X63-IL-25 cells as a source of IL-25. To evaluate the bioactivity of the produced IL-25, we established Ba/F3 cells that expressed IL-25R-MPL fusion protein (Ba/F3 IL-25R-MPL cells) and used as a responding cell for IL-25 stimulation. As shown in Fig. 1, Ba/F3 IL-25R-MPL cells proliferated in response not only to the supernatant of X63-IL-3 cells but also to the supernatant of X63-IL-25 cells in a dose-dependent manner, whereas control Ba/F3 cells proliferated in response to the supernatant of X63-IL-3 cells but not to the supernatant of X63-IL-25 cells. As expected, either Ba/F3 IL-25R-MPL cells or control Ba/F3 cells did not proliferate in response to the supernatant of X63-control cells (Fig. 1).

IL-25R cross-linking induces NF-κB activation

We next established the system that mimicked IL-25R signaling to clarify the IL-25 signaling pathway in detail. To eliminate the possible involvement of the endogenously expressed IL-25R, we used Ab-mediated cross-linking of the receptors rather than ligand-mediated activation. Either WT IL-25R or Flag-IL-25R was expressed in COS7 cells, and these cells were stimulated with the supernatant of X63-IL-25 cells or anti-Flag mAb. In cells expressing WT IL-25R, the supernatant of X63-IL-25 cells, but not stimulation with anti-Flag mAb, activated the NF-κB-responding reporter construct (Fig. 2*A*). In contrast, in cells expressing Flag-IL-25R, both the supernatant of X63-IL-25 cells and anti-Flag mAb activated NF-κB-responding reporter construct (Fig. 2*A*). These results indicate that IL-25R signaling induces NF-κB activation and that the cross-linking with anti-Flag mAb mimics the ligand-mediated signaling of IL-25 in cells expressing Flag-IL-25R.

TRAF6 is crucial for IL-25R-mediated NF-κB activation

It has been reported that TRAF6 is involved in the signaling pathways of IL-1- and IL-17-induced NF-κB activation (20, 25, 26). To determine whether TRAF6 is involved in IL-25R-mediated signaling, we investigated the effect of a DN TRAF6 on IL-25R-mediated NF-κB activation. As a control, we examined the effect of DN TRAF2 on IL-25R-mediated NF-κB activation in parallel. As shown in Fig. 2*B*, the expression of DN TRAF6, but not DN TRAF2, inhibited IL-25R-mediated NF-κB activation in a dose-dependent manner ($n = 4$, $p < 0.01$), suggesting that TRAF6 but not TRAF2 is involved in the signaling pathways of NF-κB activation under IL-25R.

To further clarify the involvement of TRAF6 in IL-25R-mediated signaling, we compared IL-25R-mediated IκB-α down-regulation in Flag-IL-25R-expressing TRAF6$^{-/-}$ MEF and in Flag-IL-25R-expressing WT MEF. As controls, these cells were stimulated

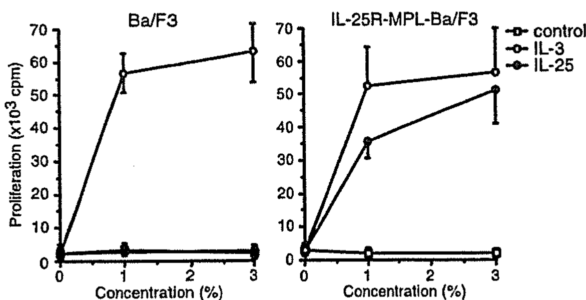

FIGURE 1. Establishment of a bioassay for IL-25. Control vector-transfected Ba/F3 cells (*left*) and IL-25R-MPL-expressing Ba/F3 cells (*right*) were cultured in the presence of the supernatant of X63-control, X63-IL-3, or X63-IL-25 cells at the indicated concentrations at 37°C for 36 h with 0.5 μCi of [^{3}H]thymidine added for the final 12 h. Data are mean ± SD of [^{3}H]thymidine incorporation for four independent experiments.

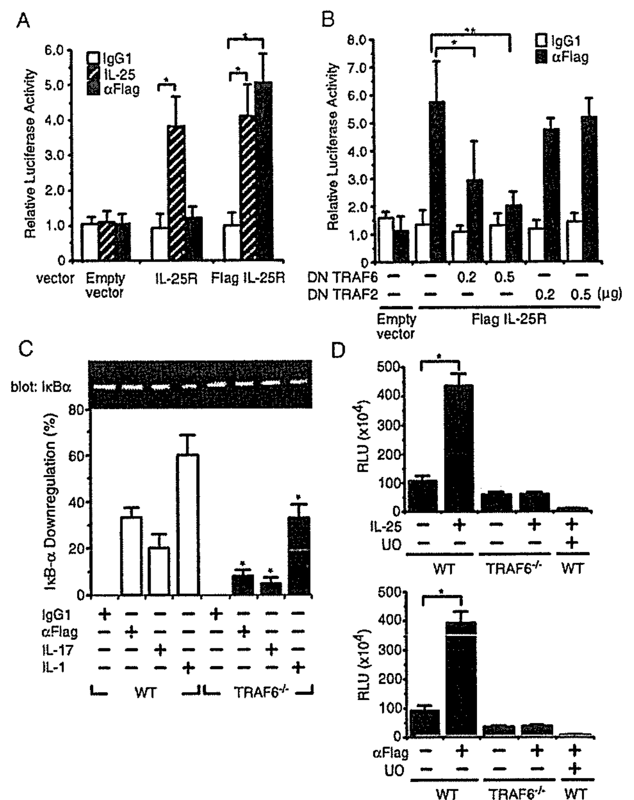

FIGURE 2. IL-25R signaling induces NF-κB activation by a TRAF6-dependent mechanism. *A*, IL-25R cross-linking induces NF-κB activation. COS7 cells were transfected with the expression vector for IL-25R or Flag-tagged-IL-25R in the presence of pNF-κB-Luc and pRL-TK. Twenty-four hours later, the cells were stimulated with the supernatant of X63-IL-25 cells (3%) or anti-Flag mAb (2 μg/ml) at 37°C for 24 h. Luciferase activities of pNF-κB-Luc were determined by a Dual-Luciferase Reporter System. Data are mean ± SD of the relative luciferase activity of pNF-κB-Luc for four experiments. Significantly different (*, $p < 0.01$) from the mean value of unstimulated cells (control IgG1). *B*, DN TRAF6 inhibits IL-25R-mediated NF-κB activation. The expression vector for Flag-tagged IL-25R was transfected to COS7 cells in the presence of pNF-κB-Luc and pRL-TK. Where indicated, amounts of expression vector for DN TRAF6 or DN TRAF2 were simultaneously transfected. Twenty-four hours later, cells were incubated with anti-Flag mAb or control IgG1 at 37°C for another 24 h, and the luciferase activities of pNF-κB-Luc were determined by the Dual-Luciferase Reporter Assay System. Data are mean ± SD for four experiments. Significant difference (*, $p < 0.05$ and **, $p < 0.01$) is shown. *C*, IL-25R-mediated IκB-α down-regulation is diminished in TRAF6$^{-/-}$ cells. WT MEF and TRAF6$^{-/-}$ MEF were infected with retrovirus of pMX-Flag-IL-25R-IRES-GFP as described in *Materials and Methods*. After infected cells were sorted and expanded, IL-25R was cross-linked with anti-Flag mAb or control IgG1 at 37°C for 20 min. As controls, MEF infected with retrovirus of pMX-Flag-IL-25R-IRES-GFP were stimulated with rIL-17 (100 ng/ml) or IL-1β (10 ng/ml) at 37°C for 20 min. Cell lysates were subjected to immunoblotting with anti-IκB-α Ab. Shown are representative blot (*top*) and mean ± SD of the percentage of IκB-α down-regulation determined by a densitometer (*bottom*) from four independent experiments. Significantly different (*, $p < 0.01$) from the mean value of the corresponding response of WT MEF. *D*, TRAF6 is required for IL-25-induced nuclear accumulation of NF-κB p65. Flag-IL-25R-expressing WT MEF or TRAF6$^{-/-}$ MEF were stimulated with X63-IL-25 condition medium (*top*) or anti-Flag mAb (*bottom*) at 37°C for 30 min. As controls, supernatant of X63-control cells (*top*) or control IgG1 (*bottom*) was used. Nuclear extracts were prepared from these cells, and the binding activity to NF-κB consensus oligonucleotides was determined as described in *Materials and Methods*. Where indicated, unlabeled competitor oligonucleotides were added to nuclear extracts to confirm specific binding. Data are mean ± SD of relative light unit (RLU) for four experiments. Significant difference (*, $p < 0.01$) are indicated.

