

Allergen-induced expression of IL-25 and IL-25 receptor in atopic asthmatic airways and late-phase cutaneous responses

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Background: IL-25 is thought to participate in allergic inflammation by propagating T_H2-type responses.

Objective: To address the hypothesis that allergen provocation increases expression of IL-25 and its receptor IL-25R in the asthmatic bronchial mucosa and skin dermis of atopic subjects. **Methods:** Sequential single and double immunostaining was used to evaluate the numbers and phenotypes of IL-25 and IL-25R immunoreactive cells in bronchial biopsies from mild atopic subjects with asthma (n = 10) before and 24 hours after allergen inhalation challenge and skin biopsies from atopic subjects (n = 10) up to 72 hours after allergen subepidermal injection.

Results: IL-25 immunoreactivity was expressed by a majority of epidermal cells in both organs at baseline and was not further augmented by challenge. IL-25R immunoreactive cells were rare in the epidermis before or after challenge. Allergen challenge was associated with significantly ($P < .01$) increased expression of IL-25 and IL-25R immunoreactivity in the submucosa of both organs. IL-25 immunoreactivity colocalized with eosinophils, mast cells, and endothelial cells, whereas IL-25R immunoreactivity colocalized with eosinophils, mast cells, endothelial cells, and T lymphocytes. In both organs, correlations were observed between increases in IL-25 expression and the magnitudes of the late-phase allergen-induced clinical responses.

Conclusion: Allergen provocation induces functionally relevant, increased expression of IL-25 and its receptor in the asthmatic bronchial mucosa and dermis of sensitized atopic subjects. In addition to T cells, eosinophils, mast cells, and endothelial cells are potential sources and targets of IL-25 in the course of allergic inflammation. (*J Allergy Clin Immunol* 2011;■■■:■■■-■■■.)

Key words: IL-25, IL-25 receptor, asthma, skin late-phase response, allergen challenge

There is increasing interest in the pivotal role likely played by the cytokine IL-25 in the maintenance of the T_H2-type immune response postulated to contribute to the pathogenesis of asthma and allergy. IL-25 (also known as IL-17E) is a member of a family of 6 structurally related but functionally distinct proteins labeled IL-17A to F. IL-17A and IL-17F are expressed, typically syngenyically, by a novel subset of CD4⁺ T_H cells and appear to play important roles in inflammation and autoimmunity. IL-25, on the other hand, is exceptional in promoting a T_H2-type immune response.¹ This has been well demonstrated in animal experiments in mice: exogenous administration of IL-25^{2,3} or transgenic expression^{4,5} induces T_H2-type inflammation. IL-25 is increased in animal bronchial challenge models,⁶ and its inhibition reduces the associated T_H2-type inflammation.^{7,8}

IL-25 acts principally on its own receptor called IL-25R, a 56-kd single transmembrane protein. We previously demonstrated⁹ that human T_H2 central memory cells selectively upregulate IL-25R when stimulated with thymic stromal lymphopoietin-activated dendritic cells or T-cell memory “homeostatic” cytokines such as IL-7 or when triggered by specific antigen. This in turn resulted in IL-25-induced, IL-4-independent, sustained expression of *GATA3*, *c-MAF*, and *JunB*. Thus, IL-25 not only augments T_H2-type memory T-cell responses but also is likely a key link in the mechanism of awakening recall responses to antigens encountered at the environmental interface. Furthermore, IL-25 acts on effector granulocytes such as eosinophils to increase the release of proinflammatory mediators¹⁰ and is itself produced by a range of these granulocytes including mast cells, basophils, and eosinophils as well as T cells.^{9,11} This raises the possibility that IL-25, produced by both memory T_H2 cells and granulocytes, creates a positive feedback amplifying loop for allergic inflammation and asthma.¹²

Notwithstanding these observations, and aside from our preliminary documentation of IL-25 and IL-25R mRNA expression in the bronchial mucosa of patients with asthma and the skin of patients

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Abbreviations used

APAAP: Alkaline phosphatase antialkaline phosphatase

HUVEC: Human vascular endothelial cell

IL-25R: IL-25 receptor

MBP: Major basic protein

UK: United Kingdom

with eczema,⁹ the kinetics and cellular origins of IL-25 and IL-25R expression in allergic inflammation and asthma have been poorly documented *in vivo* in human beings to date. Our aim was to study this by using allergen-induced late-phase reactions in the bronchial mucosa and skin of atopic subjects as a recognized surrogate of asthmatic and allergic inflammation and in particular IL-25 and IL-25R expression response to acute allergen challenge.¹³ We hypothesized that acute allergen challenge in these models is associated with increased, functionally relevant expression of IL-25 and its receptor at the relevant mucosal surfaces. We aimed to document the location and phenotypes of cells expressing these molecules and to correlate expression with the clinical response to allergen.

METHODS**Subjects and study design**

The study was approved by the Research Ethics Committees of King's College, Lewisham, and the Royal Brompton Hospitals, London, United Kingdom (UK), and subjects provided written, informed consent. Atopic subjects with asthma ($n = 10$; mean $\pm$ SEM total serum IgE, 319.8 ± 41.6 IU/mL) were recruited from King's College Hospital. The inclusion criteria were a history of mild asthma requiring inhaled β_2 -agonist only, FEV₁ $\geq 70\%$ predicted, a positive skin prick test to 1 or more of the challenge allergens, no usage of corticosteroids in the previous month, no tobacco smoking within the past 5 years, and a provocative concentration of histamine causing a 20% fall in baseline FEV₁ (PC₂₀) ≤ 8 mg/mL. Exclusion criteria were symptoms of an upper respiratory tract infection in the previous 2 weeks, allergy to pollens in subjects recruited during the pollen season, and a history of chronic illness other than asthma. Subjects underwent fiber optic bronchoscopy before allergen bronchial challenge and repeat bronchoscopy with biopsy 24 hours later as previously described.¹⁴ Briefly, the allergen used for challenge was selected on the basis of the clinical history and a positive skin prick test. Patients were selected to continue the study if they had demonstrated an early asthmatic response of at least 20% (0–2 hours after challenge) and a late asthmatic response of at least 15% (2–8 hours after challenge). The dose of allergen required to produce a late asthmatic response of 15% was computed from a dose-response curve and generated on a screening day. On a second challenge day, the computed cumulative allergen dose was administered as a bolus, using the same nebulizer, dosimeter, and dosimeter settings. Each subject received nebulized salbutamol (2.5 mg) and intravenous atropine (600 μ g) 15 minutes before fiber optic bronchoscopy. Sedation was achieved with intravenously administered diazepam, and supplemental oxygen was given throughout the procedure. Patient oxygenation was monitored by pulse oximetry. Fiber optic bronchoscopy was performed by the same operator for all subjects. After 1% lignocaine spray was applied to the nose and throat of the subject, the bronchoscope (Olympus Model OSE with a 2.2-mm-width biopsy channel; Olympus Corp, Lake Success, NY) was introduced. Local anesthesia of the larynx was produced with topical 4% lignocaine, and 2% lignocaine was used below the vocal cords. Endobronchial biopsies were then taken from either the right middle or lower lobe on segmental carinae. After the procedure, the patient was given an additional 2.5 mg salbutamol and was observed on the ward for at least 4 hours.

