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Epithelial Cell-Derived IL-25, but Not Th17 Cell-Derived IL-17 or IL-17F, Is Crucial for Murine Asthma

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IL-17A–, IL-17F–, IL-17A/F–, IL-23p19–, or retinoic acid-related orphan receptor (ROR)-γt–deficient mice—showed significant suppression of 1) the number of eosinophils and the levels of proinflammatory mediators in bronchoalveolar lavage fluids, 2) airway hyperresponsiveness to methacholine, and 3) OVA-specific IgG1 and IgE levels in the serum during OVA-induced Th2-type/eosinophilic airway inflammation. The IL-25 deficiency did not affect lung dendritic cell migration or Ag-specific memory-Th2 cell expansion during Ag sensitization. Adoptive transfer of T cells, mast cells, or bone marrow cells from IL-25–deficient mice revealed that induction of Th2-type/eosinophilic airway inflammation was dependent on activation of lung epithelial cells and eosinophils by IL-25 produced by airway structural cells such as epithelial cells but not by such hematopoietic stem-cell-origin immune cells as T cells and mast cells. Therefore, airway structural cell-derived IL-25—rather than Th1 cell-derived IL-17A and IL-17F—is responsible for induction of local inflammation by promoting activation of lung epithelial cells and eosinophils in the elicitation phase of Th2-type/eosinophilic airway inflammation. It is not required for Ag-specific Th2 cell differentiation in the sensitization phase. The Journal of Immunology, 2012, 189: 3641–3652.

Allergic asthma is considered to be a typical Th2-type cytokine-mediated chronic pulmonary disease accompanied by elevated levels of serum IgE and Th2 cytokine expression and accumulation/activation of eosinophils, mast cells, and Th2 cells in inflamed lungs of patients with atopic asthma (1, 2).

The IL-17 family of cytokines consists of six members: IL-17 (also called IL-17A), IL-17B, IL-17C, IL-17D, IL-17E (also called IL-25), and IL-17F. Both IL-17A and IL-17F, which are preferentially produced by Th17 cells, bind to the same receptor—IL-17R, consisting of IL-17RA and IL-17RC—and act as proinflammatory cytokines that play important roles in host defense against pathogens by inducing inflammation associated with neutrophils (3, 4). In contrast, excessive/inappropriate production of IL-17A and/or IL-17F is known to contribute to the development of certain autoimmune and allergic disorders, including asthma (3). Indeed, using specimens such as lung tissue, sera, sputum, and/or bronchoalveolar lavage (BAL) fluid, the levels of IL-17A...
and/or IL-17F mRNA and/or proteins were shown to be increased in asthma patients in comparison with healthy subjects (3, 5). In addition, both IL-17A and IL-17F can activate bronchial fibroblasts, epithelial cells, and/or smooth muscle cells to express various proinflammatory mediators such as IL-6 and IL-8 (3, 5). These observations suggest that IL-17A and/or IL-17F may be involved in the development of Th2-type and eosinophilic asthma, even though both cytokines are known to be potent mediators in induction of neutrophilia, rather than eosinophilia, by promoting IL-8 and IL-6 production.

On the basis of our studies using a murine Th2-type/eosinophilic allergic asthma model, however, the contributions of IL-17A and IL-17F to the pathogenesis of the disease seemed controversial. That is, we demonstrated that IL-17A−/− deficient (IL-17A−/−), IL-17F−/−, and IL-17A−/−/IL-17F−/− mice normally developed OVA-induced Th2-type/eosinophilic allergic airway inflammation (6–8). In contrast, other investigators demonstrated that IL-17RA−/− mice, which lack both IL-17A and IL-17F signals, showed attenuated OVA-induced Th2-type/eosinophilic airway inflammation (9). The contributions of IL-25 and IL-17C, which are members of the IL-17 family of cytokines, should be noted in regard to this apparent discrepancy. That is, it was recently reported that IL-17RA is a common chain for IL-17R (IL-17RA and IL-17RC), IL-25R (IL-17RA and IL-17RB) (10), and IL-17CR (IL-17RA and IL-17RE) (11, 12). This suggests that the phenotypes reported in IL-17RA−/− mice are due to functional deficiencies of at least four genes, IL-17A, IL-17C, IL-17F, and IL-25. Also, unlike IL-17A and IL-17F, IL-25 can induce production of Th2 cytokines such as IL-4, IL-5, IL-9, and/or IL-13 by Th2 cells, Th9 cells, and innate lymphoid cells, thereby contributing to the pathogenesis of Th2-type immune responses (i.e., protection against nematode infection (13, 14)). In addition, although it was also shown that IL-25 is involved in the development of Th2-type/eosinophilic airway inflammation (15, 16), the source and precise role of IL-25 in that setting remain unclear. In the current study, therefore, we aimed to elucidate the source and role of IL-25 in the pathogenesis of Th2-type/eosinophilic airway inflammation by studying IL-25−/− mice.