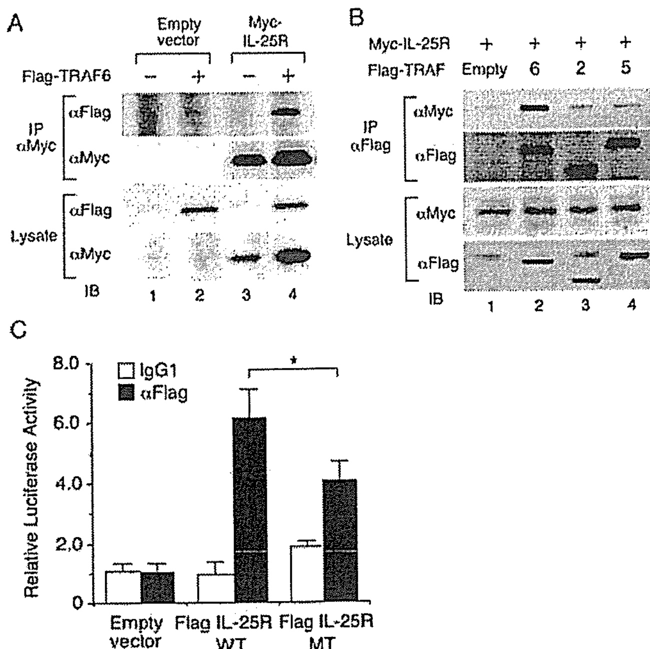

FIGURE 3. TRAF6 but not TRAF2 nor TRAF5 associates with IL-25R. *A*, TRAF6 associates with IL-25R. COS7 cells were transfected with *myc*-tagged IL-25R and/or Flag-tagged TRAF6, and the cell lysates were immunoprecipitated (IP) with anti-*myc* Ab and followed by immunoblottings (IB) with anti-Flag Ab or anti-*myc* Ab. Shown are representative data of three independent experiments. *B*, TRAF2 and TRAF5 do not associate with IL-25R. COS7 cells were transfected with *myc*-tagged IL-25R and Flag-tagged TRAF6, Flag-tagged TRAF2, or Flag-tagged TRAF5. Cell lysates were immunoprecipitated with anti-Flag Ab and followed by immunoblottings with anti-*myc* Ab or anti-Flag Ab. Shown are representative data of three independent experiments. *C*, E338A mutation of IL-25R attenuates IL-25R-mediated NF-κB activation. COS7 cells were transfected with the expression vector for Flag-tagged IL-25R WT or Flag-tagged-IL-25R E338A (Flag IL-25R MT) in the presence of pNF-κB-Luc and pRL-TK. Twenty-four hours later, the cells were stimulated with anti-Flag mAb (2 μg/ml) at 37°C for 24 h. Luciferase activities of pNF-κB-Luc were determined by a Dual-Luciferase Reporter System. Data are means ± SD of the relative luciferase activity of pNF-κB-Luc for four experiments. Significant difference (*, $p < 0.05$) is shown.

with IL-1β or IL-17, cytokines that activate the NF-κB pathway (5–7, 20, 27). As shown in Fig. 2*C*, the expression levels of IκB-α in Flag-IL-25R-expressing WT MEF were down-regulated in response to anti-Flag mAb, compared with the basal levels of IκB-α (control IgG1) ($n = 4$, $p < 0.01$). Stimulation with the supernatant of X63-IL-25 cells also down-regulated the expression levels of IκB-α in Flag-IL-25R-expressing WT MEF (data not shown). Importantly, IL-25R-mediated IκB-α down-regulation was significantly impaired in Flag-IL-25R-expressing TRAF6$^{-/-}$ MEF, compared with that in Flag-IL-25R-expressing WT MEF ($n = 4$, $p < 0.01$) (Fig. 2*C*). As expected, IL-1- or IL-17-mediated IκB-α down-regulation was also impaired in TRAF6$^{-/-}$ MEF (Fig. 2*C*).

To further examine the involvement of TRAF6 in IL-25R-mediated NF-κB activation, we compared IL-25R-mediated nuclear accumulation of NF-κB p65 in Flag-IL-25R-expressing WT MEF and TRAF6$^{-/-}$ MEF. Nuclear accumulation of NF-κB p65 was induced by IL-25 stimulation (Fig. 2*D*, *top panel*) or by anti-Flag mAb-mediated IL-25R cross-linking (Fig. 2*D*, *bottom panel*) in Flag-IL-25R-expressing WT MEF. IL-25-mediated or anti-Flag mAb-mediated nuclear accumulation of NF-κB p65 was significantly decreased in Flag-IL-25R-expressing TRAF6$^{-/-}$ MEF (Fig. 2*D*). Taken together, these results indicate that TRAF6 is involved in IL-25R-mediated NF-κB activation.

TRAF6 associates with IL-25R

We then examined whether TRAF6 associates with IL-25R by a coimmunoprecipitation assay. Flag-tagged TRAF6 was expressed with or without *myc*-tagged IL-25R in COS7 cells and the amounts of Flag-tagged TRAF6 in the immunoprecipitates with anti-*myc* mAb was evaluated. As shown in Fig. 3*A*, anti-*myc* mAb coprecipitated Flag-tagged TRAF6. We also performed the immunoprecipitation with anti-Flag mAb and confirmed that *myc*-tagged IL-25R was coimmunoprecipitated with Flag-tagged TRAF6 (Fig. 3*B*). In contrast, *myc*-tagged IL-25R was not coimmunoprecipitated with Flag-tagged TRAF2 or TRAF5 (Fig. 3*B*). These results suggest that TRAF6 but not TRAF2 or TRAF5 can associate with IL-25R and that this association occurs even in the absence of ligand binding. Furthermore, IL-25R-mediated NF-κB activation was attenuated in cells expressing IL-25R E338A, in which TRAF6-binding motif was mutated, compared with that in cells expressing WT IL-25R (Fig. 3*C*). These results suggest that the direct association between IL-25R and TRAF6 is crucial for IL-25-mediated NF-κB activation.

IL-25 induces MAPK activation by a TRAF6-independent mechanism

To determine whether IL-25 activates other intracellular signaling pathways such as MAPK pathways, we next examined the phosphorylation of ERK, JNK, and p38 in Flag-IL-25R-expressing MEF upon stimulation with anti-Flag mAb. The phosphorylation of ERK was markedly induced upon stimulation with anti-Flag mAb at similar levels to that induced by IL-17 or IL-1 stimulation (Fig. 4). The phosphorylation of JNK and p38 was also induced by the stimulation with anti-Flag mAb, although it was weaker than that induced by IL-17 or IL-1 stimulation (Fig. 4). These results indicate that IL-25 activates not only the NF-κB pathway but also ERK, JNK, and p38 pathways. Interestingly, although IL-17- or IL-1-mediated activation of JNK and p38 was impaired in TRAF6$^{-/-}$ MEF (Fig. 4, *lane 3* vs *lane 7* and *lane 4* vs *lane 8*, respectively), IL-25R-mediated activation of ERK, JNK, and p38 was not impaired in TRAF6$^{-/-}$ MEF (Fig. 4, *lane 2* vs *lane 6*). These results indicate that in contrast to IκB-α down-regulation

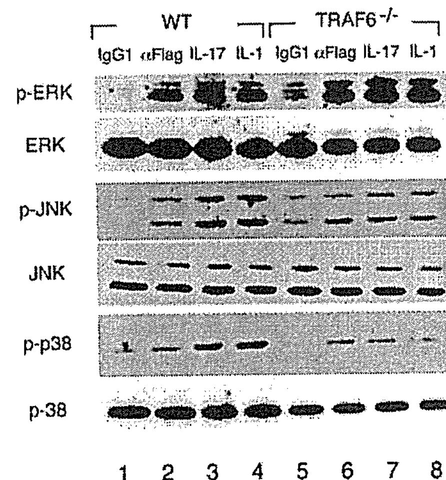

FIGURE 4. IL-25 activates ERK, JNK, and p38 by a TRAF6-independent mechanism. Similar to Fig. 2*C*, WT or TRAF6$^{-/-}$ MEF infected with retrovirus of pMX-Flag-IL-25R-IRES-GFP were incubated with control IgG1, anti-Flag mAb, IL-17, or IL-1 at 37°C for 20 min, and cell lysates were subjected to immunoblotting with anti-phospho-ERK, anti-ERK, anti-phospho-JNK, anti-JNK, anti-phospho-p38, or anti-p38 Ab. Shown are representative blots from four independent experiments.