To study the allergen-induced late-phase cutaneous response, atopic subjects ($n = 10$) were recruited from the Allergy Departments of Guy's and the Royal Brompton Hospitals, London, UK. Inclusion criteria were age 18 to 55 years,

history of seasonal and/or perennial allergic rhinitis and/or mild asthma (mean $\pm$ SEM total serum IgE, 3303.2 ± 40.2 IU/mL), absence of any other illness, and positive skin prick test (wheal diameter >5 mm at 15 minutes in the presence of negative diluent control) to 1 or more of the challenge allergens (grass pollen, house dust mite, or cat dander; ALK, Hørsholm, Denmark). Patients had not taken antihistamines or leukotriene receptor blockers for at least 2 weeks before the study. Biopsy of multiple allergen injection sites at various time points allowed construction of a time course. Comparison was with a diluent control injection biopsied after 24 hours.

Single immunohistochemistry

Single immunohistochemistry was performed by using the alkaline phosphatase antialkaline phosphatase (APAAP) technique.¹⁵ To identify cells expressing IL-25, IL-25R, and cellular phenotypic markers, we used monoclonal primary antibodies purchased from R&D Systems, Abingdon, UK (mouse antihuman IL-17E, MAB 1258, 1/20),⁵ Autogene Bioscience, Wiltshire, UK (mouse antihuman IL-25R, LB-2044, 1/50),⁹ Dako Ltd, Cambridgeshire, UK (mouse antihuman endothelial cells, CD31, 1/30; macrophages, CD68, 1/50; mast cell tryptase, 1/50; neutrophil elastase, 1/50) and Becton Dickinson, Oxford, UK (mouse antihuman T cells, CD3, 1/10). Mouse antieosinophil major basic protein (MBP) was a kind gift from Prof A. B. Kay.¹⁵ The primary antibodies were detected with second (donkey antimouse, 1/50, Jackson ImmunoResearch Laboratories, Inc, West Grove, Pa) and third layer (APAAP, 1/50, Dako) antibodies (30 minutes for each incubation) by using well established protocols that we have previously described.^{15–17} Positively staining cells were detected with Fast Red (Sigma, Poole, UK). Omission or substitution of the primary antibody with an irrelevant antibody of the same species and isotype was used as a negative control.

The slides were observed in blind fashion on an Olympus BX40 microscope (Olympus Imaging & Audio Ltd, Essex, UK) connected with a Zeiss Vision KS300 imaging system (Carl Zeiss, Göttingen, Germany) and total cells expressing immunoreactivity for IL-25, IL-25R cells, and cellular phenotypic markers enumerated in entire biopsy sections. In the bronchial submucosa and dermis, data were expressed as the total numbers of cells per unit area. In the epithelium, IL-25 immunoreactivity was expressed as percentages of the total area immunostained by using automated analysis with a preset pixel threshold. Where individual cells within the epithelium were counted, numbers were expressed per unit length of epithelial basement membrane. The mean $\pm$ SD entire cross-sectional areas (including epithelium and submucosa) of the bronchial biopsy sections before and after allergen challenge were 2.7 ± 0.4 and 2.6 ± 0.3 mm², respectively, with coefficients of variation from 7.67% (allergen challenge) to 9.45% (baseline). The mean $\pm$ SD entire cross-sectional area of the cutaneous biopsy sections was 3.6 ± 0.4 mm². The between-observer coefficients of variation for duplicate counts of all markers examined for bronchial and cutaneous biopsies were 4.6% to 6.3% and 4.9% to 7.2%, respectively.

To investigate further endothelial cellular expression of IL-25, human vascular endothelial cells (HUVECs; PromoCell GmbH, Heidelberg, Germany) were cultured in chamber slides containing complete medium (endothelial cell media kit; PromoCell) for 24 hours, then fixed in 4% paraformaldehyde/PBS for 10 minutes. After rinsing in PBS for 10 minutes, the chamber slides were incubated with monoclonal anti-IL-25 (MAB 1258 1/50; R&D Systems) at room temperature overnight. After washing with PBS, the slides were incubated with donkey antimouse antibody (1/50; Jackson ImmunoResearch) for 30 minutes, washed in PBS, incubated with APAAP, and then developed with Fast Red as described. Purified blood eosinophils were used as a positive control. Substitution of the primary antibody with an irrelevant isotype-matched immunoglobulin (mouse IgG₁; Sigma) was used as a negative control.

Double, sequential immunohistochemistry

To identify cellular sources of IL-25 and IL-25R immunoreactivity, double immunohistochemistry was used as previously described.^{14–17} Murine mAbs against cellular phenotypic markers were first used, then stained with second (donkey antimouse) and third layer (APAAP) antibodies as

TABLE I. Clinical and demographic features of subjects and their responses to allergen challenge

Lung							
		FEV ₁ , mean (range)					
		Baseline % predicted			Maximum % fall during LPR		
		92.8 (81.2 to 106.0)			−34.1 (−14.5 to −63.0)		
Asthma (n = 10)							
Sex (F:M): 4:6							
Age (y), mean (range): 28 (20-38)							
Allergen used: n/N							
HDM: 5/10							
Cat dander: 3/10							
Grass pollen: 2/10							
Skin							
		LPR (mean ± SEM diameter [mm])					
		1 h	3 h	6 h	24 h	48 h	72 h
		27.5 ± 2.6	40.3 ± 2.0	58.0 ± 3.2	38.9 ± 4.6	18.1 ± 4.3	4.7 ± 1.3
Atopic (n = 10)							
Sex (F:M): 3:7							
Age (y), mean (range): 25 (19-33)							
Allergen used: n/N							
HDM: 5/10							
Grass pollen: 5/10							

F, Female; HDM, house dust mite; LPR, late-phase response; M, male.

described. After developing with Fast Red, goat polyclonal antibodies against human IL-25 (sc-20594, 1/50; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) and IL-25R (AF1207, 1/50; R&D Systems) were used to detect IL-25 and IL-25R immunoreactivity. This was detected by incubation with horseradish peroxidase-conjugated rabbit anti-goat antibody (1/100; Jackson ImmunoResearch) followed by Fast DAB (3,3'-diaminobenzidine; Sigma-Aldrich, Gillingham, UK). Double-positive cells were stained red/brown.

Statistical analysis

Data were analyzed by using a statistical package (Minitab Release 7; Minitab Inc, State College, Pa). ANOVA was performed by using the Friedman test followed by paired comparisons between time points using the Wilcoxon signed-rank test. The data are presented as the mean ± SEM for clarity although they were analyzed nonparametrically. For all tests, *P* values less than .05 were considered significant.