Materials and Methods

Mice

C57BL/6 mice (CD45.1 and CD45.2) were purchased from Sankyo Lab (Tsukuba, Japan). IL-25−/− mice were obtained by mating chimeric mice, which were generated by using i25-targeted 129 ES cells (OYO069) by Lexicon Pharmaceuticals (The Woodlands, TX), with C57BL/6 female mice (N8). IL-25−/− mice, IL-17A−/− IL-17F−/− mice (8), IL-23p19−/− mice (17), retinoic acid-related orphan receptor (ROR)−γt−/− mice (18), TNF−/− mice (19), and KIwKd/wKd mice (20) on the C57BL/6 background mice were obtained from Taconic (Hudson, NY). Sex- and age-matched mice were used (6–12 wk old at the start of immunization). All animals were kept under specific pathogen-free conditions in an environmentally controlled clean room at the National Center for Child Health and Development, The Institute of Medical Science, The University of Tokyo, or the Animal Research Center, Tokyo Medical University. All animal experiments were approved and conducted according to the ethical and safety guidelines of those institutions.

Induction of allergic airway inflammation

Mice were sensitized with OVA, with—and in selected experiments, without—aluminum hydroxide (alum), as described previously (21), with minor modifications. Mast cells, IgE, IL-1, TNF, and CCR8 were shown to be important for development of airway inflammation in mice sensitized with OVA in the absence of alum but not required in the presence of alum (5). Mice were immunized i.p. with 100 μg OVA (grade V; Sigma-Aldrich, St. Louis, MO) emulsified with 2 mg alum (Imject Alum; Thermo Scientific, Rockford, IL) in a total volume of 200 μl PBS on days 0 and 10 or with

10 μg OVA (without alum) in 200 μl PBS on days 0, 2, 4, 6, 8, and 10. Mice sensitized with OVA with or without alum were intranasally challenged with 200 μg OVA in 20 μl PBS or PBS alone as a control on days 21, 23, and 25 or on days 40, 43, and 46, respectively.

Bronchoalveolar lavage fluids

Twenty-four hours after the last challenge, bronchoalveolar lavage fluids (BALFs) were collected as described elsewhere (21, 22). Briefly, mice were intubated with a 22-gauge blunt needle (Nipro, Osaka, Japan), and 1 ml HBSS with 2% FCS was injected through the needle into the lungs. After washing three times, BALFs were collected and centrifuged. The bronchoalveolar lavage (BAL) cells were resuspended in 100 μl HBSS with 2% FCS, and each cell type was counted with an automated hematometry analyzer, Sysmex XT-1800i (Sysmex, Hyogo, Japan), according to the manufacturer’s instructions.

Measurement of airway hyperresponsiveness

Twenty-four hours after the last challenge, bronchial responses to aerosolized methacholine (Sigma-Aldrich) were measured by an invasive approach as described elsewhere (21, 22). Briefly, mice were anesthetized with ketamine (100 mg/kg, i.p.; Dalichi Sankyo, Tokyo, Japan) and xylazine (10 mg/kg, i.p.; Sigma-Aldrich) and surgically intubated. Intubated mice were connected to plethysmograph chambers with a ventilator (Elan Series Mouse RC Site; Buxco Electronics, Sharon, CT) and mechanically ventilated at 150 breaths/min and a tidal volume of 0.2 ml. Aerosolized methacholine (10 μl) was administered for 10 s with a tidal volume of 0.2 ml. After each aerosol challenge, the data for pulmonary resistance and dynamic compliance of lungs were continuously monitored using BioSystem XA software (Buxco Electronics).

Eosinophil peroxidase assay

Eosinophil peroxidase (EPO) activities were measured as previously described (21), with minor modification. Briefly, BALFs were diluted in 50 mM PIPES (Sigma-Aldrich) containing 6 mM potassium bromide (Sigma-Aldrich), and then, an equal volume of EPO substrate buffer (50 mM PIPES, 8.8 mM H2O2, and 3 mM o-phenylenediamine) was added. After 5–10 min, 150 μl 2 M H2SO4 was added to stop the reaction, and the OD 490 nm was measured using a plate reader (Bio-Rad, Hercules, CA). Recombinant EPO (Calbiochem, Darmstadt, Germany) was used as the standard reagent.

Measurement of OVA-specific IgG in sera

Sera were collected 24 h after the last challenge. The levels of OVA-specific IgG1, IgG2a, and IgE in sera were determined by ELISA, as described elsewhere (21, 22). Biotinylated anti-mouse IgG1 (A85-1), IgG2a (R19-15), IgE (R35-118), and HRP-conjugated streptavidin were obtained from BD Biosciences and used as detection Abs/reagents.

Histology and immunohistochemistry

Twenty-four hours after the last challenge, lungs were harvested and fixed in Carnoy’s solution. The fixed tissues were embedded in paraffin and sliced into 5-μm sections, followed by H&E staining or periodic acid-Schiff staining. For immunohistochemistry, paraffin sections were incubated with 0.5% H2O2 for 15 min to quench endogenous peroxidase activity. After antigen retrieval, sections were incubated with the following Abs: rabbit anti-mouse IL-5 (1:100; Becton Dickinson, Franklin Lakes, NJ), rabbit anti-mouse IL-13 (1:100; Biologos, Clearwater, FL), IgE (R35-118), and HRP-conjugated streptavidin (1:100; Janssen Research Foundation, Spring House, PA). The peroxidase reaction was visualized with diaminobenzidine (DAB) as a substrate. For immunochemistry, slides were counterstained with hematoxylin. Sections were mounted with Permount (Fisher, Pittsburgh, PA). Immunohistochemistry was performed as described elsewhere (27).

