2, cell culture-supernatant from each condition were examined for the concentration of IL-4, IL-5 by ELISA. Media alone and APMA containing buffer that was used to activate MMP-7 (vehicle) were used as controls. * $P < 0.05$ relative to rIL-25, using the one-way ANOVA test. **b) Recombinant mouse IL-17RB-Fc fusion protein** (R&D Systems) or PBS was used to coat 96-microtiter plates. After blocking, recombinant mouse IL-25 or MMP7 cleaved IL-25 (IL-25'C) was added at different concentrations (100pg/ml to 50,000pg/ml). After washing, biotinylated polyclonal anti-mouse IL-25 antibody (R&D Systems) was added and the ability of recombinant IL-25 or IL-25'C to bind to IL-17RB-Fc fusion protein was detected by using standard ELISA protocol. Data represents mean values $\pm$ SD.

Fig. 3



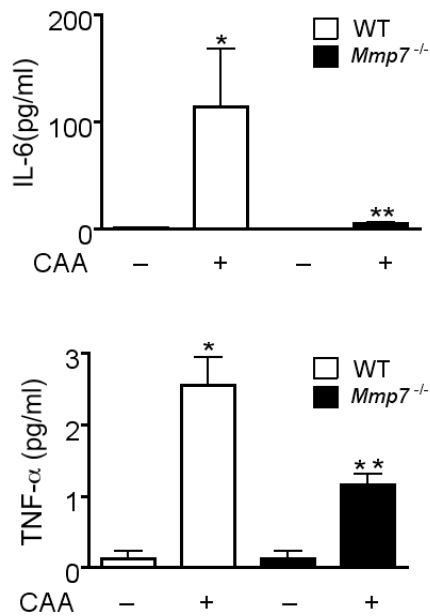
Supplementary Figure 3. Reduced total number of lung eosinophils in *Mmp7*^{-/-} mice. WT and MMP7 null mice were immunized with CAA as described in Fig. 2, and total number of lung eosinophils enumerated as SiglecF⁺MHCII⁺ granulocytes (Top and middle panel) as described in material and methods. Lower panel represents cumulative number of eosinophils in WT and *Mmp7*^{-/-} immunized mice (n=5 in each group). Data represent mean values $\pm$ SD; representative of three independent experiments.

Fig. 4



Supplementary Figure 4. Intranasal rIL-25 induces MMP7 expression in airway epithelial cells. WT (C57BL/6) mice were given intranasal PBS or rIL-25 (5 μ g) twice daily for 3 days, and 24hr following the last dose, mice were sacrificed and MMP7 expression was detected by immunohistochemistry (right panel, arrows indicate MMP7 expression; insets show 40X magnification of airway, left panel: antibody control)

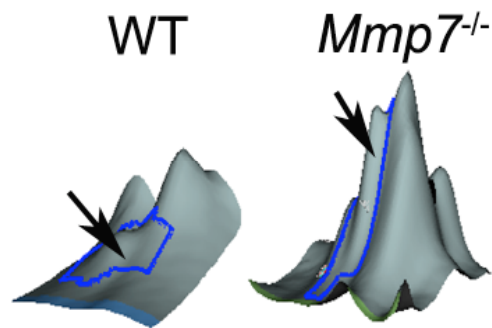
Fig. 5



Supplementary Figure 5. Reduced IL-6 and TNF-α in BAL fluid of CAA

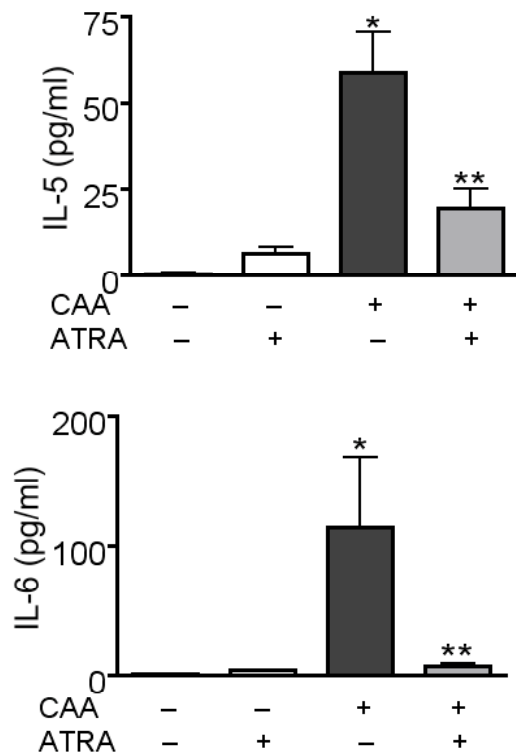
immunized *Mmp7*^{-/-} mice. Age and sex matched wild type (WT), (n=4) and MMP7 null mice (n =4) were immunized intranasally with CAA or PBS as described in Fig 2 and 24 hrs after the last immunization, BAL fluid were assessed for concentration of a) IL-6 and b) TNF-α using Luminex. Data represent mean values ± SD; *P< 0.05 relative to PBS challenged WT and **P< 0.05 relative to CAA immunized WT mice using one way ANOVA and t-test.

