

**Fig. 4** The levels of EPO and MPO activities in BALFs and serum IgE were reduced in epicutaneous OVA-sensitized ST2<sup>-/-</sup> mice after OVA challenge. (A) The levels of EPO and MPO activities in BALFs and (B) the levels of OVA-specific IgE in sera of the wild-type (WT) and ST2<sup>-/-</sup> mice used in Figure 3B. \*  $P < 0.05$  and \*\*\* $P < 0.001$  vs. the corresponding values for PBS-treated mice, and ††† $P < 0.001$  vs. the indicated groups.

OVA-sensitized ST2<sup>-/-</sup> mice after intranasal OVA challenge (Fig. 3B, 4A, 5), suggesting that the IL-33/ST2 pathway is crucial for induction of Th2-type allergic airway inflammation, especially under adjuvant-free experimental conditions.

It was recently reported that Th2-type eosinophilic airway inflammation after OVA inhalation was dramatically suppressed in IL-4<sup>-/-</sup> IL-13<sup>-/-</sup> mutant mice that had been epicutaneously sensitized with OVA, whereas Th17-type neutrophilic airway inflammation was exaggerated.<sup>12</sup> IL-17 production by T cells can be inhibited by both IL-4 and IFN- $\gamma$ ,<sup>48</sup> suggesting that IL-4-deficiency resulted in increased IL-17 production and led to the enhanced IL-17-mediated immune responses observed in IL-4<sup>-/-</sup> IL-13<sup>-/-</sup> mice. In contrast to IL-4<sup>-/-</sup> IL-13<sup>-/-</sup> mice,<sup>12</sup> we found normal levels of pulmonary IL-17 mRNA expression and airway neutrophilia in epicutaneously OVA-sensitized ST2<sup>-/-</sup> mice after the last OVA challenge (Fig. 3B, 5). It may be explained that IL-4 mRNA expression was significantly decreased, but not completely abrogated, in ST2<sup>-/-</sup> mice (Fig. 5).

Despite the normal airway neutrophilia and IL-17 levels described above, the levels of CXCL1 and CXCL2 mRNA expression and MPO activity were markedly decreased in lungs and BALFs, respectively, from epicutaneously OVA-sensitized ST2<sup>-/-</sup>

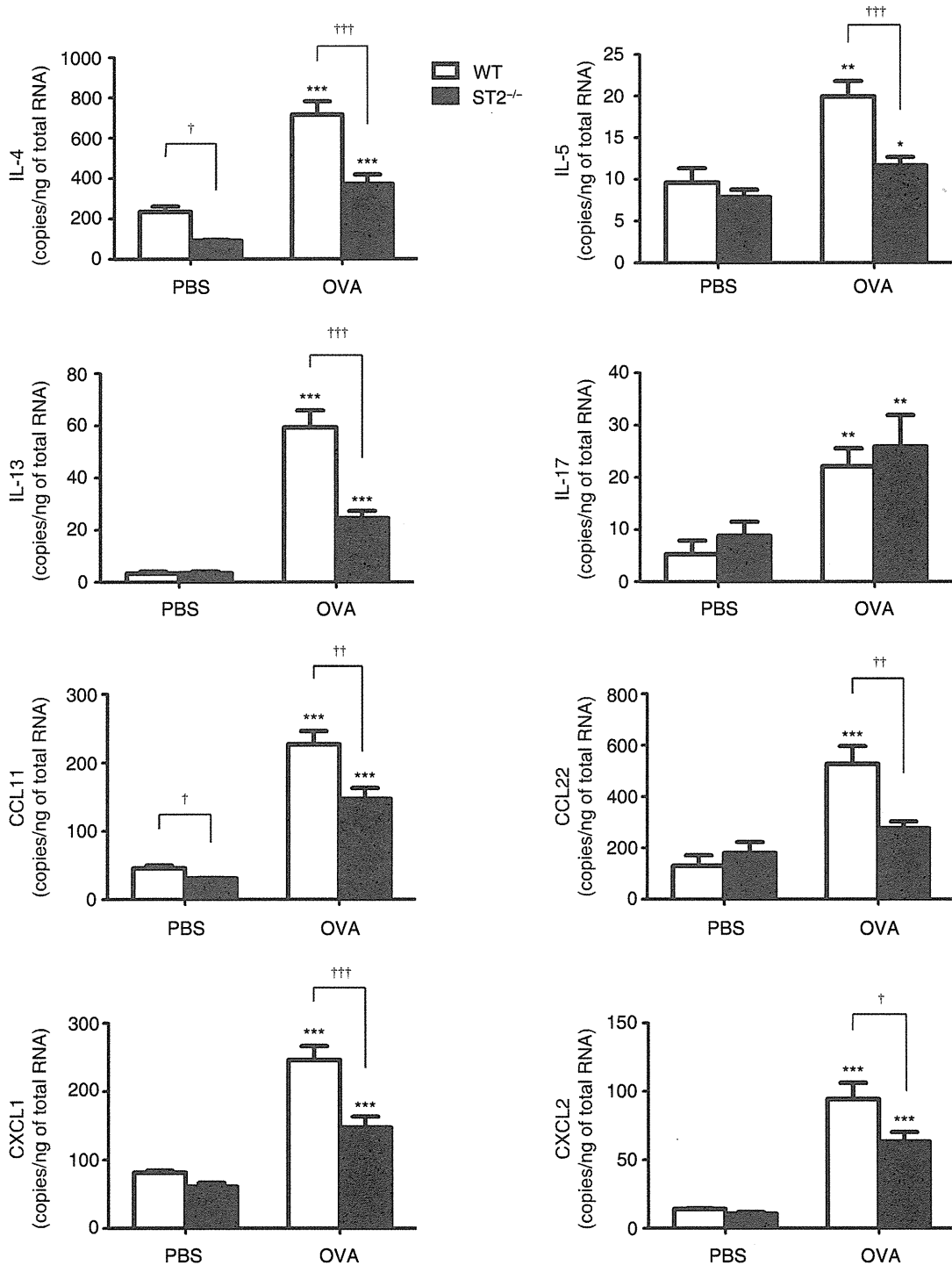
mice after the last OVA challenge (Fig. 4A, 5). This suggests that the IL-33/ST2 pathway may be partially involved in neutrophil recruitment, independently of IL-17. Indeed, we previously reported that IL-33 can directly induce production of neutrophil chemoattractants (i.e., IL-8) by both endothelial and epithelial cells.<sup>49</sup> However, since the effect of the IL-33/ST2 pathway on neutrophil recruitment was less than that of IL-17, ST2 deficiency may not exert a significant effect on airway neutrophilia in our experimental system.

In summary, we demonstrated that the IL-33/ST2 pathway is crucial for Th2-cytokine-mediated eosinophilic, rather than Th17-cytokine-mediated neutrophilic, airway inflammation in mice that had been epicutaneously sensitized with antigen. Our findings may provide a clue for development of novel therapeutics for asthma.

#### ACKNOWLEDGEMENTS

We thank Dr. Andrew N.J. McKenzie (MRC Laboratory of Molecular Biology, Cambridge, United Kingdom) for providing ST2<sup>-/-</sup> mice, and Shuhei Fukuda, Noriko Hashimoto, Yoshiko Shimamoto and Hiromi Wakita for their excellent technical assistance, and Larry Stiver for critical reading of the manuscript. This work was supported by a grant from the Na-

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**Fig. 5** The expression levels of Th2 cytokines, but not IL-17A, were reduced in lungs from epicutaneous OVA-sensitized ST2<sup>-/-</sup> mice after OVA challenge. Twenty-four hours after the last OVA challenge, lungs were harvested from the wild-type (WT) and ST2<sup>-/-</sup> mice used in Figure 3B, and the total mRNA was isolated. The expression of mRNA for cytokines and chemokines was determined by quantitative PCR. Data show the mean + SEM (WT mice: PBS, *n* = 16, and OVA, *n* = 20; ST2<sup>-/-</sup> mice: PBS, *n* = 14, and OVA, *n* = 20). \**P* < 0.05, \*\**P* < 0.01 and \*\*\**P* < 0.001 vs. the corresponding values for PBS-treated mice, and †*P* < 0.05, ††*P* < 0.01 and †††*P* < 0.001 vs. the indicated groups.

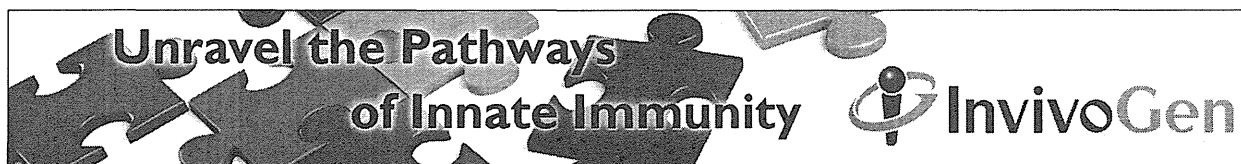
tional Institute of Biomedical Innovation, a Research on Allergic Disease and Immunology in Health Labour Sciences Research Grant, a Grant for Child Health and Development from the Ministry of Health, Labour and Welfare, and a Grant-in-Aid for Scientific Research (B) and the Program for Improvement of Research Environment for Young Researchers, The Special-Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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This information is current as of February 12, 2013.