Dendritic cell migration

Mice were intranasally administered 100 μl 10 mg/ml FITC-conjugated OVA (FITC-OVA) together with 5 μg recombinate mouse (m)IL-25 (R&D Systems) or PBS alone. Submaxillary and thoracic lymph nodes (LN) were collected separately at 10 or 24 h after the inhalation, and single-cell suspensions were prepared as described elsewhere (21, 24). After FcR blocking by incubation with anti-CD16/CD32 mAb (clone 207710; R&D Systems, Minneapolis, MN) or isotype-matched control Ab, and then, IL-25–producing cells were detected by the immunoperoxidase method, as described previously (23).

T cell transfer

T cells were purified as described elsewhere (25). Briefly, spleens and LNs (axillary, inguinal, submandibular, and cervical) were collected and pooled,
and single-cell suspensions were prepared. After lysing RBCs in RBC-lysing buffer (Sigma-Aldrich), the remaining cells were incubated with biotinylated anti-mouse B220 (RA3-6B2), CD11b (M1/70), CD11c (HL3), CD25 (PC61.5), CD49b (DX5), Gr-1 (RB6-8C5), Ter119 (Ter119), γδ TCR (GL3), FcεRIα (MAR1), and c-kit (2B8) for 20 min at 4°C. All mAbs were obtained from eBioscience. The cells were then washed and incubated with Streptavidin Particles Plus-DM (BD Biosciences). Then CD3+ T cells (95%) were isolated by negative selection using a BD iMag system (BD Biosciences). The purified T cells (1.0 × 10^7 cells in 200 μl PBS) were i.v. injected into Rag-2^−/− mice. Then, these mice were sensitized with OVA emulsified with alum and challenged with OVA as described above.

For OVA-specific Th2 cell transfer, spleen cells from OTII mice were cultured with 0.1 μM OVA peptides in the presence of 40 ng/ml rmTSLP (R&D Systems) and 40-μg/ml anti-mouse IFN-γ mAb (XMG1.2; Biolegend, San Diego, CA) for 4 d (∼20% IL-4+ Th2 cells). Then, Th2 cells were enriched using a Mouse IL-4 Secretion Assay-Cell Enrichment and Detection Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) in accordance with the manufacturer’s instructions. The enriched OTII Th2 cells (5 × 10^6 cells in 200 μl PBS and ∼80% IL-4+ Th2 cells) were i.v. injected into Rag-2^−/− and IL-25^−/− Rag-2^−/− mice. One day later, the mice were intranasally treated with OVA in PBS or PBS alone once per day for 3 d. Twenty-four hours later, BAL cells were collected as described above.

**OVA-specific cell responses**

Mice were immunized i.p. with OVA emulsified with alum on days 0 and 6. On day 13, spleen and mesenteric LN (MLN) cells were collected and cultured in the presence and absence of 40 μg/ml OV A in RPMI 1640 medium supplemented with 10% FCS at 37°C for 96 h. The OVA-specific cell proliferative responses were determined by incorporation of 0.25 μCi/ml [3H]-labeled thymidine (PerkinElmer, Wellesley, MA) for 6 h.

**Mast cell transfer**

Mouse bone marrow-derived cultured mast cells (BMCMCs) were obtained and i.v. injected (1.0 × 10^7 cells in 200 μl PBS) into KitW-sh/W-sh mice (3 wk old), as described elsewhere (21). One month after this cell transfer, the mice were used for experiments. The cells were shown to be >95% c-KithighFcεRIαhigh by flow cytometric analysis at the time of cell transfer (data not shown).

![FIGURE 1.](http://www.jimmunol.org/) IL-17, IL-17F, IL-23, and ROR-γt are not essential for development of eosinophilic airway inflammation. The number of BAL cells (A–C) and AHR to methacholine (D) in mice immunized i.p. with OVA emulsified with alum at 24 h after the last challenge. (A) C57BL/6–wild-type mice (PBS, n = 5; OVA, n = 11) and IL-17^−/− IL-17F^−/− mice (PBS, n = 5; OVA, n = 11); (B) C57BL/6–wild-type mice (PBS, n = 4; OVA, n = 10) and IL-23p19^−/− mice (PBS, n = 4; OVA, n = 10); (C) C57BL/6–wild-type mice (PBS, n = 4; OVA, n = 8) and ROR-γt^−/− (PBS, n = 5; OVA, n = 6); and (D) IL-17^−/− IL-17F^−/− (PBS, n = 5; OVA, n = 12) versus wild-type (PBS, n = 5; OVA, n = 11), IL-23p19^−/− (PBS, n = 4; OVA, n = 11) versus wild-type (PBS, n = 4; OVA, n = 12), and ROR-γt^−/− (PBS, n = 3; OVA, n = 12) versus wild-type (PBS, n = 3; OVA, n = 10) mice. Data show the mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001 versus the corresponding values for PBS-treated mice (A–D). †p < 0.05 and †††p < 0.001 versus the indicated group (A–C) or the corresponding values for knockout mice (D). RL, Pulmonary resistance.
Bone marrow cell transfer
CD45.1-C57BL/6 or IL-25−/− mice were irradiated with 8 Gy and then injected i.v. with bone marrow cells from CD45.2-C57BL/6 wild-type or IL-25−/− mice (2 × 10^7 cells). One month after this cell transfer, the mice were used for experiments (CD45.2+ cells > 95% in LN cells and spleen by FACS).

