DISCUSSION

It is widely accepted that the mechanistic basis of the hygiene hypothesis for suppression of IgE responses is an increase in the Th1/Th2 ratio (12). However, in reality, the Th1 response exacerbates allergic reactions, as human asthma is associated with the production of IFN-γ, a cytokine that appears to contribute to the pathogenesis of the disease (35). Furthermore, the adoptive transfer of allergen-specific Th1 cells causes severe airway inflammation (36). Thus, a shift in the Th1/Th2 ratio alone cannot explain all of the immunological findings observed in allergic diseases (1). Furthermore, there are several studies suggesting that BCG vaccination has little or no effect on the development and prevalence of allergic diseases (37, 38). Therefore, it is necessary to better understand the precise mechanism of IgE suppression in BCG-treated animals or humans.

In this study, neither a Th1/Th2 imbalance nor an involvement of regulatory T cells was observed in response to BCG treatment (Fig. 1). Instead, we demonstrated that IL-21–induced Bε cell apoptosis is the mechanism responsible for BCG-mediated suppression of IgE production (Figs. 1, 3, and 5). Because the human IL-21 responses to BCG vaccination were heterogeneous (Fig. 6 C), it seems likely that the magnitude of the response in each individual could cause different degrees of BCG-induced IgE suppression and might be prognostic.

Previous studies have indicated that IL-21 is preferentially expressed by activated $CD4^+$ T cells (20), the results that are partially in agreement with the present data, as half of peripheral Vα14 NKT cells are $CD4^+$ (39, 40). Interestingly, upon anti-CD3 mAb stimulation, Vα14 NKT cells, but not conventional T cells, preferentially expressed IL-21 (Fig. S2 A), similar to the results with BCG (Fig. 3 B). Therefore, the major IL-21 producers in response to BCG in mice are Vα14 NKT cells.

It has been proposed that, for full activation of Vα14 NKT cells to produce IFN-γ, two signals are required: one CD1d-dependent and the other TLR-mediated IL-12–dependent signals (31). In agreement with this, IL-21 expression by BCG-activated Vα14 NKT cells was significantly inhibited by blocking with antibodies to IL-12 and/or CD1d (Fig. 3 D). Therefore, it is likely that Vα14 NKT cells recognize endogenous antigens presented by CD1d molecules but require IL-12 signals to produce IL-21. Nevertheless, it is still possible that glycolipid BCG components such as phosphatidylinositol mannoside may directly stimulate Vα14 NKT cells to produce IL-21 in a CD1d-dependent manner (41, 42).

In terms of the receptors on DCs that are required for BCG recognition and signal transduction, we showed in this study that BCG-induced IL-12 production is IRAK-4 and MyD88 dependent (Fig. 2 E and Fig. S1). These results in mice are consistent with a recent report indicating that BCG cannot induce IL-12 or IFN-γ production by PBMCs from IRAK-4–deficient patients (43). In addition, it has been reported that BCG enhances NF-κB–dependent gene transcription through the activation of phosphatidylinositol 3 kinase and c-Jun N-terminal kinase cascades (44). The activated NF-κB is then liberated for nuclear translocation and transactivates a variety of immune response genes, including IL-12.

In contrast to a previous report that implicated TLR2 and TLR4 in the recognition of mycobacterial antigens (32), we could not identify any involvement of these receptors in IL-12 production by BCG-stimulated BM-DCs (Fig. 2, C and D). In agreement with our findings, it has recently been reported that TLR2/4 double KO mice infected with live BCG have normal adaptive immune responses and survived as long as WT mice (45). As whole BCG contains multiple components including mycobacterial glycolipids, proteins, and DNA, several receptors that use IRAK-4 and MyD88 as the signal transducer appear to be involved in the complex recognition of BCG.

In IL-21R–deficient mice, the level of circulating IgE is high, whereas that of IgG1 is low (23, 46). Similarly, in human B cells, IL-21 inhibits IgE production and stimulates IgG4 (analogous to mouse IgG1) production (19). These results suggest that IL-21 differentially regulates IgE and IgG1 (IgG4 in humans) class switching. In fact, Suto et al. (18) reported that IL-21 specifically suppresses IgE production by inhibiting germ line Cε transcripts. Our present findings do not exclude this possibility. IL-21 has also been reported to induce apoptosis in resting and activated B cells by reducing the expression levels of apoptosis-related genes (25, 26). However, in this report, we have shown that IL-21 selectively induces apoptosis in Bε, but not Bγ, cells (Fig. 4 D). Thus, our findings that BCG-activated IL-21–expressing Vα14 NKT cells suppressed IgE production even after class switching (Fig. 4 C) suggests that the role of IL-21 on Bε cells is to control cell growth and viability, rather than to regulate the differentiation and maturation of these cells.

We found that expression of a proapoptotic gene, Bmf, was significantly higher in Bε cells than in Bγ cells (Fig. 5 A). Under physiological conditions, Bmf, which is a BH3 domain–only Bcl-2 family member that inhibits Bcl-2 function and accelerates apoptosis, binds to myosin V motors via the dynein light chain 2 domain of Bmf (34). In response to certain cellular damage signals, Bmf is supposed to be released from the myosin V motors and trigger apoptosis (34). Because Bmf from Bε cells induced apoptosis and a mutation in the BH3 domain of Bmf failed to induce apoptosis (Fig. S3), we confirmed that Bmf expressed in Bε cells is functional, and that the BH3 domain is important for the binding to Bcl-2 and is essential for its proapoptotic activity. In fact, the binding of Bmf with Bcl-2 was up-regulated by IL-21R signaling (Fig. 5 C). Therefore, BCG-mediated Bε cell apoptosis is due to the augmented formation of Bmf–Bcl-2 complexes generated by IL-21R signaling in Bε cells.