Maho Suzukawa, Hideaki Morita, Aya Nambu, Ken Arae, Eri Shimura, Akiko Shibui, Sachiko Yamaguchi, Keigo Suzukawa, Wakako Nakanishi, Keisuke Oboki, Naoki Kajiwara, Tatsukuni Ohno, Akina Ishii, Heinrich Körner, Daniel J. Cua, Hajime Suto, Takayuki Yoshimoto, Yoichiro Iwakura, Tatsuya Yamasoba, Ken Ohta, Katsuko Sudo, Hirohisa Saito, Ko Okumura, David H. Broide, Kenji Matsumoto and Susumu Nakae

*J Immunol* 2012; 189:3641-3652; Prepublished online 31 August 2012;  
doi: 10.4049/jimmunol.1200461  
<http://www.jimmunol.org/content/189/7/3641>

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

Maho Suzukawa,<sup>\*,†,1</sup> Hideaki Morita,<sup>‡,§,1</sup> Aya Nambu,<sup>¶,1</sup> Ken Arae,<sup>‡,||</sup> Eri Shimura,<sup>#</sup> Akiko Shibui,<sup>\*\*</sup> Sachiko Yamaguchi,<sup>††</sup> Keigo Suzukawa,<sup>‡‡</sup> Wakako Nakanishi,<sup>‡‡</sup> Keisuke Oboki,<sup>‡</sup> Naoki Kajiwara,<sup>‡,##</sup> Tatsukuni Ohno,<sup>‡</sup> Akina Ishii,<sup>‡</sup> Heinrich Körner,<sup>§§</sup> Daniel J. Cua,<sup>¶¶</sup> Hajime Suto,<sup>#</sup> Takayuki Yoshimoto,<sup>|||</sup> Yoichiro Iwakura,<sup>##,\*\*\*</sup> Tatsuya Yamasoba,<sup>‡‡</sup> Ken Ohta,<sup>\*</sup> Katsuko Sudo,<sup>†††</sup> Hirohisa Saito,<sup>‡,#</sup> Ko Okumura,<sup>#</sup> David H. Broide,<sup>†</sup> Kenji Matsumoto,<sup>‡</sup> and Susumu Nakae<sup>¶,††,‡‡‡</sup>

IL-17A, IL-17F, and IL-25 are ligands for IL-17RA. In the current study, we demonstrated that IL-25-deficient mice—but not IL-17A-, IL-17F-, IL-17A/F-, IL-23p19-, or retinoic acid-related orphan receptor (ROR)- $\gamma$ t-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 Th17 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

\*Department of Internal Medicine, Teikyo University School of Medicine, Tokyo 173-8605, Japan; <sup>†</sup>Department of Medicine, University of California, San Diego, La Jolla, CA 92037; <sup>‡</sup>Department of Allergy and Immunology, National Research Institute for Child Health and Development, Tokyo 157-8535, Japan; <sup>§</sup>Department of Pediatrics, Keio University School of Medicine, Tokyo 160-8582, Japan; <sup>¶</sup>Laboratory of Systems Biology, Center for Experimental Medicine and Systems Biology, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan; <sup>||</sup>Department of Immunology, Faculty of Health Science, Kyorin University, Tokyo 192-8508, Japan; <sup>|||</sup>Atopy Research Center, Juntendo University School of Medicine, Tokyo 113-8421, Japan; <sup>\*\*</sup>Department of Medical Genomics, Graduate School of Frontier Sciences, The University of Tokyo, Chiba 278-0022, Japan; <sup>††</sup>Frontier Research Initiative, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan; <sup>‡‡</sup>Department of Otolaryngology Head and Neck Surgery, The University of Tokyo, Tokyo 113-8655, Japan; <sup>§§</sup>Menzies Research Institute, Hobart, Tas 7000, Australia; <sup>¶¶</sup>Merck Research Laboratories, Palo Alto, CA 94304; <sup>|||</sup>Intractable Immune System Disease Research Center, Tokyo Medical University, Tokyo 160-8402, Japan; <sup>##</sup>Laboratory of Molecular Pathogenesis, Center for Experimental Medicine and Systems Biology, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan; <sup>\*\*\*</sup>Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Saitama 332-0012, Japan; <sup>†††</sup>Animal Research Center, Tokyo Medical University, Tokyo 160-8402, Japan; and <sup>‡‡‡</sup>Precursory Research for Embryonic Science and Technology, Japan Science and Technology Agency, Saitama 332-0012, Japan

<sup>1</sup>M.S., H.M., and A.N. contributed equally to this work.

Received for publication February 6, 2012. Accepted for publication July 26, 2012.

This work was supported by the Japan Chemical Industry Association (to S.N.), Grants-in-Aid for Young Scientists (B) (to M.S., K. Suzukawa, T.O., K. Oboki, and

S.N.), a Grant-in-Aid for Scientific Research (B) (to H. Saito), the Precursory Research for Embryonic Science and Technology, Japan Science and Technology Agency (to S.N.), the Program for Improvement of Research Environment for Young Researchers, the special coordination funds for promoting science and technology (to S.N.) from the Ministry of Education, Culture, Sports, Science and Technology (Japan), a Health Labor Sciences Research grant from the Ministry of Health, Labor, and Welfare (Japan) (to K. Ohta and H. Saito), a grant from the Environmental Restoration and Conservation Agency of Japan (to K. Ohta), National Institutes of Health Grants AI 38425, AI 70535, and AI 72115 (to D.H.B.), and a grant from the National Institute of Biomedical Innovation (to H. Saito). M.S. is a Postdoctoral Fellow supported by a postdoctoral fellowship for research abroad from the Japan Society for the Promotion of Science. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Address correspondence and reprint requests to Dr. Susumu Nakae, Frontier Research Initiative, Laboratory of Systems Biology, Center for Experimental Medicine and Systems Biology, The Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato, Tokyo 108-8639, Japan. E-mail address: snakae@ims.u-tokyo.ac.jp

Abbreviations used in this article: AHR, airway hyperresponsiveness; alum, aluminum hydroxide; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; BMCMC, mouse bone marrow cell-derived cultured mast cell; DC, dendritic cell; EPO, eosinophil peroxidase; FITC-OVA, FITC-conjugated OVA; IL-17A<sup>-/-</sup>, IL-17A-deficient; LN, lymph node; MLN, mesenteric lymph node; rm, recombinant mouse; ROR, retinoic acid-related orphan receptor.

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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<sup>-/-</sup>), IL-17F<sup>-/-</sup>, and IL-17A<sup>-/-</sup>IL-17F<sup>-/-</sup> mice normally developed OVA-induced Th2-type/eosinophilic allergic airway inflammation (6–8). In contrast, other investigators demonstrated that IL-17RA<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> mice.

## Materials and Methods

### Mice

C57BL/6 mice (CD45.1 and CD45.2) were purchased from Sankyo Lab (Tsukuba, Japan). IL-25<sup>+/-</sup> mice were obtained by mating chimeric mice, which were generated by using *il25*-targeted 129 ES cells (OYC069) by Lexicon Pharmaceuticals (The Woodlands, TX), with C57BL/6 female mice (N8). IL-25<sup>-/-</sup> mice, IL-17A<sup>-/-</sup> IL-17F<sup>-/-</sup> mice (8), IL-23p19<sup>-/-</sup> mice (17), retinoic acid-related orphan receptor (ROR)- $\gamma$ <sup>-/-</sup> mice (18), TNF<sup>-/-</sup> mice (19), and *Ki1<sup>W-sh/W-sh</sup>* mice (20) on the C57BL/6 background were also used. Rag-2<sup>-/-</sup> and OTII mice 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  $\mu$ g 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  $\mu$ l PBS on days 0 and 10 or with

10  $\mu$ g OVA (without alum) in 200  $\mu$ l PBS on days 0, 2, 4, 6, 8, and 10. Mice sensitized with OVA with or without alum were intranasally challenged with 200  $\mu$ g OVA in 20  $\mu$ l 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  $\mu$ l HBSS with 2% FCS, and each cell type was counted with an automated hematology 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.; Daiichi 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  $\mu$ l) 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 H<sub>2</sub>O<sub>2</sub>, and 3 mM *o*-phenylenediamine) was added. After 5–10 min, 150 ml 2 M H<sub>2</sub>SO<sub>4</sub> 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 Igs 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- $\mu$ m sections, followed by H&E staining or periodic acid-Schiff staining. For immunohistochemistry, paraffin sections were incubated with 0.5  $\mu$ g/ml anti-mouse IL-25 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).

### Dendritic cell migration

Mice were intranasally administered 100  $\mu$ l 10 mg/ml FITC-conjugated OVA (FITC-OVA) together with 5  $\mu$ g recombinant mouse (rm)IL-25 (R&D Systems) or PBS alone. Submaxillary and thoracic lymph nodes (LNs) were collected separately at 12 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 (2.4G2; BD Biosciences, San Jose, CA), the cells were stained with PE-conjugated anti-mouse CD11c (HL3; eBioscience, San Diego, CA) and allo-PE-conjugated anti-mouse I-A/I-E (M5/114.15.2; eBioscience). The proportion of FITC<sup>+</sup> cells among 7-aminoactinomycin D-negative I-A<sup>bhi</sup>CD11c<sup>hi</sup> cells in LN cells was determined with a FACSCalibur (BD Biosciences) using CellQuest software (BD Biosciences).

### 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),  $\gamma\delta$ TCR (GL3), Fc $\epsilon$ RI $\alpha$  (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<sup>+</sup> T cells (>95%) were isolated by negative selection using a BD iMag system (BD Biosciences). The purified T cells ( $1.0 \times 10^7$  cells in 200  $\mu$ l PBS) were i.v. injected into Rag-2<sup>-/-</sup> 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  $\mu$ M OVA peptides in the presence of 40 ng/ml rmIL-4 (CeproTech, Rocky Hill, NJ), 40 ng/ml rmTSLP (R&D Systems) and 40- $\mu$ g/ml anti-mouse IFN- $\gamma$  mAb (XMG1.2; Biologend, San Diego, CA) for 4 d (~20% IL-4<sup>+</sup> 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 ( $5 \times 10^6$  cells in 200  $\mu$ l PBS and ~80% IL-4<sup>+</sup> Th2 cells) were i.v. injected into Rag-2<sup>-/-</sup>

and IL-25<sup>-/-</sup>Rag-2<sup>-/-</sup> 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.