Epithelial cell culture
Tracheas from C57BL/6 wild-type mice were harvested and digested with 1.4 mg/ml pronase (Roche Applied Sciences, Mannheim, Germany) and 0.1 mg/ml DNase I (Sigma-Aldrich) in Ca⁴⁺/Mg²⁺-free MEM at 4°C overnight. Then, the cells were suspended in DMEM/F12 supplemented with 120 mU/ml insulin and 1× nonessential amino acids and incubated at 37°C for 3 h. Nonadherent cells were collected and cultured in serum-free PCT Airway Epithelial Medium Complete (number CnT-17; Millipore, Temecula, CA) in collagen-coated plates (BD BioCoat Collagen I Cellware; BD Biosciences). The purity of the cultured epithelial cells was determined by immunohistochemical staining for cytokeratin (>99%). The isolated mouse lung epithelial cells and a mouse lung epithelial cell line (MLE-12) (2 × 10^5 cells/well in 24-well culture plates) were stimulated with various concentrations of rmIL-25, 100 ng/ml rmIL-4, or 100 ng/ml rmIL-13 (R&D Systems), as described elsewhere (26). The eosinophils were stimulated with various concentrations of rmIL-25 or 100 ng/ml rmIL-5 at 37°C overnight.

Mast cell and eosinophil culture
BMCMCs were sensitized with 2 mg/ml mouse monoclonal anti-DNP IgE (SPE-7; Sigma-Aldrich) at 37°C overnight. IgE-sensitized BMCMCs and naive BMCMCs were stimulated with DNP-conjugated human serum albumin (Sigma-Aldrich) at 37°C for 6 h. The culture supernatants were collected, and cell lysates were prepared.

Bone marrow cell-derived cultured eosinophils (92–95% by Wright-Giemsa stain) were generated by cultivation of bone marrow cells in the presence of 100 ng/ml rmflt3 ligand (PeproTech) and 100 ng/ml rmSCF (PeproTech), followed by addition of 10 ng/ml rmIL-5 (R&D Systems), as described elsewhere (26). The eosinophils were stimulated with various concentrations of rmIL-25 or 100 ng/ml rmIL-5 at 37°C overnight.

Quantitative PCR
Total RNA was extracted from cells, and cDNA was prepared as described previously (27). The expression levels of CCL1 and CCL11 in MLE12 cells were determined by quantitative PCR using an Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA). The primers and probes (CCL1, forward, 5′-GCTTACGGTCTCCAATAGCTGC-3′, and reverse, 5′-GCTTTCTCTACCTTTGTTCAGCC-3′; and CCL11, forward, 5′-TCATCCACACTTCCTGCTGCT-3′, and reverse, 5′-CTCTTTGCCCAACCCTTGTCCTTG-3′) were designed by Origene Technologies (Rockville, MD). Data were quantitated by the comparative threshold cycle method after normalization with the value of endogenous GAPDH gene expression, as

FIGURE 2. IL-25 is crucial for development of Th2-type and eosinophilic airway inflammation. The number of BAL cells, histological analysis of lungs, and AHR in C57BL/6–wild-type and IL-25−/− mice immunized i.p. with OVA emulsified with alum at 24 h after the last challenge. (A) The numbers of BAL cells. C57BL/6–wild-type mice (PBS, n = 4; OVA, n = 8) and IL-25−/− mice (PBS, n = 4; OVA, n = 11). (B and C) Histological analysis of lungs. H&E (B) and PAS staining (C). Representative data for the mice used in (A) are shown. Scale bars, 100 μm. (D) AHR to methacholine. C57BL/6–wild-type mice (PBS, n = 5; OVA, n = 13) and IL-25−/− mice (PBS, n = 5; OVA, n = 13). Data show the mean ± SEM (A, D). ***p < 0.001 and ****p < 0.0001 versus the corresponding values for PBS-treated mice (A, D). *p < 0.05 and **p < 0.01 versus the indicated group (A) or the corresponding values for IL-25−/− mice (D). RL, Pulmonary resistance.
described elsewhere (27). Data are expressed as the relative quantitation values to the expression without stimulation.

Measurement of cytokines

The levels of cytokines in BALFs, culture supernatants, and cell lysates were determined using a Bio-Plex Mouse Cytokine Group I 23-Plex (Bio-Rad) and LUMINEX 200 (Luminex, Austin, TX) or with ELISA kits, respectively, according to the manufacturers’ instructions. Mouse IFN-γ BD OptEIA ELISA set (BD Biosciences), mouse IL-4, IL-6, IL-13, and IL-17 Ready-Set-Go ELISA sets (eBioscience), and mouse IL-13, IL-17E, CCL5/RANTES, and CXCL1/KC DuoSets (R&D Systems) were used.