Finally, we defined the mechanism of BCG-induced IL-21–dependent suppression of IgE production in humans (Fig. 6). In a broader context, these findings may explain the mechanisms underlying the BCG-mediated suppression of allergic diseases and the epidemiological data indicating a reduction in the morbidity of allergic diseases in patients who

have been infected with *Mycobacterium tuberculosis*. Interestingly, IL-21–mediated B cell responses in C57BL/6 mice differ from those in BALB/c mice (26), suggesting that there is a genetic polymorphism with respect to the outcome of IL-21 signaling in B cells. In fact, a recent report indicated that polymorphisms in the IL-21R gene locus differentially affect serum IgE levels in humans (47). In this study, consistent with our data, the levels of IL-21 expression induced by BCG stimulation varied among the individuals examined (Fig. 6 C). These results suggest that the response to BCG in humans is dependent, at least in part, on genetic background. The specific genes responsible for the heterogeneity in BCG-mediated IL-21 production have not been identified. However, this observation may be applied to the development of diagnostic or therapeutic strategies in which the levels of IL-21 expression are used to evaluate the efficacy of BCG treatment, or in determining the potential benefit of therapy using bacterial products such as CpG for allergic diseases.

MATERIALS AND METHODS

Mice. 7–10-wk-old female BALB/c mice were purchased from Japan CREA Inc. Vα14 NKT-deficient (Vα14 NKT KO) mice on a BALB/c background (48), IRAK-4 KO (49), TLR2 KO, TLR4 KO, and MyD88 KO mice (50, 51) have been described. TLR2 and TLR4 double KO mice were generated by breeding. Mice were kept under specific pathogen-free conditions, maintained on an OVA-free diet, and treated in accordance with the guidelines for animal care at RIKEN Research Center for Allergy and Immunology.

Allergic sensitization and BCG. Allergic epicutaneous sensitization was performed as described previously (27). In brief, a 1-cm^2 sterile patch infused with 100 μl of PBS solution with or without 100 μg OVA (grade V; Sigma-Aldrich) was placed on the shaved back of mice and fixed in place with a bio-occlusive dressing and an elastic bandage. Patches were left on for 48 h and removed. The sensitization course was repeated at the same skin site every week for 4 wk. For BCG vaccination, mice were given a weekly i.p. injection of BCG (500 μg/mouse) or PBS at the time of OVA sensitization. The attenuated BCG (strain Tokyo) was purchased from the Japan BCG Laboratory.

Flow cytometry. Cells were stained with antibodies after adding 2.4G2 (BD Biosciences) for Fc blocking. The following antibodies were used: FITC–anti-CD19 (1D3), FITC–anti-IgE (R35-72), APC–anti-IgG1 (X59), FITC–anti-TCRβ (H57-597), APC–anti–IL-12p40/70 (C15.6), and PE–anti-CD11c (HL3; BD Biosciences). PE-conjugated α-GalCer–loaded CD1d tetramer (α-GalCer/CD1d tetramer) was prepared as described previously (52). For intracellular staining, BM-DCs were fixed and permeabilized with BD Cytofix and Cytoperm kits after staining with PE–anti-CD11c. They were then stained with APC–anti–IL-12p40/70. FACS analysis of at least 10,000 cells and cell sorting were performed with a FACSCalibur (BD Biosciences) with FlowJo software (TreeStar) or with a MoFlo cell sorter (DakoCytomation).

Cell preparations and cultures. 2×10^6 BM-DCs obtained by culturing BM for 6 d with 10 ng/ml GM-CSF were further cultured in the presence or absence of BCG, CpG, LPS (Invivogen), PGN from *Escherichia coli* (Invivogen), or 10 μg/ml anti-CD3 mAb (2C11; BD Biosciences) for 48 h at 37°C. For blocking experiments, mAb against CD1d or IL-12p40/p70 (clones 1B1 and C17.8, respectively; BD Biosciences), or an isotype control was added at a concentration of 20 μg/ml after 2.4G2 treatment. TCRβ$^+$ cells or Vα14 NKT cells with a purity of >98% were obtained from liver MNCs (52) using an Auto MACS (Miltenyi Biotec) after staining with FITC–anti-TCRβ and sorting with anti-FITC magnetic beads (Miltenyi Biotec). Vα14 NKT cells were then isolated from TCRβ$^+$ cells by MoFlo using PE–α-GalCer/CD1d tetramer. Conventional T or CD4$^+$ T cells were isolated from an α-GalCer/CD1d tetramer$^-$ fraction of TCRβ$^+$ liver MNCs. Bε and Bγ cells generated from splenic CD19$^+$ cells in the presence of 10 μg/ml sCD40L (ALX-850-075; Qbiogene) and 20 ng/ml of recombinant IL-4 (PeproTech) for 3 d (33) were cultured for 30 h for the apoptosis assay or for an additional 5 d to investigate IgE responses.

ELISA. Cytokines (IL-12p70 and IL-6) and Ig subclasses (IgG1, IgG2a, and IgE) were measured by ELISA using kits or sets of antibodies (BD Biosciences) according to the manufacturer's protocol. Specific antibodies were also measured as described previously (7).