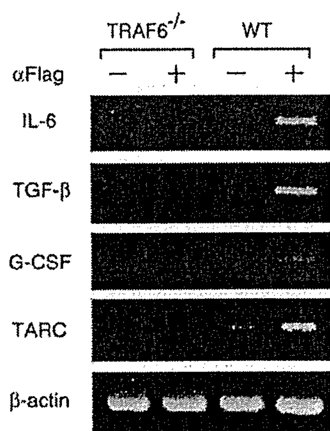

FIGURE 5. IL-25 up-regulates cytokine and chemokine mRNA expression in a TRAF6-dependent manner. WT MEF and TRAF6$^{-/-}$ MEF were infected with retrovirus of pMX-Flag-IL-25R-IRES-GFP as described in Fig. 2*C* and then incubated with anti-Flag mAb or control IgG1 at 37°C for 3 h. Total cellular RNA was prepared, and RT-PCR analysis for IL-6, TGF-β, G-CSF, TARC, and β-actin (as a control) was performed. Shown are representative data of four independent experiments.

and subsequent NF-κB activation (Fig. 2, *B* and *C*), TRAF6-independent pathways mainly contribute to the activation of ERK, JNK, and p38 under IL-25R-mediated signaling.

TRAF6 is involved in IL-25R-mediated gene expression

To determine whether TRAF6 is involved in IL-25R-mediated gene expression, we compared the mRNA induction of IL-6, TGF-β, G-CSF, and TARC in Flag-IL-25R-expressing WT MEF with Flag-IL-25R-expressing TRAF6$^{-/-}$ MEF upon stimulation with anti-Flag mAb. Interestingly, the induction of mRNA expression of IL-6, TGF-β, G-CSF, and TARC by anti-Flag cross-linking was significantly decreased in Flag-IL-25R-expressing TRAF6$^{-/-}$ MEF, compared with that in Flag-IL-25R-expressing WT MEF (Fig. 5). The induction of IL-6, TGF-β, G-CSF, and TARC mRNA was also attenuated in Flag-IL-25R E338A-expressing WT MEF, compared with that in Flag-IL-25R-expressing WT MEF (data not shown). Taken together, these results suggest that TRAF6 plays an important role in the production of cytokines and chemokines upon IL-25R-mediated signaling.

Discussion

In this study, we show that TRAF6 mediates NF-κB activation in IL-25R signaling. We found that IL-25R-mediated signaling induced NF-κB activation (Fig. 2*A*) as well as ERK, JNK, and p38 activation (Fig. 4). We also found that IL-25R-mediated NF-κB activation was down-regulated by the expression of DN TRAF6 but not of DN TRAF2 (Fig. 2*B*). Furthermore, IL-25R-mediated NF-κB activation, but not MAPK activation, was diminished in TRAF6$^{-/-}$ MEF (Figs. 2*C* and 4). In addition, coimmunoprecipitation assay revealed that TRAF6 associated with IL-25R in a ligand-independent manner (Fig. 3, *A* and *B*). Finally, we found that IL-25R-mediated gene expression of IL-6, TGF-β, G-CSF, and TARC was diminished in TRAF6$^{-/-}$ MEF (Fig. 4). Taken together, these results indicate that TRAF6 plays a critical role in IL-25R-mediated NF-κB activation and gene expression.

Our results suggest that TRAF6 directly associates with the cytoplasmic region of IL-25R and induces NF-κB activation upon ligand binding. The TRAF6-binding motif is conserved in the cytoplasmic region of mouse and human IL-25R and we showed the association between IL-25R and TRAF6 even in the absence of ligand binding (Fig. 3, *A* and *B*). We also found that the disruption of the TRAF6-binding motif attenuated IL-25R-mediated NF-κB activation (Fig. 3*C*). In contrast, although there is no TRAF6-binding motif in IL-17R, TRAF6 was coimmunoprecipitated with IL-17R (26) and IL-17-induced NF-κB activation was diminished in TRAF6$^{-/-}$ cells (26) (Fig. 2*C*). Therefore, the mechanisms underlying TRAF6 activation may be different between IL-25R- and IL-17R-mediated signaling.

In contrast, we show that IL-25R-mediated activation of ERK, JNK, and p38 is TRAF6-independent (Fig. 4). We found that IL-25R-mediated ERK, JNK, and p38 activation was similarly observed in WT and TRAF6$^{-/-}$ MEF (Fig. 4). In contrast, we found that IL-17R-mediated JNK and p38 activation was diminished in TRAF6$^{-/-}$ MEF (Fig. 4). Schwandner et al. (26) have also shown that IL-17-induced JNK activation is impaired in TRAF6$^{-/-}$ cells. These results indicate that TRAF6-independent pathways are primarily involved in the activation of JNK and p38 under IL-25R- but not IL-17R-mediated signaling.

The mechanisms by which IL-25 activates these MAPKs have not yet been elucidated. These MAPKs are activated by their specific MAPK kinases: ERK is activated by MEK1 and MEK2, JNK is activated by MAPK kinase (MKK)4 and MKK7, and p38 is activated by MKK3 and MKK6 (28). These MAPK kinases are also activated by various MAPK kinase kinases, such as Raf, TGF-β-activated protein kinase 1, MEK kinase 1, MLK, and apoptosis signal-regulating kinase 1 (28). In preliminary experiments, we found that IL-25R cross-linking modestly induced Raf-1 and MKK3 activation in Flag-IL-25R-expressing cells. However, the induction of Raf-1 and MKK3 activation by IL-25R cross-linking was weaker than that by IL-1 or IL-17. Thus, other kinases may be participated in the activation of these MAPKs under IL-25R signaling. Future studies revealing the signaling cascade of IL-25-induced MAPKs activation especially in the undefined IL-25-responding cells could help the understanding of the physiological importance of MAPKs activation through IL-25R signaling.

Our results also show that IL-25R-mediated signaling induces the production of TARC by a TRAF6-dependent mechanism (Fig. 5). We also found that rIL-25-induced TARC expression in NIH3T3 cells (data not shown). Our findings support the previous report showing that the in vivo administration of IL-25-expressing adenovirus induces the expression of chemokines including TARC in the lung (4). TARC is a specific ligand for CCR4 (29, 30) and induces chemotaxis of T cells, especially of Th2 cells (31, 32). It has also been demonstrated that TARC plays a significant role for the induction of Th2 cell-mediated eosinophil recruitment into the airways in a murine model of asthma (33). We also found that mice that specifically expressed IL-25 in the lung under the control of CC-10 (Clara cell 10-kDa) promoter exhibited the enhanced T cell recruitment into the airways after Ag inhalation (T. Tamachi, Y. Maezawa, K. Ikeda, S.-i. Kagami, M. Hatano, Y. Seto, A. Suto, K. Suzuki, N. Watanabe, Y. Saito, T. Tokuhisa, I. Iwamoto, and H. Nakajima, manuscript in preparation). Therefore, it is suggested that the induction of TARC by IL-25-induced NF-κB activation may be involved in IL-25-mediated allergic inflammation.

IL-25 is expressed in Th2-polarized CD4^{+} T cells (2) and activated mast cells (34). It has also been reported that in vivo administration of IL-25 promotes the expression of Th2-cell associated cytokines such as IL-4, IL-5, and IL-13 from a non-T/non-B cell population (2, 4). These findings suggest that IL-25 is within the amplification loop of Th2-type immune responses. In this regard, a recent study has demonstrated that APCs such as macrophages and dendritic cells express IL-25R upon IL-4 stimulation (35), suggesting that APCs may be involved in the IL-25-induced Th2-type immune responses. Further investigation is needed to

determine cell populations that respond to IL-25 and trigger Th2-type immune responses in vivo.