Statistics

Unless otherwise specified, ANOVA (measurement of airway hyper-responsiveness [AHR]) and the unpaired Student t test, two-tailed, were used for statistical evaluation of the results. All results are shown as means + SEM.

Results

IL-17A, IL-17F, IL-23, and ROR-γt are not required for development of Th2-type allergic airway inflammation

Consistent with previous observations using IL-17A−/− IL-17F−/− mice on the BALB/c background (8), we found that the increased influx of eosinophils, neutrophils, macrophages, and lymphocytes in BALFs was comparable in wild-type and IL-17A−/− IL-17F−/− mice on the C57BL/6 background during OVA/alum-induced Th2-type airway inflammation (Fig. 1A). These observations suggest that Th17 cells may not be essential in this setting. In support of this, the numbers of eosinophils in BALFs from mice deficient in IL-23 and ROR-γt—which are a key cytokine and transcription factor, respectively, for Th17 cell commitment and development—were equivalent to those in BALFs from wild-type mice 24 h after the last OVA challenge during OVA/alum-induced Th2-type airway inflammation (Fig. 1B, 1C). However, IL-23 or ROR-γt deficiency influenced the numbers of macrophages, lymphocytes, and neutrophils in BALFs (Fig. 1B, 1C). Consistent with the number of eosinophils in BALFs as well as the baseline of AHR (data not shown), AHR to methacholine in those gene-deficient mice was similar to that in wild-type mice 24 h after the last challenge (Fig. 1D). These findings suggest that IL-17A, IL-17F, IL-23, and ROR-γt are not essential for the development of OVA/alum-induced Th2-type eosinophilic airway inflammation.

FIGURE 3. IL-25−/− mice showed reduced Th2-type immune responses during Th2-type and eosinophilic airway inflammation. (A) The levels of cytokines and chemokines in BALFs; (B) the levels of EPO activities in BALFs; and (C) the levels of OVA-specific IgG1, IgE, and IgG2a in sera from mice, as shown in Fig. 2, were determined. Data show the mean + SEM. C57BL/6−/− wild-type mice (PBS, n = 4; OVA, n = 8) and IL-25−/− mice (PBS, n = 4; OVA, n = 11). *p < 0.05, **p < 0.01, and ***p < 0.001 versus the corresponding values for PBS-treated mice. †p < 0.05, ††p < 0.01, or †††p < 0.001 versus the indicated group.
IL-25 is responsible for development of Th2-type allergic airway inflammation

OVA/alum-induced Th2-type eosinophilic allergic inflammation was attenuated in mice deficient in IL-17RA, which is a component of receptors for IL-17A and IL-17F (9), but it developed normally in IL-17A−/−IL-17F−/− mice (Fig. 1A) (8). This suggests that IL-25, which can bind to IL-17RA (10), rather than IL-17A and IL-17F, may contribute to the responses. Indeed, we found that the numbers of inflammatory cells such as eosinophils, neutrophils, macrophages, and lymphocytes in BALFs were profoundly reduced in OVA-challenged IL-25−/− mice in comparison with OVA-challenged wild-type mice during OVA/alum-induced airway inflammation (Fig. 2A). In accordance with this, pathological changes such as airway inflammation and mucin deposition on the airway walls were strongly reduced in the lungs of IL-25−/− mice after the last OVA challenge (Fig. 2B, 2C). In addition, AHR was also significantly suppressed in IL-25−/− mice compared with wild-type mice 24 h after the last OVA challenge (Fig. 2D). Moreover, the levels of Th2 cytokines such as IL-4, IL-5, IL-9, and IL-13—but not the levels of Th1 cytokines such as IFN-γ, Th17 cytokines such as IL-17A, chemokines such as CXCL1/KC, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/RANTES, or the activity of eosinophil peroxidase (EPO)—were significantly reduced in BALFs from OVA-challenged IL-25−/− mice compared with OVA-challenged wild-type mice (Fig. 3A, 3B). Although the levels of total IgE in sera were comparable between wild-type and IL-25−/− mice, irrespective of the OVA challenge (data not shown), the levels of OVA-specific IgE, but not IgG1 or IgG2a, were also significantly lower in sera from OVA-challenged IL-25−/− mice than in sera from OVA-challenged wild-type mice (Fig. 3C). However, the proportion of CD4+PD-1−CXCR5+ follicular Th cells (28) in the spleen was similar in both groups (wild-type mice versus IL-25−/− mice = 0.20 ± 0.08 versus 0.13 ± 0.02; each n = 5). These observations clearly indicate that IL-25, rather than IL-17A and IL-17F, is responsible for the development of OVA/alum-induced Th2-type eosinophilic airway inflammation.