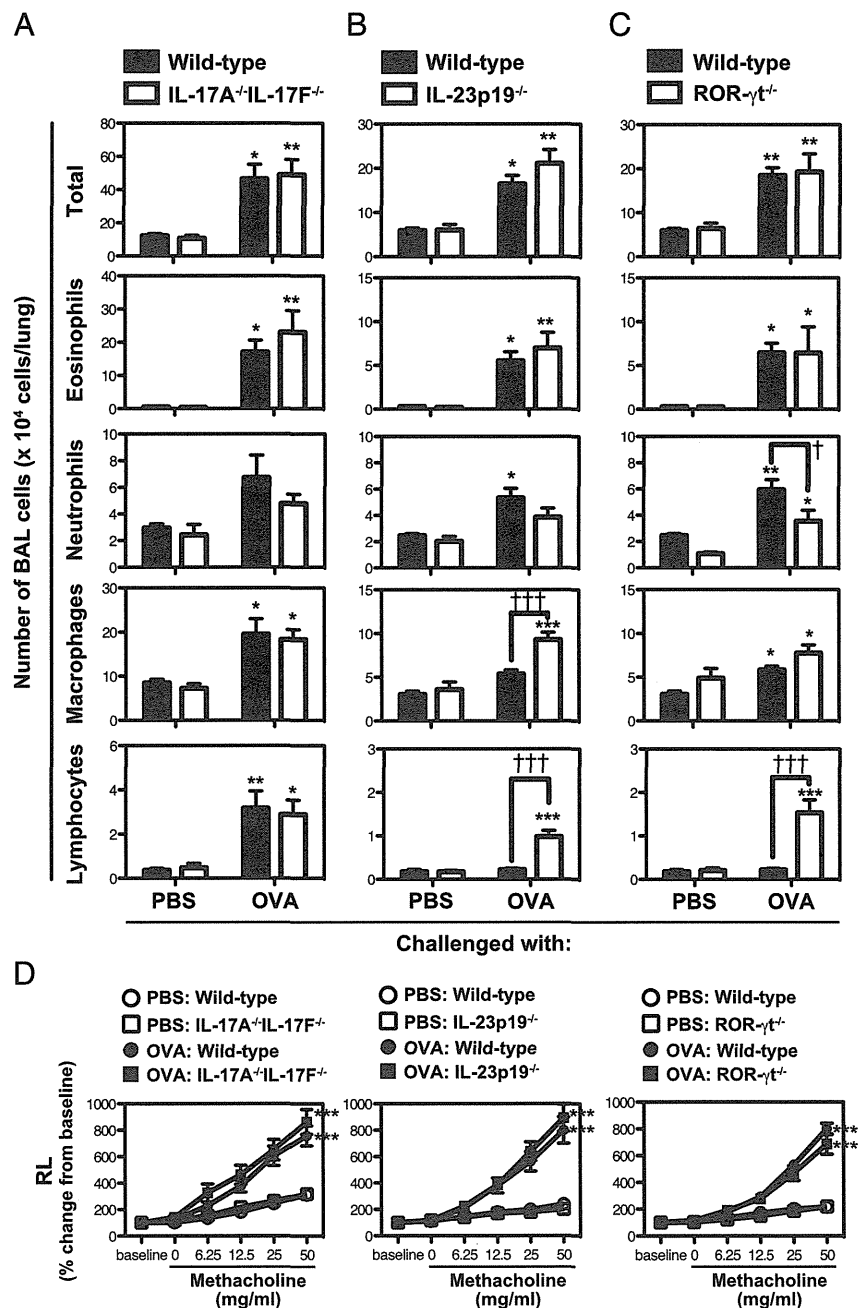
*OVA-specific cell responses*

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  $\mu$ g/ml OVA 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  $\mu$ Ci/ml [<sup>3</sup>H]-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 ( $1.0 \times 10^7$  cells in 200  $\mu$ l PBS) into *Kit*<sup>W-sh/W-sh</sup> 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-Kit<sup>high</sup>Fc $\epsilon$ RI $\alpha$ <sup>high</sup> by flow cytometric analysis at the time of cell transfer (data not shown).

**FIGURE 1.** IL-17, IL-17F, IL-23, and ROR- $\gamma$ t 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<sup>-/-</sup>IL-17F<sup>-/-</sup> mice (PBS, n = 5; OVA, n = 11); (B) C57BL/6-wild-type mice (PBS, n = 4; OVA, n = 10) and IL-23p19<sup>-/-</sup> mice (PBS, n = 4; OVA, n = 10); (C) C57BL/6-wild-type mice (PBS, n = 4; OVA, n = 8) and ROR- $\gamma$ t<sup>-/-</sup> (PBS, n = 5; OVA, n = 6); and (D) IL-17<sup>-/-</sup>IL-17F<sup>-/-</sup> (PBS, n = 5; OVA, n = 12) versus wild-type (PBS, n = 5; OVA, n = 11), IL-23p19<sup>-/-</sup> (PBS, n = 4; OVA, n = 11) versus wild-type (PBS, n = 4; OVA, n = 12), and ROR- $\gamma$ t<sup>-/-</sup> (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<sup>-/-</sup> 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<sup>-/-</sup> mice ( $2 \times 10^7$  cells). One month after this cell transfer, the mice were used for experiments (CD45.2<sup>+</sup> 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 Ca<sup>2+</sup>/Mg<sup>2+</sup>-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) ( $2 \times 10^5$  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) in the presence and absence of PI3K inhibitor LY294002, p38MAPK inhibitor SB203580, MEK1/2 inhibitor U0126, MAPK/ERK1 (MEK1) inhibitor PD98059, NF-κB inhibitor Bay11-7082, or JNK inhibitor SP600125 (Cayman Chemical, Ann Arbor, MI) for 4 h (for quantitative PCR) or 24 h (for ELISA).

### Mast cell and eosinophil culture

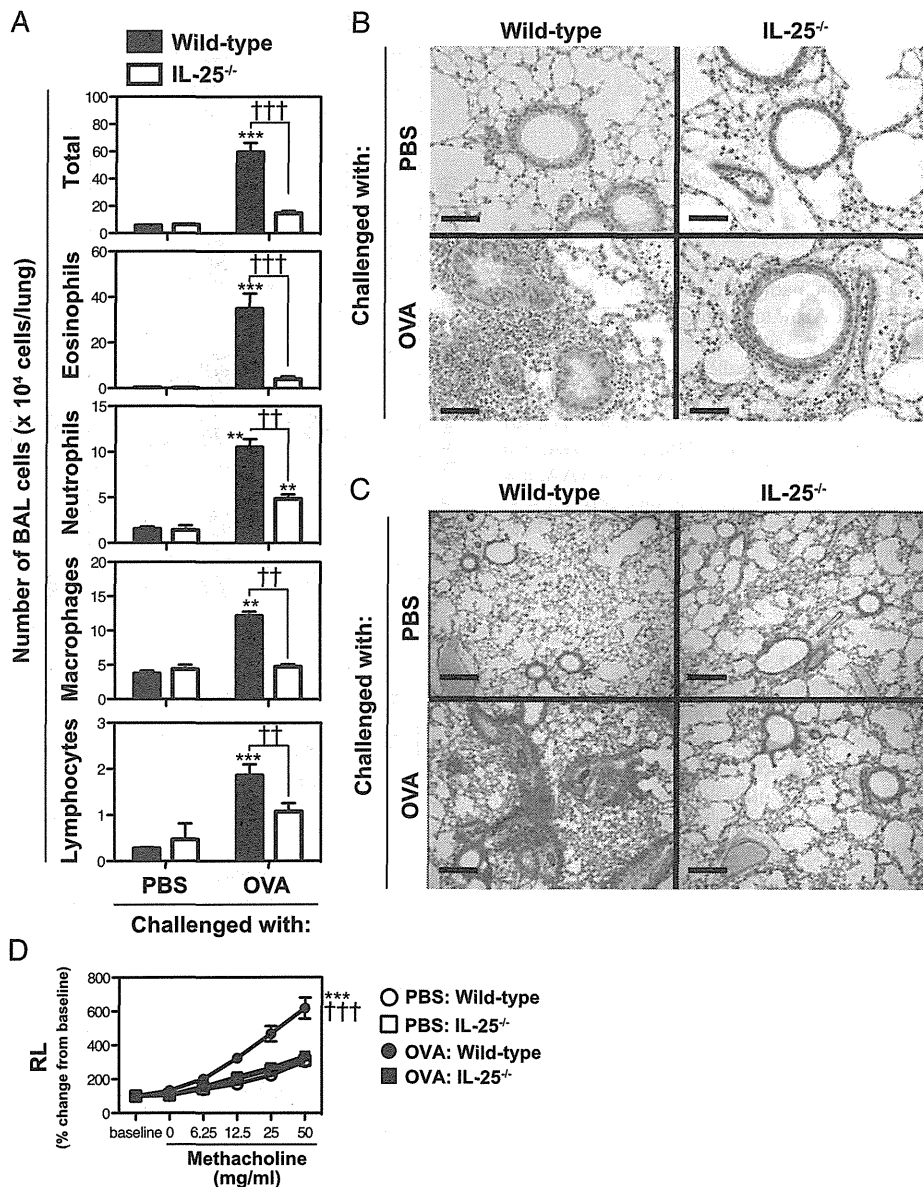
BMCMCs were sensitized with 2 μg/ml mouse monoclonal anti-DNP IgE (SPE-7; Sigma-Aldrich) at 37°C overnight. IgE-sensitized BMCMCs and naive BMCMCs were stimulated with DNP-conjugated human serum albumin (Sigma-Aldrich) at 37°C for 6 h. The culture supernatants were collected, and cell lysates were prepared.

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'-TC-CATCCAACTTCCTGCTGCT-3', and reverse, 5'-CTCTTTGCCAA-CCTGGTCTTG-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<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> mice (PBS,  $n = 5$ ; OVA,  $n = 13$ ). Data show the mean + SEM (A, D). \*\* $p < 0.01$  and \*\*\* $p < 0.001$  versus the corresponding values for PBS-treated mice (A, D). †† $p < 0.01$  and ††† $p < 0.001$  versus the indicated group (A) or the corresponding values for IL-25<sup>-/-</sup> 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- $\gamma$  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.