RESULTS

Allergen-induced late-phase airways responses and cutaneous reactions

Allergen inhalation challenge induced both early (up to 2 hours after challenge) and late (4-24 hours after challenge) phase bronchoconstriction in all 10 atopic subjects with asthma. Cutaneous allergen injection induced late-phase reactions in all atopic subjects. Further details are provided in Table I.

Allergen-induced inflammatory cellular infiltration

Comparison of bronchial biopsies taken before and 24 hours after bronchial allergen challenge showed significantly increased numbers of CD3⁺ T cells, MBP⁺ eosinophils, and tryptase⁺ mast cells in the epithelium and submucosa (Fig 1). In addition, there were significant increases in the numbers of elastase⁺ neutrophils and CD31⁺ endothelial cells in the submucosa. In the dermis of the skin, the numbers of CD3⁺ T cells, CD68⁺ macrophages, MBP⁺ eosinophils, elastase⁺ neutrophils, and CD31⁺ endothelial cells were significantly increased 24 hours after allergen

compared with diluent challenge, whereas tryptase⁺ mast cell numbers did not change significantly (Fig 2).

Allergen-induced IL-25 and IL-25R expression in the bronchial mucosa

Typical examples of bronchial mucosal immunostaining for IL-25 and IL-25R are shown in Fig 3. IL-25 immunoreactivity was observed in the epithelium and submucosa (Fig 3, *B* and *E*), whereas IL-25R immunoreactive cells were predominantly located in the submucosa (Fig 3, *C* and *F*). A majority of bronchial epithelial cells expressed IL-25 protein even at baseline with no significant further increase after allergen challenge (23.75% ± 4.2% vs 26.3% ± 4.5% of the total area, respectively). Allergen inhalation challenge was associated with significant increases in the numbers of IL-25⁺ cells (*P* = .006) and IL-25R⁺ cells (*P* = .008) in the bronchial mucosa (Fig 4). Increased numbers of submucosal IL-25 but not IL-25R immunoreactive cells correlated inversely with the maximum percentage change in FEV₁ during the late-phase response (*r* = −0.734; *P* = .007).

Allergen-induced IL-25 and IL-25R expression in the skin

Typical examples of IL-25 and IL-25R immunostaining in the skin sections are shown in Fig 3. A majority of epidermal keratinocytes expressed IL-25 protein even at diluent-challenged sites, and there was no significant further increase (78.5% ± 2.4% vs 83.6% ± 4.1% of the total area, respectively) after allergen challenge. In the dermis, few cells expressed IL-25 and IL-25R immunoreactivity after diluent challenge, but there was a significant, time-dependent increase in IL-25 and IL-25R protein expression after allergen challenge, maximal after 24 hours, and persisting up to 48 hours (IL-25) and 72 hours (IL-25R; Fig 5). Again, increased numbers of IL-25, but not IL-25R, immunoreactive cells significantly correlated with the sizes of late-phase reactions 3 hours after

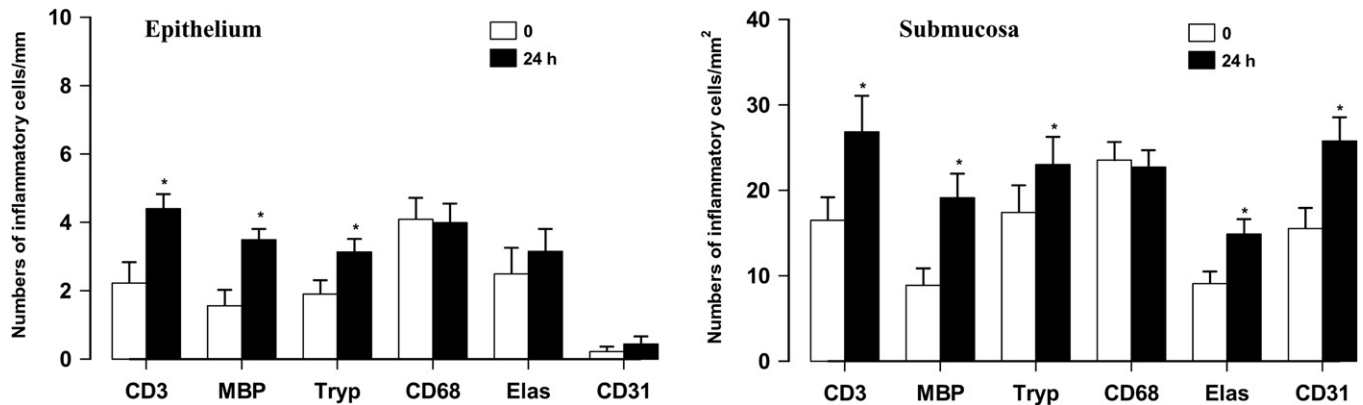


FIG 1. Numbers of inflammatory and endothelial cells in epithelium (left, mm length) and submucosa (right, mm²) of bronchial biopsies from atopic patients with asthma before and 24 hours after allergen inhalation challenge (n = 10). **P* < .05 compared with paired baseline (Wilcoxon signed-rank test). CD3, CD3⁺ T cells; CD31, CD31⁺ endothelial cells; CD68, CD68⁺ macrophages; Elas, elastase⁺ neutrophils; MBP, MBP⁺ eosinophils; Tryp, tryptase⁺ mast cells.

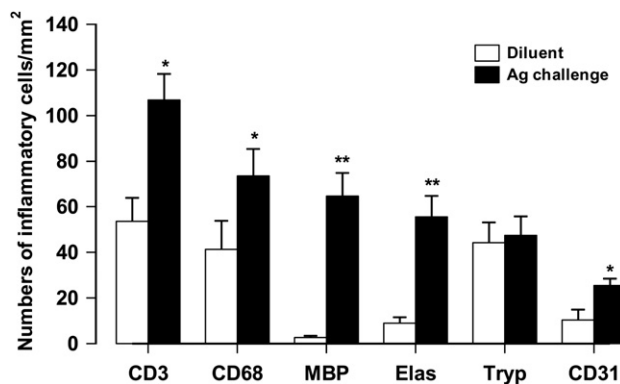


FIG 2. Numbers of inflammatory cells in the dermis of atopic subjects 24 hours after allergen or diluent challenge (n = 10). **P* < .05, ***P* < .01 compared with paired baseline (Wilcoxon signed-rank test). Ag, Antigen; CD3, CD3⁺ T cells; CD31, CD31⁺ endothelial cells; CD68, CD68⁺ macrophages; Elas, elastase⁺ neutrophils; MBP, MBP⁺ eosinophils; Tryp, tryptase⁺ mast cells.

allergen challenge ($r = 0.727$; $P = .017$). The mean numbers of IL-25 (mean $\pm$ SEM, $9.6 \pm 2.5/\text{mm}^2$) and IL-25R (mean $\pm$ SEM, $3.9 \pm 0.9/\text{mm}^2$) immunoreactive cells in the dermis of skin biopsies (n = 3) obtained from normal controls in the absence of any challenge were very similar to those observed after diluents challenge.

