

IL-25 enhances allergic airway inflammation by amplifying a T_H2 cell-dependent pathway in mice

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Background: A novel IL-17 family cytokine, IL-25, has been reported to induce IL-4, IL-5, and IL-13 production from undefined non-T/non-B cells and then induce T_H2-type immune responses. However, the roles of IL-25 in inducing allergic airway inflammation remain unknown.

Objective: We sought to determine whether IL-25 is involved in causing allergic airway inflammation.

Methods: We examined the expression of IL-25 mRNA in the lungs of sensitized mice on antigen inhalation. We also examined the effect of IL-25 neutralization by soluble IL-25 receptor on antigen-induced airway inflammation. We then generated IL-25 transgenic mice that express IL-25 specifically in the lung under the control of the Clara cells–10-kd protein promoter and investigated the effect of enforced IL-25 expression on antigen-induced airway inflammation.

Results: IL-25 mRNA was expressed in the lungs of sensitized mice on antigen inhalation, and the neutralization of IL-25 by soluble IL-25 receptor decreased antigen-induced eosinophil and CD4⁺ T-cell recruitment into the airways. The enforced expression of IL-25 in the lung itself failed to induce allergic airway inflammation, whereas the expression of IL-25 significantly enhanced antigen-induced T_H2 cytokine production, eosinophil and CD4⁺ T cell recruitment, and goblet cell hyperplasia in the airways. Moreover, IL-25-induced enhancement of allergic airway inflammation was inhibited by the depletion of CD4⁺ T cells or by the absence of signal transducer and activator of transcription 6.

Conclusion: IL-25 enhances antigen-induced allergic airway inflammation by amplifying a T_H2 cell-dependent pathway. **Clinical implications:** IL-25 might be involved in the enhancement, prolongation, or both of T_H2 cell-mediated allergic diseases, such as asthma. (J Allergy Clin Immunol 2006;117:1016–1021.)

Key words: Allergic inflammation, IL-25, eosinophils, T_H2 cells

Allergic airway inflammation is a pathognomonic feature of asthma that is characterized by intense eosinophil and CD4⁺ T-cell infiltrates in the airways, mucus hypersecretion, airway remodeling, and airway hyperreactivity.^{1–4} It is well known that antigen-induced allergic airway inflammation is mediated by T_H2 cells and their cytokines, IL-4, IL-5, and IL-13.^{1–4} It has been shown that IL-5 mediates antigen-induced eosinophil recruitment into the airways of sensitized mice.^{5,6} It has also been shown that IL-13 is a key cytokine that induces goblet cell hyperplasia, airway remodeling, and airway hyperreactivity.^{7,8}

IL-25 is a recently cloned, T_H2 cell-derived cytokine that is structurally related to IL-17⁹ and is produced by activated T_H2 cells⁹ and mast cells.¹⁰ The biologic activities of IL-25 are markedly different from those described for IL-17 and other IL-17 family cytokines.^{9,11–15} Systemic administration of IL-25 protein⁹ or the systemic expression of IL-25 by transgene¹¹ induces the production of IL-4, IL-5, and IL-13 from undefined non-T/non-B cells and the resultant T_H2-type immune responses, including increased serum IgE levels, blood eosinophilia, and pathologic changes in the lung and other tissues.^{9,11} In contrast, other IL-17 family cytokines induce the production of IL-1β and TNF-α and neutrophilia.^{11,14,15} In addition, it has been shown that IL-25–responding non-T/non-B cells are lineage-negative accessory cells expressing class II MHC molecules,^{9,11} although the exact cell types have not yet been identified. These findings suggest that IL-25 might be involved in causing allergic inflammation. However, the regulatory roles of IL-25 in inducing allergic airway inflammation remain unknown.

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Abbreviations used

BALF: Bronchoalveolar lavage fluid
 CC10: Clara cells–10-kd protein
 IL-25R: IL-25 receptor
 OVA: Ovalbumin
 PBST: PBS containing 0.05% Tween 20
 sIL-25R: Soluble IL-25 receptor
 Stat6: Signal transducer and activator of transcription 6
 TARC: Thymus and activation-regulated chemokine
 WT: Wild-type

Therefore in this study we determined whether IL-25 is involved in causing allergic airway inflammation. We found that IL-25 mRNA was expressed in the lungs of antigen-sensitized and antigen-inhaled mice and that neutralization of endogenously produced IL-25 by soluble IL-25 receptor decreased antigen-induced eosinophil and CD4⁺ T-cell recruitment into the airways. The enforced expression of IL-25 in the lung itself failed to induce allergic airway inflammation in Clara cells–10-kd protein (CC10) IL-25 mice that express IL-25 specifically in the lung, whereas the expression of IL-25 significantly enhanced antigen-induced T_H2 cytokine production and eosinophil and CD4⁺ T-cell recruitment in the airways. Moreover, IL-25-induced enhancement of antigen-induced eosinophil recruitment into the airways was inhibited by the depletion of CD4⁺ T cells or by the absence of signal transducer and activator of transcription 6 (Stat6). Our results indicate that IL-25 plays an important role in enhancing antigen-induced allergic airway inflammation by amplifying a T_H2 cell-dependent pathway, but IL-25 itself does not significantly induce allergic inflammation.

METHODS**Generation of CC10 IL-25 mice**

Expression vector for IL-25 (BCMGS Flag–IL-25) was described previously.¹⁶ The DNA fragment coding IL-25 was subcloned into the *NdeI/BglII* site of transgenic construct pCC10-SV40 (a kind gift from Dr R. Flavell, Yale University School of Medicine)¹⁷ to generate pCC10–IL-25–SV40. Transgenic mice (CC10 IL-25 mice) were generated by using standard procedures with pCC10–IL-25–SV40. CC10 IL-25 mice were genotyped by means of PCR and backcrossed to BALB/c mice (Charles River Laboratories, Atsugi, Japan) for 7 generations. In some experiments CC10 IL-25 mice were crossed with Stat6^{−/−} mice¹⁸ to obtain CC10 IL-25 Stat6^{−/−} mice. All mice were housed in microisolator cages under pathogen-free conditions, and all experiments were performed according to the guidelines of Chiba University.