Fig 6



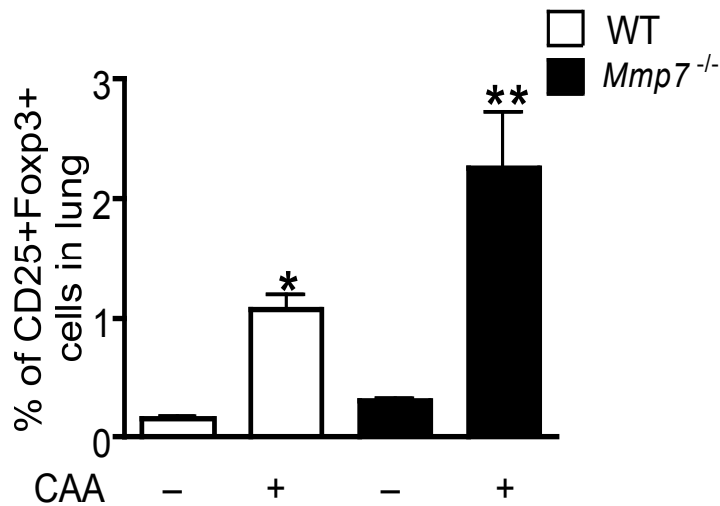
Supplementary Figure 6. Increased abundance of RALDH-1 in MMP7 null CAA immunized mice. Peaks (as indicated by the arrows) represent relative abundance of RALDH-1 in BAL fluid. Spot volumes were standardized and log transformed using DeCyder software, Biological Variation Analysis. Each point represents the spot volume from a pooled sample (n=3) in each group.

Fig 7



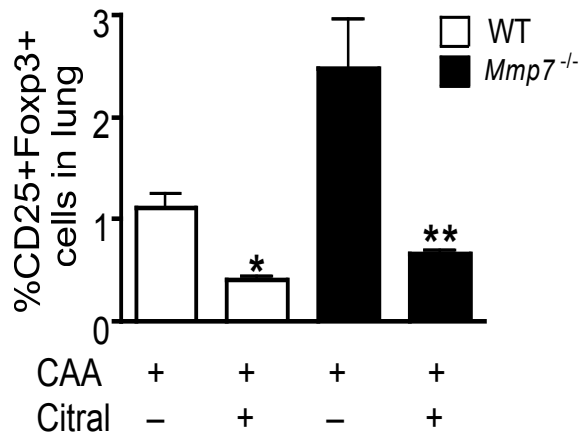
Supplementary Figure 7. ATRA reduces IL-5 and IL-6 in CAA immunized mice. WT mice (n=5 in each group) were treated with liposomal ATRA as described in Fig 4. IL-5 and IL-6 levels in BAL fluid were measured by Luminex. (Data represent mean values $\pm$ SD; *P<0.05 relative to PBS challenged WT and **P<0.05 relative to CAA immunized WT mice using One way ANOVA test).

Fig. 8



Supplementary Figure 8. Increased relative abundance of regulatory T cells MMP7 null immunized mice. Single cell suspensions from the lung of WT (n=4) and MMP7 null (n=4) CAA or sham (PBS) immunized (n=4 in each group) mice were stained with anti-CD4, -CD25 and -FoxP3 to determine the abundance of T regulatory cells. * P < 0.05 relative to PBS challenged WT and ** indicates P < 0.05 relative to CAA immunized WT mice using one way ANOVA and t-test. Data represent mean values $\pm$ SD; representative of two independent experiments.

Fig. 9



Supplementary Figure 9. Citral reduces T regulatory cells in the lungs of CAA immunized mice. Abundance of T regulatory cells were determined from WT (n=4; open bars) and MMP7 null (n=4; filled bars) null mice that were given citral, 1 hours before CAA intranasal immunization. * $P < 0.05$ relative to CAA immunized WT and ** indicates $P < 0.05$ relative to CAA immunized MMP7 null mice using one way ANOVA and t-test. Data represent mean values $\pm$ SD; representative of three independent experiments.