Cellular sources of IL-25 and IL-25R in the bronchial mucosa

The cellular provenance of IL-25 and IL-25R expression in the bronchial subepithelial mucosa was investigated by using double sequential immunohistochemistry (Fig 6). MBP⁺ eosinophils, tryptase⁺ mast cells, and CD31⁺ endothelial cells were the principal sources of IL-25 immunoreactivity, accounting for (baseline vs allergen challenge) $39.2\% \pm 3.5\%$ vs $39.4\% \pm 2.1\%$, $26.3\% \pm 2.0\%$ vs $30.5\% \pm 1.3\%$, and $20.4\% \pm 1.2\%$ vs $18.5\% \pm 1.9\%$ (mean $\pm$ SEM) of all the IL-25⁺ cells, respectively. Relatively few CD68⁺ macrophages, elastase⁺ neutrophils, and CD3⁺ T cells expressed IL-25 immunoreactivity ($2.9\% \pm 0.6\%$ vs $1.9\% \pm 0.5\%$, $2.8\% \pm 0.7\%$ vs $1.4\% \pm 0.4\%$, and $2.0\% \pm 0.6\%$ vs $2.5\% \pm 0.4\%$ of the total, respectively). Together these

cells accounted for nearly all (mean, 93.6% at baseline and 94.2% after allergen challenge) of the IL-25⁺ cells. Although eosinophils, mast cells, and endothelial cells expressed most of the IL-25 in the sections, this was observed in subsets of only 40% to 70% of the total cells (Fig 6). This overall picture was not significantly altered by allergen challenge despite the increased overall number of infiltrating leukocytes (Fig 6).

Major basic protein⁺ eosinophils, CD31⁺ endothelial cells, tryptase⁺ mast cells, and CD3⁺ T cells were the principal cell types expressing IL-25R immunoreactivity, accounting for (baseline vs allergen challenge) $29.8\% \pm 4.3\%$ versus $30.7\% \pm 2.1\%$, $24.7\% \pm 2.6\%$ versus $25.9\% \pm 2.3\%$, $18.2\% \pm 0.8\%$ versus $13.9\% \pm 1.4\%$, and $16.7\% \pm 1.7\%$ versus $18.1\% \pm 1.5\%$ (mean $\pm$ SEM) of all the IL-25R⁺ cells, respectively. Very few CD68⁺ macrophages and elastase⁺ neutrophils expressed IL-25R ($1.2\% \pm 0.5\%$ vs $1.2\% \pm 0.5\%$ and $1.1\% \pm 0.4\%$ vs $1.2\% \pm 0.5\%$ of the total, respectively; Fig 6). Again these cells accounted for the majority (mean, 92.6% at baseline and 93.2% after allergen challenge) of all the submucosal IL-25R⁺ cells. Although there was no significant variation in the percentages of cells making up the total IL-25R⁺ population between baseline and 24 hours after allergen challenge (Fig 6), the percentages of MBP⁺ eosinophils and CD3⁺ T cells expressing IL-25R were significantly increased.

Cellular sources of IL-25 and IL-25R in skin biopsies

As mentioned, a majority of epidermal keratinocytes expressed IL-25 immunoreactivity even before allergen challenge. In the dermis, MBP⁺ eosinophils, tryptase⁺ mast cells, and CD31⁺ endothelial cells were the principal cells expressing IL-25 immunoreactivity, accounting for $40.8\% \pm 2.6\%$, $25.1\% \pm 2.9\%$, and $24.0\% \pm 3.0\%$ (mean $\pm$ SEM) of the total, respectively. As in the bronchial submucosa, very few CD3⁺ T cells, CD68⁺ macrophages, and elastase⁺ neutrophils contributed to IL-25 immunoreactivity ($2.1\% \pm 0.6\%$, $1.9\% \pm 0.5\%$, and $1.9\% \pm 0.7\%$ of the total immunoreactive cells; Fig 7).

Cells expressing IL-25R immunoreactivity were located principally in the dermis. As in the bronchial submucosa, MBP⁺ eosinophils, CD31⁺ endothelial cells, CD3⁺ T cells, and tryptase⁺ mast cells were the principal cell types expressing IL-25R (29.2%