Antigen-induced allergic inflammation in the airways

Mice (age 7–8 weeks) were immunized intraperitoneally with 4 μ g of ovalbumin (OVA) in 4 mg of aluminum hydroxide. Two weeks after the immunization, the sensitized mice were twice administered aerosolized OVA (50 mg/mL) dissolved in 0.9% saline through a DeVilbiss 646 nebulizer (DeVilbiss Corp, Somerset, Pa) for 20 minutes at a 48-hour interval. As a control, 0.9% saline alone was

administered through the nebulizer. Where indicated, mice were injected intraperitoneally with anti-CD4 antibody (clone GK1.5, 1 mg/mouse; BD PharMingen, San Diego, Calif) or isotype-matched control antibody (BD PharMingen) at 24 hours before the first OVA inhalation. Forty-eight hours after the last inhalation, a sagittal block of the left lung was excised, fixed in 10% buffered formalin, and embedded in paraffin. Lung sections (3- μ m thick) were stained with hematoxylin and eosin and periodic acid–Schiff according to standard protocols. The number of goblet cells was counted on periodic acid–Schiff–stained lung sections, as described elsewhere.¹⁹ The number of eosinophils and CD4⁺ T cells recovered in the bronchoalveolar lavage fluid (BALF) was evaluated as described previously.²⁰

Cytokine levels in BALF

The amounts of IL-4, IL-5, IL-13, and IFN- γ in the BALF were determined by using an enzyme immunoassay, as described previously.²⁰ The detection limits of these assays were 15 pg/mL for IL-4 and IL-5, 50 pg/mL for IFN- γ , and 30 pg/mL for IL-13.

ELISA for IL-25

ELISA plates were coated with monoclonal anti-mouse IL-25 antibody (50 mg/mL; R&D Systems, Minneapolis, Minn) for 16 hours at 4°C, washed 3 times with PBS containing 0.05% Tween 20 (PBST), and blocked with PBS containing 10% FCS (blocking buffer). After washing with PBST, samples were added to the wells and incubated for 90 minutes at room temperature. As a standard, serial dilutions of recombinant mouse IL-25 (R&D Systems) were used. After washing, biotinylated anti-mouse IL-25 antibody (300 ng/mL in blocking buffer, R&D Systems) was added to each well and incubated at room temperature for 2 hours. Wells were washed with PBST, incubated with avidin–horseradish peroxidase, and then developed with substrate solution according to the manufacturer's instructions (BD PharMingen). The detection limit of this assay was 300 pg/mL IL-25.

RT-PCR

Total cellular RNA was prepared from various tissues, and RT-PCR analysis for IL-25 was performed as described previously.¹⁰ Primer pairs for Muc5AC and thymus and activation-regulated chemokine (TARC) were described previously.^{16,21} The following primers were used for eotaxin: sense primer, CAACAGATGCACCCTGAAAGC; antisense primer, TCCCTCAGAGCACGCTCTTAGG. RT-PCR for β -actin was performed to control the sample-to-sample variation in RNA isolation and integrity, RNA input, and reverse transcription. All PCR amplifications were performed at least 3 times with multiple sets of experimental RNAs.

Taqman PCR analysis

Expression of IL-25 mRNA was determined by means of real-time quantitative Taqman PCR with a standard protocol on an ABI PRISM 7000 instrument (Applied Biosystems, Foster City, Calif). PCR primers and a fluorogenic probe were described previously.¹⁰ The levels of IL-25 were normalized to the levels of GAPDH mRNA (Applied Biosystems).

Preparation of soluble IL-25 receptor

DNA fragment coding the extracellular domain of murine IL-25 receptor (IL-25R; a kind gift from Dr J. D. Shaughnessy, University of Arkansas for Medical Sciences)²² was fused to the fragment coding an Fc portion of human IgG1 by means of PCR, as described previously,²³ and then cloned into expression vector pCDNA3 (pCDNA3 soluble IL-25R [sIL-25R]). The construction was confirmed by means of DNA sequencing. CHO cells that stably express pCDNA3 sIL-25R (sIL-25R CHO cells) were obtained by using a

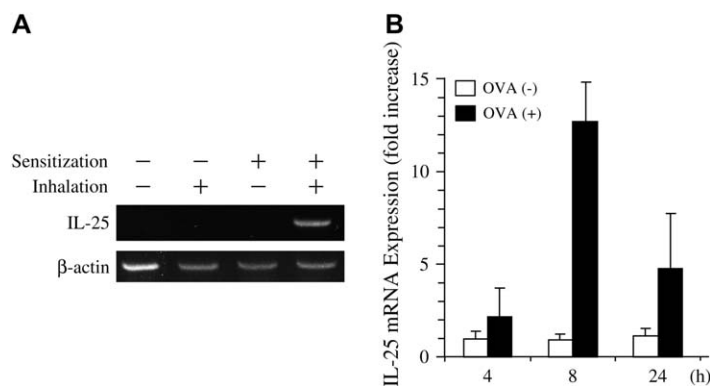


FIG 1. IL-25 mRNA is expressed in the lungs of sensitized mice on antigen inhalation. **A**, BALB/c mice were immunized with or without OVA in aluminum hydroxide, and 2 weeks after the immunization, mice were challenged twice with inhaled OVA or saline at a 48-hour interval. Twenty-four hours after the last OVA inhalation, total RNA was prepared from the lung tissues, and RT-PCR for IL-25 mRNA, as well as β -actin mRNA (as a control), was performed. Shown are representative data from 5 independent experiments. **B**, Total RNA was prepared from lung tissues of OVA-sensitized BALB/c mice at the indicated time after the last OVA or saline inhalation. Real-time PCR analysis for IL-25 mRNA, as well as GAPDH (as a control) mRNA, was performed. The levels of IL-25 mRNA are normalized to the levels of GAPDH mRNA and then expressed as a fold increase to the baseline level (without the inhalation). Data are presented as means $\pm$ SD from 5 experiments.

standard protocol with FuGENE6 transfection reagent (Roche Diagnostics, Indianapolis, Ind), and sIL-25R CHO cells were cultured in CELLLine AD1000 (INTEGRA Biosciences AG, Chur, Switzerland) to obtain the concentrated sIL-25R in culture supernatants. The supernatants of sIL-25R CHO cells were collected, and sIL-25R was purified by using a HiTrap protein G HP column (Amersham Biosciences, Piscataway, NJ).