RT-PCR. Total RNA was extracted by RNAeasy (QIAGEN), and cDNA was synthesized with random primers after DNase treatment. The following RT-PCR primer sets were used for mouse genes: IL-21, 5′-CCCTTGTCTGTCTGGTAGTCATC-3′ and 5′-ATCACAGGAAGGGCATTTAGC-3′; IgE (Cε), 5′-AGGAACCCTCAGCTCTACCC-3′ and 5′-GCCAGCTGACAGAGACATCA-3′; mIL-21R, 5′-TGTCAATGTGACGGACCAGT-3′ and 5′-CAGCATAGGGGTCTCTGAGG-3′; γc, 5′-GTCGACAGAGCAAGCACCATGTTGAAACTA-3′ and 5′-GGATCCTGGGATCACAAGATTCTGTAGGTT-3′; Bmf, 5′-CAGACCCTCAGTCCAGCTTC-3′ and 5′-CGTATGAAGCCGATGGAACT-3′; Bcl-2, 5′-GGTGGTGGAGGAACTCTTCA-3′ and 5′-CATGCTGGGGCCATATAGTT-3′; and HPRT, 5′-AGCGTCGTGATTAGCGATG-3′ and 5′-CTTTTATGTCCCCCGTTGAC-3′. The numbers of PCR cycles were as follows: 30 for HPRT; 35 for IgE, γc, and IL-21R; 40 for IL-21 and Bmf; and 45 for Bcl-2. The amounts of cDNA were standardized by quantification of the housekeeping gene HPRT using primers for mouse samples. The human IL-21 mRNA levels were quantified by real-time quantitative PCR on the ABI Prism 7000 sequence detection system (Applied Biosystems) by using TaqMan assay kits and TaqMan Gene Expression Assays (primers and TaqMan probes).

Electrophoretic mobility shift assay. 2×10^6 BM-DCs were stimulated with 50 μg/ml BCG or 1.0 μM CpG-B for the indicated periods. Nuclear extracts were prepared and used for Gel Shift Assay Systems (Promega) as described previously (50).

Bε cell–derived Bmf and its mutants. cDNAs encoding bmf were amplified from Bε cells by PCR using primers 5′-CCGAATTCGGATGGAGCCACCTCAGTGTGT-3′ and 5′-GCGGCCGCCTGCATTCCTGGTGATCCAT-3′ (EcoRI and NotI sites for cloning are underlined). The amplified products were cloned using the pGEM-T Easy Vector System (Promega). Mutant cDNAs were generated by PCR using point-mutated primer pairs.

Immunoprecipitation and Western blotting. Interaction of Bmf with Bcl-2 in Bε cells was detected by immunoprecipitation with anti–Bcl-2 mAb (clone 7; BD Transduction Laboratories) and subsequent immunoblotting with anti-Bmf rabbit antibody (Cell Signaling). The protein levels were visualized by ECL (GE Healthcare) using horseradish peroxidase–conjugated Protein A/G (Pierce Chemical Co.).

Human studies. All human specimens were obtained under informed consent. The protocol for the human research project has been approved by the Ethics Committee of Chiba University and RIKEN, and conformed to the provisions of the Declaration of Helsinki in 1995. 10^8 PBMCs from healthy volunteers were prepared by Ficoll-Paque density gradient centrifugation and used for the cultures. Human recombinant IL-21 was purchased from BIOSOURCE Inc. Human total IgE was measured with a sensitive immune assay (GE Healthcare).

Statistical analysis. Statistical analyses were performed using the Student's *t* test or matched pairs *t* test. $P < 0.05$ was considered statistically significant.

Online supplemental material. Fig. S1 provides data demonstrating that MyD88 signaling in DCs is required for BCG-induced activation. Fig. S2 contains data demonstrating IL-21 mRNA expression by NKT cells, $CD4^+$ T cells, and $CD8^+$ T cells of murine and human origin. Fig. S3 provides the data indicating proapoptotic activity of Bε cell–derived Bmf and functional domain analysis using mutant Bmf in Baf3 cells. The online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20062206/DC1.

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Involvement of TNF Receptor-Associated Factor 6 in IL-25 Receptor Signaling[1]

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IL-25 (IL-17E) induces IL-4, IL-5, and IL-13 production from an unidentified non-T/non-B cell population and subsequently induces Th2-type immune responses such as IgE production and eosinophilic airway inflammation. IL-25R is a single transmembrane protein with homology to IL-17R, but the IL-25R signaling pathways have not been fully understood. In this study, we investigated the signaling pathway under IL-25R, especially the possible involvement of TNFR-associated factor (TRAF)6 in this pathway. We found that IL-25R cross-linking induced NF-κB activation as well as ERK, JNK, and p38 activation. We also found that IL-25R-mediated NF-κB activation was inhibited by the expression of dominant negative TRAF6 but not of dominant negative TRAF2. Furthermore, IL-25R-mediated NF-κB activation, but not MAPK activation, was diminished in TRAF6-deficient murine embryonic fibroblast. In addition, coimmunoprecipitation assay revealed that TRAF6, but not TRAF2, associated with IL-25R even in the absence of ligand binding. Finally, we found that IL-25R-mediated gene expression of IL-6, TGF-β, G-CSF, and thymus and activation-regulated chemokine was diminished in TRAF6-deficient murine embryonic fibroblast. Taken together, these results indicate that TRAF6 plays a critical role in IL-25R-mediated NF-κB activation and gene expression. *The Journal of Immunology,* **2006, 176: 1013–1018.**

Interleukin-25 has recently been identified as the fifth member of the IL-17 cytokine family (IL-17E) by database searching (1–4). The IL-17 family now consists of six family members, namely IL-17 (IL-17A), IL-17B, IL-17C, IL-17D, IL-25, and IL-17F (5–7). Among IL-17 family members, IL-25 is less homologic to other IL-17 family members, e.g., 18% homology to IL-17A at amino acid level. Accordingly, in vivo biologic activities of IL-25 are markedly different from those described for IL-17 and other IL-17 family cytokines (2–4, 8–10). Remarkably, it has been shown that the enforced expression of IL-25 induces IL-4, IL-5, and IL-13 production from an unidentified non-T/non-B cell population and subsequently induces Th2-type immune responses such as eosinophilic airway inflammation, mucus production, and airway hyperreactivity (2–4, 8).

IL-25R, which is also called IL-17BR, IL-17Rh1, or Evi27, is a 56-kDa single transmembrane protein with homology to IL-17R (1, 11, 12). IL-25R was first identified as a receptor for IL-17B (11) but IL-25R has subsequently been shown to exhibit a higher affinity for IL-25 than for IL-17B (1). IL-25 has also been demonstrated to activate NF-κB and induce IL-8 production in a human renal carcinoma cell line (1). However, the molecular components consisting of IL-25R signaling pathways and their regulation are still largely unknown.