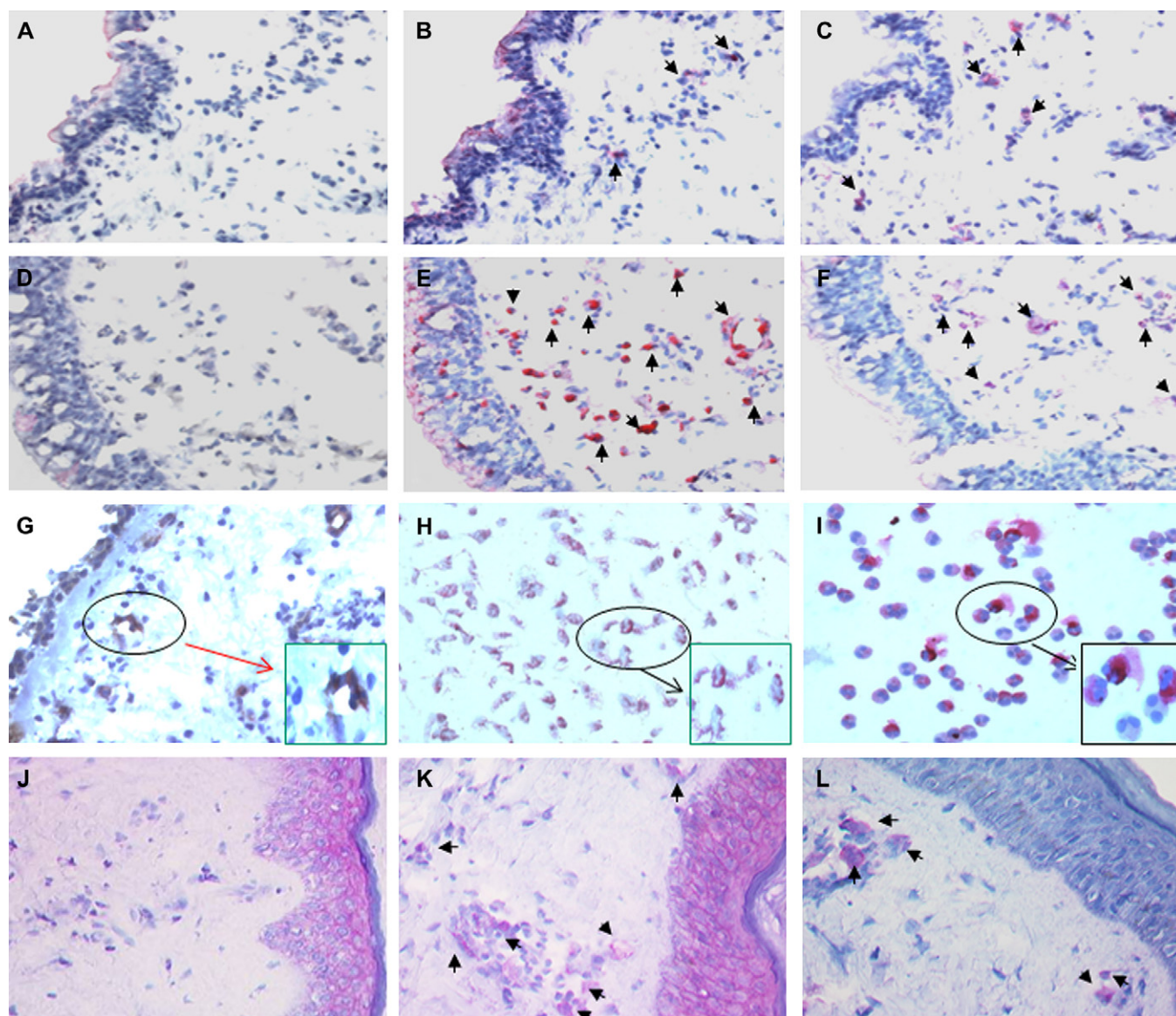


FIG 3. Immunohistochemistry of bronchial biopsies stained with isotype-control (A), anti-IL-25 (B), and anti-IL-25R (C) before and 24 hours after allergen challenge (D-F; arrows). IL-25⁺/CD31⁺ endothelial cell (red arrow; G). IL-25⁺ endothelial cells (H) and eosinophils (I). IL-25⁺ cells in the skin 24 hours after diluent (J) and allergen challenge (K). IL-25R⁺ cells after allergen challenge (L). Magnification (×20; insert, ×40).

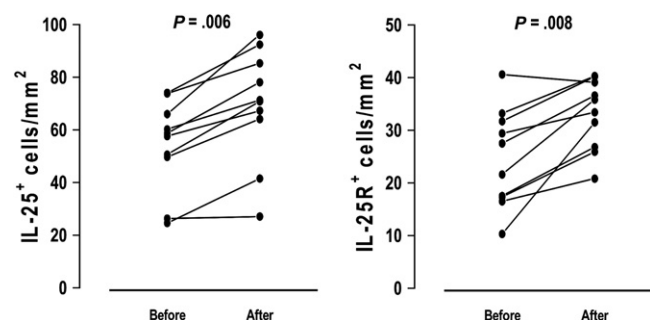


FIG 4. Numbers of IL-25 and IL-25R immunoreactive cells per unit area of the atopic asthmatic bronchial submucosa before and 24 hours after allergen inhalation challenge (n = 10). Wilcoxon signed-rank test.

$\pm 2.0\%$, $26.6\% \pm 2.0\%$, $19.4\% \pm 1.7\%$, and $15.4\% \pm 2.0\%$, respectively; Fig 7). The contribution of CD68⁺ macrophages and elastase⁺ neutrophils to IL-25R⁺ cells was minimal ($1.1\% \pm 0.4\%$ and $1.0\% \pm 0.4\%$ of total cells). Taken together, these cells accounted for the majority of the total IL-25R⁺ cells (mean, 92.7% of the total), although IL-25R was expressed only by a minority (8% to 35%) of these cell types (Fig 7).

To confirm further expression of IL-25 protein by HUVECs, single immunocytochemistry was performed on HUVECs cultured in chamber slides. IL-25 immunoreactivity was detectable in $80.5\% \pm 3.4\%$ of resting HUVECs (n = 3; Fig 3, H), comparable to purified human blood eosinophils used as a positive control (Fig 3, I; $85.8\% \pm 3.9\%$ of cells positive; n = 3).

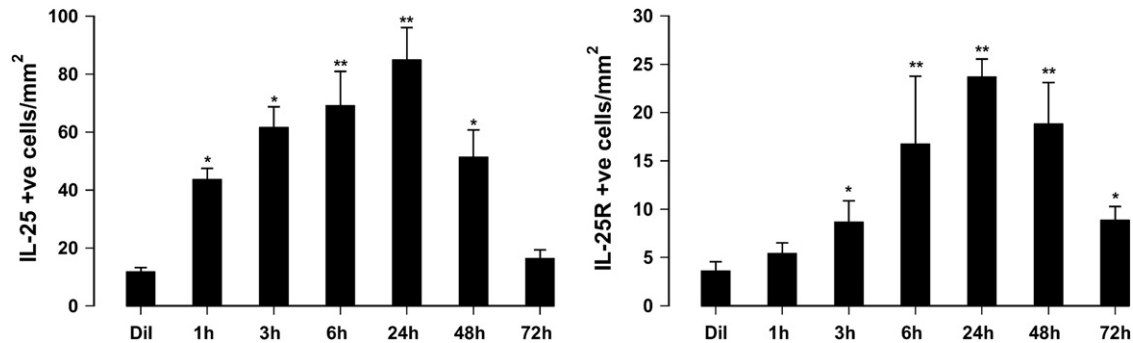


FIG 5. Time course of expression of IL-25 (left) and IL-25R (right) immunoreactivity in skin biopsies from atopic subjects ($n = 10$) at 1, 3, 6, 24, 48, and 72 hours after challenge with allergen or diluent (Dil; 24 hours only). Data are expressed as mean $\pm$ SEM numbers of positive cells per square millimeter of dermis. * $P < .01$, ** $P < .001$ versus diluent. Wilcoxon signed-rank test.