Measurement of neutralizing activity of sIL-25R on IL-25

X63 cells that stably express BCMGS Flag-IL-25 were generated (X63 IL-25 cells), and the supernatants of X63 IL-25 cells were used as a source of IL-25.¹⁶ The supernatants of empty vector (BCMGS neo)-transfected X63 cells or those of IL-3-producing X63 cells (X63 IL-3 cells)²⁴ were used as negative and positive controls, respectively. The neutralizing activity of sIL-25R against IL-25 was evaluated by measuring the effect of sIL-25R on IL-25-induced proliferation of Ba/F3 IL-25R-MPL cells, which stably express a fusion protein of an extracellular domain of IL-25R and a transmembrane and cytoplasmic domain of MPL.¹⁶ Proliferation of Ba/F3 IL-25R-MPL cells was measured by using CellTiter-Glo reagent, according to the manufacturer's instructions (Promega, Madison, Wis).

Effect of sIL-25R on antigen-induced airway inflammation

BALB/c mice were immunized intraperitoneally with OVA-alum and then challenged with inhaled OVA twice at 14 and 16 days after immunization. Where indicated, the sensitized mice were injected intraperitoneally with purified sIL-25R (100, 300, or 900 mg) or human IgG1 (900 mg; Chemicon Inc, Temecula, Calif) at 8 hours before each OVA inhalation. The number of eosinophils and CD4⁺ T cells recovered in the BALF, the levels of cytokines in the BALF, and the number of goblet cells in the airways were evaluated at 48 hours after the last OVA inhalation.

Data analysis

Data are summarized as means $\pm$ SD. The statistical analysis of the results was performed by using the unpaired *t* test. *P* values of less than .05 were considered significant.

RESULTS

IL-25 mRNA is expressed in the lung of sensitized mice on antigen inhalation

To determine whether a novel T_H2 cytokine, IL-25, is involved in the regulation of allergic airway inflammation, we first examined the expression of IL-25 mRNA in the lungs of sensitized mice on antigen inhalation. BALB/c mice were immunized intraperitoneally with OVA-alum, and 2 weeks later, they were challenged twice with inhaled OVA at a 48-hour interval. We found that IL-25 mRNA was expressed in the lung at 24 hours after the last inhalation (Fig 1, A). In the absence of immunization with OVA or inhaled OVA challenge, no IL-25 mRNA was detected in the lung tissues (Fig 1, A). The induction of IL-25 mRNA by means of antigen inhalation was confirmed by using Taqman PCR analysis, and the peak of the expression was at 8 hours after the inhaled OVA challenge (Fig 1, B).

Neutralization of IL-25 by sIL-25R inhibits antigen-induced eosinophil and CD4⁺ T-cell recruitment into the airways

We next examined the effect of IL-25 neutralization on antigen-induced eosinophil and CD4⁺ T-cell recruitment into the airways. Because no neutralizing antibody against IL-25 or IL-25R is available, we neutralized the bioactivity of IL-25 by using a fusion protein of the extracellular domain of IL-25R and an Fc portion of human IgG1 (sIL-25R). We first estimated the neutralizing activity of sIL-25R against IL-25 by using a system in which the bioactivity of IL-25 was evaluated on the basis of the proliferation of BA/F3 IL-25R-MPL cells that stably express a fusion protein of an extracellular domain of IL-25R and a transmembrane and cytoplasmic domain of thrombopoietin receptor MPL.¹⁶ IL-25 induced proliferation of BA/F3

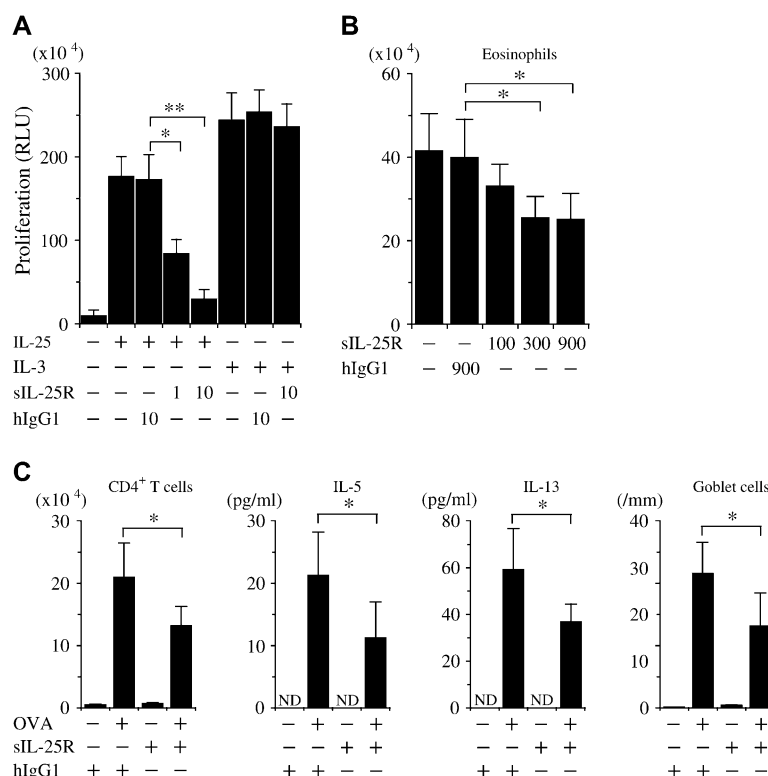


FIG 2. Neutralization of IL-25 by sIL-25R inhibits antigen-induced eosinophil and CD4⁺ T-cell recruitment into the airways. **A**, Ba/F3 IL-25R-MPL cells that stably express IL-25R-MPL fusion protein were cultured with X63-IL-25 or X63-IL-3 conditioning medium (10% vol/vol). Where indicated, sIL-25R (1 or 10 μ g/mL) and human IgG1 (*hIgG1*; 10 μ g/mL [as a control]) were added to the culture. Forty-eight hours later, the proliferation of Ba/F3 IL-25R-MPL cells was evaluated by using CellTiter-Glo reagent. The proliferation is expressed as a relative light unit (RLU). Data are presented as means $\pm$ SD from 4 independent experiments. Significantly different from the mean value of control response: * P < .01, ** P < .001. **B**, BALB/c mice were immunized with OVA-alum and then challenged with inhaled OVA. sIL-25R (100, 300, or 900 μ g per mouse) or human IgG1 (900 μ g per mouse) was injected intraperitoneally twice at 8 hours before each OVA inhalation. The number of eosinophils in BALF was evaluated at 48 hours after the last OVA inhalation. Data are presented as means $\pm$ SD for 6 mice in each group. * P < .05. **C**, OVA-sensitized BALB/c mice were challenged twice with inhaled OVA or saline. sIL-25R (900 μ g per mouse) or human IgG1 (900 μ g per mouse) was injected intraperitoneally twice at 8 hours before each OVA inhalation. The number of CD4⁺ T cells and the levels of IL-5 and IL-13 in the BALF and the number of goblet cells in the airway were evaluated at 48 hours after the last OVA inhalation. Data are presented as means $\pm$ SD for 6 mice in each group. * P < .05.