It has recently been shown that TNFR-associated factor (TRAF)[3] family proteins play a critical role in a number of signaling pathways that activate NF-κB (13–16). TRAF family proteins contain a conserved TRAF-C domain that is essential for the interaction with their cognate receptors or cytoplasmic signaling proteins (13–16). Among TRAF family proteins, TRAF6 exhibits the unique properties in that its TRAF-C domain interacts with a peptide motif distinct from that recognized by other TRAF proteins (17), supporting the findings that TRAF6 exhibits various functions in regulating adaptive and innate immunity, bone metabolism, and cell apoptosis (13–16). The structural analysis of the peptide-TRAF6 interaction has clarified the TRAF6-binding motif as X-X-Pro-X-Glu-X-X-(aromatic/acidic residue) (17). The TRAF6-binding motif is found not only in adaptor proteins such as IL-1R-associated kinase (17) and TIFA (18) but also in membrane-bound proteins such as CD40 and the receptor activator of NF-κB RANK (17). Importantly, the TRAF6-binding motif is present in human and murine IL-25R.

In the present study, we investigated whether TRAF6 is involved in IL-25R signaling. Our results have clearly demonstrated a critical involvement of TRAF6 in IL-25R-mediated NF-κB activation and gene expression.

Materials and Methods

Cell culture

X63 cells were maintained in RPMI 1640 medium with 10% FCS, 50 μM 2-ME, and antibiotics (complete RPMI 1640 medium). Ba/F3 cells were cultured in complete RPMI 1640 medium supplemented with 10% (v/v) of the supernatant of murine IL-3-producing X63 cells (X63-IL-3; a gift from Dr. H. Karasuyama, Tokyo Medical and Dental University, Tokyo, Japan) (19). COS7 cells were cultured in DMEM supplemented with 10% FCS

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[3] Abbreviations used in this paper: TRAF, TNFR-associated factor; TARC, thymus and activation-regulated chemokine; MEF, murine embryonic fibroblast; DN, dominant negative; MKK, MAPK kinase; WT, wild type.

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and antibiotics (complete DMEM). Wild-type (WT) murine embryonic fibroblast (MEF), TRAF6-deficient ($TRAF6^{-/-}$) MEF (20), and Plat-E cells (21) were established and maintained as described elsewhere.

Plasmids

DNA fragment coding the extracellular domain of murine IL-25R, a gift from Dr. J. D. Shaughnessy (University of Arkansas for Medical Sciences, Little Rock, AR) (12) was fused to the fragment coding C-terminal 187 aa of MPL, a receptor for thrombopoietin (22), and cloned into expression vector pCDNA3 (pCDNA3 IL-25R-MPL). Expression vectors for WT TRAF2, dominant negative (DN) TRAF2, Flag-tagged WT TRAF6, and Flag-tagged DN TRAF6 were previously described (23). Expression vectors for Flag-tagged IL-25 (BCMGS Flag-IL-25), Flag-tagged IL-25R (pCMV1 Flag-IL-25R), and *myc*-tagged intracellular region of IL-25R (pCDNA3 *myc*-IL-25R) were constructed by PCR amplification using PFU polymerase (Stratagene). The DNA fragment coding Flag-tagged IL-25R was subsequently subcloned into the retrovirus vector pMX IRES-GFP to generate pMX Flag-IL-25R-IRES-GFP. Alanine substitution of IL-25R on glutaminic acid at aa 338 (IL-25R E338A) was generated by using a PCR-based site-directed mutagenesis kit (Stratagene). The mutation was confirmed by DNA sequencing.

Cytokines

X63 cells were transfected with BCMGS Flag-IL-25 to generate murine IL-25-producing X63 cells (X63-IL-25). The supernatant of X63-IL-25 cells was collected and used as a source of IL-25. The supernatant of murine IL-3-producing X63 cells (X63-IL-3) and the empty vector (BCMGS neo)-transfected X63 cells (X63-control) were also used as controls.

Bioassay for IL-25

IL-3-dependent Ba/F3 cells were transfected with pCDNA3 IL-25R-MPL and Ba/F3 cells that stably expressed IL-25R-MPL were selected by G418 (Ba/F3 IL-25R-MPL cells). The expression of IL-25R-MPL was evaluated not only at mRNA levels by RT-PCR analysis but also at protein levels with the response to the supernatant of X63-IL-25 cells. Subsequently, bioactivity of IL-25 was assessed by the proliferative response of Ba/F3 IL-25R-MPL cells. Briefly, Ba/F3 IL-25R-MPL cells (2×10^3 cells/well) were cultured in triplicate at 37°C in 96-well plates in the complete RPMI 1640 medium in the presence of X63-IL-25 conditioned medium or X63-IL-3 conditioned medium (as a positive control) for 36 h with 0.5 μCi of [^{3}H]thymidine added for the final 12 h. Empty vector (pCDNA3)-transfected Ba/F3 cells were used as a negative control.

Retrovirus-mediated expression of IL-25R in MEF

A transient retrovirus packaging cell line of Plat-E cells (2×10^6) was transfected with 3 μg of pMX Flag-IL-25R-IRES-GFP using FuGENE6 transfection reagents (Roche Diagnostics). At 24 h after the transfection, the medium was once changed and another 24 h later, the supernatant was harvested as virus stocks and stored at −80°C until use. WT MEF or $TRAF6^{-/-}$ MEF (1×10^6) were infected with 2 ml of virus stocks for 4 h in the presence of polybrene (1 μg/ml) and then diluted and maintained in the complete DMEM. Under these conditions, the efficiency of infection was 90% as assessed by GFP^+ cells by FACS.