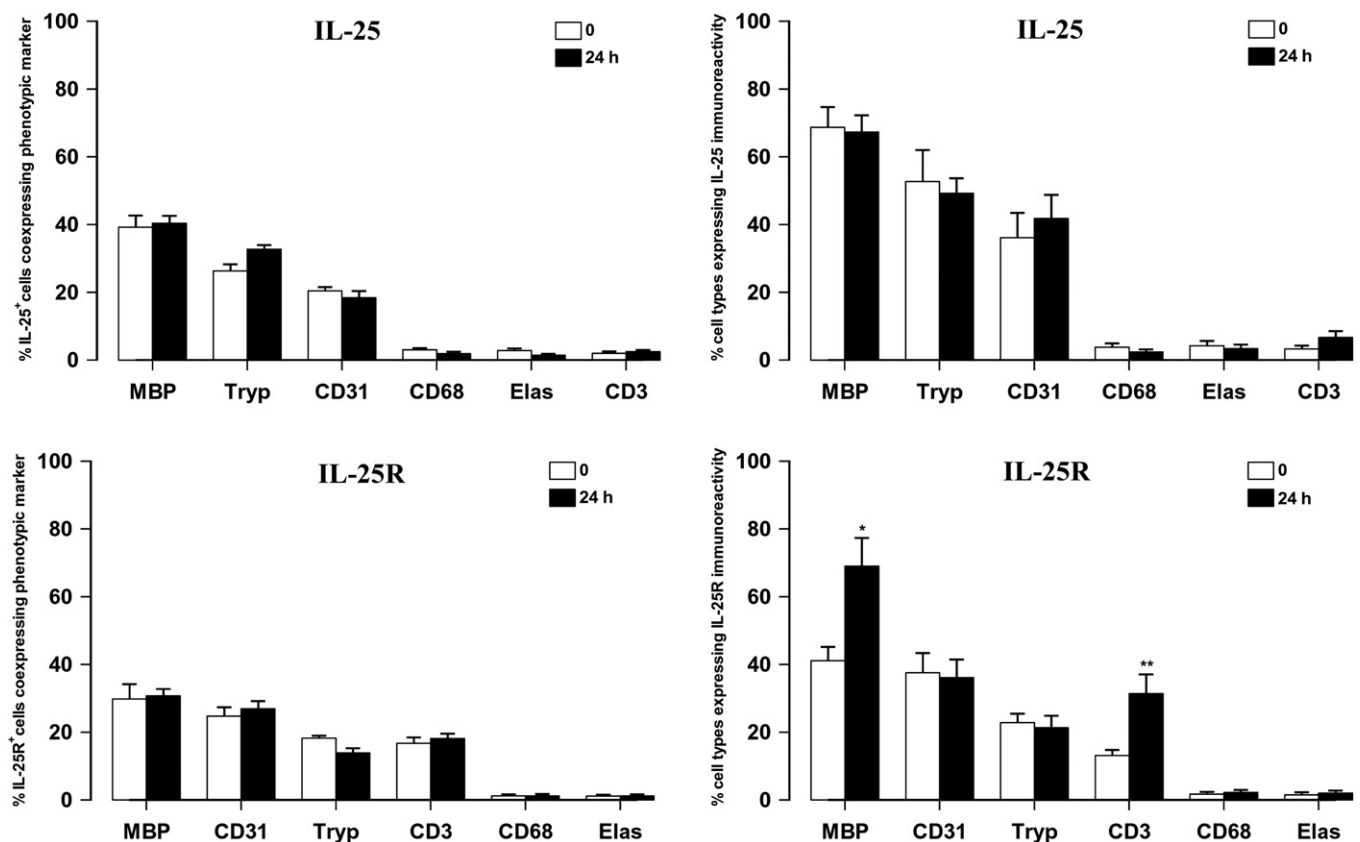


FIG 6. Left panel, Mean ($\pm$ SEM)% total IL-25⁺ and IL-25R⁺ cells accounted for the stated phenotypes. Right panel, Percentages of total cells of each phenotype expressing IL-25 and IL-25R immunoreactivity in bronchial biopsies 24 hours after allergen-challenge ($n = 10$). CD3, CD3⁺ T cells; CD31, CD31⁺ endothelial cells; CD68, CD68⁺ macrophages; Elas, elastase⁺ neutrophils; MBP, MBP⁺ eosinophils; Tryp, tryptase⁺ mast cells. * $P < .05$, ** $P < .01$ versus paired baseline (Wilcoxon signed-rank test).

DISCUSSION

By using a well established, dynamic model of human allergic inflammation *in vivo*, we have clearly demonstrated an acute increase in the local reservoir of cells with the potential to secrete and respond to IL-25 in the asthmatic bronchial mucosa and dermis of atopic subjects after allergen exposure. Fascinatingly, both epithelial and endothelial cells were a rich potential source of IL-25 protein in both organs even before exposure. In the bronchial

submucosa, the increase in IL-25⁺ cells after allergen exposure most likely reflected recruitment or local proliferation/differentiation of eosinophils, mast cells, and endothelial cells. It is difficult to conclude with certainty that IL-25 was increased in individual cells because the proportions of positive cells of each type did not markedly change after challenge. This study was neither designed nor powered to study relationships between IL-25/IL-25R expression and asthma severity (ethically one is obliged to challenge