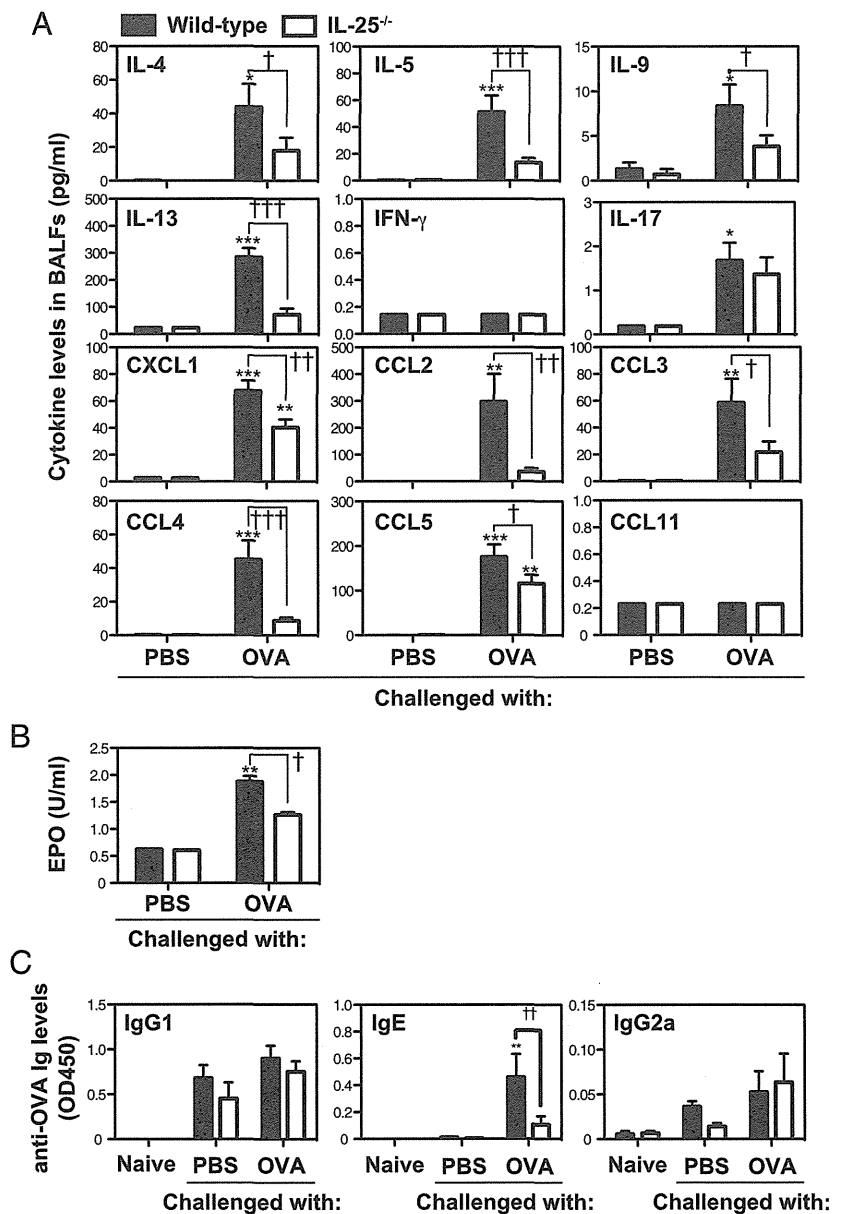
**Results**

*IL-17A, IL-17F, IL-23, and ROR- $\gamma$ t are not required for development of Th2-type allergic airway inflammation*

Consistent with previous observations using IL-17A<sup>-/-</sup>IL-17F<sup>-/-</sup> 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<sup>-/-</sup>IL-17F<sup>-/-</sup> 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- $\gamma$ t—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- $\gamma$ t 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- $\gamma$ t are not essential for the development of OVA/alum-induced Th2-type/eosinophilic airway inflammation.

**FIGURE 3.** IL-25-deficient 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<sup>-/-</sup> 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.



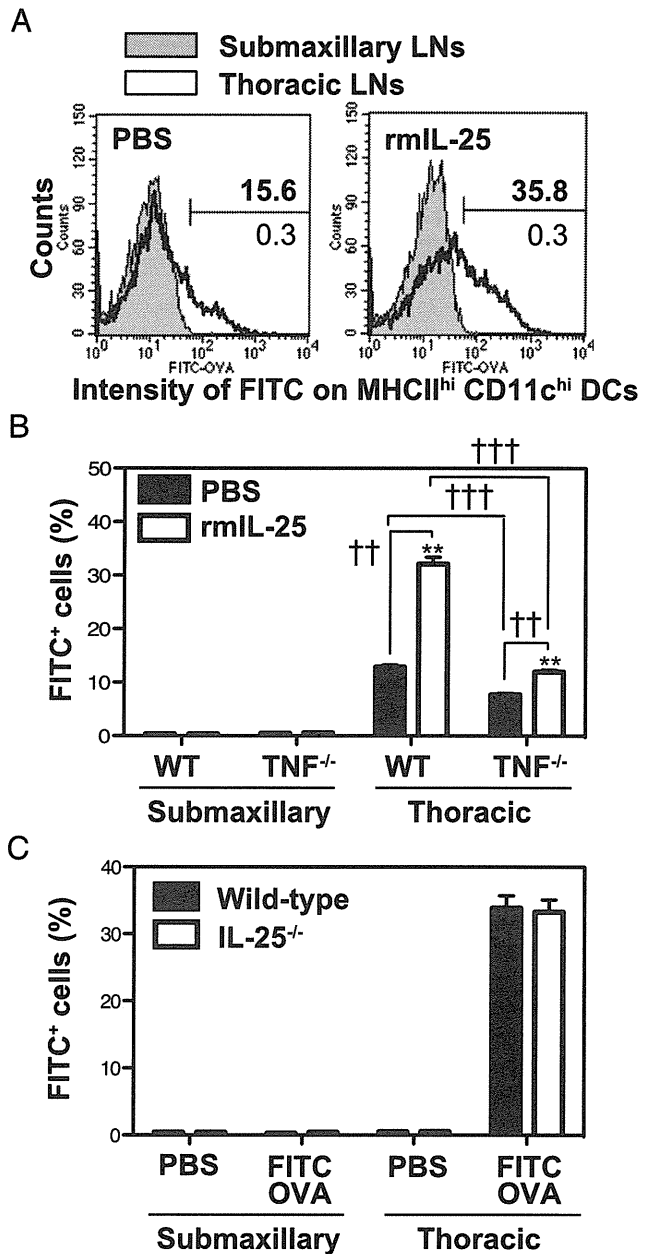
*IL-25 is responsible for development of Th2-type allergic airway inflammation*

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<sup>-/-</sup>IL-17F<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> mice compared with wild-type mice after the last OVA challenge (Fig. 2B, 2C). In addition, AHR was also significantly suppressed in IL-25<sup>-/-</sup> 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- $\gamma$ , Th17 cytokines such as IL-17A, chemokines such as CXCL1/KC, CCL2/MCP-1, CCL3/MIP-1 $\alpha$ , CCL4/MIP-1 $\beta$ , and CCL5/RANTES, or the activity of eosinophil peroxidase (EPO)—were significantly reduced in BALFs from OVA-challenged IL-25<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> mice than in sera from OVA-challenged wild-type mice (Fig. 3C). However, the proportion of CD4<sup>+</sup>PD-1<sup>+</sup>CXCR5<sup>+</sup> follicular Th cells (28) in the spleen was similar in both groups (wild-type mice versus IL-25<sup>-/-</sup> mice = 0.20  $\pm$  0.08 versus 0.13  $\pm$  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<sup>+</sup> DCs in thoracic LNs, but not in submaxillary LNs, 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<sup>-/-</sup> mice, it was impaired in TNF<sup>-/-</sup> 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<sup>-/-</sup> mice. Twenty-four hours after FITC-