In summary, we have demonstrated that TRAF6 is involved in IL-25R-mediated NF-κB activation and gene expression. Because IL-25 is suggested to be involved in Th2 cell-mediated allergic inflammation by inducing Th2 cytokine production from an unidentified non-T/non-B cell population, the elucidation of IL-25R-mediated signaling provides a new tool for the treatment of allergic diseases such as bronchial asthma, atopic rhinitis, and atopic dermatitis.

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Disclosures

The authors have no financial conflict of interest.

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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;118:606-14.)

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

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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-17[9] and is produced by activated T_H2 cells[9] 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 protein[9] or the systemic expression of IL-25 by transgene[11] 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.

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

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

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 $Stat6^{-/-}$ mice[18] 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 μg 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-μm 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, TCCCTCAGAGCACGTCTTAGG. 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 GADPH 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 standard protocol with FuGENE6 transfection reagent (Roche Diagnostics, Indianapolis, Ind), and sIL-25R CHO cells were cultured in CELLine AD1000 (INTEGRA Biosciences AG, Chur, Switzerland)

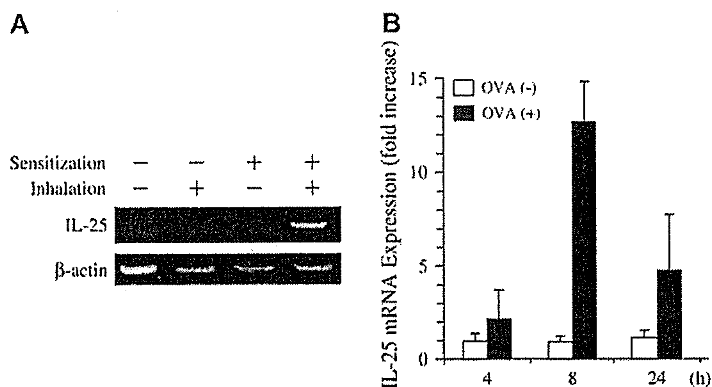

FIG 1. IL-25 mRNA is expressed in the lungs of sensitized mice on antigen inhalation. **A,** BALB/c mice were immunized with or without OVA in aluminum hydroxide, and 2 weeks after the immunization, mice were challenged twice with inhaled OVA or saline at a 48-hour interval. Twenty-four hours after the 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 GADPH (as a control) mRNA, was performed. The levels of IL-25 mRNA are normalized to the levels of GADPH mRNA and then expressed as a fold increase to the baseline level (without the inhalation). Data are presented as means ± SD from 5 experiments.

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 ± 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

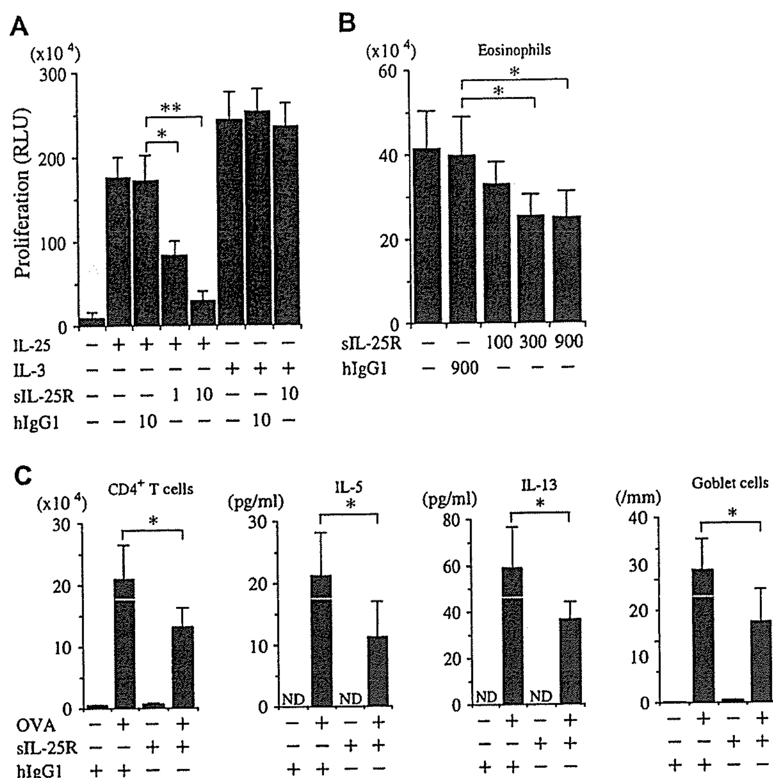

FIG 2. Neutralization of IL-25 by sIL-25R inhibits antigen-induced eosinophil and CD4+ T-cell recruitment into the airways. **A**, Ba/F3 IL-25R–MPL cells that stably express IL-25R–MPL fusion protein were cultured with X63–IL-25 or X63–IL-3 conditioning medium (10% vol/vol). Where indicated, sIL-25R (1 or 10 μg/mL) and human IgG1 (*hIgG1*; 10 μg/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 ± 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 μg per mouse) or human IgG1 (900 mg 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 ± SD for 8 mice in each group. *$P < .05$. **C**, OVA-sensitized BALB/c mice were challenged twice with inhaled OVA or saline. sIL-25R (900 μg per mouse) or human IgG1 (900 μg 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 ± SD for 6 mice in each group. *$P < .05$.

transmembrane and cytoplasmic domain of thrombopoietin receptor MPL.[16] IL-25 induced proliferation of BA/F3 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

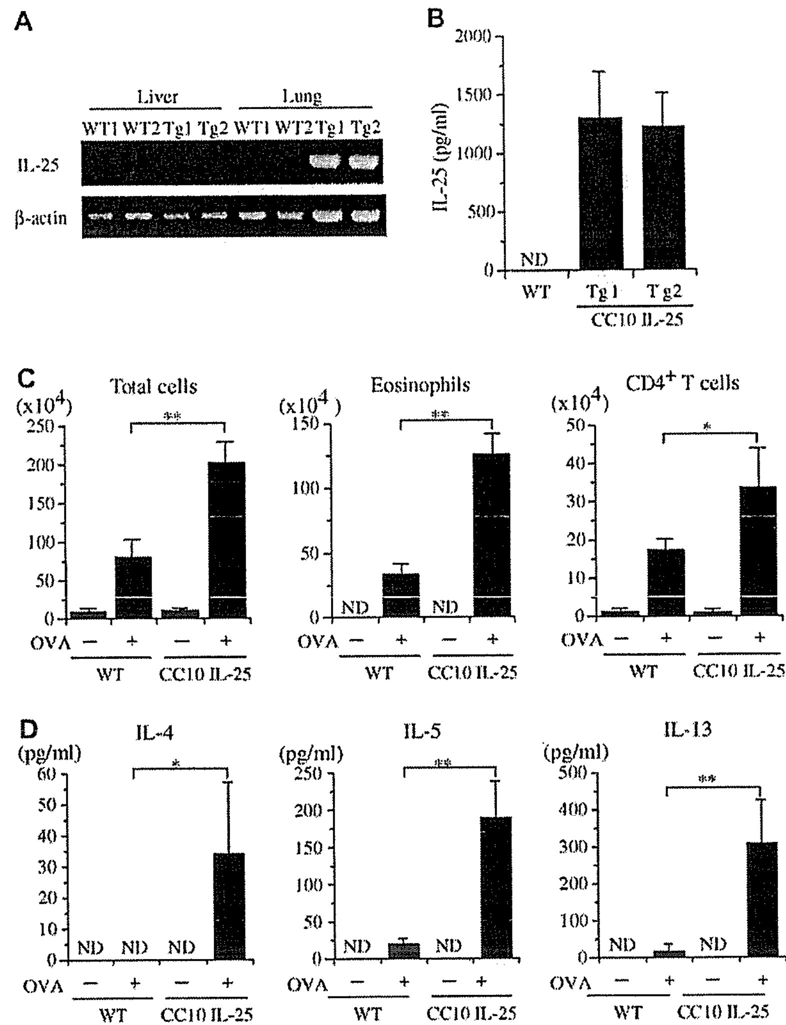

FIG 3. Antigen-induced eosinophil and $CD4^+$ T-cell recruitment and T_H2 cytokine production in the airways are enhanced in CC10 IL-25 mice. **A,** Total RNA was prepared from lung or liver tissues of CC10 IL-25 mice (transgenic line 1 *[Tg1]* and transgenic line 2 *[Tg2]*) and WT littermates (*WT1* 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 ± 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 ± SD for 6 to 10 mice in each group. *$P < .05$, **$P < .01$.