Table 1. IL-25 amino acid sequences that are identified as the cleavage sites for MMP7. Recombinant IL-25 was incubated with either MMP7 *in vitro* and partial sequencing of cleavage products was performed using N-terminal sequencing to confirm their identity. Pairs of amino acids highlighted in red are cleavage sites for MMP7, and we identified the gray highlighted fragments from cleavage products using N-terminal sequencing.

1 MYQAVAF~~LA~~M IVGTHTVSLR IQEGCSHLPS CCPSKEQEPP EEWLKWSSAS VSPPE~~PL~~SHT
61 HHAESCRA~~SK~~ DGPLNSRAIS PWSYELDRDL NRVPQ~~DL~~YHA RCLCPHCVSL QTGSHMDPLG
121 NSVPLYHNQT VFYRRPCHGE EGTHRRYCLE RRLYRVSLAC VCVRPRVMA

Table 2: Identification of selected proteins uniquely altered in BAL fluid of MMP7 null CAA immunized mice and their putative functions.

Protein	Function	t-test p value	Abundance ratio
Serine proteinase inhibitor A3K precursor (Contrapsin)	Contrapsin inhibits trypsin-like proteases	0.00082	4.28
Annexin A5	Calcium/phospholipid-binding protein which promotes membrane fusion and is involved in exocytosis	0.00005	-1.88
Peroxiredoxin 6	The enzyme participates directly in eliminating hydrogen peroxide and neutralizing other reactive oxygen species	0.00280	-2.35
Peroxiredoxin 5	Peroxiredoxin 6 is an enzyme that detoxifies hydrogen peroxide and various organic peroxides	0.04100	-1.61
Superoxide Dismutase (Cu-Zn)	Converts free oxygen radicals to peroxide and to molecular oxygen	0.00530	-3.30
Ubiquitin	label proteins for proteasomal degradation. Ubiquitination also controls the stability, function, and intracellular localization of a wide variety of proteins	0.02400	-3.43

Abundance ratio is the relative spot volume from wild-type to MMP7 null mice, where positive values >1.5 were considered significantly higher in CAA immunized MMP7 null BAL relative to similarly treated wild-type mice, and negative values <1.5 were significantly lower in MMP7 null BAL compared to similarly treated wild-type mice.

Supplementary Methods

Mice. *Mmp7*^{-/-} mice were genotyped using standard PCR techniques (Neo R: 5'-TTGAGCCTGGCGAACAGT-3', Neo F: 5'-TGGATTGCACGCAGGTTCT-3', WT F: 5'-ACTTACCTCGGATCGTAGTG-3', WT R: 5'-GTCCAGTACTCATCCTTGTC-3'). Wild-type C57BL/6 mice, and/or heterozygote littermates were used as controls and were purchased from the Baylor Center for Comparative Medicine facility.

Experimental model of asthma. Briefly, liposome formulation of ATRA (Sigma) and citral (Tokyo Chemical Industry Chemical Co.) were made in dilauroylphosphatidylcholine (DLPC) (Avanti Polar Lipid Inc.) as we have previously described¹. Aerosol was generated from an Aerotech II nebulizer (CIS USA; flow rate 10 L/Min) and was discharged from an opening to the entry orifice. Following a dose titration study that determined efficacy of aerosol delivery, mice received a total concentration of 5 µg/gram of body weight of ATRA, 5 mg of citral, or vehicle control (empty liposomes) over 1 h before each of the CAA challenges.

Experimental ragweed allergen challenge in human volunteers. Otherwise healthy volunteers with symptoms of seasonal allergic rhinitis (congestion, nasal discharge, sneezing) and positive prick skin test to ragweed extract were recruited for this study. Volunteers received intranasal saline challenges delivered with a nasal spray bottle on one day. The next day, the same volunteers received a graded intranasal challenge with ragweed (saline, 0.33, 1, 3, 9, 27 µg/ml *Ambrosia artemisiifolia* a 1, (Greer laboratories) every 15 min until sneezing, rhinorrhea and nasal congestion were observed. On both

days, 5 ml nasal lavage fluids were collected using previously described techniques 30 min and 5 h post nasal challenge. The fluids were centrifuged and cell-free supernatants were analyzed for MMP7 response at each time point using ELISA (R&D).

Serial sections of bronchial biopsies samples were collected under protocols approved by the UCSF Committee on Human Research and with written informed consent for future use as part of the UCSF Airway Tissue Bank from untreated asthmatic and control volunteers were stained for the presence of MMP7 and IL-25 using standard immunohistochemical techniques as described below.