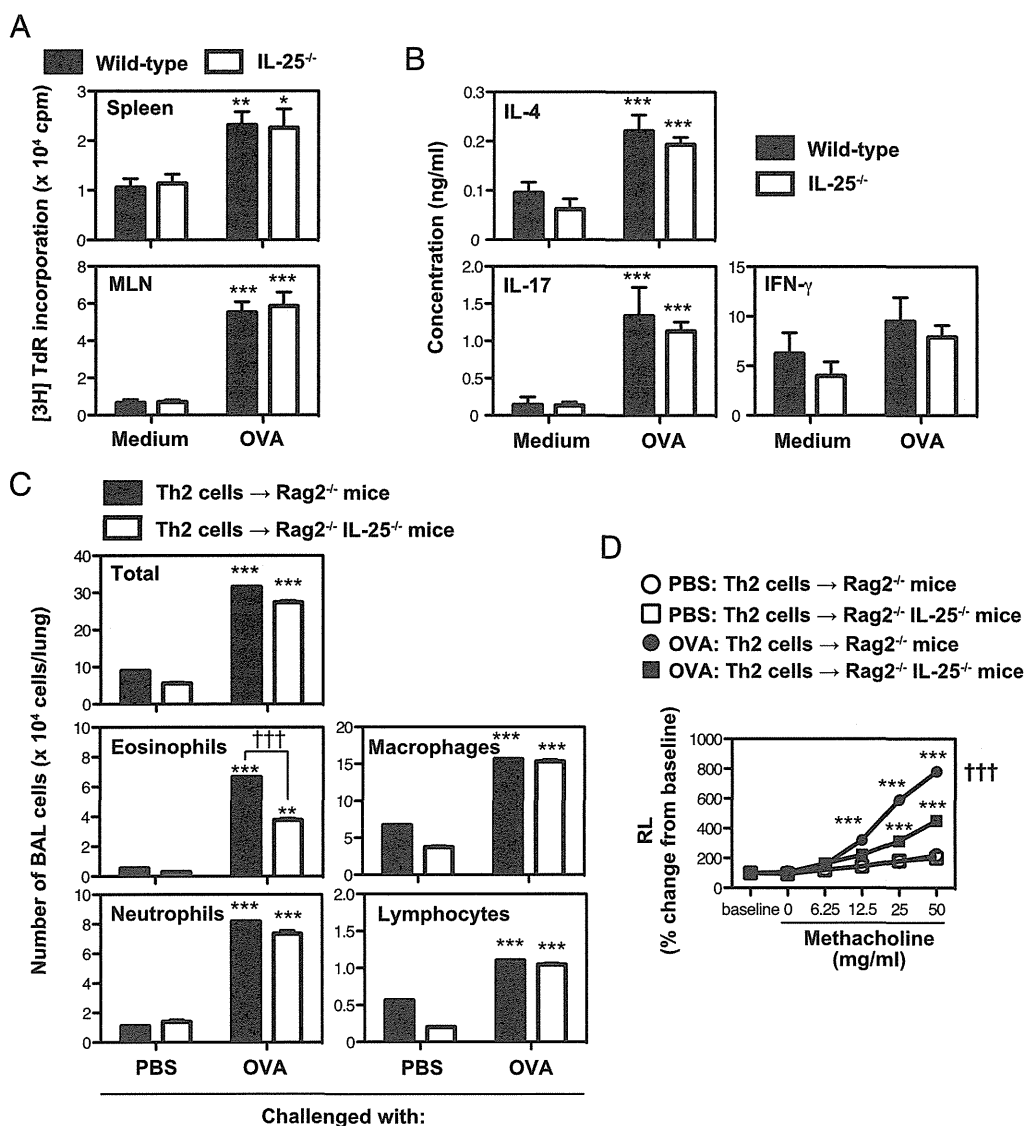
**FIGURE 4.** IL-25 enhances, but is not essential for, DC migration from the lungs to draining LNs. Mice were intranasally treated with FITC-OVA or PBS in the presence and absence of rmIL-25. Submaxillary and thoracic LNs were collected at 12 h (A, B) or 24 h (C) after the inhalation. The proportion of FITC<sup>+</sup> cells in 7-aminoactinomycin D-negative I-A<sup>b</sup>hiCD11c<sup>hi</sup> 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<sup>+</sup> cells among the thoracic and submaxillary LNs, respectively. (B) C57BL/6-WT and TNF<sup>-/-</sup> mice treated intranasally with FITC-OVA plus rmIL-25 or PBS alone ( $n$  = 5). (C) C57BL/6-WT and IL-25<sup>-/-</sup> mice treated intranasally with FITC-OVA ( $n$  = 10) or PBS ( $n$  = 6). Data show the mean  $\pm$  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<sup>+</sup> DCs in thoracic LNs were comparable in wild-type and IL-25<sup>-/-</sup> 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 is not essential for expansion of Ag-specific memory T cells*

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<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> mice, the number of eosinophils in BALFs and mucus secretion in the lungs from IL-25<sup>-/-</sup> Rag-2<sup>-/-</sup> mice injected with OVA-specific OTII mouse-derived Th2 cells was significantly decreased compared with Rag-2<sup>-/-</sup> 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<sup>-/-</sup> Rag-2<sup>-/-</sup> mice injected with OVA-specific OTII mouse-derived Th2 cells was also reduced compared with Rag-2<sup>-/-</sup> 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.



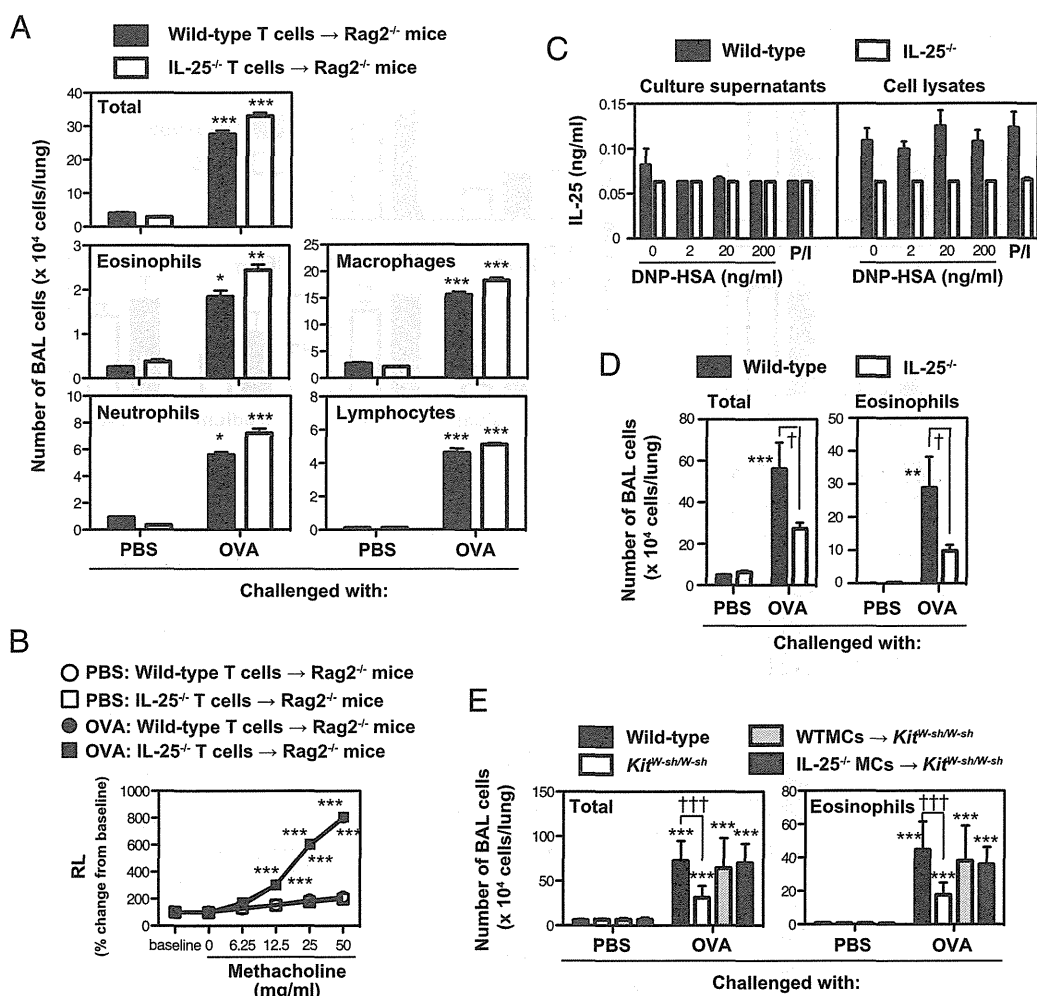
**FIGURE 5.** IL-25 is not essential for induction of Ag-specific T cell expansion in the sensitization phase. (A and B) Spleen and MLNs were collected from mice immunized i.p. with OVA emulsified with alum. Spleen and MLN cells were cultured in the presence and absence of OVA. (A) Proliferative responses of spleen cells and MLN cells. (B) The levels of IL-4, IL-17, and IFN-γ in culture supernatants of the spleen cells in (A). Data show the mean + SEM (n = 5). \*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001 versus the corresponding values for medium alone (-). The numbers of BAL cells (C) and AHR to methacholine (D) in Rag-2<sup>-/-</sup> (for BAL cells: PBS, n = 6; OVA, n = 12. For AHR: PBS, n = 3; OVA, n = 5) and IL-25<sup>-/-</sup> Rag-2<sup>-/-</sup> (for BAL cells: PBS, n = 5; OVA, n = 13. For AHR: PBS, n = 3; OVA, n = 5) mice adoptively transferred with Th2-skewed OTII T cells 24 h after the last challenge. Data show the mean + SEM and are representative results from two independent experiments. \*\*\*p < 0.001 versus the corresponding values for PBS-treated mice, †††p < 0.001 versus the indicated group. RL, Pulmonary resistance.

*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<sup>-/-</sup> mice to Rag-2-deficient mice (wild-type T cells or IL-25<sup>-/-</sup> T cells → Rag-2<sup>-/-</sup> mice). The levels of IL-4, IL-17, and IFN- $\gamma$  in the culture supernatants and proliferation of purified

splenic CD3<sup>+</sup> T cells in response to plate-coated anti-CD3 and anti-CD28 mAbs were similar in wild-type and IL-25<sup>-/-</sup> 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<sup>-/-</sup> mice and IL-25<sup>-/-</sup> T cells → Rag-2<sup>-/-</sup> 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