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, *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

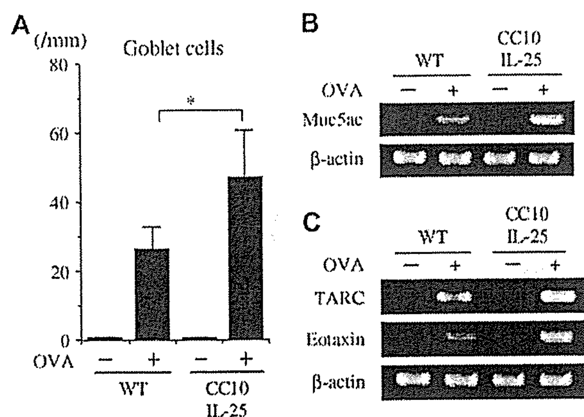

FIG 4. Antigen-induced goblet cell hyperplasia and chemokine production in the airways are enhanced in CC10 IL-25 mice. **A,** OVA-sensitized CC10 IL-25 mice (transgenic line 1) and littermate WT mice were challenged twice with OVA or saline inhalation. Forty-eight hours after the last OVA or saline inhalation, the number of goblet cells was evaluated on periodic acid–Schiff–stained sections of the lung. Data are presented as means ± 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.

mice and littermate wild-type (WT) mice were immunized with OVA-alum and then challenged twice with inhaled 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,

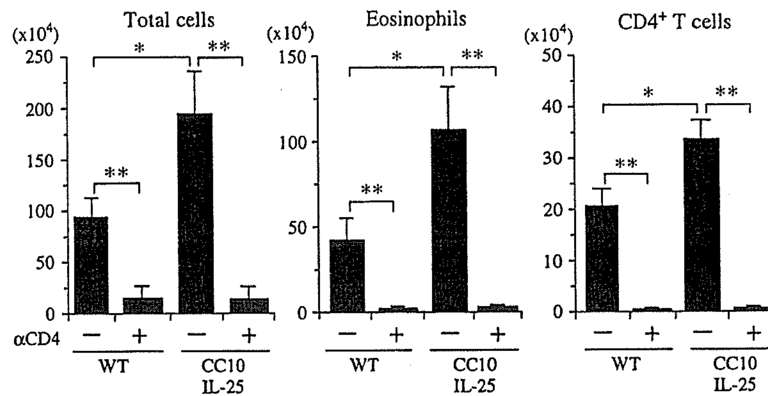

FIG 5. $CD4^+$ T cells are required for the enhanced eosinophil recruitment into the airways in CC10 IL-25 mice. OVA-sensitized CC10 IL-25 mice and WT mice were challenged twice with inhaled OVA. Where indicated, mice were injected intraperitoneally with anti-CD4 antibody (1 mg) or control antibody (1 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 ± SD for 6 mice in each group. $*P < .01$, $**P < .001$.

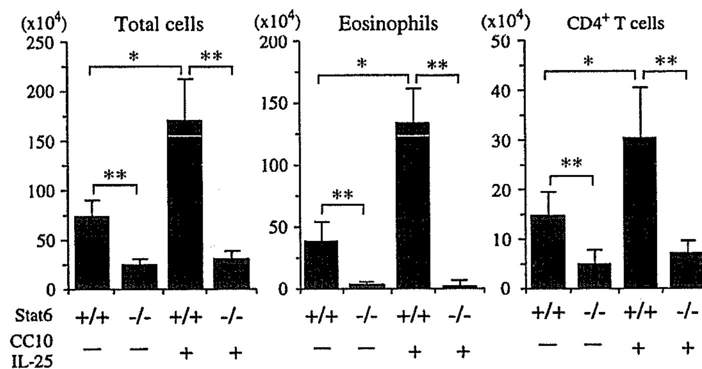

FIG 6. Stat6 is required for the enhanced eosinophil recruitment into the airways in CC10 IL-25 mice. CC10 IL-25 $Stat6^{-/-}$ mice, CC10 IL-25 $Stat6^{+/+}$ mice, $Stat6^{-/-}$ mice, and $Stat6^{+/+}$ mice were immunized with OVA-alum and then challenged twice with inhaled OVA. The number of total cells, eosinophils, and $CD4^+$ T cells in the BALF was evaluated at 48 hours after the last inhalation. Data are presented as means ± 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 al[11] 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 al[27] 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-25[9] or the systemic expression of IL-25 by transgene[11] 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 al[9] 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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Review

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Interleukin 25 in Allergic Airway Inflammation

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Key Words
Interleukin 25 · Asthma · Allergic inflammation · CD4+ T cells

Abstract
T helper 2 (Th2) cells induce allergic inflammation through the production of cytokines such as interleukin (IL)-4, IL-5 and IL-13. Recently, it has been demonstrated that a novel IL-17 family cytokine IL-25 (IL-17E) is a product of activated Th2 cells and mast cells. Interestingly, when systemically administered to mice, IL-25 induces IL-4, IL-5 and IL-13 production from undefined non-T/non-B cells and then induces Th2-type immune responses such as blood eosinophilia and increased serum immunoglobulin E levels. In addition, we have recently shown that IL-25 mRNA is expressed in the lung after an inhaled antigen challenge in sensitized mice and that neutralization of the produced IL-25 by soluble IL-25 receptor decreases antigen-induced eosinophil and CD4+ T cell recruitment into the airways. Moreover, we have shown that the enforced expression of IL-25 in the lung significantly enhances antigen-induced Th2 cytokine production and eosinophil recruitment into the airways, and that the IL-25-mediated enhancement of antigen-induced eosinophil recruitment is inhibited by the depletion of CD4+ T cells. Thus, it is suggested that IL-25 plays an important role in enhancing allergic airway inflammation by a CD4+ T-cell-dependent mechanism.

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Interleukin 25 as a Novel T Helper 2 Cell-Derived Cytokine

Recently, five new cytokines homologous to interleukin (IL)-17 (IL-17A) have been identified by database searching, namely IL-17B, IL-17C, IL-17D, IL-25 (IL-17E) and IL-17F [1, 2]. Among the IL-17 family cytokines, IL-25 is less homologic to other IL-17 family members and has been reported to be expressed specifically in activated T helper 2 (Th2) cells [3]. In addition, it has been shown that in vivo and in vitro biological activities of IL-25 are markedly different from those described for IL-17 and other IL-17 family cytokines [1, 2]. For example, it has been shown that the systemic administration of IL-25 results in eosinophilia through the production of IL-5 [3–5], whereas other IL-17 family cytokines induce neutrophilia [6–8]. Moreover, IL-25 induces elevated gene expression of IL-4 and IL-13 in multiple tissues and the resultant Th2-type immune responses, including increased serum immunoglobulin (Ig)E levels

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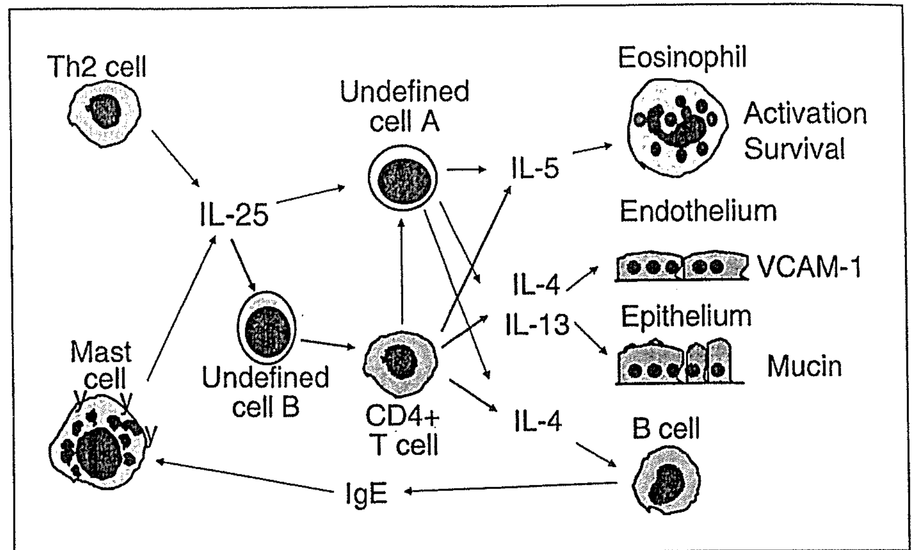