Luciferase assay

COS7 cells (1×10^5) were transfected with 1.0 μg of pCMV1 Flag-IL-25R and 0.3 μg of NF-κB-responding *Photinus pyralis* luciferase reporter vector pNF-κB-Luc (Stratagene) using FuGENE6. In some experiments, expression vector for DN TRAF6 or DN TRAF2 was cotransfected. Empty vector was added to adjust the total amount of plasmid DNA for transfection. To normalize for transfection efficiency, 10 ng of *Renilla reniformis* luciferase reporter vector pRL-TK was added to each transfection. At 24 h after the transfection, cells were stimulated with X63-IL-25 condition medium or anti-Flag M2 mouse mAb (2 μg/ml; Sigma-Aldrich) at 37°C for 24 h, and the luciferase activity of *Photinus pyralis* and *Renilla reniformis* were determined by the Dual-Luciferase Reporter Assay System (Promega). *Photinus pyralis* luciferase activity of pNF-κB-Luc was normalized by *Renilla reniformis* luciferase activity of pRL-TK. Condition medium of X63-control cells or mouse monoclonal IgG1 (Ancell) was used as controls.

Nuclear accumulation of NF-κB p65

WT MEF or $TRAF6^{-/-}$ MEF were infected with retrovirus of pMX Flag-IL-25R-IRES-GFP as described earlier and Flag-IL-25R-expressing WT MEF or $TRAF6^{-/-}$ MEF were stimulated with X63-IL-25 condition medium or anti-Flag M2 mAb (2 μg/ml) at 37°C for 30 min. Nuclear extracts were prepared as described elsewhere (24), and DNA-binding activity of NF-κB p65 in the nuclear extracts was detected by Transfactor NF-κB chemiluminescent kit (BD Biosciences) according to the manufacturer's instruction. Briefly, nuclear extracts (5 μg) were added to wells coated with NF-κB consensus oligonucleotides and incubated for 1 h at room temperature. After washing, the wells were incubated with anti-NF-κB p65 rabbit polyclonal Ab, followed by anti-rabbit IgG HRP and then chemiluminescent substrate mixture. Chemiluminescent intensities were measured with Arvo 1420 multilabel counter (Wallac). For DNA competition experiments, 0.5 μg of unlabeled competitor oligonucleotide was added to the nuclear extracts.

Immunoblotting

MEF (1×10^5) were starved from FCS for over 12 h and then stimulated with anti-Flag mAb (2 μg/ml), mouse rIL-17 (100 ng/ml; R&D Systems), or mouse rIL-1β (10 ng/ml; PeproTech) for 30 min. The cells were then lysed with cell lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 0.875% Brij97, 0.125% Nonidet P-40, 8 mM DTT, and 1% protease inhibitor mixture (Sigma-Aldrich)) supplemented with 1 mM Na_3VO_4, 10 mM NaF, and 60 mM β-glycerophosphate. The aliquot of lysates was applied for SDS-PAGE. The following Abs were used for immunoblotting: anti-IκB-α (MBL Japan), anti-p38α/SAP2a, anti-p38 (pT180/pY182), anti-ERK1, anti-ERK1/2 (pT202/pT204), anti-pan JNK/SAPK1, and anti-JNK (pT183/pT185) (BD Transduction).

Coimmunoprecipitation assay

COS7 cells (4×10^5) were transfected with pCDNA3 *myc*-IL-25R (1.0 μg) and/or pME18S Flag-TRAF6 (1.0 μg), pME18S Flag-TRAF2 (1.0 μg), or pME18S Flag-TRAF5 (1.0 μg) using FuGENE6. Twenty-four hours after the transfection, cells were harvested, lysed with cell lysis buffer, and centrifuged to remove cellular debris. After preclearation, the supernatants were immunoprecipitated with either anti-*myc* mAb (9E10; Santa Cruz Biotechnology) or anti-Flag M2 mAb and 100 μl of protein G-Sepharose (Pharmacia). The immunoprecipitates or the aliquot of whole cell lysates were applied for immunoblotting with rabbit polyclonal anti-Flag Ab (Sigma-Aldrich) or biotin-labeled anti-*myc* mAb (9E10; Santa Cruz Biotechnology).

RT-PCR

Total cellular RNA was prepared, and RT-PCR analysis was performed as previously described (24). In brief, Flag-IL-25R-expressing WT MEF or $TRAF6^{-/-}$ MEF were stimulated with anti-Flag mAb (2 μg/ml) at 37°C for 3 h and the total cellular RNA was isolated using Isogen solution (Nippon Gene) according to the manufacturer's instruction. The following primer pairs were used for PCR: IL-6 (ATGAAGTTCCTCTCTGCAA GAG and GTTTGCCGAGTAGATCTCAAAG), G-CSF (GCTGTG GCAAAGTGCACTATG and AAGCCCTGCAGGTACGAAATG), TGF-β (ATTCAGCGCTCACTGCTCTTG and TCAGCTGCACTTG CAGGAGC), and thymus and activation-regulated chemokine (TARC) (TGAGGTCACTTCAGATGCTGC and ACCAATCTGATGGCCT TCTTC). RT-PCR for β-actin was performed as a control. All PCR amplifications were performed at least three times with multiple sets of experimental RNAs.

Data analysis

Data are summarized as mean ± SD. The statistical analysis of the results was performed by the unpaired *t* test. Values for $p < 0.05$ were considered significant.