IL-25 enhances, but is not essential for, lung dendritic cell migration

Dendritic cells (DCs) in the bronchi and lungs are considered to be the major APCs in allergic pulmonary disorders. After mice inhale FITC-OVA, lung DCs, which have captured FITC-OVA, are known to migrate into the draining LNs, with peak responses observed at 24 h after FITC-OVA inhalation (29). Overexpression of IL-25 in mice resulted in elevation of serum IL-5, IL-13, and TNF, leading to increased blood eosinophils and neutrophils (30). We previously demonstrated that TNF is important for lung DC migration after FITC-OVA inhalation (24), suggesting that IL-25 may be involved in that migration, at least by promoting TNF production. Indeed, the number of FITC+ DCs in thoracic LNs, dependent at least in part on TNF, was significantly increased in wild-type mice treated with FITC-OVA plus rmIL-25 compared with those treated with FITC-OVA plus PBS, even at 12 h after treatment (Fig. 4A). In addition, although this enhancement of DC migration by rmIL-25 treatment was observed even in TNF−/− mice, it was impaired in TNF−/− mice compared with wild-type mice (Fig. 4B). Therefore, exogenous IL-25 (or excessive IL-25 production) can enhance DC migration from the lungs to draining LNs, dependent at least in part on TNF.

We next investigated the role of intrinsic IL-25 in lung DC migration using IL-25−/− mice. Twenty-four hours after FITC-

![FIGURE 4. IL-25 enhances, but is not essential for, DC migration from the lungs to draining LNs. Mice were intranasally treated with FITC-OVA or PBS in the presence and absence of rmIL-25. Submaxillary and thoracic LNs were collected at 12 h (A, B) or 24 h (C) after the inhalation. The proportion of FITC+ cells in 7-aminoactinomycin D-negative I-AbhiCD11chi cells among LN cells was determined by flow cytometry. (A) C57BL/6−wild-type (WT) mice treated intranasally with FITC-OVA plus rmIL-25 or PBS alone. Representative data from five mice are shown. The upper and lower numbers are the percentages of FITC+ cells in the thoracic and submaxillary LNs, respectively. (B) C57BL/6−WT and TNF−/− mice treated intranasally with FITC-OVA plus rmIL-25 or PBS alone (n = 5). (C) C57BL/6−WT and IL-25−/− mice treated intranasally with FITC-OVA (n = 10) or PBS (n = 6). Data show the mean + SEM (B, C). **p < 0.01 versus the corresponding values for submaxillary LNs. †p < 0.01 and ††p < 0.01 versus the indicated group.

OVA or PBS treatment, the numbers of FITC+ DCs in thoracic LNs were comparable in wild-type and IL-25−/− mice after FITC-OVA inhalation (Fig. 4C). These observations indicate that IL-25 can enhance, but is not essential for, DC migration from the lungs to draining LNs after allergen challenge.
IL-25 was reported to enhance the differentiation of Th2 cells from naive T cells directly (31) and/or indirectly through modulation of DC function (32). This suggests that IL-25 deficiency may result in impaired induction of Ag-specific memory T cells, particularly Th2 cells, causing attenuation of OVA/alum-induced Th2-type eosinophilic airway inflammation. To elucidate this, spleen cells and MLN cells from OVA/alum-sensitized wild-type and IL-25−/− mice were cultured in the presence and absence of OVA. However, the OVA-specific proliferative responses were comparable between spleen and MLN cells from wild-type and IL-25−/− mice after OVA stimulation (Fig. 5A). The levels of IL-4 as well as IFN-γ and IL-17A in the culture supernatants of MLN cells from IL-25−/− mice were also identical to those from wild-type mice (Fig. 5B). These observations indicate that IL-25 is not essential for OVA-specific T cell expansion, at least in the present setting. In contrast, like intact IL-25−/− mice, the number of eosinophils in BALFs and mucus secretion in the lungs from IL-25−/− Rag-2−/− mice injected with OVA-specific OTII mouse-derived Th2 cells was significantly decreased compared with Rag-2−/− mice injected with OVA-specific OTII mouse-derived Th2 cells (Fig. 5C; data not shown). In association with this, AHR to methacholine in IL-25−/− Rag-2−/− mice injected with OVA-specific OTII mouse-derived Th2 cells was also reduced compared with Rag-2−/− mice injected with OVA-specific OTII mouse-derived Th2 cells (Fig. 5D). Thus, these results suggest that IL-25 is not essential for induction of Ag-specific Th2 cells in the sensitization phase but is crucial for activation of Th2 cells in the elicitation phase of Th2-mediated allergic airway inflammation.
Immune cell-derived IL-25 is not required for development of Th2-type allergic airway inflammation

Although it was also shown that IL-25 is involved in the development of Th2-type/eosinophilic airway inflammation (15, 16), the source of IL-25 remains to be elucidated. As described above, IL-25 deficiency did not influence OVA-specific T cell expansion in the sensitization phase of OVA/alum-induced Th2-type airway inflammation. In contrast, because IL-25 mRNA is known to be expressed in Th2 cells (33, 34), T cell-derived IL-25 may be important for activation of such target cells in local inflammatory sites, contributing to development of allergic airway inflammation. Therefore, we next examined the role of T cell-derived IL-25 in the pathogenesis of OVA/alum-induced Th2-type airway inflammation by performing adoptive transfer of T cells from wild-type or IL-25−/− mice to Rag-2−/− mice (wild-type T cells → Rag-2−/− mice). The levels of IL-4, IL-17, and IFN-γ in the culture supernatants and proliferation of purified splenic CD3+ T cells in response to plate-coated anti-CD3 and anti-CD28 mAbs were similar in wild-type and IL-25−/− mice (data not shown), suggesting that IL-25 deficiency did not influence the intrinsic functions of T cells before adoptive transfer. The number of inflammatory cells in BALFs, mucus secretion in the lungs, and AHR to methacholine did not differ significantly between wild-type T cells → Rag-2−/− mice and IL-25−/− T cells → Rag-2−/− mice after the last OVA challenge (Fig. 6A, 6B; data not shown). This indicates that T-cell-derived IL-25 is not crucial for induction of OVA/alum-induced Th2-type eosinophilic airway inflammation.