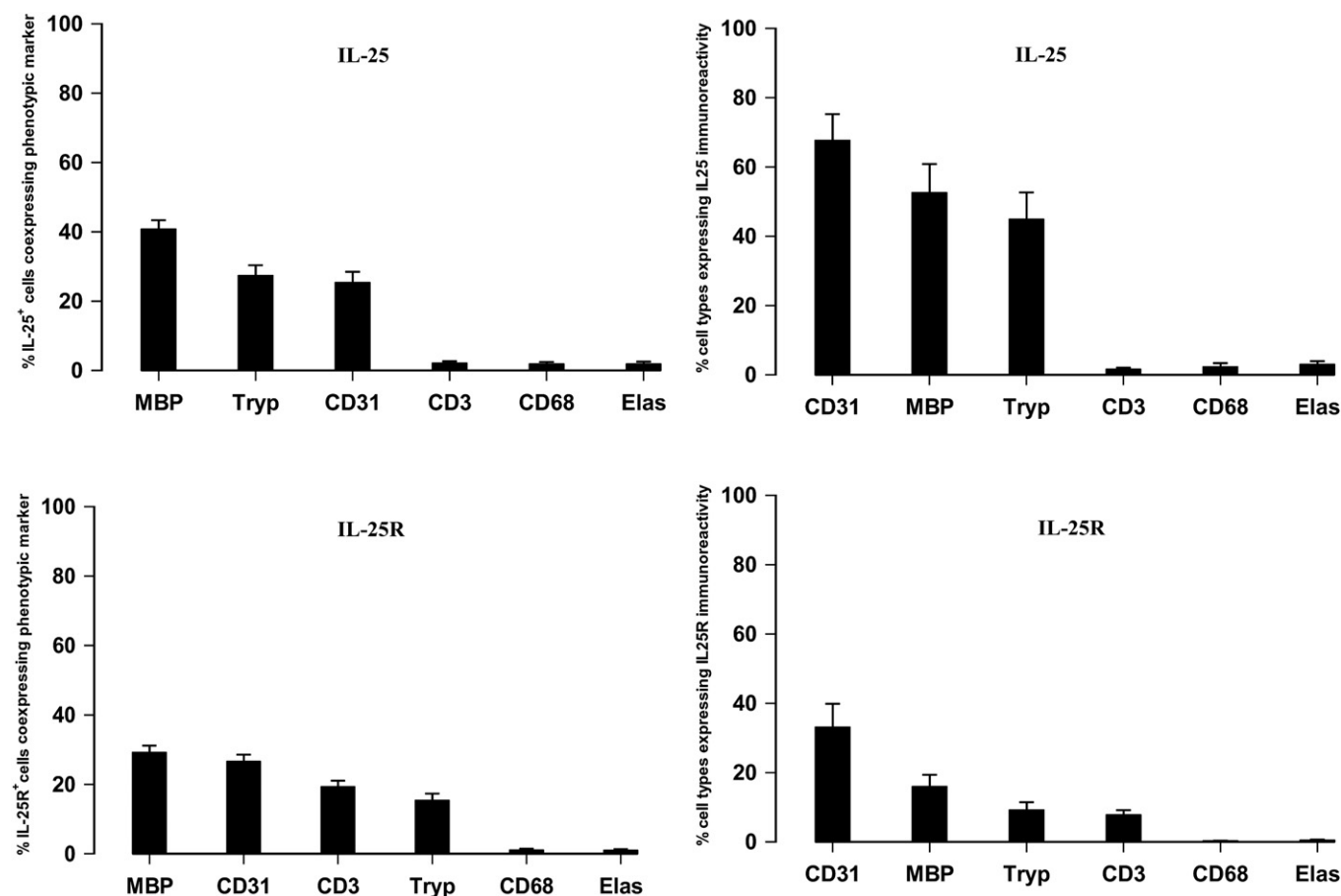


FIG 7. Left panel, Mean ($\pm$ SEM) percentages of the total IL-25⁺ and IL-25R⁺ cells accounted for by cells of the stated phenotypes. Right panel, Percentages of total cells of each phenotype expressing IL-25 and IL-25R immunoreactivity in skin biopsies from 24-hour allergen-challenged sites ($n = 10$). CD3, CD3⁺ T cells; CD31, CD31⁺ endothelial cells; CD68, CD68⁺ macrophages; Elas, elastase⁺ neutrophils; MBP, MBP⁺ eosinophils; Tryp, tryptase⁺ mast cells.

only patients with very mild atopic asthma with allergen). In a separate recent study,¹⁸ however, we have shown that expression of IL-25 but not IL-25R inversely correlated with FEV₁ at baseline in a group of patients with more severe asthma. This and our current observation that submucosal IL-25 but not IL-25R immunoreactive cells correlated inversely with the maximum percentage change in FEV₁ during the late-phase response together suggest a significant functional role for IL-25 in regulating asthma severity through its actions on inflammatory and structural cells. Increased bronchial mucosal IL-25R⁺ immunoreactivity most likely reflected increased expression on local T cells and eosinophils after allergen exposure because the proportions of these cells expressing IL-25R increased, although again, other mechanisms may have contributed. A similar situation pertained in the skin except that dermal mast cells were not increased in this model. We were unable reliably to measure percentages of cells showing IL-25⁺ and IL-25R⁺ immunoreactivity in the skin before allergen exposure because of the very small numbers of some of these cells. Although it was not possible to measure increases in IL-25 expression synchronously with the peak magnitudes of the late-phase allergic reactions in the bronchi and skin, the fact that a correlation was observed between the 2 supports the hypothesis that IL-25 plays some role of itself in the genesis of

late-phase allergic inflammation. This is congruent with animal studies⁷ in which it was reported that exogenous IL-25 induced airways inflammation and hypersensitivity even in gene-deleted mice bereft of the classic T_H2 cytokines (*il4*^{-/-}*il5*^{-/-}*il9*^{-/-}*il13*^{-/-} mice). Allergen-induced overall production of IL-25 rather than its production by specific cells may be more important in the pathogenesis of allergic inflammation.

Although these experiments show cells with the potential to secrete IL-25 and/or respond to it, they do not gauge its production by individual cells: immunohistochemical analysis is well known preferentially to detect cells that store cytokines in granules, rather than cells that may be synthesizing and actively secreting them but not storing them. Nevertheless, our data clearly earmark eosinophils, mast cells, and endothelial cells as well as T cells, but not neutrophils or macrophages, as potential sources as well as targets of IL-25 in acute exacerbation of allergic inflammation. Although these cells accounted for most of the IL-25⁺ and IL-25R⁺ immunoreactivity observed in the subepidermal regions of both organs, contribution from other cell types cannot be ruled out (basophils, for example, are known to express IL-25,¹⁹ but we did not have suitable antibodies with which to identify these in double, sequential immunohistochemistry experiments).

In addition to leukocytes and endothelial cells, we have identified epidermal cells as a reservoir of IL-25 immunoreactivity. Similarly, increased IL-25 expression in epidermal keratinocytes was reported in a study of atopic dermatitis²⁰ in which the authors also showed that IL-25 inhibited filaggrin synthesis by these cells (although we were unable to detect short-term decreases in filaggrin expression after allergen challenge of “virgin” skin in atopic subjects²¹). These studies further suggest a role for IL-25 in environmental interactions with allergens not only in initiating and reawakening Th2 inflammatory responses but also in regulating factors such as mucosal permeability. In further support of this, recent studies in animals suggest that allergens, and particularly those with protease activity, induce IL-25 (as well as thymic stromal lymphopoietin) production in primary lung epithelial cells.²² In addition, it was shown that matrix metalloproteinases may regulate the biological function of IL-25.²³ These data together suggest that different types of allergen, differing routes for allergen exposure, and many other undefined factors may affect the expression of molecules involved in the pathogenesis of asthma. In the current study, we did not observe marked variation in the expression of IL-25/IL-25R induced by different challenge allergens, although the study was neither designed nor powered to detect such differences.

Our data re-emphasize the importance of IL-25 as central mediator of allergic and asthmatic inflammation and open new vistas of investigation into its range of activities. Here we extend our preliminary data⁹ in confirming eosinophils as a potential source of IL-25. A recent study of Churg-Strauss vasculitis²⁴ also confirms this and implicates eosinophil-derived IL-25 as a prime activator of effector Th2 T cells in this disease. In addition, IL-25 has been shown to increase the survival and adhesion of human eosinophils²⁵ and increase their antitumor activity secondary to increased tumor infiltration.²⁶ These findings strongly echo the positive T-cell/eosinophil/T-cell feedback loop that we postulate drives allergic inflammation, and once again suggest that IL-25 may be more important than established cytokines such as IL-5 in this regard. In contrast, there is little direct evidence for a direct effect of IL-25 on neutrophils; these cells have not been shown to express IL-25R or respond to IL-25, and our own data concur with this. Although administration of IL-25 was reported to induce infiltration of neutrophils in a mouse model,²⁷ the potential mechanisms remain to be clearly identified.

Our observation that both IL-25 and its receptor are expressed by significant numbers of capillary vascular endothelial cells within the bronchial submucosa and dermis is to our knowledge the first such demonstration in human beings. Interestingly, IL-25 was reported to be expressed by brain capillary endothelial cells in mice, where it was postulated to play a role in maintaining the integrity of the blood-brain barrier.²⁸ In view of further reports^{29,30} of IL-25R expression on human airways smooth muscle cells and lung fibroblasts, we speculate that IL-25 also has a significant role to play in the regulation of capillary permeability in allergic inflammation and in airways remodeling changes effected by smooth muscle cells and fibroblasts in patients with asthma.

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Clinical implications: IL-25 produced in response to allergen challenge of atopic subjects may be a key molecular target to reduce local inflammation and anamnestic immunologic responses to allergen.