Results

Establishment of a bioassay for IL-25

It has been reported that IL-25 activates NF-κB in a renal carcinoma cell line (1), but the signaling pathway under IL-25R is largely unknown. To examine IL-25 signaling in detail, we first prepared rIL-25 and an assay that verifies the bioactivity of rIL-25. Because IL-25 belongs to the cystine knot family and correct refolding and dimer formation seem to be required for its biological activity (6, 7), we used the mammalian cell-based cytokine expression system (19) rather than the *Escherichia coli*-based expression system. We first established X63 cells that stably produced mouse IL-25 (X63-IL-25 cells) and used the supernatant of

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

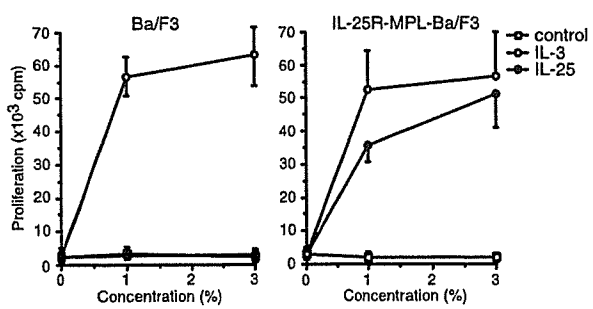

FIGURE 1. Establishment of a bioassay for IL-25. Control vector-transfected Ba/F3 cells (*left*) and IL-25R-MPL-expressing Ba/F3 cells (*right*) were cultured in the presence of the supernatant of X63-control, X63-IL-3, or X63-IL-25 cells at the indicated concentrations at 37°C for 36 h with 0.5 μCi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

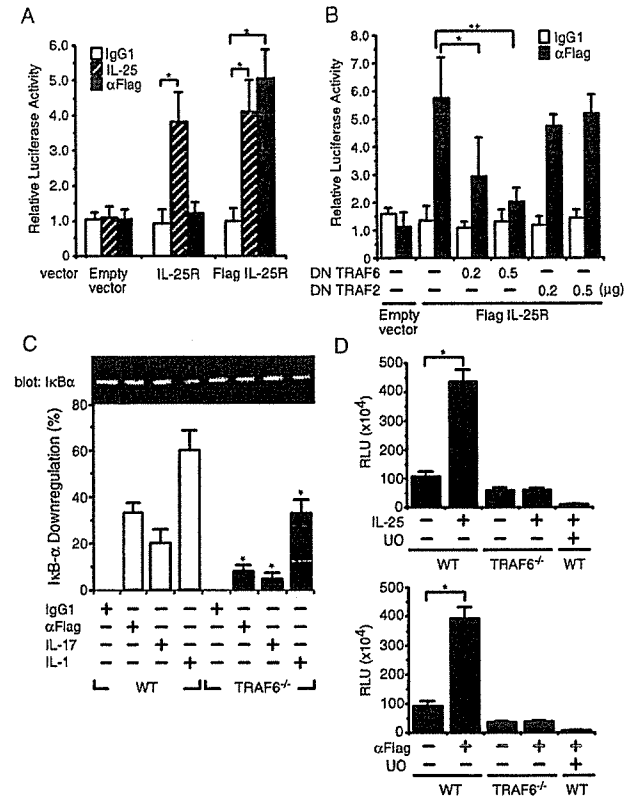

FIGURE 2. IL-25R signaling induces NF-κB activation by a TRAF6-dependent mechanism. *A*, IL-25R cross-linking induces NF-κB activation. COS7 cells were transfected with the expression vector for IL-25R or Flag-tagged-IL-25R in the presence of pNF-κB-Luc and pRL-TK. Twenty-four hours later, the cells 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.

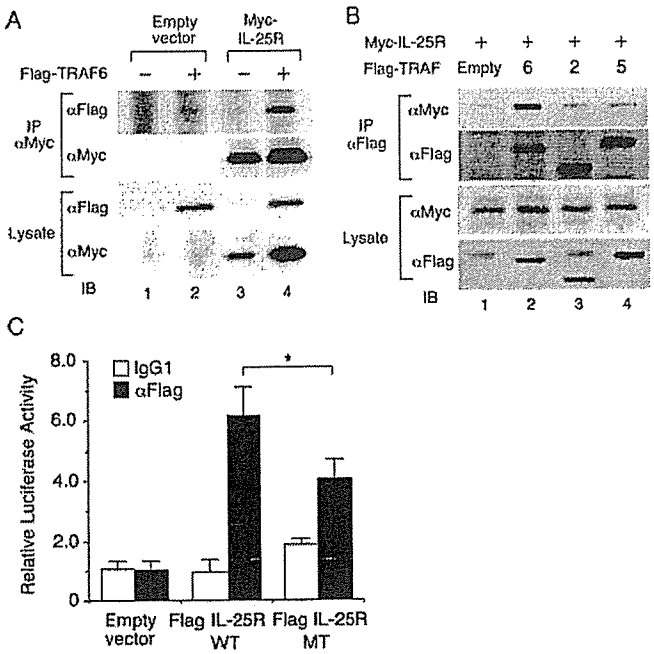

FIGURE 3. TRAF6 but not TRAF2 nor TRAF5 associates with IL-25R. *A*, TRAF6 associates with IL-25R. COS7 cells were transfected with *myc*-tagged IL-25R and/or Flag-tagged TRAF6, and the cell lysates were immunoprecipitated (IP) with anti-*myc* Ab and followed by immunoblottings (IB) with anti-Flag Ab 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

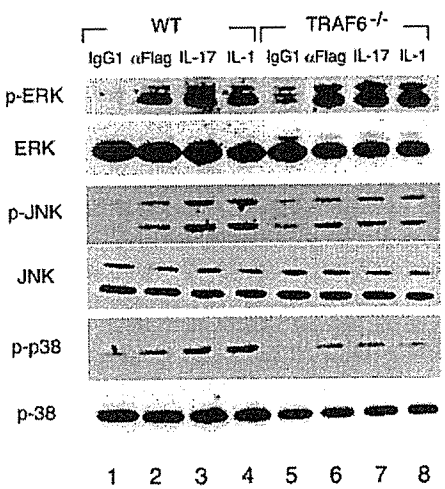

FIGURE 4. IL-25 activates ERK, JNK, and p38 by a TRAF6-independent mechanism. Similar to Fig. 2*C*, WT or TRAF6$^{-/-}$ MEF infected with retrovirus of pMX-Flag-IL-25R-IRES-GFP were incubated with control IgG1, anti-Flag mAb, IL-17, or IL-1 at 37°C for 20 min, and cell lysates were subjected to immunoblotting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