Fig. 1. Possible mechanisms for IL-25-mediated enhancement of Th2-type immune responses. Th2 cells and mast cells produce IL-25 upon activation. In a situation where IL-25 is abundant, IL-25 directly functions on a lineage-negative antigen-presenting cell (undefined cell A) to produce Th2 cytokines and then causes allergic inflammation [3, 4]. In contrast, in other situations where the amounts of IL-25 are limited, collaboration with CD4+ T cells is required for IL-25-mediated allergic inflammation. In this situation, IL-25 may induce the recruitment of antigen-specific CD4+ T cells to the site of allergic inflammation through the production of chemokines such as TARC by the undefined cell B and then may enhance CD4+ T-cell-mediated allergic inflammation. Because the amounts of the endogenously produced IL-25 in the lung of antigen-sensitized, antigen-inhaled mice seem to be limited, we speculate that the latter mechanism is mainly involved in the induction of allergic airway inflammation in a physiological setting. VCAM-1 = Vascular cell adhesion molecule 1.

and pathological changes in the multiple tissues [3–5]. Interestingly, previous studies have demonstrated that IL-25-induced Th2 cytokine production was observed even in mice lacking T cells [3, 5]. These findings suggest that once IL-25 is produced, IL-25 is capable of enhancing allergic inflammation even in the absence of Th2 cells.

Mast Cells Produce IL-25 upon Activation

Regarding IL-25-producing cells, a previous report has demonstrated that IL-25 mRNA is exclusively expressed in polarized Th2 cells [3]. However, quantitative RT-PCR analyses have revealed that IL-25 mRNA is detected in multiple tissues, including the colon, uterus, stomach, small intestine, kidney and lung [3, 4, 9]. Thus, it is suggested that, in addition to Th2 cells, other cell types would produce IL-25. In this regard, we have shown that when bone-marrow-derived mast cells are stimulated with IgE crosslinking, IL-25 mRNA is induced within 30 min and the levels of IL-25 mRNA are comparable with that of activated Th2 cells [10]. Therefore, mast cells are potent IL-25 producers, and mast-cell-derived IL-25 may be involved in the augmentation and/or prolongation of Th2-type immune response (fig. 1).

Role of IL-25 in Antigen-Induced Eosinophil Recruitment into the Airway

Antigen-induced allergic inflammation is induced by Th2 cells through the production of Th2 cytokines [11, 12]. In a murine model of allergic airway inflammation, we and others have provided evidence that IL-5-producing CD4+ T cells mediate antigen-induced eosinophil recruitment into the airways of sensitized mice [13, 14]. In addition, it has been shown that IL-13 is a key cytokine that induces goblet cell hyperplasia and airway hyperreactivity [15, 16]. Regarding the role of IL-25 in allergic inflammation, we have recently shown that IL-25 mRNA is detected in the airways of antigen-sensitized, antigen-inhaled mice [Tamachi et al., submitted], although we have not identified the IL-25-producing cells in the site of allergic airway inflammation. Furthermore, we have investigated the effect of soluble IL-25 receptor (sIL-

25R), which is able to neutralize the bioactivity of IL-25, on antigen-induced eosinophil and CD4+ T cell recruitment into the airways and have found that the injection of sIL-25R significantly inhibits antigen-induced eosinophil and CD4+ T cell recruitment into the airways [Tamachi et al., submitted]. Although we could not formally exclude the possibility that the other ligand IL-17B for IL-25R [8] is involved in the sIL-25R-mediated inhibition of antigen-induced eosinophil recruitment into the airways, it is suggested that IL-25 is the main target of sIL-25R, because the in vivo administration of IL-17B induces neutrophil but not eosinophil recruitment [8], whereas IL-25 induces eosinophil recruitment in many experimental systems including ours [3–5; Tamachi et al., submitted]. Further experiments using a neutralizing antibody specific for IL-25 or mice lacking IL-25 are needed to define the specific role of IL-25 in causing allergic airway inflammation.

Mechanisms Underlying IL-25-Mediated Eosinophil Recruitment into the Airways

To address the role of IL-25 in allergic airway inflammation, we generated CC10 IL-25 mice that express murine IL-25 specifically in the lung under the control of a rat CC10 promoter [Tamachi et al., submitted]. CC10 IL-25 mice exhibited no apparent abnormalities in cell populations in the thymus, spleen and peripheral blood. In addition, without the inhaled antigen challenge, no inflammatory cell infiltration was observed in the lung in CC10 IL-25 mice. However, antigen-induced eosinophil and CD4+ T cell recruitment into the airway was significantly enhanced in CC10 IL-25 mice as compared with that in wildtype mice. Th2 cytokine production in the airways was also increased in antigen-inhaled CC10 IL-25 mice. Importantly, depletion of CD4+ T cells by the in vivo administration of anti-CD4 antibody inhibited IL-25-mediated enhancement of eosinophil recruitment into the airways, indicating that CD4+ T cells are required for IL-25-mediated allergic inflammation. In contrast, previous studies have demonstrated that the intranasal administration of large amounts of recombinant IL-25 (5 μg/body) or the systemic expression of IL-25 induces Th2 cytokine production and eosinophil infiltration even in the absence of CD4+ T cells [3–5]. Together, these results suggest that IL-25 could induce allergic inflammation by two different mechanisms. In a situation where IL-25 is abundant, IL-25 itself is sufficient for causing allergic inflammation through the induction of IL-4, IL-5 and IL-13 from non-T/non-B cells. In contrast, in other situations where the amounts of IL-25 are limited, collaboration with CD4+ T cells is required for IL-25-mediated allergic inflammation. Because the levels of the endogenously produced IL-25 in the lung of antigen-sensitized, antigen-inhaled mice are lower than those of CC10 IL-25 mice, it is suggested that in a physiological setting, IL-25 needs antigen-activated CD4+ T cells to exert its function on allergic inflammation.

The mechanisms by which CD4+ T cells are required for IL-25-mediated allergic airway inflammation have not yet been elucidated. Because IL-25 does not directly enhance Th2 cell differentiation in vitro [3; our unpubl. data], it is unlikely that IL-25 enhances allergic inflammation through the induction of Th2 cell differentiation. On the other hand, it has been demonstrated that the expression of thymus and activation-regulated chemokine (TARC), a specific ligand for CC chemokine receptor 4 [17, 18], is enhanced in the lung upon IL-25 stimulation [4]. Because it has been shown that TARC induces chemotaxis of T cells, especially of Th2 cells [19, 20], and plays a significant role in the induction of Th2-cell-mediated eosinophil recruitment into the airways in a murine model of asthma [21], IL-25 may induce the recruitment of CD4+ T cells through TARC expression and may then enhance CD4+ T-cell-mediated allergic airway inflammation. However, it is also possible that some cytokines and/or chemokines produced by CD4+ T cells are required for the recruitment, differentiation and/or activation of undefined IL-25-responding cells (fig. 1). Future studies identifying IL-25-responding cells could help the understanding of the role of CD4+ T cells in IL-25-mediated enhancement of allergic airway inflammation.

In conclusion, although there has been no information available on the expression levels of IL-25 in allergic diseases in humans, accumulating evidence including ours raises the possibility that IL-25 may be involved in the enhancement and/or prolongation of Th2-cell-mediated allergic diseases such as asthma and allergic rhinitis and thus suggests that IL-25 could be a possible target of these diseases.

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T-bet inhibits both T_H2 cell–mediated eosinophil recruitment and T_H17 cell–mediated neutrophil recruitment into the airways

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Background: Previous studies have shown that mice lacking T-bet, a critical transcription factor for T_H1 cell differentiation, spontaneously develop airway inflammation with intense eosinophil infiltrates. However, the mechanism underlying T-bet–mediated inhibition of allergic airway inflammation is still unknown.

Objective: To determine the regulatory role of T-bet in antigen-induced allergic airway inflammation.

Methods: We examined the role of T-bet in antigen-induced allergic airway inflammation using T-bet$^{-/-}$ mice on a BALB/c background that did not develop spontaneous airway inflammation. We also examined the role of T-bet expression of CD4$^+$ T cells in airway inflammation by adoptive transfer experiments.