REFERENCES

- Dong C. Regulation and pro-inflammatory function of interleukin-17 family cytokines. *Immunol Rev* 2008;226:80-6.
- Fort MM, Cheung J, Yen D, Li J, Zurawski SM, Lo S, et al. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. *Immunity* 2001;15: 985-95.
- Hurst SD, Muchamuel T, Gorman DM, Gilbert JM, Clifford T, Kwan S, et al. New IL-17 family members promote Th1 or Th2 responses in lung: in vivo function of the novel cytokine IL-25. *J Immunol* 2002;169:443-53.
- Pan G, French D, Mao W, Maruoka M, Risser P, Lee J, et al. Forced expression of murine IL-17E induces growth retardation, jaundice, a Th2-biased response, and multiorgan inflammation in mice. *J Immunol* 2001;167: 6559-67.
- Kim MR, Manoukian R, Yeh R, Silbiger SM, Danilenko DM, Scully S, et al. Transgenic overexpression of human IL-17E results in eosinophilia, B-lymphocyte hyperplasia, and altered antibody production. *Blood* 2002;100:2330-40.
- Tamachi T, Maezawa Y, Ikeda K, Kagami S, Hatano M, Seto Y, et al. IL-25 enhances allergic airway inflammation by amplifying a Th2 cell-dependent pathway in mice. *J Allergy Clin Immunol* 2006;118:606-14.
- Ballantyne SJ, Barlow JL, Jolin HE, Nath P, Williams AS, Chung KF, et al. Blocking IL-25 prevents airway hyperresponsiveness in allergic asthma. *J Allergy Clin Immunol* 2007;120:1324-31.
- Angkasekwinai P, Park H, Wang YH, Wang YH, Chang SH, Corry DB, et al. Interleukin 25 promotes the initiation of proallergic type 2 responses. *J Exp Med* 2007;204:1509-17.
- Wang YH, Angkasekwinai P, Lu N, Voo KS, Arima K, Hanabuchi S, 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;204:1837-47.
- Wong CK, Cheung PF, Ip WK, Lam CW. Interleukin 25-induced chemokines and interleukin-6 release from eosinophils is mediated p38 mitogen-activated protein kinase, c-Jun, N-terminal kinase, and nuclear factor-kappa B. *Am J Respir Cell Mol Biol* 2005;33:186-94.
- Ikeda K, Nakajima H, Suzuki K, Kagami S, Hirose K, Suto A, et al. Mast cells produce interleukin-25 upon Fc epsilon RI-mediated activation. *Blood* 2003;101: 3594-6.
- Wang YH, Liu YJ. Thymic stromal lymphopoietin, OX40-ligand, and interleukin-25 in allergic responses. *Clin Exp Allergy* 2009;39:798-806.
- Kay AB. Allergy and allergic diseases. *N Engl J Med* 2001;344:30-7.
- Phipps S, Benyahia F, Ou TT, Barkans J, Robinson DS, Kay AB. Acute allergen-induced airway remodelling in atopic asthma. *Am J Respir Cell Mol Biol* 2004;31: 626-32.
- Corrigan CJ, Jayaratnam A, Wang Y, Liu Y, de Waal Malefyt R, Meng Q, et al. Early production of thymic stromal lymphopoietin precedes infiltration of dendritic cells expressing its receptor in allergen-induced late phase cutaneous responses in atopic subjects. *Allergy* 2009;64:1014-22.
- Ying S, O'Connor B, Ratoff J, Meng Q, Mallett K, Cousins D, et al. Thymic stromal lymphopoietin (TSLP) expression is increased in asthmatic airways correlates with expression of Th2-attracting chemokines and disease severity. *J Immunol* 2005;174:8183-90.
- Ying S, O'Connor B, Ratoff J, Meng Q, Fang C, Cousins D, et al. Expression and cellular provenance of thymic stromal lymphopoietin and chemokines in patients with severe asthma and chronic obstructive pulmonary disease. *J Immunol* 2008; 181:2790-8.
- Corrigan CJ, Wang W, Meng Q, Fang CL, Lv Z, Fan YQ, et al. T-helper cell type 2 (Th2) memory T cell-potentiating cytokine 25 has the potential to promote angiogenesis in asthma. *Proc Natl Acad Sci U S A* 2011;108:1579-84.
- Schneider E, Thieblemont N, De Moraes ML, Dy M. Basophils: new players in the cytokine network. *Eur Cytokine Netw* 2010;21:142-53.
- Hvid M, Vestergaard C, Kemp K, Christensen GB, Deleuran B, Deleuran M. IL-25 in atopic dermatitis: a possible link between inflammation and skin barrier dysfunction? *J Invest Dermatol* 2010;131:150-7.
- Ying S, Meng Q, Corrigan CJ, Lee TH. Lack of filaggrin expression in the human bronchial mucosa. *J Allergy Clin Immunol* 2006;118:1386-8.
- Yu HS, Angkasekwinai P, Chang SH, Chung Y, Dong C. Protease allergens induce the expression of IL-25 via Erk and p38 MAPK pathway. *J Korean Med Sci* 2010; 25:829-34.

23. Goswami S, Angkasekwinai P, Shan M, Greenlee KJ, Barranco WT, Polikepahad S, et al. Divergent functions for airway epithelial matrix metalloproteinase 7 and retinoic acid in experimental asthma. *Nat Immunol* 2009;10:496-503.
24. Terrier B, Bièche I, Maisonnobe T, Laurendeau I, Rosenzweig M, Kahn JE, et al. IL-25: a cytokine linking eosinophils and adaptive immunity in Churg-Strauss syndrome. *Blood* 2010;116:4523-31.
25. Cheung PF, Wong CK, Ip WK, Lam CW. IL-25 regulates the expression of adhesion molecules on eosinophils: mechanism of eosinophilia in allergic inflammation. *Allergy* 2006;61:878-85.
26. Benatar T, Cao MY, Lee Y, Li H, Feng N, Gu X, et al. Virulizin induces production of IL-17E to enhance antitumor activity by recruitment of eosinophils into tumors. *Cancer Immunol Immunother* 2008;57:1757-69.
27. Claudio E, Sønder SU, Saret S, Carvalho G, Ramalingam TR, Wynn TA, et al. The adaptor protein CIKS/Act1 is essential for IL-25-mediated allergic airway inflammation. *J Immunol* 2009;182:1617-30.
28. Sonobe Y, Takeuchi H, Kataoka K, Li H, Jin S, Mimuro M, et al. Interleukin-25 expressed by brain capillary endothelial cells maintains blood-brain barrier function in a protein kinase Cepsilon-dependent manner. *J Biol Chem* 2009;284:31834-42.
29. Lajoie-Kadoch S, Joubert P, Létuvé S, Halayko AJ, Martin JG, Soussi-Gounni A, et al. TNF-alpha and IFN-gamma inversely modulate expression of the IL-17E receptor in airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2006;290:L1238-46.
30. Létuvé S, Lajoie-Kadoch S, Audusseau S, Rothenberg ME, Fiset PO, Ludwig MS, et al. IL-17E upregulates the expression of proinflammatory cytokines in lung fibroblasts. *J Allergy Clin Immunol* 2006;117:590-6.