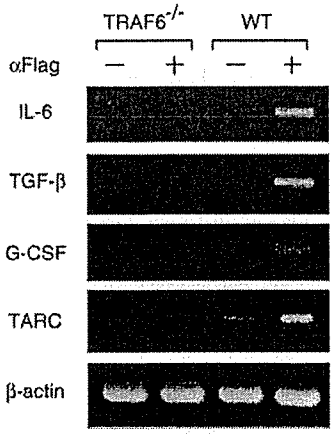

FIGURE 5. IL-25 up-regulates cytokine and chemokine mRNA expression in a TRAF6-dependent manner. WT MEF and TRAF6$^{-/-}$ MEF were infected with retrovirus of pMX-Flag-IL-25R-IRES-GFP as described in Fig. 2*C* and then incubated with anti-Flag mAb or control IgG1 at 37°C for 3 h. Total cellular RNA was 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

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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*

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

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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

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)

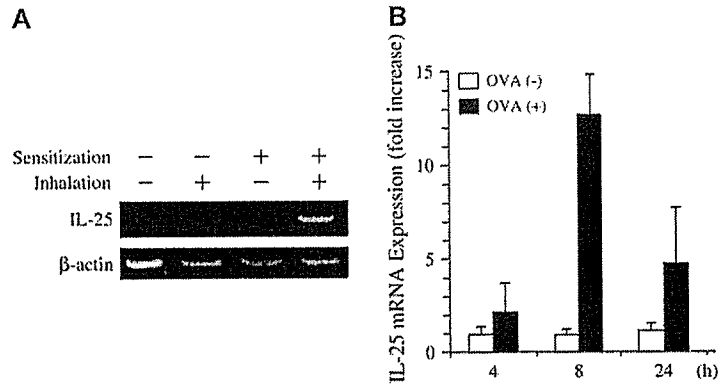

FIG 1. IL-25 mRNA is expressed in the lungs of sensitized mice on antigen inhalation. **A**, BALB/c mice were immunized with or without OVA in aluminum hydroxide, and 2 weeks after the immunization, mice were challenged twice with inhaled OVA or saline at a 48-hour interval. Twenty-four hours after the 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

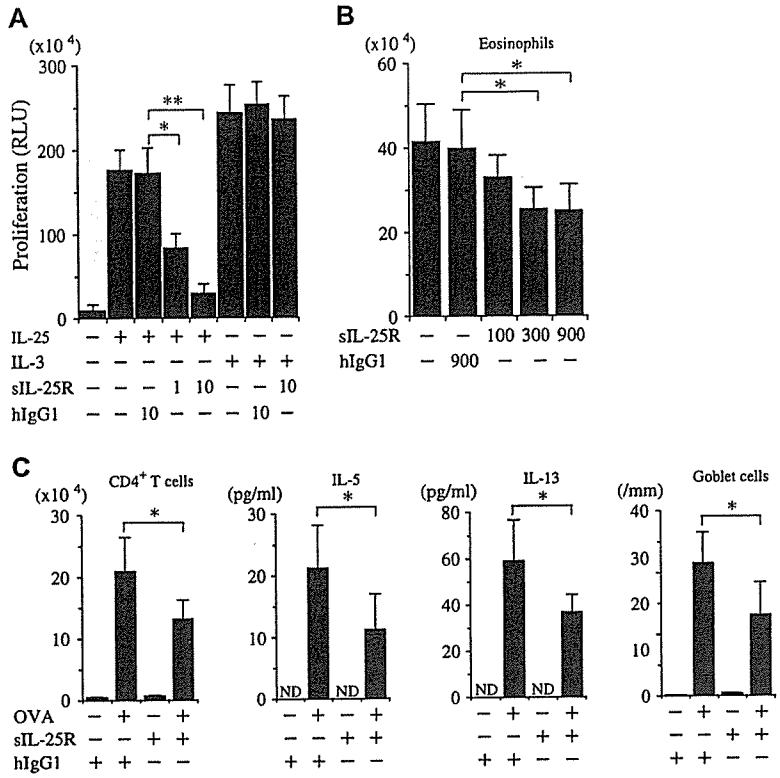

FIG 2. Neutralization of IL-25 by sIL-25R inhibits antigen-induced eosinophil and $CD4^+$ T-cell recruitment into the airways. **A**, Ba/F3 IL-25R–MPL cells that stably express IL-25R–MPL fusion protein were cultured with X63–IL-25 or X63–IL-3 conditioning medium (10% vol/vol). Where indicated, sIL-25R (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