Mast cells (i.e., BMCMCs) were reported to express/produce IL-25 mRNA/proteins in response to PMA + ionophore and IgE + Ags (34). Although the levels of IL-25 proteins in the culture supernatants of BMCMCs after stimulation with PMA + ionophore and IgE + Ags were below the limit of detection by ELISA, the lysates of wild-type BMCMCs contained ELISA-detectable...
levels of IL-25 proteins, irrespective of stimulation, compared with lysates of IL-25−/− BMCMCs as negative controls (Fig. 6C). These observations suggest that mast cell-derived IL-25 may contribute to the development of OVA-induced Th2-type/eosinophilic airway inflammation. However, mast cells were shown not to be essential for development of OVA-induced airway inflammation in mice sensitized with OVA emulsified with alum (hereinafter, mast cell-independent airway inflammation) (5). Therefore, mast cells and mast cell-derived IL-25 do not seem to be important in this setting.

In contrast, mast cells are known to be responsible for the development of OVA-induced airway inflammation in mice repeatedly sensitized with OVA alone (without alum) (called mast cell-dependent airway inflammation) (5). Because mast cell-dependent airway inflammation (Fig. 6D) and also mast cell-independent airway inflammation (Fig. 2A–C) were significantly impaired in IL-25−/− mice, mast cell-derived IL-25 may be involved in the pathogenesis of mast cell-dependent airway inflammation. To elucidate the role of mast cell-derived IL-25 in this setting, we used mast cell-specific IL-25-deficient mice generated by injection of IL-25−/− BMCMCs to mast cell-deficient KitW-sh/W-sh mice (IL-25−/− BMCMCs → KitW-sh/W-sh mice), as described elsewhere (35). IL-25 deficiency did not exert any influence on 1) the development of BMCMCs, 2) expression of c-kit and FcεRI on the cell surface of BMCMCs, or 3) β-hexosaminidase release and IL-6, IL-13, and TNF production by BMCMCs after stimulation by PMA + ionophore or IgE + Ags and after stimulation with IgE, LPS, and IL-33, respectively (data not shown).

The number of eosinophils in BALFs was decreased in the mast cell-deficient KitW-sh/W-sh mice compared with wild-type mice during mast cell-dependent OVA-induced airway inflammation (Fig. 6E). That decreased eosinophilia returned to the wild-type mouse level in wild-type BMCMCs → KitW-sh/W-sh mice after the last OVA challenge (Fig. 6E). Similar recovery was observed even in IL-25−/− BMCMCs → KitW-sh/W-sh mice in the setting (Fig. 6E). These observations indicate that mast cell-derived IL-25 is dispensable for mast cell-dependent OVA-induced airway inflammation. Moreover, in contrast to intact IL-25−/− mice, the number of BAL cells was comparable between CD45.1−/− wild-type mice and CD45.1−/− IL-25−/− mice that had been reconstituted with CD45.2−/− wild-type bone marrow cells or IL-25−/− bone marrow cells after irradiation (Fig. 7A) 24 h after OVA challenge during mast cell-independent airway inflammation.

Thus, these observations indicate that IL-25, which is produced by immune cells derived from hematopoietic stem cells, is not essential for induction of Th2-type allergic airway inflammation. In support of this, our immunohistochemical studies showed that bronchial and alveolar epithelial cells in naive and OVA-inhaled lungs from wild-type, but not IL-25−/−, mice were IL-25+ cells (Fig. 7B). Thus, taken together, our findings suggest that IL-25 produced by epithelial cells, but not immune cells, is crucial for induction of Th2-type allergic airway inflammation.

**IL-25 is important for activation of epithelial cells and eosinophils**

In allergic airway inflammation, NKT cells (36), lung macrophages (37), innate lymphoid cells (38), and epithelial cells (39) are considered to be target cells in the response to IL-25. In addition, we demonstrated that IL-25 is important for Th2 cell activation in the elicitation phase—but not Th2 cell differentiation in the sen-
Allergic airway inflammation.

Immune cells such as Th2 cells and eosinophils during Th2-type allergic airway inflammation. IL-25 plays important roles in the induction of local inflammation by bone marrow cell-derived eosinophils from wild-type BALB/c mice. IL-25 partially, but dose dependently, suppressed by addition of dexamethasone (Fig. 9D). These observations suggest that IL-25–mediated CCL5/RANTES and CXCL1/KC production in epithelial cells is regulated by distinct signal cascades. In addition to epithelial cells, IL-25 was able to induce IL-13, but not IL-4, production by bone marrow cell-derived eosinophils from wild-type mice (Fig. 9E). Taken all together, these observations suggest that IL-25 plays important roles in the induction of local inflammation by activating epithelial cells. Thus, IL-25 can induce chemokine production by airway epithelial cells. The levels of CCL5 and CXCL1 in the culture supernatants and cell lysates of airway epithelial cells after stimulation with the indicated concentrations of rmIL-25, 100 ng/ml rmIL-4, or 100 ng/ml rmIL-13 for 24 h. Data show the mean + SEM (n = 4). *p < 0.05, **p < 0.01, or ***p < 0.001 versus the corresponding values for unstimulated cells (IL-25 = 0).