Results: We found that antigen-induced eosinophil recruitment, goblet cell hyperplasia, and T_H2 cytokine production in the airways were enhanced in T-bet$^{-/-}$ mice. However, in the absence of signal transducer and activator of transcription 6 (STAT6), T-bet deficiency could not induce the antigen-induced eosinophilic airway inflammation. Adoptive transfer of T-bet$^{-/-}$ or T-bet$^{+/+}$ CD4$^+$ T cells to T-bet$^{-/-}$Rag-2$^{-/-}$ mice revealed that the expression of T-bet in CD4$^+$ T cells was vital for the inhibition of antigen-induced eosinophilic airway inflammation. Interestingly, antigen-induced neutrophil recruitment in the airways was also enhanced in T-bet$^{-/-}$ mice. Moreover, T-bet$^{-/-}$ CD4$^+$ T cells preferentially differentiated into IL-17–producing cells that mediated neutrophilic airway inflammation.

Conclusion: T-bet inhibits both T_H2 cell–mediated eosinophilic inflammation and T_H17 cell–mediated neutrophilic inflammation in the airways.

Clinical implications: The dysfunction of T-bet may be involved in the pathogenesis of severe asthma, in which accumulation of neutrophils as well as eosinophils in the airways is a hallmark of disease. (J Allergy Clin Immunol 2007;119:662-70.)

Key words: Allergic inflammation, T-bet, Eosinophils, T_H2 cells, T_H17 cells

Asthma is characterized by allergic airway inflammation with intense eosinophil and CD4$^+$ T-cell infiltrates.[1-3] It has been demonstrated that T_H2 cell–derived cytokines such as IL-4, IL-5, IL-9, and IL-13 play critical roles in orchestrating and amplifying allergic inflammation in asthma.[1-3] On the other hand, IFN-γ, the principal T_H1 cell–derived cytokine, has been shown to prevent the development of antigen-induced allergic airway inflammation.[4,5] Thus, allergic airway inflammation is regulated by the balance between T_H2 cells and T_H1 cells.

It has recently been shown that mice lacking T-bet, a T-box transcription factor required for T_H1 cell differentiation and IFN-γ production,[6,7] spontaneously develop airway inflammation with intense eosinophil infiltrates and airway hyperreactivity.[8] It has also been shown that the number of CD4$^+$ T cells expressing T-bet is decreased in the airways of patients with asthma.[8] Moreover, polymorphisms of the T-bet gene have been reported to be associated with asthma and airway hyperresponsiveness.[9] These findings suggest that T-bet may be a negative regulator for allergic airway inflammation and airway hyperreactivity in both human beings and mice. However, the mechanism underlying T-bet–mediated inhibition of allergic airway inflammation is still largely unknown. Particularly, the spontaneous airway inflammation in T-bet–deficient (T-bet$^{-/-}$) mice makes it difficult to adress the role of T-bet in the regulation of allergic airway inflammation provoked by the specific antigen.

Therefore, we examined the regulatory role of T-bet in antigen-induced allergic airway inflammation using T-bet$^{-/-}$ mice on a BALB/c background in which we unexpectedly found that spontaneous airway inflammation did not develop. We show here that the expression of T-bet in CD4$^+$ T cells is crucial not only for the inhibition of T_H2 cell–mediated eosinophil recruitment but also for the inhibition of IL-17–mediated neutrophil recruitment into the airways.

METHODS

Mice

T-bet$^{-/-}$ mice[7,8] on a BALB/c background (BALB/c T-bet$^{-/-}$ mice) and on a C57BL/6 background (C57BL/6 T-bet$^{-/-}$ mice) were

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Abbreviations used

BALF: Bronchoalveolar lavage fluid
FACS: Fluorescence-activated cell sorting
HE: Hematoxylin-eosin
mEAR2: Mouse eosinophil-associated ribonuclease 2
OVA: Ovalbumin
PAS: Periodic acid-Schiff
WT: Wild-type

obtained from Jackson Laboratory (Bar Harbor, Me). BALB/c T-bet$^{-/-}$ mice were crossed with STAT6$^{-/-}$ mice[10] or Rag-2$^{-/-}$ mice to obtain T-bet$^{-/-}$ STAT6$^{-/-}$ mice or T-bet$^{-/-}$ Rag-2$^{-/-}$ mice, respectively. Ovalbumin (OVA)-specific DO11.10$^+$ T-cell receptor transgenic mice were backcrossed over 10 generations onto BALB/c mice and then crossed with BALB/c T-bet$^{-/-}$ mice to obtain DO11.10$^+$ T-bet$^{-/-}$ mice. All mice were housed in micro-isolator 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 twice with 6 μg OVA (Sigma Chemical Co, St Louis, Mo) in 4 mg aluminum hydroxide (alum) at 2-week intervals. Two weeks after the second immunization, the sensitized mice were given with aerosolized OVA (50 mg/mL) dissolved in 0.9% saline by a DeVilbiss 646 nebulizer (DeVilbiss Corp, Somerset, Pa) for 20 minutes. As a control, 0.9% saline alone was administered by the nebulizer. Thirty-six hours after the inhalation, a sagittal block of left lung and trachea was excised, fixed in 10% buffered formalin, and embedded in paraffin. Sections of lung and trachea (3 μm thick) were stained with hematoxylin-eosin (HE), Luna, and periodic acid-Schiff (PAS) according to standard protocols. The number of eosinophils in the submucosal tissue of trachea was counted in Luna-stained sections and expressed as the number of eosinophils per the length of the basement membrane of trachea as described previously.[11] The number of lymphocytes, eosinophils, and neutrophils recovered in the bronchoalveolar lavage fluid (BALF) was evaluated as described previously.[12] A fraction of the cells was subjected to a flow cytometric analysis for the lymphocyte surface phenotyping.

Cytokine levels in BALF

The amounts of IL-4, IL-5, and IFN-γ in the BALF were determined by ELISA kits from BD PharMingen (San Diego, Calif). The amounts of IL-13 and IL-17 in the BALF were determined by ELISA kits from R&D Systems Inc (Minneapolis, Minn). The detection limits of these assays were 15 pg/mL of IL-4 and IL-5, 1.5 pg/mL of IL-13, 5 pg/mL of IL-17, and 50 pg/mL of IFN-γ.

Flow-cytometric analysis

Cells from the BALF were stained and analyzed on a FACS Calibur (Becton Dickinson, San Jose, Calif) by using BD CellQuest Pro software (Becton Dickinson) as described previously.[12]

RT-PCR

Total cellular RNA was prepared from lung and RT-PCR for CD3ε and eosinophil-associated RNase 2 (mEAR2) was performed.[13] Primer pairs for mEAR2 were described previously.[14] Primer pairs for CD3ε were 5′caggacgatgccgagaacattgaa3′ and 5′tcatagtctgggttgggaacaggt3′. 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.

Antigen-induced T_H cell differentiation

Splenocytes (2 × 10^6/mL) from DO11.10$^+$ T-bet$^{-/-}$ mice and the littermate DO11.10$^+$ mice were stimulated with OVA323-339 peptide (50 μmol/L) in a 24-well microtiter plate at 37°C for 48 hours. Where indicated, IL-12 (20 ng/mL; R&D Systems Inc) was added to polarize toward T_H1 cells (T_H1 condition), and IL-4 (20 ng/mL; R&D Systems Inc) and anti–IFN-γ antibody (20 μg/mL; XMG1.2) was added to polarize toward T_H2 cells (T_H2 condition). Cells were washed with PBS and cultured for another 3 days in the same condition except that IL-2 (5 ng/mL; R&D Systems Inc) was added in nonpolarizing T_H0 condition and T_H1 condition. Intracellular cytokine analyses for IL-4, IL-17, and IFN-γ were performed by using anti–IL-4 phycoerythrin (BVD4-1D11; BD PharMingen), anti–IL-17 phycoerythrin (TC11-18H10.1; BD PharMingen), and anti–IFN-γ allophycocyanin (APC) (XMG1.2; BD PharMingen) according to the manufacturer's instructions. Detection of intracellular IL-13 was performed with biotinylated anti–IL-13 antibody (R&D Systems Inc) and streptavidin-APC (BD PharMingen).