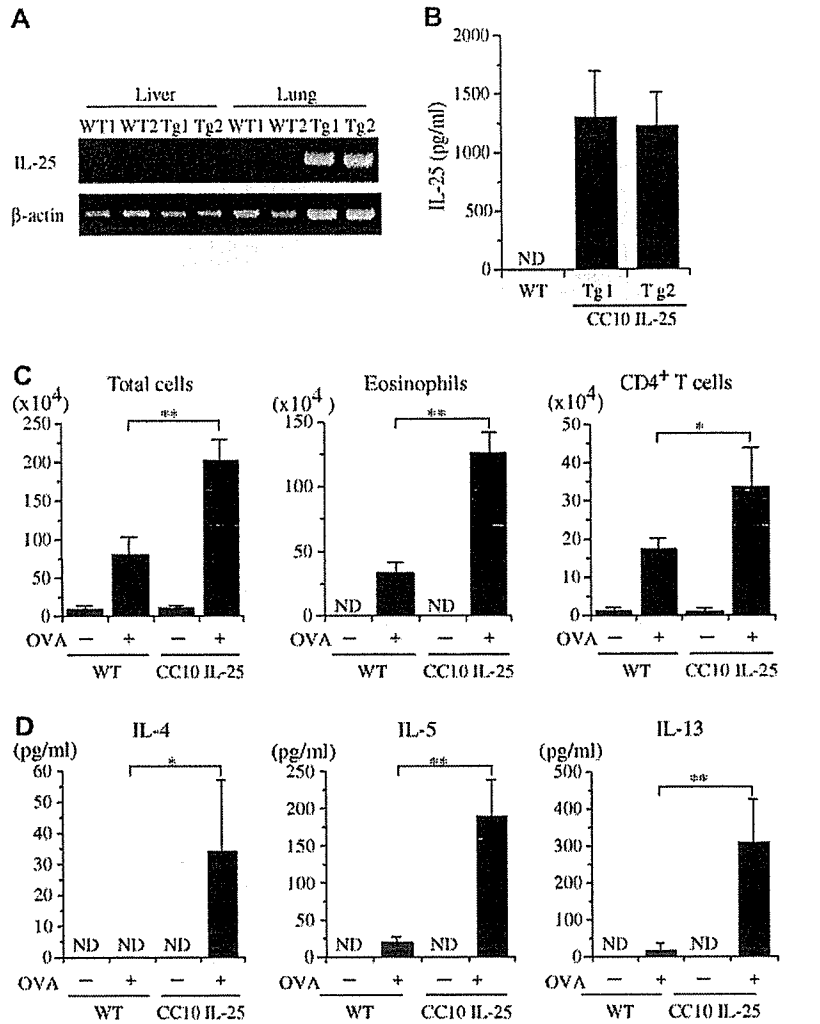

FIG 3. Antigen-induced eosinophil and $CD4^+$ T-cell recruitment and T_H2 cytokine production in the airways are enhanced in CC10 IL-25 mice. **A**, Total RNA was prepared from lung or liver tissues of CC10 IL-25 mice (transgenic line 1 *[Tg1]* and transgenic line 2 *[Tg2]*) and WT littermates (*WT1*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

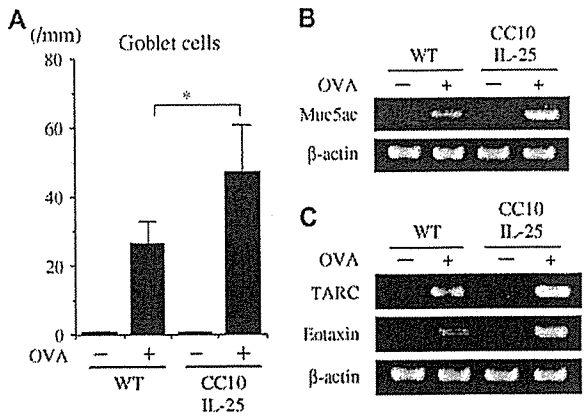

FIG 4. Antigen-induced goblet cell hyperplasia and chemokine production in the airways are enhanced in CC10 IL-25 mice. A, OVA-sensitized CC10 IL-25 mice (transgenic line 1) and littermate WT mice were challenged twice with OVA or saline inhalation. Forty-eight hours after the last OVA or saline inhalation, 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,

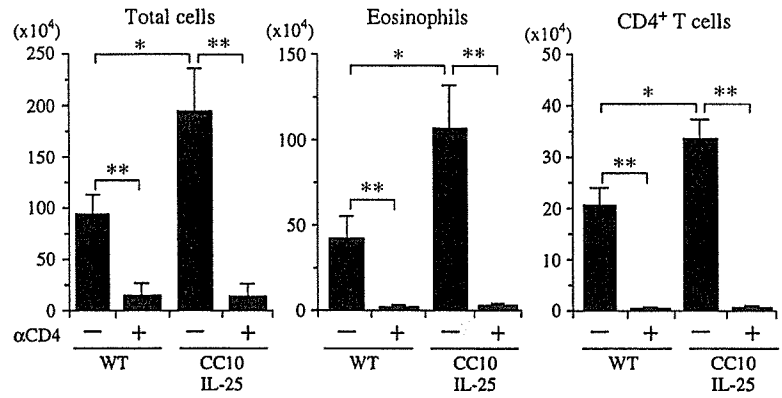

FIG 5. $CD4^+$ T cells are required for the enhanced eosinophil recruitment into the airways in CC10 IL-25 mice. OVA-sensitized CC10 IL-25 mice and WT mice were challenged twice with inhaled OVA. Where indicated, mice were injected intraperitoneally with anti-CD4 antibody (1 mg) or control antibody (1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

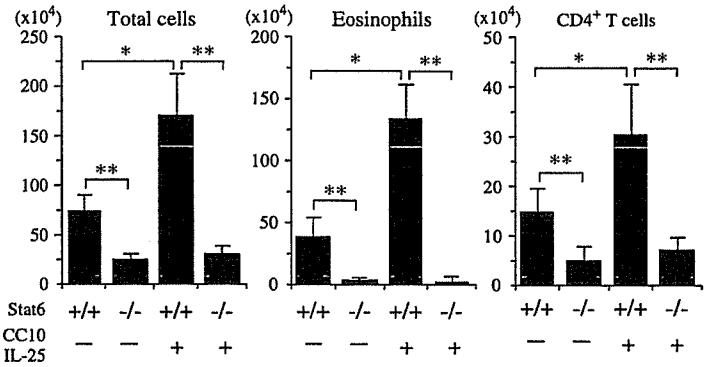

FIG 6. Stat6 is required for the enhanced eosinophil recruitment into the airways in CC10 IL-25 mice. CC10 IL-25 $Stat6^{-/-}$ mice, CC10 IL-25 $Stat6^{+/+}$ mice, $Stat6^{-/-}$ mice, and $Stat6^{+/+}$ mice were immunized with OVA-alum and then challenged twice with inhaled OVA. The number of total cells, 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.

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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