IL-25R-MPL cells (Fig 2, A) but not of parent BA/F3 cells (data not shown), whereas IL-3 induced proliferation of BA/F3 IL-25R-MPL cells (Fig 2, A) and parent BA/F3 cells (data not shown). The addition of sIL-25R inhibited IL-25-induced proliferation of BA/F3 IL-25R-MPL cells in a dose-dependent manner (Fig 2, A). As expected, sIL-25R did not inhibit IL-3-induced proliferation of BA/F3 IL-25R-MPL cells (Fig 2, A). These results suggest that sIL-25R is able to neutralize the bioactivity of IL-25.

We therefore examined the effect of sIL-25R on antigen-induced airway inflammation in sensitized mice. OVA-sensitized mice were injected intraperitoneally with sIL-25R or human IgG1 and then challenged with inhaled OVA. The administration of sIL-25R significantly inhibited antigen-induced eosinophil recruitment into the airways in a dose-dependent manner (n = 8 mice in each group, P < .05; Fig 2, B). The administration of sIL-25R also significantly inhibited antigen-induced CD4⁺ T-cell

recruitment into the airways (n = 6, P < .05; Fig 2, C). Moreover, sIL-25R inhibited antigen-induced IL-5 and IL-13 production and goblet cell hyperplasia in the airways (n = 6, P < .05; Fig 2, C). IL-4 was undetectable in the BALF in both antigen-inhaled control IgG1-treated mice and sIL-25R-treated mice (data not shown). These results suggest that endogenously produced IL-25 is involved in T_H2-type responses in the airways on antigen inhalation.

Generation of CC10 IL-25 mice that express IL-25 specifically in the lung

To address the mechanisms underlying IL-25-mediated allergic airway inflammation, we generated CC10 IL-25 mice that express murine IL-25 specifically in the lung under the control of rat CC10 promoter. We obtained 5 lines of CC10 IL-25 mice, and all of the offspring expressed IL-25 mRNA in the lung but not in the liver

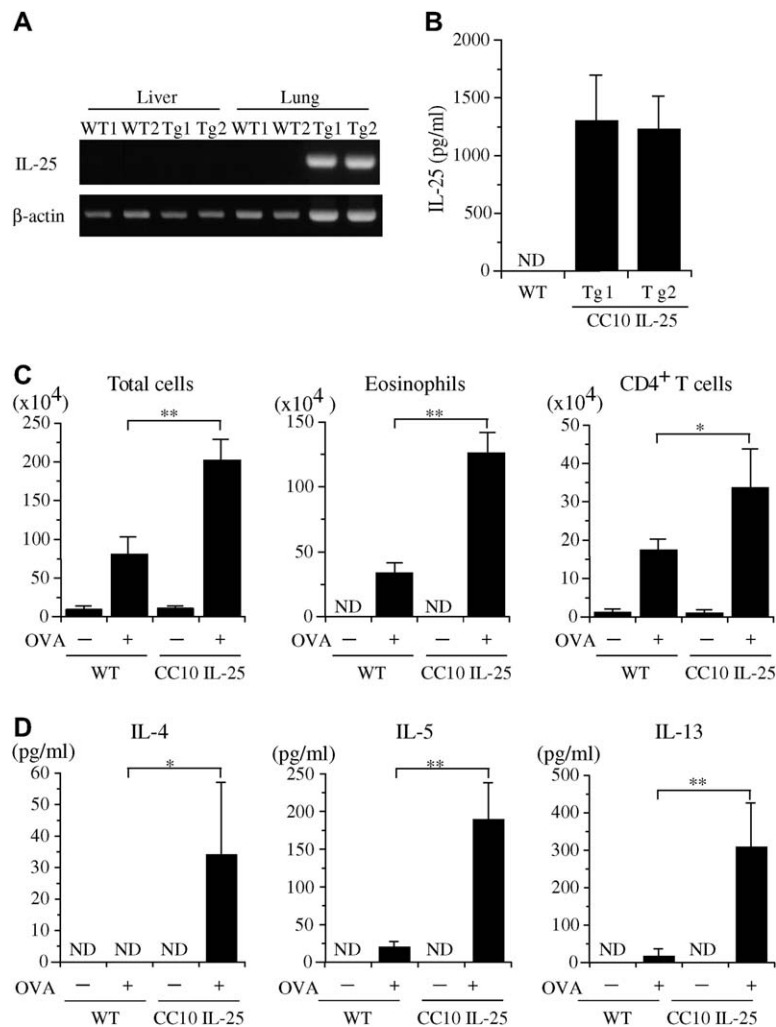


FIG 3. Antigen-induced eosinophil and CD4⁺ T-cell recruitment and TH2 cytokine production in the airways are enhanced in CC10 IL-25 mice. **A**, Total RNA was prepared from lung or liver tissues of CC10 IL-25 mice (transgenic line 1 [Tg1] and transgenic line 2 [Tg2]) and WT littermates (WT1 and WT2). RT-PCR for IL-25 mRNA, as well as β -actin mRNA (as a control), was performed. Shown are representative data from 5 independent experiments. Similar results were obtained in lines 3, 4, and 5 of CC10 IL-25 mice (data not shown). **B**, CC10 IL-25 mice (transgenic line 1 [Tg1] and transgenic line 2 [Tg2]) and littermate WT mice were subjected to bronchoalveolar lavage, and the amounts of IL-25 in the BALF were determined by means of ELISA. Data are presented as means $\pm$ SD for 5 mice in each group. ND, Not detectable. **C** and **D**, CC10 IL-25 mice (transgenic line 1) and littermate WT mice were immunized with OVA-alum, and 14 days and 16 days after the immunization, mice were challenged with inhalation of OVA or saline (as a control). Forty-eight hours after the last inhalation, the numbers of total cells, eosinophils, and CD4⁺ T cells (Fig 3, C), as well as the levels of IL-4, IL-5, and IL-13 (Fig 3, D), in the BALF were evaluated. Data are presented as means $\pm$ SD for 6 to 10 mice in each group. * $P < .05$, ** $P < .01$.