Adoptive transfer experiments for antigen-induced airway inflammation

Splenic CD4$^+$ T cells were purified (>95% pure by flow cytometry) using T cell enrichment columns (R&D Systems Inc) from DO11.10$^+$ T-bet$^{-/-}$ mice and DO11.10$^+$ mice as described previously.[13] CD4$^+$ T cells (2 × 10^6 cells/mouse) were transferred intravenously to T-bet$^{-/-}$ Rag-2$^{-/-}$ mice, and these mice were then immunized twice with 6 μg OVA in 4 mg alum at 2-week intervals. Two weeks after the second immunization, the sensitized mice were given aerosolized OVA (50 mg/mL) in saline. Where indicated, anti-murine IL-17 antibody (150 μg/mouse; clone 50104; R&D Systems Inc) or control rat IgG_{2a} (150 μg/mouse) was administered intraperitoneally at 12 hours before the inhaled OVA challenge. At 36 hours after the OVA inhalation, the number of lymphocytes, eosinophils, and neutrophils in the BALF and cytokine levels in the BALF was evaluated.

Data analysis

Data are summarized as means ± SDs. The statistical analysis of the results was performed by the unpaired *t* test. *P* values < .05 were considered significant.

RESULTS

Spontaneous airway inflammation does not develop in BALB/c T-bet$^{-/-}$ mice

It has been shown that T-bet$^{-/-}$ mice develop peribronchial inflammation with eosinophil and lymphocyte infiltrates in the absence of inhaled antigen challenge.[8] To determine the role of T-bet in the regulation of allergic airway inflammation in more detail, we first examined airway inflammation in T-bet$^{-/-}$ mice on a T_H2-biased BALB/c background (BALB/c T-bet$^{-/-}$ mice) in parallel with T-bet$^{-/-}$ mice on a T_H1-biased C57BL/6 background (C57BL/6 T-bet$^{-/-}$ mice). Consistent with the previous report,[8] C57BL/6 T-bet$^{-/-}$ mice developed peribronchial inflammation with eosinophil and lymphocyte infiltrates in the absence of antigen challenge (Fig 1, *A*). However, unexpectedly, BALB/c T-bet$^{-/-}$ mice did not develop peribronchial inflammation in the absence of

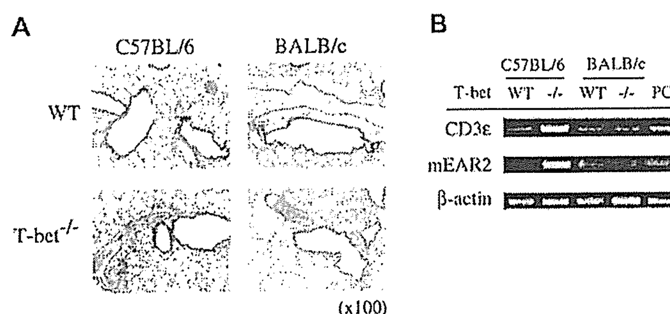

FIG 1. Spontaneous airway inflammation develops in T-bet$^{-/-}$ mice on a C57BL/6 background but not on a BALB/c background. **A,** Representative photomicrographs of HE-stained lung sections from 12-week-old T-bet$^{-/-}$ mice and littermate WT mice on either C57BL/6 or BALB/c background (x100; n = 5 mice in each group). **B,** RT-PCR analyses for CD3ε and mEAR2 mRNA were performed on lung tissues of T-bet$^{-/-}$ mice and WT mice on either C57BL/6 or BALB/c background (n = 5). A positive control *(PC)* is lung tissues of OVA-sensitized, OVA-inhaled BALB/c mice.

antigen challenge (Fig 1, *A*). RT-PCR analysis for CD3ε and mEAR2, one of orthologues of human eosinophil–derived neurotoxin,[15] of the lung tissue confirmed the presence of T cells and eosinophils in C57BL/6 T-bet$^{-/-}$ mice but not in BALB/c T-bet$^{-/-}$ mice (Fig 1, *B*). These results indicate that the induction of allergic airway inflammation by the absence of T-bet depends on the genetic backgrounds of mice.

Antigen-induced allergic airway inflammation is enhanced in BALB/c T-bet$^{-/-}$ mice

We then addressed the role of T-bet in antigen-induced airway inflammation in BALB/c T-bet$^{-/-}$ mice. OVA-sensitized BALB/c T-bet$^{-/-}$ mice and littermate wild-type (WT) mice were challenged with inhaled OVA, and histologic analysis of the lung was performed at 36 hours after the OVA inhalation. As shown in Fig 2, *A*, antigen-induced inflammatory cell infiltration in the peribronchial and perivascular areas was significantly enhanced in T-bet$^{-/-}$ mice compared with WT mice, and the majority of cells infiltrating into the lung were eosinophils (Fig 2, *A, a vs b*). Consequently, the number of eosinophils infiltrating into the submucosal tissue of trachea was significantly increased by 88% in T-bet$^{-/-}$ mice (n = 8 mice in each group; $P < .01$; Fig 2, *A, c vs d*). Antigen-induced eosinophil recruitment into the trachea at 72 hours after the OVA inhalation was also significantly increased in T-bet$^{-/-}$ mice (data not shown). In addition, antigen-induced epithelial goblet cell hyperplasia was increased in T-bet$^{-/-}$ mice compared with that in WT mice (Fig 2, *A, e vs f*).

Consistent with the histologic analysis, the number of eosinophils recovered in BALF at 36 hours after the OVA inhalation was significantly increased in T-bet$^{-/-}$ mice (n = 8 mice in each group; $P < .01$; Fig 2, *B*). The number of neutrophils recovered in the BALF at 36 hours was also increased in T-bet$^{-/-}$ mice (n = 8; $P < .05$; Fig 2, *B*). On the other hand, the number of lymphocytes in the BALF was not significantly increased in T-bet$^{-/-}$ mice (Fig 2, *B*), and fluorescence-activated cell sorting (FACS) analysis of lymphocytes in the BALF revealed that the majority of lymphocytes were T-cell receptor (TCR)$\alpha\beta^+$CD4$^+$ T cells in both T-bet$^{-/-}$ mice and WT mice (data not shown).

The levels of IL-4, IL-5, and IL-13 in the BALF at 36 hours after the OVA challenge were significantly increased in T-bet$^{-/-}$ mice (n = 5; $P < .01$, $P < .05$, and $P < .05$, respectively; Fig 2, *C*). On the other hand, IFN-γ was undetectable in both WT mice and T-bet$^{-/-}$ mice at 36 hours after the OVA challenge (data not shown). Taken together, these results suggest that T-bet is crucial for the inhibition of antigen-induced T_H2 cytokine production and eosinophil recruitment in the airways.

The enhanced antigen-induced eosinophil recruitment but not neutrophil recruitment into the airways of T-bet$^{-/-}$ mice depends on STAT6

We next examined whether STAT6, a critical transcription factor for T_H2 cell differentiation under IL-4 signaling,[16] was involved in the enhanced antigen-induced allergic airway inflammation in T-bet$^{-/-}$ mice by generating mice lacking both T-bet and STAT6 (T-bet$^{-/-}$STAT6$^{-/-}$ mice). Again, the number of eosinophils and neutrophils recovered in the BALF was significantly increased in T-bet$^{-/-}$ mice as compared with that in littermate WT mice (n = 5 each; $P < .01$ and $P < .05$, respectively; Fig 3, *A*). Antigen-induced eosinophil recruitment into the airways in T-bet$^{-/-}$STAT6$^{-/-}$ mice was significantly decreased compared with that in T-bet$^{-/-}$ mice (n = 5 each; $P < .001$; Fig 3, *A*), suggesting that STAT6 is essential for the enhanced antigen-induced eosinophil recruitment in T-bet$^{-/-}$ mice. Histological analysis of the lung confirmed that STAT6 was essential not only for the enhancement of antigen-induced peribronchial inflammation with eosinophil infiltrates (Fig 3, *B, upper panels*) but also for the goblet cell hyperplasia (Fig 3, *B, bottom panels*) in T-bet$^{-/-}$ mice. These results indicate that STAT6 plays a dominant role over T-bet–mediated inhibition in the induction of T_H2 cell–mediated airway inflammation. In contrast, antigen-induced neutrophil recruitment into the airways of T-bet$^{-/-}$ mice was not