**FIGURE 6.** T cell- and mast cell-derived IL-25 is dispensable for development of eosinophilic airway inflammation. The number of BAL cells (**A**) and AHR to methacholine (**B**) in OVA/alum-immunized Rag-2<sup>-/-</sup> mice, which had been injected with C57BL/6-wild-type (wild-type T cells → Rag-2<sup>-/-</sup> mice) or IL-25<sup>-/-</sup> T cells (IL-25<sup>-/-</sup> T cells → Rag-2<sup>-/-</sup> mice) at 24 h after the last OVA challenge. The data show the mean + SEM (wild-type T cells → Rag-2<sup>-/-</sup> mice: PBS, *n* = 6, and OVA, *n* = 13, for BAL cells; and PBS, *n* = 4, and OVA, *n* = 5, for AHR. IL-25<sup>-/-</sup> T cells → Rag-2<sup>-/-</sup> mice: PBS, *n* = 8, and OVA, *n* = 16, for BAL cells; and PBS, *n* = 4, and OVA, *n* = 5, for AHR). (**C**) The production of IL-25 by IgE-sensitized C57BL/6-wild-type (*n* = 12) or IL-25<sup>-/-</sup> (*n* = 3) BMCMCs in response to Ags (DNP-conjugated human serum albumin [DNP-HSA]) or PMA + ionomycin (Iono). (**D**) The numbers of total cells and eosinophils in BALFs from C57BL/6-wild-type and IL-25<sup>-/-</sup> mice sensitized with OVA in the absence of alum (mast cell-dependent airway inflammation) at 24 h after the last OVA challenge (PBS, *n* = 8; OVA, *n* = 19). (**E**) The numbers of total cells and eosinophils in BALFs from C57BL/6-wild-type, mast cell-deficient *Kit*<sup>W-sh/W-sh</sup> mice, *Kit*<sup>W-sh/W-sh</sup> mice engrafted with C57BL/6-wild-type BMCMCs (wild-type [WT] MCs → *Kit*<sup>W-sh/W-sh</sup>), and *Kit*<sup>W-sh/W-sh</sup> mice engrafted with IL-25<sup>-/-</sup> BMCMCs (IL-25<sup>-/-</sup> MCs → *Kit*<sup>W-sh/W-sh</sup>). PBS, *n* = 6 and OVA, *n* = 10. Data show the mean + SEM and are representative results from two independent experiments. \**p* < 0.05, \*\**p* < 0.01, or \*\*\**p* < 0.001 versus the corresponding values for PBS-treated mice. †*p* < 0.05 versus the indicated group, †††*p* < 0.001.

levels of IL-25 proteins, irrespective of stimulation, compared with lysates of IL-25<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> BMCMCs to mast cell-deficient *Kit*<sup>W-sh/W-sh</sup> mice (IL-25<sup>-/-</sup> BMCMCs → *Kit*<sup>W-sh/W-sh</sup> 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 FcεRIα on the cell surface of BMCMCs, or 3) β-hexosaminidase release and IL-6, IL-13, and TNF production by BMCMCs after stimulation with PMA + ionophore or IgE + Ags and after stimulation with IgE, LPS, and IL-33, respectively (data not shown).

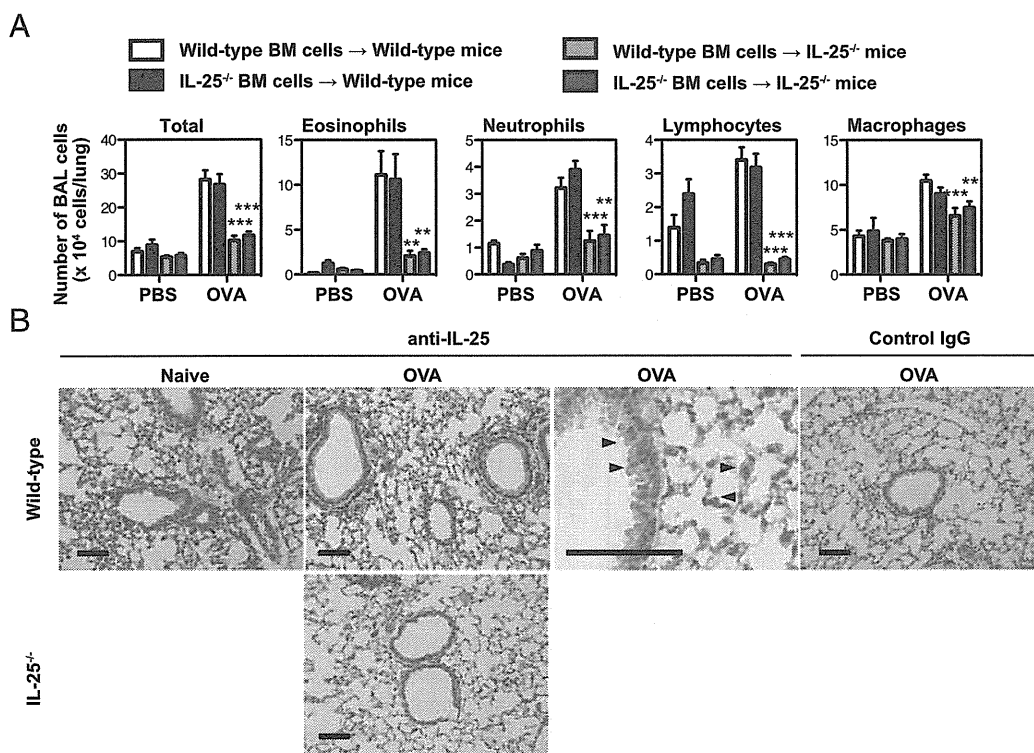
The number of eosinophils in BALFs was decreased in the mast cell-deficient *Kit*<sup>W-sh/W-sh</sup> mice compared with wild-type mice

during mast cell-dependent OVA-induced airway inflammation (Fig. 6E). That decreased eosinophilia returned to the wild-type mouse level in wild-type BMCMCs → *Kit*<sup>W-sh/W-sh</sup> mice after the last OVA challenge (Fig. 6E). Similar recovery was observed even in IL-25<sup>-/-</sup> BMCMCs → *Kit*<sup>W-sh/W-sh</sup> 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<sup>-/-</sup> mice, the number of BAL cells was comparable between CD45.1-wild-type mice and CD45.1-IL-25<sup>-/-</sup> mice that had been reconstituted with CD45.2-wild-type bone marrow cells or IL-25<sup>-/-</sup> 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<sup>-/-</sup>, mice were IL-25<sup>+</sup> 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

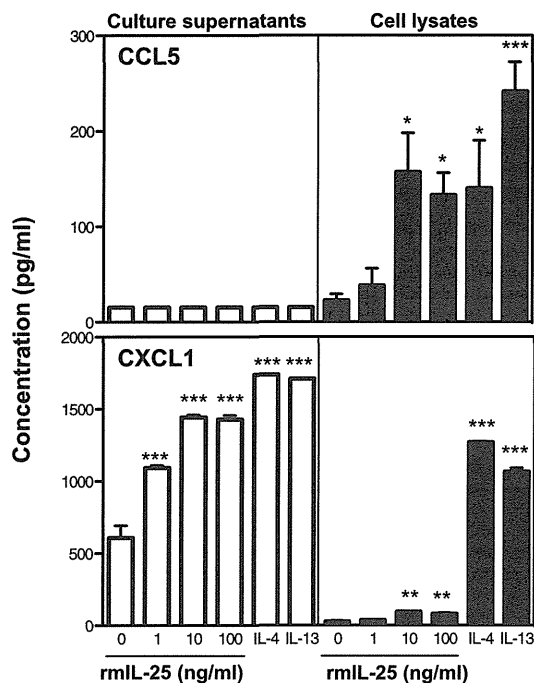
In allergic airway inflammation, NKT cells (36), lung macrophages (37), innate lymphoid cells (38), and epithelial cells (39) are considered to be target cells in the response to IL-25. In addition, we demonstrated that IL-25 is important for Th2 cell activation in the elicitation phase—but not Th2 cell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



**FIGURE 7.** Bone marrow cell-origin immune cell-derived IL-25 is dispensable for development of eosinophilic airway inflammation. **(A)** The number of BAL cells from CD45.1-C57BL/6-wild-type mice that had been injected with CD45.2-C57BL/6-wild-type BM cells (PBS,  $n = 8$ ; OVA,  $n = 19$ ) or -IL-25<sup>-/-</sup> BM cells (PBS,  $n = 8$ ; OVA,  $n = 14$ ) and CD45.1-IL-25<sup>-/-</sup> mice that had been injected with CD45.2-C57BL/6-wild-type BM cells (PBS,  $n = 8$ ; OVA,  $n = 14$ ) or IL-25<sup>-/-</sup> BM cells (PBS,  $n = 8$ ; OVA,  $n = 14$ ) after irradiation during mast cell-independent airway inflammation at 24 h after the last OVA challenge. Data show the mean + SEM. \*\*\* $p < 0.001$  versus the corresponding values for PBS-treated mice. † $p < 0.05$  versus the indicated group. **(B)** Detection of IL-25-producing cells (indicated by arrowheads) in mouse airway by immunohistochemistry. Representative data for the mice used in Fig. 2 are shown. Scale bars, 100 μm.



sitization phase—of Th2-type allergic airway inflammation (Fig. 5). In support of this, IL-25 enhanced mRNA expression for, or production of, CCL1/I-309, CCL11/eotaxin, CCL5/RANTES, and/or CXCL1/KC, which are potential chemoattractants for Th2 cells and/or eosinophils (40, 41) by primary epithelial cells isolated from mouse trachea (Fig. 8) and by a mouse airway epithelial cell line, MLE-12 (Fig. 9A, 9B). Regarding the signal transduction of IL-25R, which consists of IL-17RA and IL-17RB, like IL-17RA (42, 43), Act1 is recruited to SEFIR of IL-17RB through interaction of SEFIR between IL-17RB and Act1 after IL-25 binding (35). In contrast to IL-17RA, IL-17RB—which has a TNFR-associated factor 6-binding motif in the cytoplasmic region—can directly associate with TNFR-associated factor 6 irrespective of IL-25 binding, resulting in activation of NF- $\kappa$ B, but not MAPK, after IL-25 binding (44). Production of CCL5/RANTES by MLE-12 cells in response to IL-25 was significantly reduced in the presence of inhibitors of PI3K or NF- $\kappa$ B but not MEK1/2, MEK1, JNK, or p38MAPK, whereas production of CXCL1/KC was suppressed in the presence of those inhibitors, except the p38MAPK inhibitor (Fig. 9C). Moreover, IL-25-mediated CCL5/RANTES, but not CXCL1/KC, production by MLE-12 cells was 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 airway structural cells such as epithelial cells and immune cells such as Th2 cells and eosinophils during Th2-type allergic airway inflammation.