(Fig 3, A, and data not shown). In addition, the secretion of IL-25 protein into the BALF was confirmed in CC10 IL-25 mice by means of immunoassay ($n = 5$; Fig 3, B), whereas IL-25 was undetectable in the sera of CC10 IL-25 mice. CC10 IL-25 mice were fertile and did not show any gross phenotypic abnormalities, including lungs under specific pathogen-free housing conditions (data not shown). In addition, no apparent abnormalities were found in cell populations in the thymus, spleen, and peripheral blood in CC10 IL-25 mice (data not shown). Because the expression levels of IL-25 mRNA in the lung were

similar among 5 lines of CC10 IL-25 mice, we used transgenic line 1 in the following experiments.

Lung-specific expression of IL-25 enhances antigen-induced eosinophil and CD4⁺ T-cell recruitment into the airways

We next investigated the effect of enforced expression of IL-25 in the airways on antigen-induced eosinophil and CD4⁺ T-cell recruitment into the airways. CC10 IL-25 mice and littermate wild-type (WT) mice were immunized with OVA-alum and then challenged twice with inhaled

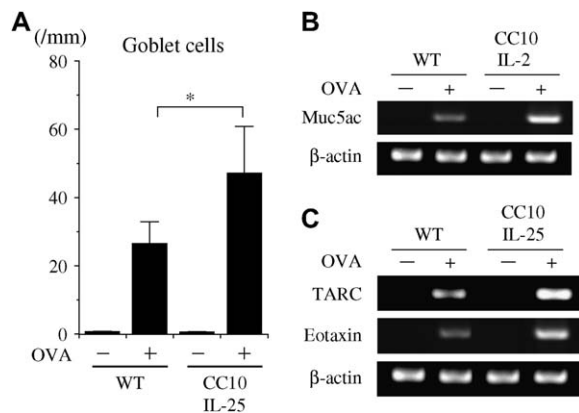


FIG 4. Antigen-induced goblet cell hyperplasia and chemokine production in the airways are enhanced in CC10 IL-25 mice. **A**, OVA-sensitized CC10 IL-25 mice (transgenic line 1) and littermate WT mice were challenged twice with OVA or saline inhalation. Forty-eight hours after the last OVA or saline inhalation, the number of goblet cells was evaluated on periodic acid-Schiff-stained sections of the lung. Data are presented as means $\pm$ SD for 8 mice in each group. $*P < .01$. **B** and **C**, OVA-sensitized CC10 IL-25 mice and WT mice were challenged with inhaled OVA or saline. Total RNA was prepared from lung tissues, and RT-PCR for Muc5ac and β -actin mRNA (as a control; Fig 4, B), as well as RT-PCR for TARC, eotaxin, and β -actin mRNA (Fig 4, C), was performed. Shown are representative data from 5 independent experiments.

OVA or saline. With inhaled saline challenge, no significant inflammatory cell infiltration was observed in the airways in WT mice and even in CC10 IL-25 mice (Fig 3, C). The inhaled OVA challenge induced eosinophil recruitment into the airways in WT mice and in CC10 IL-25 mice, but the antigen-induced eosinophil recruitment into the airways was significantly more enhanced in CC10 IL-25 mice than that in WT mice (CC10 IL-25 mice: $124.5 \pm 16.0 \times 10^4$ vs WT mice: $32.0 \pm 7.7 \times 10^4$; $n = 10$ mice in each group, $P < .01$; Fig 3, C). Antigen-induced lymphocyte recruitment into the airways was also significantly enhanced in CC10 IL-25 mice compared with that seen in WT mice (CC10 IL-25 mice: $56.4 \pm 18.3 \times 10^4$ vs WT mice: $35.7 \pm 13.3 \times 10^4$; $n = 10$ mice in each group, $P < .05$). FACS analysis of cells in the BALF revealed that the majority of lymphocytes in the BALF were CD4⁺ T cells, and consequently, the number of CD4⁺ T cells was increased by 94% in CC10 IL-25 mice ($n = 6$, $P < .05$; Fig 3, C). Histologic analysis showed that inflammatory cell infiltration in the lung was also significantly enhanced in CC10 IL-25 mice compared with that in WT mice at 48 hours after OVA inhalation (data not shown). Even at 96 hours after the inhaled OVA challenge, the number of eosinophils and CD4⁺ T cells in the BALF was also significantly increased in CC10 IL-25 mice than in WT mice (data not shown). In contrast, the number of neutrophils and macrophages in the BALF of CC10 IL-25 mice was similar to that in WT mice (data not shown). In addition, no significant difference was observed in the levels of OVA-specific IgE between CC10 IL-25 mice and WT mice (data not shown).

Antigen-induced T_H2 cytokine production in the airways is enhanced in CC10 IL-25 mice

We next examined cytokine levels in the BALF of OVA-sensitized CC10 IL-25 mice and WT mice with or without the inhaled OVA challenge. IL-4 and IL-5 levels in the BALF were significantly increased in CC10 IL-25 mice at 48 hours after the inhaled OVA challenge compared with those in WT mice ($n = 6$; $P < .05$ and $P < .01$, respectively; Fig 3, D). IL-13 levels were also increased in the BALF in CC10 IL-25 mice compared with those in WT mice ($n = 6$, $P < .01$; Fig 3, D). On the other hand, a representative T_H1 cytokine, IFN- γ , was undetectable in the BALF of CC10 IL-25 mice and WT mice (data not shown). With inhaled saline challenge, no cytokine production was observed in CC10 IL-25 mice and WT mice ($n = 6$; Fig 3, D). As expected, without OVA sensitization, the inhaled OVA did not significantly induce cytokine production in CC10 IL-25 mice and WT mice (data not shown). These results indicate that the enforced expression of IL-25 in the airways enhances T_H2 cytokine production on antigen inhalation, suggesting that the enhanced antigen-induced eosinophil recruitment in CC10 IL-25 mice results in part from the enhanced T_H2 cytokine production in the airways.