Analysis of asthmatic phenotype. BAL fluid was collected by instilling and withdrawing 1.6 ml of sterile phosphate buffered saline (PBS) through the tracheal cannula in two aliquots of 0.8 ml. BAL fluid total and differential cell count were measured using the standard hemocytometer and hematoxylin and eosin (H&E) stain of cytopsin slides as described previously². In each experiment, mouse lungs were fixed with intra-tracheal instillations of 800 µl of 4% paraformaldehyde solution via a tracheal cannula and were embedded in paraffin and 4–5 µm sections of paraffin embedded lung tissues were either stained with H&E, periodic acid-Schiff (PAS), and/or were prepared for immunohistochemistry.

Immunohistochemistry. Primary antibodies were used in 1:50 to 1:100 dilution, followed by incubation with corresponding biotinylated secondary antibodies (1:200 dilution) and Vectastain Elite ABC reagent (Vector) at 25 °C. Slides were then incubated

with DAB substrate (Vector) and counterstained with hematoxylin. After addition of bluing reagent (0.08% NH₄OH), slides were mounted in xylene.

BAL Cytokine and Chemokine Measurement. All recombinant protein, capture antibodies, and their corresponding biotin-conjugated detection antibodies were purchased from R&D system. IL-4, IL-5, IL-13, IL-6 and TNF concentrations in BAL fluid were measured by Lincoplex (Millipore). In each case, the same volumes of human nasal washings were used to detect MMP7 using ELISA kit purchased from R&D system.

Proteomics analysis of BAL protein. Internal standard was made by mixing equal amounts of protein from wild-type and *Mmp7*^{-/-} samples before CyDye labeling. Wild-type, *Mmp7*^{-/-} and the internal standard (IS) were labeled and loaded for separation on triplicate gels as follows: WT-Cy3, *Mmp7*^{-/-} Cy5, IS- Cy2. First dimension separation was performed based on isoelectric focusing using the GE Healthcare IPGPhor II. The second dimension separation was performed using the same IPG strips that were secured in place with agarose on top of a 15% 18 x 16 cm SDS gel and were electrophoresed for 4 h at 20 mA. Electrophoresed gels were scanned using a laser-based image instrument (Typhoon 9400, GE Healthcare) and were digitized using ImageQuant software (GE Healthcare). Gel images were imported into the DeCyder (v. 5.5) Differential In-gel Analysis (GE Healthcare) program and resulting spots were matched among the gels. Using DeCyder Biological Variation Analysis software (GE Healthcare), spot differences were quantified and protein spots that exhibited a significant normalized change in

volume, with respect to the internal standard sample (1.5 fold; $p < 0.05$), were picked for identification. For spot picking, spot map coordinates were transferred to the ETTAN spot handling work-station (GE Healthcare) for automated picking, in-gel trypsin digestion, and MALDI plate spotting.

Quantitative RT-PCR. Expression of mRNA samples were normalized to 18S rRNA, and relative abundance of the target genes were calculated as described previously^{3,4}. Two-step real time q-RT-PCR was performed to determine relative expression of *Il25*; expression was normalized with *Actb*. cDNA was generated using oligo-dT, random hexamers, and SuperScript RT II (Invitrogen).

MMP activation and *in vitro* cleavage assay. Alternatively, cleaved IL-25 protein was separated as above, electroblotted in Tris-glycine buffer (25 mM Tris, 192 mM glycine, 10% MeOH (pH 8.3) to a polyvinylidene difluoride (PVDF) membrane and stained with 0.05% Coomassie Blue in 1% acetic acid, 50% methanol. The PVDF membranes were destained in 50% methanol and deionized water. The visible bands were cut out with a clean scalpel blade and air-dried.

Flow cytometry. Abundance of lung T regulatory cells was estimated by staining the cells with Mouse Regulatory T cell Staining Kit (eBioscience); samples were acquired in BD LSRII flow cytometer. Analysis was performed with FlowJo software (version 8.5.3).

IL-25-IL-17RB-Fc binding assay. Recombinant mouse IL-17RB-Fc fusion protein (R&D Systems) or PBS was used to coat 96-microtiter plates. After blocking, recombinant mouse IL-25 or MMP7 cleaved rIL-25 (rIL-25'C) was added at different concentrations (100 pg/ml to 50,000 pg/ml). After washing, biotinylated anti-mouse IL-25 antibody (R&D Systems) was added and the ability of rIL-25 or rIL-25'C to bind to IL-17RB-Fc fusion protein was detected by using standard ELISA protocol.

References

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