**Figure 8.** IL-25 can induce chemokine production by airway epithelial cells. The levels of CCL5 and CXCL1 in the culture supernatants and cell lysates of airway epithelial cells after stimulation with the indicated concentrations of rmIL-25, 100 ng/ml rmIL-4, or 100 ng/ml rmIL-13 for 24 h. Data show the mean + SEM (n = 4). *p < 0.05, **p < 0.01, or ***p < 0.001 versus the corresponding values for unstimulated cells (IL-25 = 0).

**Discussion**

We previously demonstrated that IL-17A, but not IL-17F, was crucial for induction of allergen-induced neutrophilic airway inflammation (6, 8, 21). In contrast, using IL-17AΔIL17RA, IL-17FΔIL17RA, and IL-17AΔIL17FΔIL17RA mice, we also demonstrated that neither IL-17A nor IL-17F was essential for development of Th2-type/eosinophilic airway inflammation (6-8). In contrast, mice deficient in IL-17RA, which is a component of receptors for both IL-17A and IL-17F, showed attenuated development of Th2-type/eosinophilic airway inflammation (9). Because it was recently reported that IL-17RA is a common chain for IL-17R (IL-17RA and IL-17RC) and IL-25R (IL-17RA and IL-17RB) (10), the distinctly different phenotypes of IL-17AΔIL17RA, IL-17FΔIL17RA, and IL-17RAΔIL17RA mice may be dependent on the function of IL-25. Indeed, we demonstrated that IL-25ΔIL17RA mice, but not IL-17AΔIL17FΔIL17RA, IL-23p19ΔIL17RA, or ROR-γtΔIL17RA mice, showed attenuated induction of Th2-type/eosinophilic airway inflammation (Figs. 1, 2). Thus, IL-25, rather than IL-17A and IL-17F, is important for the development of Th2-type/eosinophilic airway inflammation. Moreover, others demonstrated that development of Th2-type/eosinophilic airway inflammation is attenuated in mice treated with anti–IL-25 mAb (18).

IL-25 enhances the differentiation of Th2 cells from naive T cells directly (31) and/or indirectly through modulation of DC function (32). In the current study, we demonstrated that IL-25 inhalation enhanced DC migration from the lungs into draining LNs, at least partly dependent on TNF (Fig. 4). However, IL-25 deficiency did not influence that DC migration (Fig. 4). In addition, OVA-specific proliferation of, and IL-4 production by, LN cells from IL-25ΔIL17RA mice after OVA sensitization were similar to in wild-type mice, suggesting that IL-25 is not essential for Ag-specific memory T cell (including Th2-cell) expansion in the sensitization phase of Th2-type/eosinophilic airway inflammation (Fig. 5A, 5B). In addition, the eosinophil number in BALFs was significantly decreased in IL-25ΔIL17RA mice compared with Rag-2ΔIL17RA mice after the last OVA inhalation, even though these mice had been similarly engraved with in vitro-skewed OVA–specific OTIH Th2 cells (Fig. 5C). In contrast, IL-25 inhalation resulted in development of airway eosinophilia even in T and B cell-deficient Rag-2ΔIL17RA mice (45) by activating macrophage-like cells (37) and epithelial cells (39) in the lungs. These observations suggest that IL-25 is involved in induction of local inflammation in the elicitation phase, rather than the sensitization phase, of Th2-type/eosinophilic airway inflammation.

IL-25 mRNA/proteins are expressed in immune cells such as T cells and mast cells (33, 34, 46), whereas in the airway, alveolar macrophages (47), eosinophils, basophils (32), and airway epithelial cells (31) are also known to be potential producers of IL-25. The contribution of IL-25 produced by these cell types to the development of Th2-type/eosinophilic airway inflammation has not previously been investigated. Using adoptive transfer of IL-25–deficient cells, we demonstrated that IL-25 produced by T cells, mast cells, and other hematopoietic stem-cell-origin immune cells was not essential for the development of Th2-type/eosinophilic airway inflammation, suggesting that IL-25 produced by nonimmune cells such as airway epithelial cells is crucial for its development. Supporting this, we found that IL-25 was expressed in bronchial and alveolar epithelial cells (Fig. 7B). Moreover, in addition to inducing lung eosinophilia, IL-25 might activate alveolar epithelial cells to produce chemoattractants for Th2 cells. Thus, epithelial cell–derived IL-25 may activate epithelial cells through autocrine or paracrine mechanisms to promote activation of Th2 cells and eosinophil localization in the airway during Th2-type allergic airway inflammation.
In summary, our studies suggest that IL-25, rather than IL-17A and IL-17F, is responsible for the induction of Th2-type/eosinophilic airway inflammation.

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Disclosures

D.J.C. is an employee of Merck. The other authors have no financial conflicts of interest.

References

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