Antigen-induced mucus secretion and chemokine production in the airways are enhanced in CC10 IL-25 mice

Because the levels of IL-13, a key cytokine that induces goblet cell hyperplasia,^{7,8} in the airways were increased in antigen-sensitized, antigen-inhaled CC10 IL-25 mice (Fig 3, D), we next compared the number of epithelial goblet cells in OVA-sensitized CC10 IL-25 mice and WT mice after the inhaled OVA challenge. As shown in Fig 4, A, antigen-induced epithelial goblet cell hyperplasia was significantly increased in CC10 IL-25 mice compared with that in WT mice ($n = 8$ mice each, $P < .01$). In addition, antigen-induced mRNA induction of Muc5ac, one of the mucin genes that are mainly produced by goblet cells in response to IL-13,²⁵ was increased in CC10 IL-25 mice (Fig 4, B). Taken together, these results suggest that IL-25 enhances antigen-induced goblet cell hyperplasia and mucus production in the airways.

Given that T_H2 cytokines are also involved in the expression of a number of chemokines,²⁶ we next examined the expression of chemokines in the lungs of antigen-sensitized, antigen-inhaled CC10 IL-25 mice. The expression of TARC, as well as eotaxin, was significantly enhanced in the lungs of CC10 IL-25 mice (Fig 4, C). These results suggest that the increased chemokine expression might also be involved in the enhanced antigen-induced allergic airway inflammation in CC10 IL-25 mice.

CD4⁺ T cells are required for the enhanced eosinophil recruitment into the airways in CC10 IL-25 mice

To determine whether IL-25-induced enhancement of allergic airway inflammation is mediated by CD4⁺ T cells,

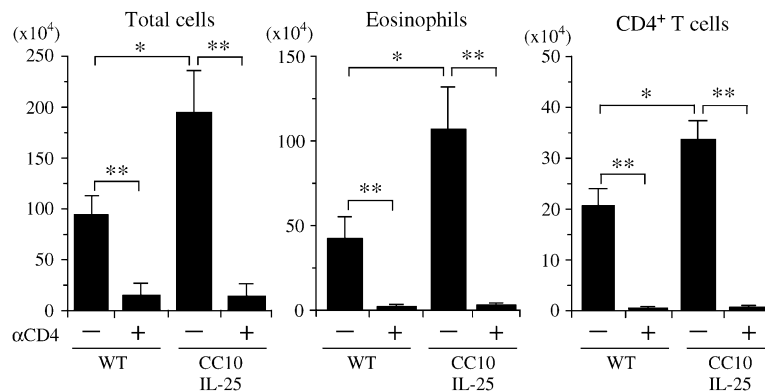


FIG 5. CD4⁺ T cells are required for the enhanced eosinophil recruitment into the airways in CC10 IL-25 mice. OVA-sensitized CC10 IL-25 mice and WT mice were challenged twice with inhaled OVA. Where indicated, mice were injected intraperitoneally with anti-CD4 antibody (1 mg) or control antibody (1 mg) at 24 hours before the first OVA inhalation. The number of total cells, eosinophils, and CD4⁺ T cells in the BALF was evaluated at 48 hours after the last inhalation. Data are presented as means $\pm$ SD for 6 mice in each group. * P < .01, ** P < .001.

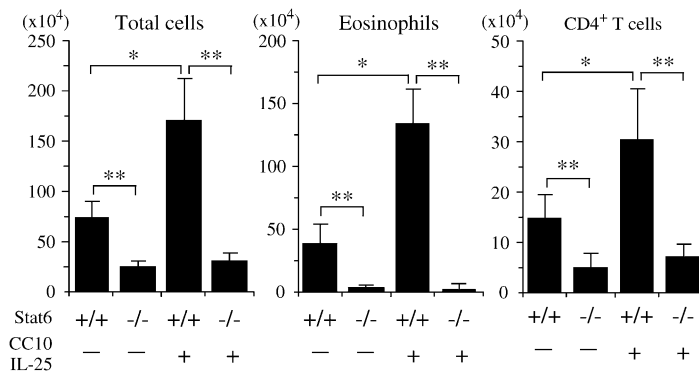


FIG 6. Stat6 is required for the enhanced eosinophil recruitment into the airways in CC10 IL-25 mice. CC10 IL-25 Stat6^{-/-} mice, CC10 IL-25 Stat6^{+/+} mice, and Stat6^{+/+} mice were immunized with OVA-alum and then challenged twice with inhaled OVA. The number of total cells, eosinophils, and CD4⁺ T cells in the BALF was evaluated at 48 hours after the last inhalation. Data are presented as means $\pm$ SD for 4 mice in each group. * P < .01, ** P < .001.

we next examined the effect of CD4⁺ T-cell depletion on antigen-induced eosinophil recruitment into the airways in CC10 IL-25 mice. OVA-sensitized CC10 IL-25 mice or littermate WT mice were injected intraperitoneally with anti-CD4 antibody or control antibody 24 hours before the first OVA inhalation and then challenged with the inhaled OVA. Consistent with data shown in Fig 3, C, the number of eosinophils in the BALF at 48 hours after the inhaled OVA challenge was significantly increased in CC10 IL-25 mice compared with WT mice when these mice were administered with control antibody ($n = 6$, $P < .01$, Fig 5). Importantly, the number of eosinophils recovered in the BALF was significantly decreased by the depletion of CD4⁺ T cells not only in WT mice but also in CC10 IL-25 mice ($n = 6$, $P < .001$, Fig 5). As expected, the number of CD4⁺ T cells in the BALF was near zero in mice administered anti-CD4 antibody ($n = 6$, Fig 5). The levels of IL-4 and IL-5 in the BALF were also significantly inhibited in WT mice, as well as in CC10 IL-25 mice, by the depletion of CD4⁺ T cells (data not shown). These results indicate that although previous studies

reported that CD4⁺ T cells were not required for IL-25-induced T_H2 cytokine production and subsequent eosinophil-rich inflammation,^{9,11} CD4⁺ T cells are essential for IL-25-induced enhancement of antigen-induced eosinophil recruitment into the airways in our experimental system.