**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).

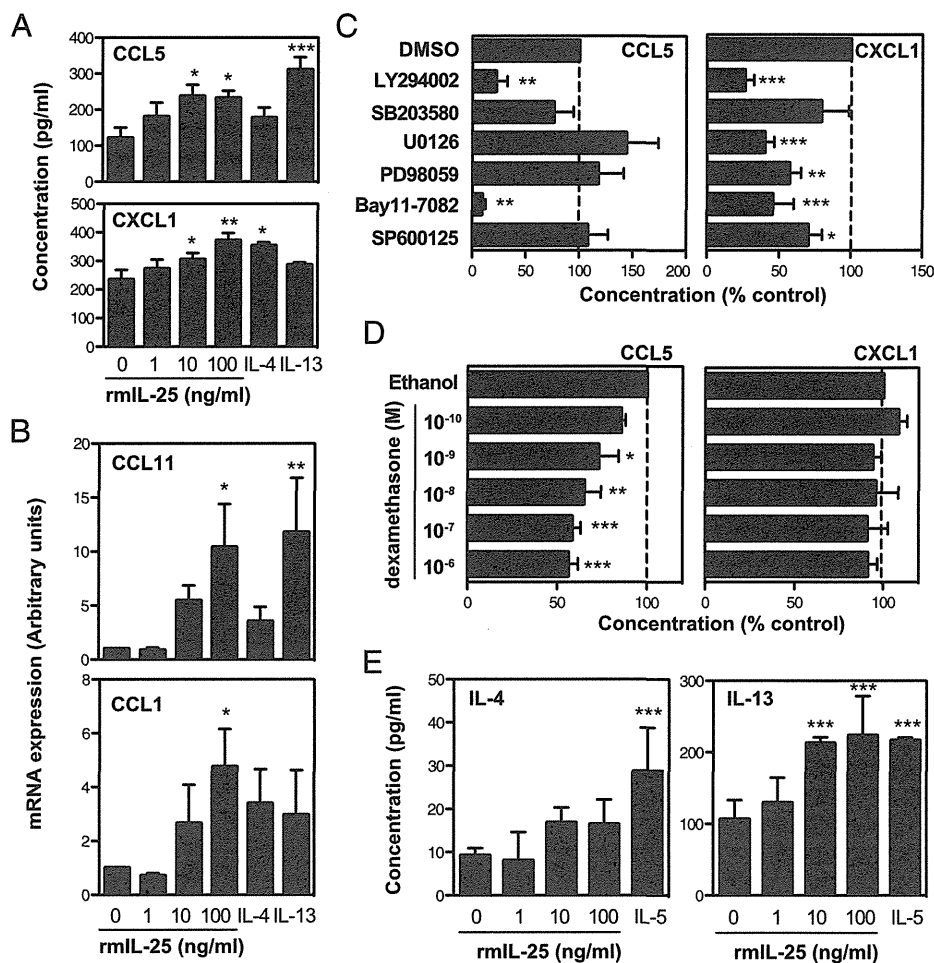
## Discussion

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-17A<sup>-/-</sup>, IL-17F<sup>-/-</sup>, and IL-17A<sup>-/-</sup>IL-17F<sup>-/-</sup> 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-17A<sup>-/-</sup>, IL-17F<sup>-/-</sup>, and IL-17RA<sup>-/-</sup> mice may be dependent on the function of IL-25. Indeed, we demonstrated that IL-25<sup>-/-</sup> mice, but not IL-17A<sup>-/-</sup>IL-17F<sup>-/-</sup>, IL-23p19<sup>-/-</sup>, or ROR- $\gamma$ t<sup>-/-</sup> 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-25<sup>-/-</sup> 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-25<sup>-/-</sup>Rag-2<sup>-/-</sup> mice compared with Rag-2<sup>-/-</sup> mice after the last OVA inhalation, even though these mice had been similarly engrafted with in vitro-skewed OVA-specific OTII Th2 cells (Fig. 5C). In contrast, IL-25 inhalation resulted in development of airway eosinophilia even in T and B cell-deficient Rag-2<sup>-/-</sup> 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.

**FIGURE 9.** IL-25 can potently induce airway epithelial cell and eosinophil activation. **(A and B)** Chemokine expression/production by MLE-12 cultured in the presence and absence of the indicated concentrations of rmIL-25, 100 ng/ml rmIL-4 or 100 ng/ml rmIL-13 for 4 h (for quantitative PCR) or 24 h (for ELISA). **(C and D)** Chemokine levels produced by MLE-12 cells cultured with 100 ng/ml rmIL-25 in the presence and absence (DMSO (C) or ethanol (D) alone) of 10  $\mu$ M of the respective inhibitor for PI3K (LY294002), p38MAPK (SB203580), MEK1/2 (U0126), MAPK/ERK1 (MEK1) (PD98059), NF- $\kappa$ B (Bay11-7082), or JNK (SP600125) (C) or dexamethasone alone (D) for 24 h. Data are expressed as the percentage of the levels of control groups [DMSO (C) or ethanol (D)]. **(E)** IL-4 and IL-13 production by mouse bone marrow cell-derived eosinophils cultured in the presence and absence of the indicated concentrations of rmIL-25 or 100-ng/ml rmIL-5 for 24 h. Data show the mean  $\pm$  SEM (A–C,  $n = 6$ ; D, E,  $n = 3$ ). \* $p < 0.05$ , \*\* $p < 0.01$ , or \*\*\* $p < 0.001$  versus the corresponding values for unstimulated cells (A, B, E) and control DMSO- or ethanol-stimulated cells (C, D).



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.

**Acknowledgments**

We thank Drs. Dan R. Littman (New York University School of Medicine, New York, NY) and Ichiro Taniuchi (RIKEN, Saitama, Japan) for providing ROR- $\gamma$ t-deficient mice and Hiromi Wakita, Shuhei Fukuda, Noriko Hashimoto, Michiko Yamada, and Ikuko Ogahara for excellent technical assistance. We also thank Lawrence W. Stiver (Tokyo, Japan) for critical reading of the manuscript.

**Disclosures**

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

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# The Association between Oxytocin and Social Capital

Takeo Fujiwara<sup>1\*</sup>, Laura D. Kubzansky<sup>2</sup>, Kenji Matsumoto<sup>3</sup>, Ichiro Kawachi<sup>2</sup>

**1** Department of Social Medicine, National Research Institute for Child Health and Development, Tokyo, Japan, **2** Department of Society, Human Development, and Health, Harvard School of Public Health, Massachusetts, United States of America, **3** Department of Allergy and Immunology, National Research Institute for Child Health and Development, Tokyo, Japan

## Abstract

**Background:** Oxytocin is known to be related to social behaviors, including trust. However, few studies have investigated the association between oxytocin levels and social capital. Thus, we tested the hypothesis that endogenous oxytocin levels are positively associated with social capital. We also considered whether the association differed across gender because previous studies have shown differential effects of OT on social behaviors depending on gender.

**Methods:** We recruited a convenience sample of 50 women and 31 men in Japan via community sampling from whom we obtained urine sample with which to measure oxytocin levels. Individual-level cognitive social capital (social trust and mutual aid) and structural social capital (community participation) were assessed using a questionnaire. We used multivariate regression, adjusted for covariates (age, number of children, self-rated health, and education), and stratified by gender to consider associations between oxytocin and social capital.

**Results:** Among women, oxytocin was inversely associated with social trust and mutual aid ( $p < 0.05$ ). However, women participating in only 1 organization in the community showed higher oxytocin than women who participated in either no organizations ( $p < 0.05$ ) or 2 or more organization (i.e. inverse-U shape association). Among men, no association was observed between oxytocin and either form of cognitive and structural social capital.

**Conclusion:** Women who perceived low cognitive social capital showed higher oxytocin levels, while structural social capital showed inverse-U shape association with oxytocin. No association between oxytocin and social capital was found among men. Further study is needed to elucidate why oxytocin was inversely associated with cognitive social capital only among women.

**Citation:** Fujiwara T, Kubzansky LD, Matsumoto K, Kawachi I (2012) The Association between Oxytocin and Social Capital. PLoS ONE 7(12): e52018. doi:10.1371/journal.pone.0052018

**Editor:** Petter Holme, Umeå University, Sweden

**Received:** August 22, 2012; **Accepted:** November 7, 2012; **Published:** December 19, 2012

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**Funding:** This research was supported by Research Development Grant for Child Health and Development from National Center for Child Health and Development (21 shi –10). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

\* E-mail: fujiwara-tk@ncchd.go.jp

## Introduction

Oxytocin (OT), a neuropeptide composed of nine amino acids [1], is known to have both peripheral effects (e.g., uterine contraction during labor and milk ejection during lactation) [2] and central effects (e.g., maternal behavior, pair bonding, and social recognition) [3]. Previous studies employing exogenous OT administration have shown links between OT and social behaviors in both animals [4,5] and humans [6,7], and a number of studies have reported associations between endogenous circulating levels of OT and social behaviors. For example, plasma OT levels in early pregnancy and the postpartum period were significantly positively correlated with maternal behaviors towards the offspring (e.g., gaze, affectionate touch, and frequent infant checking) [8]. Also, momentary experiences of romantic love were associated with pulsatile increases in OT levels among heterosexual romantic couples [9]. Further, OT is associated with trust in others: in the “trust game,” which is characterized by sequential, anonymous monetary payoffs, the trustee’s plasma OT level was significantly higher when the trustee received money intentionally than

randomly; further, the amount of money returned to the investor was correlated with OT level [10].

According to Fukuyama, trust is defined as the expectation of regular, honest, and cooperative behavior from others that arises within a community; such expectations are based on commonly shared norms [11]. Thus, a property that can be called “social trust” arises from the prevalence of trust in a given community. Social trust is an aspect of social capital that has shown consistent positive effect on self-rated physical health [12,13,14,15,16,17,18,19,20], mental health [15,18,21,22,23,24,25,26], and health behaviors [27,28]. Social capital is defined as the resources that individuals can access through their social connections to others. The resources available through social networks can take several distinct forms, including: a) levels of trust between individuals, b) reciprocity exchanges, c) ability to undertake coordinated, collective action. For example, a community with high social capital is one in which members frequently exchange favors for one another. These reciprocity exchanges in turn hinge on high levels of interpersonal trust (i.e. trust that a recipient of a good deed will return the favor in the future). Therefore, it is reasonable assumption that OT might be

positively associated with individual-level social capital, ie, perception of social trust among neighbors.