Stat6 is required for the enhanced eosinophil recruitment into the airways in CC10 IL-25 mice

Finally, we examined whether Stat6 is required for IL-25-induced enhancement of antigen-induced eosinophil recruitment into the airways by generating CC10 IL-25 Stat6-deficient (Stat6^{-/-}) mice. CC10 IL-25 Stat6^{-/-} mice, CC10 IL-25 Stat6^{+/+} mice, Stat6^{-/-} mice, and Stat6^{+/+} mice were immunized with OVA-alum and then challenged twice with inhaled OVA at a 48-hour interval. At 48 hours after the last inhalation, the number of eosinophils and CD4⁺ T cells in the BALF was evaluated. Again, the number of eosinophils and CD4⁺ T cells recovered in the BALF was significantly increased in CC10

IL-25 Stat6^{+/+} mice compared with that in Stat6^{+/+} mice ($n = 4$ each, $P < .01$, Fig 6). However, almost no eosinophil and CD4⁺ T-cell recruitment was observed in CC10 IL-25 Stat6^{-/-} mice and Stat6^{-/-} mice, even after antigen inhalation ($n = 4$ each, $P < .001$, Fig 6), suggesting that Stat6 is essential for IL-25-induced enhancement of antigen-induced eosinophil and CD4⁺ T-cell recruitment into the airways.

DISCUSSION

In this study we demonstrate that IL-25 acts in amplifying T_H2 cell-mediated allergic airway inflammation, but IL-25 itself does not significantly induce allergic inflammation *in vivo*. We found that IL-25 was produced at the site of allergic airway inflammation (Fig 1) and that IL-25 actually contributed to the enhancement of allergic airway inflammation, as indicated by the inhibition of antigen-induced eosinophil and CD4⁺ T-cell recruitment into the airways by sIL-25R (Fig 2). However, we also found that the expression of IL-25 in the lung itself did not significantly induce allergic airway inflammation in CC10 IL-25 mice, whereas the expression of IL-25 indeed enhanced antigen-induced T_H2 cytokine production, eosinophil and CD4⁺ T-cell recruitment, and goblet cell hyperplasia in the airways (Figs 3 and 4). Finally, we found that CD4⁺ T cells and Stat6 expression were required for IL-25-induced enhancement of antigen-induced eosinophil recruitment into the airways (Figs 5 and 6). Taken together, these results indicate that IL-25 enhances antigen-induced allergic airway inflammation by amplifying a T_H2 cell-dependent pathway.

We show that IL-25 is produced by antigen inhalation in the lungs of sensitized mice (Fig 1). Hurst et al¹¹ have reported that the expression of IL-25 mRNA is increased during fungal and helminth infection in the lung and gut, respectively. More recently, Letuve et al²⁷ have demonstrated that IL-25 is expressed in the airways of asthmatic patients. Together, these findings suggest that IL-25 is produced not only at the site of fungal or helminth infection but also at the site of allergic airway inflammation. Regarding cell types that produce IL-25, a previous report has demonstrated that T_H2-polarized CD4⁺ T cells express IL-25 mRNA on stimulation.⁹ In addition, we have recently shown that mast cells produce IL-25 on FcεR1-mediated activation.¹⁰ As yet, the cell types that produce IL-25 at the site of allergic airway inflammation remain to be determined.

We also show that IL-25 produced by antigen inhalation enhances antigen-induced allergic airway inflammation in sensitized mice (Fig 2, B and C). We found that the administration of sIL-25R, which neutralizes the bioactivity of IL-25 (Fig 2, A), inhibited antigen-induced allergic inflammation and goblet cell hyperplasia in the airways (Fig 2, B and C), suggesting that endogenously produced IL-25 is involved in antigen-induced T_H2-type immune responses in the airways. On the other hand, although sIL-25R tended to inhibit antigen-induced airway

hyperreactivity, the inhibition did not reach statistical significance in this experimental setting (unpublished observation). Further studies will be necessary to determine the precise role of IL-25 in the regulation of airway hyperreactivity.

We demonstrate that CD4⁺ T cells are required for IL-25-induced enhancement of allergic airway inflammation. We found that the enforced expression of IL-25 in the lung itself, in the absence of antigen challenge, failed to induce T_H2 cytokine production or allergic inflammation in the airways of sensitized mice, whereas the expression of IL-25 significantly enhanced antigen-induced T_H2 cytokine production and eosinophil and CD4⁺ T-cell recruitment in the airways of sensitized mice (Fig 3). We also showed that the depletion of CD4⁺ T cells abolished IL-25-induced enhancement of antigen-induced eosinophil recruitment into the airways of sensitized mice (Fig 5). Furthermore, we showed that the absence of Stat6, a key transcription factor for T_H2 cell development and subsequent T_H2 cell-mediated allergic inflammation,^{1-4,28} similarly diminished IL-25-induced enhancement of antigen-induced eosinophil and CD4⁺ T-cell recruitment into the airways (Fig 6). These results suggest that among CD4⁺ T cells, T_H2 cells are required for IL-25-induced enhancement of allergic airway inflammation.

By contrast, previous studies have demonstrated that the administration of a large amount of IL-25⁹ or the systemic expression of IL-25 by transgene¹¹ itself induces T_H2 cytokine production and inflammation with eosinophil infiltrates in the lung and other tissues, even in the absence of CD4⁺ T cells. The different requirement of CD4⁺ T cells for IL-25-induced allergic inflammation between previous studies and ours could result from the difference in the amount, tissue distribution, or both of IL-25. We speculate that in a situation in which IL-25 is abundant, IL-25 can easily reach IL-25-responding non-T cells and directly induce T_H2 cytokine production from these cells because it has recently been shown that IL-25 itself can expand the as-yet-undefined IL-25-responding non-T cells that secrete T_H2 cytokines.²⁹ On the other hand, in a situation in which IL-25 is in a physiologic amount, IL-25 plays a significant role in inducing allergic inflammation in cooperation with T_H2 cells at effector cell levels (Fig 4, C).²⁷ Therefore it is suggested that in a physiologic setting IL-25 needs antigen-activated CD4⁺ T cells, especially T_H2 cells, to exert its enhancing function on inducing allergic inflammation.

The mechanisms by which CD4⁺ T cells are required for IL-25-induced enhancement of allergic airway inflammation can be explained in several ways. It is possible that T_H2 cell-derived cytokines might be required for the recruitment of undefined IL-25-responding non-T cells that produce T_H2 cytokines, which might not normally exist in the lung, into the site of allergic airway inflammation. It is also possible that IL-25 might induce the production of chemokines, such as TARC, from lung resident cells, such as epithelial cells and fibroblasts, and then might enhance the recruitment of T_H2 cells and subsequent T_H2 cell-mediated allergic airway inflammation. On the other

hand, it is unlikely that IL-25 enhances allergic inflammation through the induction of T_H2 cell differentiation because Fort et al⁹ have shown that IL-25 does not directly induce T_H2 cytokine production from naive and memory T cells *in vitro*. We also found that using OVA-specific DO11.10 T cells, IL-25 did not enhance antigen-specific T_H2 cell differentiation (data not shown). Future studies identifying IL-25–responding non-T cells that produce T_H2 cytokines could help in the understanding of the precise role of $CD4^+$ T cells in IL-25–induced enhancement of allergic airway inflammation.

In summary, we have shown that IL-25 enhances T_H2 cell–mediated allergic airway inflammation in mice, but IL-25 itself does not significantly induce allergic inflammation. Our results raise the possibility that IL-25 might be involved in the enhancement, prolongation, or both of T_H2 cell–mediated allergic diseases, such as asthma and allergic rhinitis, and suggest that IL-25 could be a possible target of these diseases.

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REFERENCES

- Wills-Karp M. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu Rev Immunol* 1999;17:255-81.
- Herrick CA, Bottomly K. To respond or not to respond: T cells in allergic asthma. *Nat Rev Immunol* 2003;3:405-12.
- Larche M, Robinson DS, Kay AB. The role of T lymphocytes in the pathogenesis of asthma. *J Allergy Clin Immunol* 2003;111:450-63.
- Kay AB, Phipps S, Robinson DS. A role for eosinophils in airway remodelling in asthma. *Trends Immunol* 2004;25:477-82.
- Nakajima H, Iwamoto I, Tomoe S, Matsumura R, Tomioka H, Takatsu K, et al. $CD4^+$ T-lymphocytes and interleukin-5 mediate antigen-induced eosinophil infiltration into the mouse trachea. *Am Rev Respir Dis* 1992;146:374-7.
- Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J Exp Med* 1996;183:195-201.
- Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, et al. Interleukin-13: central mediator of allergic asthma. *Science* 1998;282:2258-61.
- Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, et al. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 1998;282:2261-3.
- Fort MM, Cheung J, Yen D, Li J, Zurawski SM, Lo S, et al. IL-25, a novel molecule that induces IL-4, IL-5, and IL-13 and $Th2$ -associated pathologies *in vivo*. *Immunity* 2001;15:985-95.
- Ikeda K, Nakajima H, Suzuki K, Kagami S-i, Hirose K, Suto A, et al. Mast cells produce interleukin-25 upon FcεRI-mediated activation. *Blood* 2003;101:3594-6.
- 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 the 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.
- Schwarzenberger P, La Russa V, Miller A, Ye P, Huang W, Zieske A, et al. IL-17 stimulates granulopoiesis in mice: use of an alternate, novel gene therapy-derived method for *in vivo* evaluation of cytokines. *J Immunol* 1998;161:6383-9.
- Shi Y, Ullrich SJ, Zhang J, Connolly K, Grzegorzewski KJ, Barber MC, et al. A novel cytokine receptor-ligand pair: identification, molecular characterization, and *in vivo* immunomodulatory activity. *J Biol Chem* 2000;275:19167-76.
- Maezawa Y, Nakajima H, Suzuki K, Tamachi T, Ikeda K, Inoue J-I, et al. Involvement of TNF receptor-associated factor (TRAF) 6 in IL-25 receptor signaling. *J Immunol* 2006;176:1013-8.
- Rankin JA, Picarella DE, Geba GP, Temann UA, Prasad B, DiCosmo B, et al. Phenotypic and physiologic characterization of transgenic mice expressing interleukin 4 in the lung: lymphocytic and eosinophilic inflammation without airway hyperreactivity. *Proc Natl Acad Sci U S A* 1996;93:7821-5.
- Takeda K, Tanaka T, Shi W, Matsumoto M, Minami M, Kashiwamura S, et al. Essential role of Stat6 in IL-4 signaling. *Nature* 1996;380:627-30.
- Leung SY, Eynott P, Nath P, Chung KF. Effects of ciclesonide and fluticasone propionate on allergen-induced airway inflammation and remodeling features. *J Allergy Clin Immunol* 2005;115:989-96.
- Kagami S, Nakajima H, Kumano K, Suzuki K, Suto A, Imada K, et al. Both Stat5a and Stat5b are required for antigen-induced eosinophil and T cell recruitment into the tissue. *Blood* 2000;95:1370-7.
- Louahed J, Toda M, Jen J, Hamid Q, Renauld JC, Levitt RC, et al. Interleukin-9 upregulates mucus expression in the airways. *Am J Respir Cell Mol Biol* 2000;22:649-56.
- Tian E, Sawyer JR, Largaespada DA, Jenkins NA, Copeland NG, Shaughnessy JD Jr. Evi27 encodes a novel membrane protein with homology to the IL17 receptor. *Oncogene* 2000;19:2098-109.
- Aruffo A, Stamenkovic I, Melnick M, Underhill CB, Seed B. CD44 is the principal cell surface receptor for hyaluronate. *Cell* 1990;61:1303-13.
- Karasuyama H, Melchers F. Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4 or 5, using modified cDNA expression vectors. *Eur J Immunol* 1988;18:97-104.
- Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, Zhu Z, et al. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat Med* 2002;8:885-9.
- Zimmermann N, Hershey GK, Foster PS, Rothenberg ME. Chemokines in asthma: cooperative interaction between chemokines and IL-13. *J Allergy Clin Immunol* 2003;111:227-42.
- Letuve 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.
- Glimcher LH, Murphy KM. Lineage commitment in the immune system: the T helper lymphocyte grows up. *Gene Dev* 2000;14:1693-711.
- Fallon PG, Ballantyne SJ, Mangan NE, Barlow JL, Dasvarma A, Hewett DR, et al. Identification of an interleukin (IL)-25–dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. *J Exp Med* 2006;203:809-12.