Prior research has suggested that, social capital, especially community participation, tends to be higher among women than men [29,30]. Prior work in humans has often found effects of oxytocin to differ by gender, although levels of OT in urine do not differ by gender [31]. For example, in one study, a significant positive association between warm contact and OT was found among women, but not among men [32]; another study showed that the effects of OT on social behaviors (e.g., negative engagement and interactive stress) differ by gender [31]. Based on prior hints of gender differences in the effects of oxytocin, we hypothesized that a stronger association between social capital and OT would be observed among women than men.

Social capital can be decomposed into two dimensions: cognitive social capital and structural social capital [33]. The cognitive dimensions of social capital includes perceptions of trust as well as beliefs about the extent to which neighbours can be called upon to provide social support, and structural dimensions of social capital refers to reports of actual behaviours, such as participating in locally based associations [34]. These dimensions might be differently associated with OT, because the structural dimension requires actual behaviors in order to participate in some associations, while the cognitive dimension does not include actual behaviors. As several factors affect actual behaviors rather than perception per se, we hypothesized that cognitive social capital is more likely to be associated with OT than structural social capital. Therefore, the purpose of this study is to examine the association between circulating OT levels and individual-level

social capital, decomposed by cognitive and structural dimensions and stratified by gender.

## Methods

### Participants

The study was approved by the Ethics Committee of National Institute for Public Health, and all participants signed informed consent forms prior to enrollment in the study. Eighty-one participants (50 women and 31 men [the men were spouses of participating women]) were recruited through personal connections with research coordinators or the participants themselves (i.e., a convenience snowball sample). The eligibility criteria restricted the sample to women and men with 18–48-month-old children, because a previous study reported that OT levels were stable among women and men with children [31].

**Table 2.** Summary of oxytocin measurements.

	Mean	SD	Median	Min	Max
Female (n = 50)	108.9	38.2	98.1	53.5	227.8
Male (n = 31)	112.8	42.1	109.3	54.0	236.2

unit:  $\mu\text{U/ml}$  per creatinin  $\text{g/L}$ .

doi:10.1371/journal.pone.0052018.t002

**Table 1.** Distribution of demographics, oxytocin, and social capital variables by gender.

		Female (n = 50)				Male (n = 31)			
		N or Mean	% or SD	Min	Max	N or Mean	% or SD	Min	Max
Age	Year	35.9	3.9	24	44	36.9	2.8	31	42
Number of children	1	26	52.0			18	58.1		
	2	16	32.0			11	35.5		
	3	8	16.0			2	6.5		
Self-rated health	1 (excellent)	20	40.0			14	45.2		
	2 (very good)	19	38.0			10	32.3		
	3 (good)	6	12.0			7	22.6		
	4 (fair)	5	10.0			0	0		
	5 (poor)	0	0.0			0	0		
Education	High school or less	7	14.0			6	19.4		
	Some college	27	54.0			3	9.7		
	College or more	16	32.0			22	71		
Social trust	High	10	20.0			7	22.6		
	Middle	29	58.0			15	48.3		
	Low	11	22.0			9	29		
Mutual aid	High	13	26.0			7	22.6		
	Middle	21	42.0			16	51.6		
	Low	16	32.0			8	25.8		
Community participation	0 organization	15	30.0			11	35.5		
	1 organization	14	28.0			10	32.3		
	2 or more organizations	21	42.0			10	32.3		

doi:10.1371/journal.pone.0052018.t001

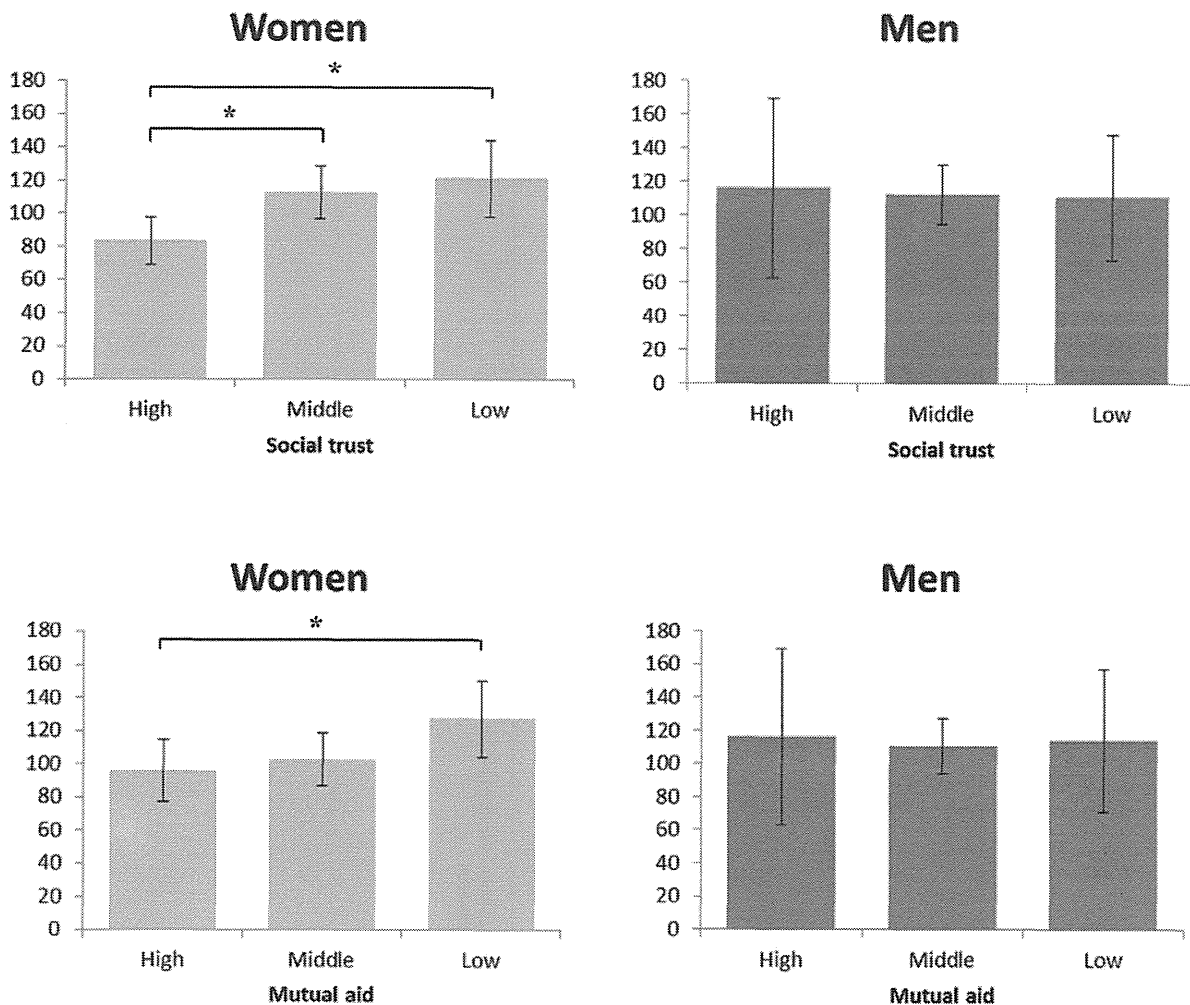
## Procedure

Research coordinators visited the participants' home for approximately 1 hour between 11 a.m. and 2 p.m. A questionnaire was sent before the home visit and was collected during the home visit. We collected urine samples, because they are noninvasive and easy to collect and assay. In animal models, urinary oxytocin assays have been reported to provide a valid biomarker [35]. A 1-mL urine sample was collected in a tube to which a 40- $\mu$ l aliquot of sodium citrate buffer (0.03 M sodium citrate, 25 mM EDTA and 0.35 mM 1,10-phenanthroline) was added; samples were immediately kept in a cooler box at 4°C for maximum of 2 hours and kept at -20°C in the laboratory.

## Oxytocin Analysis

OT concentrations in the urine samples were measured by a competitive radioimmunoassay, as described elsewhere [36]. The radioimmunoassay method to measure urinary OT per mg creatinine showed a strong correlation with plasma OT measurements in a previous study ( $r = 0.89$ ) [37]. Briefly, rabbit antiserum specific for human OT was generated by immunizing a rabbit four

times with recombinant human OT (ASKA Pharmaceutical Co., Ltd., Tokyo, Japan) conjugated with water-soluble carbodiimide (Nakarai Tesque, Tokyo, Japan). The urine sample was decomplexed at 56°C for 30 min, and the supernatant was extracted after centrifugation (3,000 rpm, 10 min, 4°C). The decomplexed sample and the same amount of  $^{125}$ I-labeled OT (Perkin Elmer Life Sciences, Inc., Boston MA) solution were allocated for use in an assay tube (Shionogi, Tokyo, Japan). Then, rabbit anti-OT serum was added to each assay tube, followed by incubation for 2 days at 4°C. Next, goat anti-rabbit IgG serum (ASKA Pharmaceutical Co., Ltd., Tokyo, Japan) was added to each assay tube, followed by incubation for one day at 4°C. After centrifugation, the radioactivity of the pellet was measured by a gamma counter (Auto Well Gamma System ARC-1000 M, Aloka, Tokyo Japan). According to the standard curve, the minimal detection limit of this assay was 3  $\mu$ U/ml (1  $\mu$ U of OT is equivalent to 1.776 pg). All assays were performed in duplicate. The assay's intra- and inter-assay coefficients of variability were <14.2%. The concentration of OT in the urine was standardized according to the urinary creatinine concentration. Urinary creatinine was mea-



\* $p < 0.05$ .

**Figure 1. Association between social capital and urine oxytocin by gender.**  
doi:10.1371/journal.pone.0052018.g001